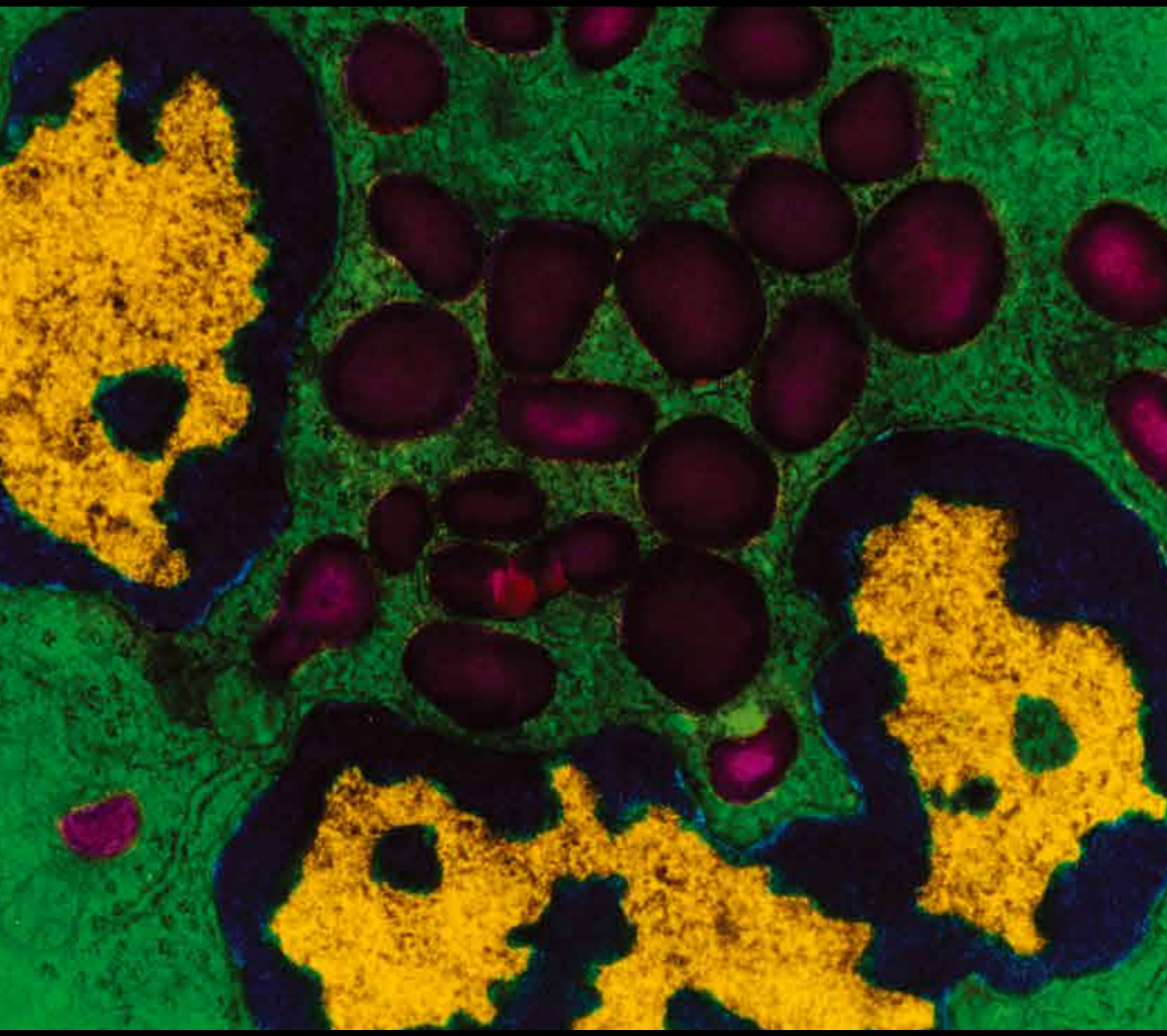
Mediators of Inflammation

Mediators of Interorgan Crosstalk in Metabolic Inflammation

Guest Editors: Massimo Collino, Assaf Rudich, and Daniel Konrad



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Editorial

Mediators of Interorgan Crosstalk in Metabolic Inflammation

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The concept proposing a major role for interorgan crosstalk as a link between dysmetabolism and inflammation largely builds on two major conceptual advances that developed mainly in the last two decades. The first one is the recognition that endocrine-type crosstalk exists between virtually all organs and tissues, extending way beyond very specific, "classical" endocrine glands. This has relied on various lines of experimental approaches, including "omics" types of data, revealing that a large fraction of the expressed genome of many tissues encodes known and predicted secreted proteins. Complementarily, tissue-specific genetic manipulations provided *in vivo* evidence for the ability to alter the function of a tissue distant from the site directly affected by the genetic intervention. The second conceptual advance is the huge expansion in our understanding of the homeostatic-physiological roles, as well as the pathophysiological involvement, of the immune system in diverse types of diseases. In this context, two decades ago, the finding that tumour necrosis factor-α (TNF-α) is overexpressed in adipose tissue of obese mice provided the first clear link between obesity and chronic inflammation [1]. Almost at the same time, the discovery of leptin, the first adipokine that signals through a cytokine receptor family member, sparked the understanding that inflammatory changes within adipose tissue may alter the way this tissue communicates with various organs, even the brain [2]. More recently, a wealth of data established the concept that obesity is associated with systemic and tissue-level low-grade chronic inflammation, initially recognized by adipose tissue infiltration with inflammatory cells in response to an excessive energetic nutrient load [3, 4]. The concept of "metabolic inflammation" or "metaflammation" was eventually formulated and fueled by examples of specific mechanisms tying metabolic mediators to inflammatory cascades and inflammatory mediators to alterations in metabolic regulation [5]. The metabolic inflammation, possibly initiated in response to excess calories/high fat feeding in the adipose tissue, may subsequently involve other metabolic organs such as the liver, skeletal muscle, and pancreas, contributing to metabolic dysfunction and insulin resistance. This multiorgan involvement of obesity-associated inflammation presents a challenge to researchers attempting to clarify the complex signaling mechanisms of the interorgan crosstalk leading to metabolic dysfunction. Alongside inflammation pertaining to "metabolic" organs, studies published during the past decade have convincingly demonstrated a pathophysiological role for the inflammatory response in the development of cardiovascular disease (CVD), the most important complication of the metabolic syndrome and diabetes [6]. In addition, a multitude of mediators of metabolic inflammation have been found to modulate vascular homeostasis, suggesting further mechanisms of interorgan crosstalk between metabolic tissues and the vasculature. Therefore, the low chronic inflammatory state may be responsible for both insulin resistance and endothelial dysfunction, providing deleterious connections between inflammation and metabolic/CVD processes.

This special issue aims to highlight selected topics projecting on advances in our current understanding of the players involved in interorgan communication in obesity-associated metabolic diseases.

In their review, S. Raschke and J. Eckel present an update on bioactive proteins secreted by adipose tissue and skeletal muscles (termed "adipokines" and "myokines", resp.), revealing their peculiar interplay, which represents a yin/yang-type balance: adipokines, upregulated in the obese state, tend to contribute to establish a chronic inflammatory environment that promotes pathological processes such as atherosclerosis and insulin resistance, whereas myokines seem to largely counteract these effects of adipokines, likely reflecting their essential regulatory role in muscle metabolism during contraction. Yet, as more than half of the described myokines are also secreted by adipocytes, the authors suggest to term these cytokines "adipo-myokines," whose beneficial or adverse effects are strictly dependent on their local concentration and kinetics.

A confirmation of this general principle can be found in the review by T. Romacho and colleagues describing the cellular effects of the recently identified adipokine Visfatin/Nampt. Within the adipose tissue, Visfatin/Nampt is not only synthesized and released by adipocytes but also by inflammatory cells, like activated macrophages. Very recently, Visfatin/Nampt expression has been reported in cardiomyocytes, hepatocytes, and myoblasts, thus suggesting its potential role in an even more intricate interorgan metabolic crosstalk. The authors summarize convincing evidence for its deleterious effects on the cardiovascular system, including monocyte/macrophage activation, vascular inflammation, and remodeling. At the same time, the authors quote several papers on beneficial actions of Visfatin/Nampt in ischemia-related clinical conditions.

In their research article, R. Schlich and colleagues investigate the role of vascular endothelial growth factor (VEGF). This well-known growth factor is also released from visceral adipose tissue in obesity and from perivascular adipose tissue of patients with type 2 diabetes and may constitute yet another interorgan mediator, in this instance, linking adipose tissue inflammation to abnormal vascular smooth muscle cells (VSMC) proliferation. Using a model of VSMC proliferation induced by adipocyte-conditioned medium (CM), the authors clearly show that this effect was mainly mediated by VEGF. Moreover, they demonstrate that CM also induced the release of VEGF by VSMC, which in turn could stimulate proliferation via "second-level" autocrine/paracrine communication.

Another immune system (complement-related) mediator, adipose-derived factor with potential autocrine/paracrine and endocrine functions is the acylation stimulating protein (ASP). Using an *in vivo* model of diet-induced obesity, which mimics a typical unhealthy western diet with both high fat and high sucrose intakes, A. Fisette and colleagues demonstrate the paradoxical effects of acute administration of ASP. Particularly, they show that ASP supplementation improved glucose clearance through a short-term insulin sensitizing process in spite of its proinflammatory properties, as demonstrated by increased serum and tissue levels of IL-6 and TNF-α. This may represent a growing current notion expanding our view of the links between inflammation and metabolic regulation [7].

E. Benetti and colleagues focus their attention on the deleterious effects of high sugar intake. They demonstrated that mice chronically fed a sugar-enriched diet develop dyslipidemia and hyperinsulinemia. These metabolic effects were associated with increased infiltration of inflammatory cells into skeletal muscle. Most notably, dyslipidemia and hyperinsulinemia could be prevented by oral administration of a selective agonist of the nuclear receptor peroxisome proliferator activated receptor-δ, which positively correlated with increased muscular levels of the myokine FGF-21, a mediator with expanding roles in metabolic and energy regulation [8].

The role of diet components/additives in affecting subclinical inflammation and insulin resistance is further investigated by S. Bhattacharyya and colleagues. They provide novel insights into the mechanisms of carrageenan-induced inflammation. Interestingly, mice lacking Bcl10, a key molecule in the activation of the inflammatory cascade in epithelial and immune cells, were partly protected from carrageenan-induced inflammation, and such effect was related to an inhibition of both canonical and noncanonical pathways of NF-κB activation.

A potential intestinal participation in the pathogenesis of insulin resistance is more widely addressed in the review by B. M. Carvalho and colleagues, showing how gut microbiota modulation promoted by a bacterial diversity shift mediated by overnutrition increases intestinal permeability, culminating in inflammation and insulin resistance. Herein, activation of inflammatory pathways by gut-derived bacteria products and metabolites is discussed, with particular reference to the key role of the inflammasome multiprotein complex. This recently identified novel player of the intercellular crosstalk linking chronic inflammation to metabolic disease pathogenesis is also the main topic of the review article by B. Benetti et al.

The role of selective inflammatory mediators in pancreatic β-cell dysfunction has been investigated in two different research articles. L. Quintana-Lopez and colleagues demonstrate in cultured islet β-cells that nitric oxide (NO) is an important mediator of the antiproliferative effect induced by proinflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α. In keeping with these *in vitro* data, the same authors report that early treatment of biobred rats (inbred laboratory rats spontaneously developing autoimmune type 1 diabetes) with the NO synthase inhibitor L-NMMA improved β-cell proliferation compared to untreated animals. In addition, A. Puddu and colleagues demonstrate that the detrimental effects evoked by exposing the hamster pancreatic β-cell line HIT-T15 to a pool of advanced glycation end products are prevented by cell treatment with the incretin hormone glucagon-like peptide-1 (GLP-1), which was shown here to restore selective pathways regulating proinsulin production.

Two additional articles discuss the molecular mechanisms underlying the positive association between obesity-associated insulin resistance and cardiac hypertrophy or destructive periodontal disease. Specifically, M. Asrih and colleagues report evidence on the dual role of the cytosolic signaling proteins mitogen-activated protein kinases in inducing synthesis of both specific mediators of cardiac

hypertrophy (such as the gap junction protein connexin 43 and the cardiotrophin-1) and glucose transporters (by affecting the insulin signaling pathway) in cardiomyocytes, thus suggesting selective intracellular mechanisms of cardiac adverse remodeling associated with insulin resistance. R. Khosravi and colleagues offer in their overview potential explanations for the higher risk of destructive periodontal disease in obesity and insulin resistance; that is, they provide evidence for TNF-α and IL-6 as crucial inflammatory mediators connecting these different diseases.

Finally, R. Murphy and colleagues propose an intriguing approach to better elucidate the firm cause-effect relationships between obesity and insulin resistance development. In fact, although obesity is often reported to confer the highest risk for the development of metabolic diseases, the key events in this process are difficult to disentangle from compensatory effects and epiphenomena. The authors of this review support the suggestion that a deep investigation on key monogenic disorders may significantly contribute to unravel the pathogenic links between the defined molecular defect and the related exaggerated metabolic disorders, without the confounding effects of all the other components. For instance, they show how the key role of components of pancreatic β-cell function can be confirmed by evidence, demonstrating that common monogenic pancreatic β-cell defects lead to early onset of diabetes even in the absence of obesity-related insulin resistance.

Overall, we hope that readers will find the articles in this issue not only interesting but also instructive. They might even serve as a source of inspiration for future investigations aiming to further elucidate the intricate inflammatory mechanisms operational in the pathogenesis, and potentially also the resolution, of obesity-associated insulin resistance and type 2 diabetes. Such lines of research may raise hope for discovering new strategies to battle one of the key drivers of human morbidity in modern sedentary times.

Massimo Collino
Assaf Rudich
Daniel Konrad

References

[1] G. S. Hotamisligil, N. S. Shargill, and B. M. Spiegelman, "Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance," *Science*, vol. 259, no. 5091, pp. 87–91, 1993.

[2] M. Maffei, H. Fei, G. H. Lee et al., "Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 6957–6960, 1995.

[3] H. Xu, G. T. Barnes, Q. Yang et al., "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance," *The Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1821–1830, 2003.

[4] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante Jr., "Obesity is associated with macrophage accumulation in adipose tissue," *The Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, 2003.

[5] J. I. Odegaard and A. Chawla, "Pleiotropic actions of insulin resistance and inflammation in metabolichomeostasis," *Science*, vol. 339, no. 6116, pp. 172–177, 2013.

[6] A. Taube, R. Schlich, H. Sell, K. Eckardt, and J. Eckel, "Inflammation and metabolic dysfunction: links to cardiovascular diseases," *American Journal of Physiology*, vol. 302, no. 11, pp. H2148–H2165, 2012.

[7] J. Ye and O. P. McGuinness, "Inflammation during obesity is not all bad: evidence from animal and human studies," *American Journal of Physiology*, vol. 304, no. 5, pp. E466–E477, 2013.

[8] A. C. Adams and A. Kharitonenkov, "FGF21: the center of a transcriptional nexus in metabolic regulation," *Current Diabetes Reviews*, vol. 8, no. 4, pp. 285–293, 2012.

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Review Article

Tumor Necrosis Factor-α and Interleukin-6: Potential Interorgan Inflammatory Mediators Contributing to Destructive Periodontal Disease in Obesity or Metabolic Syndrome

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Obesity has become a worldwide health burden in the last two decades. Obesity has been associated with increased comorbidities, such as coronary artery disease, diabetes, and destructive periodontal disease. Obesity is also part of a group of risk factors occurring together in an individual, which is referred to as metabolic syndrome. Clinical studies have shown higher risk for destructive periodontal disease in obesity and metabolic syndrome. However, the role of obesity and metabolic syndrome in the onset and development of destructive periodontal disease has not yet been fully understood. In this review, we discuss a working model, which focuses on interorgan inflammation as a common etiological factor for destructive periodontal disease associated with obesity and metabolic syndrome. Specifically, we suggest that elevated levels of tumor necrosis factor-α (TNF-α) or interleukin 6 (IL-6)—both adipokines and known risk factors for destructive periodontal disease—in obesity and metabolic syndrome contribute to the onset and development of destructive periodontal disease. The connections between destructive periodontal disease and systemic conditions, such as obesity or metabolic syndrome, are complex and potentially multidirectional. This review largely focuses on TNF-α and IL-6, inflammatory mediators, as potential common risk factors and does not exclude other biological mechanisms.

1. Periodontal Diseases

Periodontal diseases are inflammatory diseases affecting the surrounding and supporting tissues of teeth—the periodontium. Gingivitis and destructive periodontal disease (periodontitis) are the two most common forms of periodontal diseases. Gingivitis is an inflammatory reaction often induced by the pathogens residing in dental plaque (biofilm), which forms on the adjacent tooth surfaces [1]. Destructive periodontal disease results in an apical loss of epithelial attachment along with the periodontal soft and hard tissues [2]. Unlike gingivitis, which is cured following the removal of local etiological factors, destructive periodontal disease is irreversible. Destructive periodontal disease is mediated by various intrinsic and acquired factors; two individuals with similar microbiological profile could show different susceptibility to periodontal diseases [3]. Several case-control and cohort studies have reported the contribution of systemic conditions and diseases in the onset and exacerbation of destruction periodontal disease. Preterm birth [4], cardiovascular diseases [5], and diabetes [6] are examples of these conditions. In addition, a growing body of evidence during the last decade suggests obesity as a risk factor for destructive periodontal disease [7, 8]. Metabolic syndrome has also been shown to be positively associated with destructive periodontal disease [9–22]. Although the majority of studies on destructive periodontal disease in individuals with obesity or metabolic syndrome concentrated on adults, some studies reported on evidence proposing that this potential link in children and adolescence exists [23]. In this paper, we review

the evidence suggesting that destructive periodontal disease is linked to obesity and metabolic syndrome, as an example of interorgan crosstalk under inflammatory conditions. Additionally, we discuss a working biological model on the onset of destructive periodontal disease in individuals with obesity or metabolic syndrome based on elevated levels of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) in these conditions.

2. Obesity

Obesity is a multifactorial condition with a wide range of etiological factors including genetic, biological, social, and behavioral factors, all of which likely interact to ultimately lead to a chronic imbalance between energy intake and energy expenditure. This imbalance could cause excessive fat accumulation and result in adverse health consequences. Obesity has reached epidemic proportion worldwide, largely because of increased consumption of high caloric diet and a sedentary lifestyle. According to the World Health Organization [24], approximately 2.3 billion adults will be overweight and more than 700 million will be obese by 2015. This phenomenon affects particularly developed countries. Over the past two decades, the overall obesity rates have reached 24.1% and 34.4% in Canada and the United States, respectively [25].

Based on current Health Canada guidelines, a body mass index (BMI, kg/m^2) of 25–30 and over 30 are considered overweight and obese, respectively [26]. Obesity is categorized into 3 classes according to the increased health risks associated with increasing BMI levels: class I (BMI 30–34.9), class II (BMI 35–39.9), and class III (BMI $\geq$ 40) [27]. Pediatric obesity has also become a public health concern since it is more common for children to experience the negative health consequences of obesity, which used to be only seen in adulthood. In 2010, more than 40 million children under the age of five were estimated to be overweight worldwide [28]. In 2004, 26% of Canadian children and adolescents aged 2 to 17 years were overweight or obese [29]. Obesity has been associated with a wide spectrum of comorbidities, such as coronary artery disease, strokes, diabetes, arthritis, reproductive dysfunctions, and various cancers [30].

3. Metabolic Syndrome

Metabolic syndrome is a cluster of risk factors (abdominal obesity, diabetes, high cholesterol, high blood pressure, and raised fasting glucose) that increases the chance of developing type 2 diabetes and cardiovascular diseases [31]. Metabolic syndrome is also known as syndrome X, insulin resistance syndrome, dysmetabolic syndrome X, Reaven's syndrome, multiple metabolic syndrome, and metabolic cardiovascular syndrome [18, 32–34]. Several definitions for metabolic syndrome in adult populations have been proposed by different organizations including the World Health Organization (WHO), the International Diabetes Federation (IDF), the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), the National Cholesterol Education Program Adult Treatment Panel III P(NCEP-ATP III), and the European Group for the Study of Insulin Resistance (EGIR) [15]. Although these diagnostic criteria are similar, there is variability with the different cut-off values set for each criterion. The unified criteria to diagnose metabolic syndrome include at least 3 of the following: abdominal obesity, high level of plasma triglycerides, low level of high-density lipoprotein (HDL) cholesterol, high blood pressure, and impaired fasting glucose or insulin resistance [35].

The reported prevalence of metabolic syndrome varies depending on the diagnostic criteria opted by different studies, nonetheless the prevalence of metabolic syndrome has increased at epidemic rates for the past decade. Findings from the National Health and Nutrition Examination Survey (NHANES) 2003–2006 showed that 34% of Americans, 20 years and older, met the criteria to be diagnosed with metabolic syndrome based on the NCEP-ATP III definition [36]. Canadian Health Measures Survey revealed that about one in five Canadian adults (20% of the population) had metabolic syndrome [37]. No standard diagnostic criteria for metabolic syndrome in children and adolescents have been developed, which makes studying metabolic syndrome in children challenging. Nonetheless, the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) reported on a 4.2% prevalence of metabolic syndrome among 12- to 19-year-old American adolescents (approximately 1 million adolescents) [38]. In addition, nearly 30% of overweight adolescents met the criteria of metabolic syndrome according the definition proposed by the NCEP-ATPIII [38]. De Ferranti and colleagues reported an increase of 38% in the prevalence of metabolic syndrome in adolescents of the same age group between 1988 and 2000 using the same definition [39].

4. Periodontal Diseases in Obesity and Metabolic Syndrome

Periodontal diseases, a chronic inflammation by its nature, have been linked to many systemic conditions. The findings from meta-analyses suggest that cardiovascular diseases [40–42] and obesity [43–45] increase the chance of destructive periodontal disease by 20% and 35%, respectively. Moreover, studies on diabetic patients indicate that type 2 diabetes is a risk factor for advanced form of destruction periodontal disease [46]. Metabolic syndrome is also positively correlated with destructive periodontal disease [9–22]. A recent meta-analysis reported that the odds of destructive periodontal disease to occur was 1.71 to 2.09 higher in individuals with metabolic syndrome compared to those without this syndrome [47]. Despite all these evidence indicating the higher risk of destructive periodontal disease in obesity and metabolic syndrome, the underlying biological mechanism(s) is yet to be fully understood. Studying the common etiological factors in obesity, metabolic syndrome, and destructive periodontal disease would be a potential approach to delineate biological mechanisms explaining the higher risk of destructive periodontal disease under these conditions. Inflammation is indeed one of the common factors in the pathogenesis of destructive periodontal disease, obesity, and metabolic syndrome. Figure 1 represents

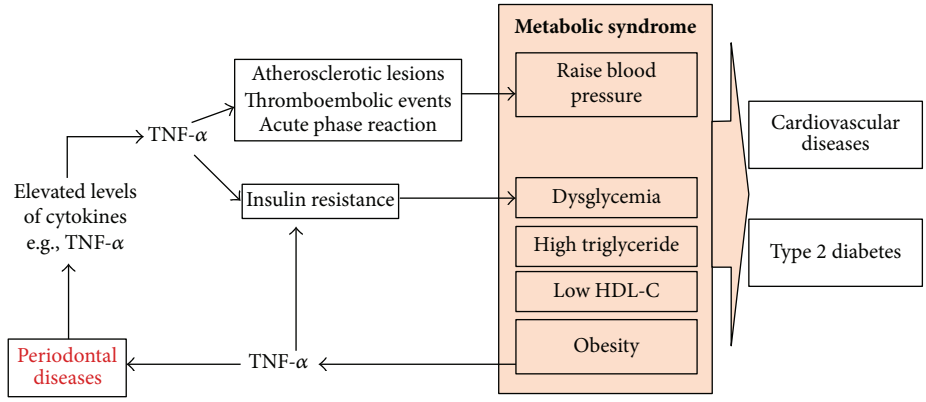

Figure 1: A biological working model on the onset of destructive periodontal disease in obesity and metabolic syndrome adapted from Nishimura et al., 2003 [8]. This scheme represents a potential working model in which the systemic elevated levels of TNF-α in obesity and metabolic syndrome potentially contribute to the onset and development of destructive periodontal disease. Specifically, the elevated levels of systemic inflammatory mediators, such as TNF-α or IL-6, in obesity or metabolic syndrome enhance the host response to periodontal pathogens hence increase the chance to develop destructive periodontal disease. In destructive periodontal disease, periodontal pathogens induce inflammation prompting the destruction of connective tissues and bone in the periodontium. The connections between destructive periodontal disease and systemic conditions, such as obesity, are complex and often multidirectional; the working model presented here is a simplified picture of these connections.

a simplified working model explaining how inflammation connects these diseases. Indeed, this working model does not exclude other potential biological mechanisms.

In this review we focus on a potential interorgan inflammatory mechanism explaining the higher prevalence of destructive periodontal disease in obesity and metabolic syndrome. Higher risk of destructive periodontal disease associated with obesity and/or metabolic syndrome is most likely bidirectional, and here we only concentrate on how obesity or metabolic syndrome induces or enhances destructive periodontal disease.

Tumor necrosis factor-α (TNF-α) is the best candidate connecting higher destructive periodontal disease in obesity or metabolic syndrome. TNF-α levels are systemically elevated in both obesity and metabolic syndrome [48]. Studies on human and animal models in the 1990s indicated that adipocytes secrete TNF-α, and hence the excess of fat in obesity leads to a systemic chronic inflammation [49, 50]. TNF-α was also reported to induce insulin resistance in both diabetes and obesity [51]. It is known that other cytokines from adipose tissues also contribute to the chronic systemic inflammation in obesity [48].

TNF-α is one of the key periodontal pathogens-induced early inflammatory cytokines in destructive periodontal disease [52]. Elevated levels of TNF-α are a well-known risk factor for destruction periodontal disease [52]. Increased levels of TNF-α contribute to the onset of destructive periodontal disease via several mechanisms. Examples of these mechanisms are (i) TNF-α prompts the destruction of alveolar bone by stimulating the formation of bone-resorbing cells (osteoclasts) [53]; (ii) TNF-α, as one of the early promoters of host response to periodontal bacterial pathogens, regulates matrix metalloproteinases (MMPs), which are capable of degrading the connective tissues. Interestingly, studies on the immune response to periodontal pathogens showed that TNF-α enhanced the immune response to these pathogens [54]. Previous studies on human subjects reported on the elevated levels of TNF-α in gingival crevicular fluid (GCF) of obese individuals. The authors reported on a 0.74 pg increase in GCF TNF-α with an increase of one BMI unit. 32 obese subjects aged between 13 and 24 years was recruited in this study [55]. In a larger scale study on the third National Health and Nutrition Examination Survey (NHANES II) dataset, Genco and colleagues showed that serum TNF-α levels were not correlated with the severity of destructive periodontal disease in BMI over 30 kg/m, and proposed the notion that TNF-α mainly contributes to the initial stage of destructive periodontal disease development [56]. In addition, our group has been investigated the potential development of destructive periodontal disease in children at risk of obesity and metabolic syndrome as they age. We are studying the Oral Health component within a cohort of 600 children in Quebec (the Quebec Adipose and Lifestyle Investigation in Youth; QUALITY cohort), which aims to investigate the natural history of obesity and its vascular and metabolic consequences [57]. Our results from the baseline visit of the QUALITY cohort so far indicate that obese boys, but not girls, have 37% higher levels of TNF-α, a risk factor for destructive periodontal disease, in their gingival crevicular fluid (GCF) compared to nonobese boys [58]. We also found that metabolic syndrome in boys was associated with 49 percent increase in the levels of TNF-α in gingiva crevicular fluid (Ka et al.; unpublished data).

The second candidate explaining a mechanism for the higher prevalence of destructive periodontal diseases in obesity or metabolic syndrome is interleukin 6 (IL-6). IL-6 is a multifunctional cytokine produced by variety of cells including macrophages, neutrophils, and endothelial cells (reviewed in [59]). The double edge effects (i.e., pro- and anti-inflammatory) of IL-6 create a complexity in investigating its

roles in normal or diseased conditions [59]. IL-6 systemic and GCF levels increase in destructive periodontal diseases (reviewed in [60]). Additionally, controversial studies suggested an increase or no change in serum IL-6 levels in obesity and metabolic syndrome (reviewed in [61]). Unfortunately, to the best of our knowledge, no evidence from animal or human studies supports the hypothetical model in which obesity or metabolic syndrome-induced IL-6 increases the risk of destructive periodontal disease.

Taken together, these evidence support the notion that, in obesity and metabolic syndrome, the elevated levels of TNF-α, and possibly IL-6, may increase the chance of destructive periodontal diseases development, which could be directly through mechanisms discussed earlier and also indirectly by enhancing the bacterial-induced host immune response in obesity and metabolic syndrome.

5. Conclusion

The obesity epidemic in North America and worldwide is an alarming public health issue. The prevalence of childhood obesity and overweight is rising rapidly in Canada [62, 63]. The total direct costs attributable to overweight and obesity in Canada was $6.0 billion in 2006, representing 4.1% of the total health expenditures in the country [64]. Similarly, data from American adults showed an increase in the prevalence of metabolic syndrome from 24 to 27% between 1988–1994 and 1999–2000 [65]. Similar trends were seen in American adolescents particularly in overweight and obese subjects, for whom the prevalence of metabolic syndrome was 32.1% [32]. Moreover, higher health care cost with increased number of metabolic syndrome components has been reported [66–68]. The annual health care cost associated for an individual with 1 component was estimated to be $5564 compared to $12,287 for someone with 4 components [68].

Understanding the multidirectional and dynamic links among obesity, metabolic syndrome, and destructive periodontal disease can improve current preventive and therapeutic modalities for these conditions. For example, one could screen the GCF TNF-α levels of obese individuals to identify a subgroup of obese subjects, who are more susceptible to develop destructive periodontal disease.

Authors' Contribution

Roozbeh Khosravi and Khady Ka equally contributed to this publication.

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References

[1] B. L. Pihlstrom, B. S. Michalowicz, and N. W. Johnson, "Periodontal diseases," *The Lancet*, vol. 366, no. 9499, pp. 1809–1820, 2005.

[2] C. L. Drisko, D. L. Cochran, T. Blieden et al., "Position paper: sonic and ultrasonic scalers in periodontics. Research, Science and Therapy Committee of the American Academy of Periodontology," *Journal of Periodontology*, vol. 71, no. 11, pp. 1792–1801, 2000.

[3] T. E. Van Dyke and D. Sheilesh, "Risk factors for periodontitis," *Journal of the International Academy of Periodontology*, vol. 7, no. 1, pp. 3–7, 2005.

[4] S. Offenbacher, S. Lieff, K. A. Boggess et al., "Maternal periodontitis and prematurity. Part I: obstetric outcome of prematurity and growth restriction," *Annals of Periodontology*, vol. 6, no. 1, pp. 164–174, 2001.

[5] F. A. Scannapieco, R. B. Bush, and S. Paju, "Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease, and stroke. A systematic review," *Annals of Periodontology*, vol. 8, no. 1, pp. 38–53, 2003.

[6] G. W. Taylor, "Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective," *Annals of Periodontology*, vol. 6, no. 1, pp. 99–112, 2001.

[7] M. Alabdulkarim, N. Bissada, M. Al-Zahrani, A. Ficara, and B. Siegel, "Alveolar bone loss in obese subjects," *Journal of the International Academy of Periodontology*, vol. 7, no. 2, pp. 34–38, 2005.

[8] F. Nishimura, Y. Iwamoto, J. Mineshiba, A. Shimizu, Y. Soga, and Y. Murayama, "Periodontal disease and diabetes mellitus: the role of tumor necrosis factor-α in a 2-way relationship," *Journal of Periodontology*, vol. 74, no. 1, pp. 97–102, 2003.

[9] C. Benguigui, V. Bongard, J.-B. Ruidavets et al., "Metabolic syndrome, insulin resistance, and periodontitis: a cross-sectional study in a middle-aged French population," *Journal of Clinical Periodontology*, vol. 37, no. 7, pp. 601–608, 2010.

[10] P. K. D. O. Borges, S. G. A. Gimeno, N. E. Tomita et al., "Prevalence and characteristics associated with metabolic syndrome in Japanese-Brazilians with and without periodontal disease," *Cadernos de Saude Publica*, vol. 23, no. 3, pp. 657–668, 2007.

[11] F. D'Aiuto, W. Sabbah, G. Netuveli et al., "Association of the metabolic syndrome with severe periodontitis in a large U.S. population-based survey," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 10, pp. 3989–3994, 2008.

[12] D.-H. Han, S.-Y. Lim, B.-C. Sun, D. Paek, and H.-D. Kim, "The association of metabolic syndrome with periodontal disease is confounded by age and smoking in a Korean population: the Shiwha-Banwol environmental health study," *Journal of Clinical Periodontology*, vol. 37, no. 7, pp. 609–616, 2010.

[13] Y. S. Khader, B. Khassawneh, B. Obeidat et al., "Periodontal status of patients with metabolic syndrome compared to those without metabolic syndrome," *Journal of Periodontology*, vol. 79, no. 11, pp. 2048–2053, 2008.

[14] M. Kushiyama, Y. Shimazaki, and Y. Yamashita, "Relationship between metabolic syndrome and periodontal disease in japanese adults," *Journal of Periodontology*, vol. 80, no. 10, pp. 1610–1615, 2009.

[15] Y.-E. Kwon, J.-E. Ha, D.-I. Paik, B.-H. Jin, and K.-H. Bae, "The relationship between periodontitis and metabolic syndrome among a Korean nationally representative sample of adults," *Journal of Clinical Periodontology*, vol. 38, no. 9, pp. 781–786, 2011.

[16] P. Li, L. He, Y.-Q. Sha, and Q.-X. Luan, "Relationship of metabolic syndrome to chronic periodontitis," *Journal of Periodontology*, vol. 80, no. 4, pp. 541–549, 2009.

[17] T. Morita, Y. Yamazaki, A. Mita et al., "A cohort study on the association between periodontal disease and the development of metabolic syndrome," *Journal of Periodontology*, vol. 81, no. 4, pp. 512–519, 2010.

[18] L. Nibali, F. D'Aiuto, G. Griffiths, K. Patel, J. Suvan, and M. S. Tonetti, "Severe periodontitis is associated with systemic inflammation and a dysmetabolic status: a case-control study," *Journal of Clinical Periodontology*, vol. 34, no. 11, pp. 931–937, 2007.

[19] M. M. Pozharitskaia, T. G. Simakova, L. K. Starosel'tseva, and V. V. Kirienko, "Inflammatory diseases of the parodontium in patiens with metabolic syndrome," *Stomatologiia*, vol. 83, no. 6, pp. 13–16, 2004.

[20] T. Saito, Y. Shimazaki, Y. Kiyohara et al., "The severity of periodontal disease is associated with the development of glucose intolerance in non-diabetics: the hisayama study," *Journal of Dental Research*, vol. 83, no. 6, pp. 485–490, 2004.

[21] Y. Shimazaki, T. Saito, K. Yonemoto, Y. Kiyohara, M. Iida, and Y. Yamashita, "Relationship of metabolic syndrome to periodontal disease in Japanese women: the hisayama study," *Journal of Dental Research*, vol. 86, no. 3, pp. 271–275, 2007.

[22] P. Timonen, M. Niskanen, L. Suominen-Taipale, A. Jula, M. Knuuttila, and P. Ylöstalo, "Metabolic syndrome, periodontal infection, and dental caries," *Journal of Dental Research*, vol. 89, no. 10, pp. 1068–1073, 2010.

[23] M. S. Al-Zahrani, N. F. Bissada, and E. A. Borawski, "Obesity and periodontal disease in young, middle-aged, and older adults," *Journal of Periodontology*, vol. 74, no. 5, pp. 610–615, 2003.

[24] WHO, *Unhealthy Diets & Physical Inactivity*, World Health Organization, 2009.

[25] M. Shields, M. D. Carroll, and C. L. Ogden, "Adult obesity prevalence in Canada and the United States," *NCHS Data Brief*, no. 56, pp. 1–8, 2011.

[26] Health Canada, *Canadian Guidelines for Body Weight Classification in Adults*, 2003.

[27] M. Tjepkema, "Adult obesity," *Health Reports*, vol. 17, no. 3, pp. 9–25, 2006.

[28] WHO, *Obesity and Overweight*, World Health Organization, 2010.

[29] M. Shields, *Nutrition: Findings From the Canadian Community Health Survey*, Statistics Canada Publication, Ottawa, Canada, 2005.

[30] D. P. Guh, W. Zhang, N. Bansback, Z. Amarsi, C. L. Birmingham, and A. H. Anis, "The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis," *BMC Public Health*, vol. 9, article 88, 2009.

[31] International Diabetes Federation, *The IDF Concensus Definition of the Metabolic Syndrome in Children and Adolescents*, 2007.

[32] G. E. Duncan, S. M. Li, and X.-H. Zhou, "Prevalence and trends of a metabolic syndrome phenotype among U.S. adolescents, 1999–2000," *Diabetes Care*, vol. 27, no. 10, pp. 2438–2443, 2004.

[33] I. Hjermann, "The metabolic cardiovascular syndrome: syndrome X, Reaven's syndrome, insulin resistance syndrome, atherothrombogenic syndrome," *Journal of Cardiovascular Pharmacology*, vol. 20, no. 8, pp. S5–S10, 1992.

[34] A. D. Liese, E. J. Mayer-Davis, and S. M. Haffner, "Development of the multiple metabolic syndrome: an epidemiologic perspective," *Epidemiologic Reviews*, vol. 20, no. 2, pp. 157–172, 1998.

[35] K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy et al., "Harmonizing the metabolic syndrome: a joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International atherosclerosis society; and international association for the study of obesity," *Circulation*, vol. 120, no. 16, pp. 1640–1645, 2009.

[36] R. B. Ervin, "Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003–2006," *National Health Statistics Reports*, no. 13, pp. 1–7, 2009.

[37] N. D. Riediger and I. Clara, "Prevalence of metabolic syndrome in the Canadian adult population," *Canadian Medical Association Journal*, vol. 183, no. 15, pp. E1127–E1134, 2011.

[38] S. Cook, M. Weitzman, P. Auinger, M. Nguyen, and W. H. Dietz, "Prevalence of a metabolic syndrome phenotype in adolescents: findings from the third National Health and Nutrition Examination Survey, 1988–1994," *Archives of Pediatrics and Adolescent Medicine*, vol. 157, no. 8, pp. 821–827, 2003.

[39] S. D. De Ferranti, K. Gauvreau, D. S. Ludwig, J. W. Newburger, and N. Rifai, "Inflammation and changes in metabolic syndrome abnormalities in US adolescents: findings from the 1988–1994 and 1999–2000 National Health and Nutrition Examination Surveys," *Clinical Chemistry*, vol. 52, no. 7, pp. 1325–1330, 2006.

[40] S.-J. Janket, A. E. Baird, S.-K. Chuang, and J. A. Jones, "Meta-analysis of periodontal disease and risk of coronary heart disease and stroke," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 95, no. 5, pp. 559–569, 2003.

[41] Y. S. Khader, Z. S. M. Albashaireh, and M. A. Alomari, "Periodontal diseases and the risk of coronary heart and cerebrovascular diseases: a meta-analysis," *Journal of Periodontology*, vol. 75, no. 8, pp. 1046–1053, 2004.

[42] Z. You, M. Cushman, N. S. Jenny, and G. Howard, "Tooth loss, systemic inflammation, and prevalent stroke among participants in the reasons for geographic and racial difference in stroke (REGARDS) study," *Atherosclerosis*, vol. 203, no. 2, pp. 615–619, 2009.

[43] B. W. Chaffee and S. J. Weston, "Association between chronic periodontal disease and obesity: a systematic review and meta-analysis," *Journal of Periodontology*, vol. 81, no. 12, pp. 1708–1724, 2010.

[44] J. Katz and E. Bimstein, "Pediatric obesity and periodontal disease: a systematic review of the literature," *Quintessence International*, vol. 42, no. 7, pp. 595–599, 2011.

[45] J. Suvan, F. D'Aiuto, D. R. Moles, A. Petrie, and N. Donos, "Association between overweight/obesity and periodontitis in adults. A systematic review," *Obesity Reviews*, vol. 12, no. 501, pp. e381–e404, 2011.

[46] N. G. M. Chávarry, M. V. Vettore, C. Sansone, and A. Sheiham, "The relationship between diabetes mellitus and destructive periodontal disease: a meta-analysis," *Oral Health & Preventive Dentistry*, vol. 7, no. 2, pp. 107–127, 2009.

[47] L. Nibali, N. Tatarakis, I. Needleman et al., "Association between metabolic syndrome and periodontitis: a systematic review and meta-analysis," *Journal of Clinical Endocrinology and Metabolism*, vol. 98, no. 3, p. 913, 2013.

[48] M. F. Gregor and G. S. Hotamisligil, "Inflammatory mechanisms in obesity," *Annual Review of Immunology*, vol. 29, pp. 415–445, 2011.

[49] G. S. Hotamisligil, N. S. Shargill, and B. M. Spiegelman, "Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance," *Science*, vol. 259, no. 5091, pp. 87–91, 1993.

[50] G. S. Hotamisligil, P. Arner, J. F. Caro, R. L. Atkinson, and B. M. Spiegelman, "Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance," *The Journal of Clinical Investigation*, vol. 95, no. 5, pp. 2409–2415, 1995.

[51] G. S. Hotamisligil and B. M. Spiegelman, "Tumor necrosis factor α: a key component of the obesity-diabetes link," *Diabetes*, vol. 43, no. 11, pp. 1271–1278, 1994.

[52] D. T. Graves and D. Cochran, "The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction," *Journal of Periodontology*, vol. 74, no. 3, pp. 391–401, 2003.

[53] K. Kobayashi, N. Takahashi, E. Jimi et al., "Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction," *Journal of Experimental Medicine*, vol. 191, no. 2, pp. 275–285, 2000.

[54] D. T. Graves, R. Liu, M. Alikhani, H. Al-Mashat, and P. C. Trackman, "Diabetes-enhanced inflammation and apoptosis—impact on periodontal pathology," *Journal of Dental Research*, vol. 85, no. 1, pp. 15–21, 2006.

[55] M. Lundin, T. Yucel-Lindberg, G. Dahllöf, C. Marcus, and T. Modéer, "Correlation between TNFα in gingival crevicular fluid and body mass index in obese subjects," *Acta Odontologica Scandinavica*, vol. 62, no. 5, pp. 273–277, 2004.

[56] R. J. Genco, S. G. Grossi, A. Ho, F. Nishimura, and Y. Murayama, "A proposed model linking inflammation to obesity, diabetes, and periodontal infections," *Journal of Periodontology*, vol. 76, no. 11, pp. 2075–2084, 2005.

[57] M. Lambert, A. Van Hulst, J. O'Loughlin et al., "Cohort profile: the quebec adipose and lifestyle investigation in youth cohort," *International Journal of Epidemiology*, vol. 41, pp. 1533–1544, 2012.

[58] R. Khosravi, S. D. Tran, M. Lambert et al., "Adiposity and gingival crevicular fluid tumour necrosis factor-α levels in children," *Journal of Clinical Periodontology*, vol. 36, no. 4, pp. 301–307, 2009.

[59] T. Kishimoto, "Interleukin-6: discovery of a pleiotropic cytokine," *Arthritis Research and Therapy*, vol. 8, no. 2, 2006.

[60] L. Nibali, S. Fedele, F. D'Aiuto, and N. Donos, "Interleukin-6 in oral diseases: a review," *Oral Diseases*, vol. 18, no. 3, pp. 236–243, 2012.

[61] K. Eder, N. Baffy, A. Falus, and A. K. Fulop, "The major inflammatory mediator interleukin-6 and obesity," *Inflammation Research*, vol. 58, no. 11, pp. 727–736, 2009.

[62] S. Amar and X. Han, "The impact of periodontal infection on systemic diseases," *Medical Science Monitor*, vol. 9, no. 12, pp. RA291–RA299, 2003.

[63] M. S. Tremblay and J. D. Willms, "Secular trends in the body mass index of Canadian children," *Canadian Medical Association Journal*, vol. 163, no. 11, pp. 1429–1433, 2000.

[64] A. H. Anis, W. Zhang, N. Bansback, D. P. Guh, Z. Amarsi, and C. L. Birmingham, "Obesity and overweight in Canada: an updated cost-of-illness study," *Obesity Reviews*, vol. 11, no. 1, pp. 31–40, 2010.

[65] E. S. Ford, W. H. Giles, and A. H. Mokdad, "Increasing prevalence of the metabolic syndrome among U.S. adults," *Diabetes Care*, vol. 27, no. 10, pp. 2444–2449, 2004.

[66] D. M. Boudreau, D. C. Malone, M. A. Raebel et al., "Health care utilization and costs by metabolic syndrome risk factors," *Metabolic Syndrome and Related Disorders*, vol. 7, no. 4, pp. 305–313, 2009.

[67] G. A. Nichols and E. J. Moler, "Metabolic syndrome components are associated with future medical costs independent of cardiovascular hospitalization and incident diabetes," *Metabolic Syndrome and Related Disorders*, vol. 9, no. 2, pp. 127–133, 2011.

[68] L. Tamariz, A. Palacio, Y. Yang, D. Parris, R. Ben-Joseph, and H. Florez, "Health economics perspective of the components of the cardiometabolic syndrome," *Journal of Clinical Hypertension*, vol. 12, no. 7, pp. 549–555, 2010.

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Research Article

VEGF in the Crosstalk between Human Adipocytes and Smooth Muscle Cells: Depot-Specific Release from Visceral and Perivascular Adipose Tissue

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Adipose tissue secrets adipokines and fatty acids, which may contribute to obesity-associated vascular dysfunction and cardiovascular risk. This study investigated which factors are responsible for the synergistic effect of adipokine and oleic acid- (OA-) induced proliferation of human vascular smooth muscle cells (VSMC). Adipocyte-conditioned medium (CM) from human adipocytes induces proliferation of VSMC in correlation to its vascular endothelial growth factor (VEGF) content. CM increases VEGF-receptor (VEGF-R) 1 and 2 expression and VEGF secretion of VSMC, while OA only stimulates VEGF secretion. VEGF neutralization abrogates CM- and OA-induced proliferation and considerably reduces proliferation induced by CM and OA in combination. VEGF release is higher from visceral adipose tissue (VAT) of obese subjects compared to subcutaneous adipose tissue (SAT) and VAT from lean controls. Furthermore, VEGF release from VAT correlates with its proliferative effect. Perivascular adipose tissue (PAT) from type 2 diabetic patients releases significantly higher amounts of VEGF and induces stronger proliferation of VSMC as compared to SAT and SAT/PAT of nondiabetics. In conclusion, VEGF is mediating CM-induced proliferation of VSMC. As this adipokine is released in high amounts from VAT of obese patients and PAT of diabetic patients, VEGF might link adipose tissue inflammation to increased VSMC proliferation.

1. Introduction

Obesity has become a major worldwide health problem, especially in industrial countries, and is associated with a number of metabolic diseases and secondary complications, including insulin resistance, type 2 diabetes, atherosclerosis, and cardiovascular disease [1]. It is well established that adipose tissue is an endocrine organ releasing lipid mediators and a variety of proteins, the so-called adipokines [2]. Increasing evidence indicates that obesity is causally linked to a chronic low-grade systemic inflammatory state [3, 4] that contributes to obesity-associated vascular dysfunction and cardiovascular risk [5]. Obesity is strongly related to the development of atherosclerosis in humans as well as in various animal models [6, 7]. In this context, visceral obesity confers the highest risk for the development of metabolic and cardiovascular diseases [8]. Specifically in the pathophysiology of vascular diseases, perivascular adipose tissue might play an important role because almost all blood vessels are surrounded by this fat depot, and due to the fact

that perivascular adipocytes are not separated from the blood vessel wall by an anatomic barrier, the secretion of adipokines by this fat depot may provide a new link between obesity and vascular complications [9]. However, till now the mechanism how visceral and perivascular fat enhances the risk of metabolic and cardiovascular disease is not completely unraveled. Besides endothelial cells, vascular smooth muscle cells (VSMC) represent one of the major cell types of the vascular wall retaining homeostasis of the vessel wall. Arterial wall thickening is mediated by migration of VSMC from the media to intima and their concomitant proliferation. We previously established a model of adipocyte-conditioned medium (CM) inducing VSMC proliferation [10]. The combination of CM with oleic acid (OA) increased the proliferation in a synergistic way via induction of iNOS expression, NO production, and proinflammatory signaling. However, until now the mechanisms and factors, which are responsible for aberrant VSMC proliferation induced by lipid mediators and adipokines, are not fully understood. Therefore, the main objective of this study was to identify potential candidates that mediate the crosstalk between adipose tissue and VSMC potentially linking obesity and atherosclerosis. Using paired biopsies of visceral and subcutaneous adipose tissue (VAT and SAT) as well as perivascular adipose tissue (PAT) derived from lean, obese, and obese type 2 diabetic patients, this study is also aimed at relating the *in vitro* findings to a more physiological setting. Our study demonstrates that the vascular endothelial growth factor (VEGF) content of CM is positively correlated with CM-induced VSMC proliferation and that VEGF neutralization completely abrogates CM- and OA-induced proliferation. CM and VEGF regulate expression of VEGF-receptor (VEGF-R) 1 and 2. The secretory output from VAT and PAT of patients with type 2 diabetes and/or obesity contains high amounts of VEGF and stimulates proliferation and VEGF-R1/2 expression as compared to SAT.

2. Materials and Methods

2.1. Materials. Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (Munich, Germany). Polyclonal antibodies raised against VEGF-R 1 and 2 were supplied by cell signalling technology (Frankfurt, Germany), and the anti-actin antibody came from Abcam (Cambridge, Great Britain). Vascular endothelial growth factor (VEGF) neutralization was achieved by pretreatment of CM with 1 μg/mL VEGF-neutralizing antibody from R&D Systems (Wiesbaden-Nordenstadt, Germany) for 1 hour. HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies came from Promega (Mannheim, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). Troglitazone and BSA (fraction V, fatty acid free, low endotoxin) were obtained from Sigma (München, Germany). Human recombinant VEGF was purchased from Millipore GmbH (Schwalbach, Germany). The cell proliferation ELISA (BrdU, chemiluminescent) and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). VEGF ELISA kits were purchased from BioVendor GmbH (Heidelberg, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, USA). Sodium salt of OA (Sigma, München, Germany) was dissolved in water as a 6 mM stock solution and was further diluted in sterile serum-free VSMC medium containing 4% (wt/v) BSA for coupling. OA was applied to VSMC at a final concentration of 100 μmol/L for 18 h. All controls of experiments involving fatty acids were treated with BSA alone. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

2.2. Culture of Human Adipocytes and CM Generation. Subcutaneous adipose tissue was obtained from healthy lean or moderately overweight women ($n = 23$, body mass index (BMI) 26.1 ± 1.1, and aged 36.6 ± 2.0 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). All subjects were healthy and free of medication and had no evidence of metabolic diseases according to routine laboratory tests. Preadipocytes were isolated by collagenase digestion of adipose tissue as previously described by us [11]. Seeded preadipocytes were induced to differentiation into adipocytes over 15 days as previously described by us [10]. The degree of differentiation was determined by oil red staining and induction of adiponectin. Differentiated adipocytes were used for the generation of adipocyte-CM, as recently described by us [12]. Briefly, CM was generated by culturing adipocytes for 48 h in VSMC basal medium (PromoCell) with addition of 50 ng/mL amphotericin b and 50 μg/mL gentamycin. Each CM was tested for its proliferative effect and the content of adiponectin (negatively correlated to proliferation) [10]. A more-detailed characterization of CM was described previously by us [12, 13]. CM generated from *in vitro* differentiated adipocytes was used for all experiments presented in Figures 1 and 2.

2.3. Generation of CM from Explants of SAT, VAT, and PAT. Paired biopsies from subcutaneous (SAT) und visceral adipose tissue (VAT) were obtained from lean patients without diabetes ($n = 10$, body mass index (BMI) of 21 ± 1 kg/m^2 aged 67 ± 6), obese patients without diabetes ($n = 11$, BMI of 38 ± 3 kg/m^2 aged 56 ± 6 years) and obese patients with type 2 diabetes ($n = 6$, BMI of 37 ± 6 kg/m^2 aged 56 ± 5 years) undergoing elective surgery such as hernia, gall bladder surgery, and other noninflammatory and nonmalignant causes. Human perivascular (PAT) and SAT biopsies were obtained from patients with type 2 diabetes ($n = 5$, BMI 29 ± 7 kg/m^2, aged 68 ± 9 years) and patients without type 2 diabetes ($n = 9$, BMI 28 ± 4 kg/m^2, aged 69 ± 12 years) undergoing coronary artery bypass surgery. Information on the diabetes status was obtained from the medical records of the patients. PAT was collected from the coronary artery and used to generate CM as described [14, 15] using 100 mg of adipose tissue explant to generate 1 mL of CM. After 24 h, CM was collected and stored in aliquots at −80°C until further use. CM generated from SAT and VAT was used for all experiments presented in Figure 3, and CM generated from SAT and PAT was used for all experiments presented in Figure 4.

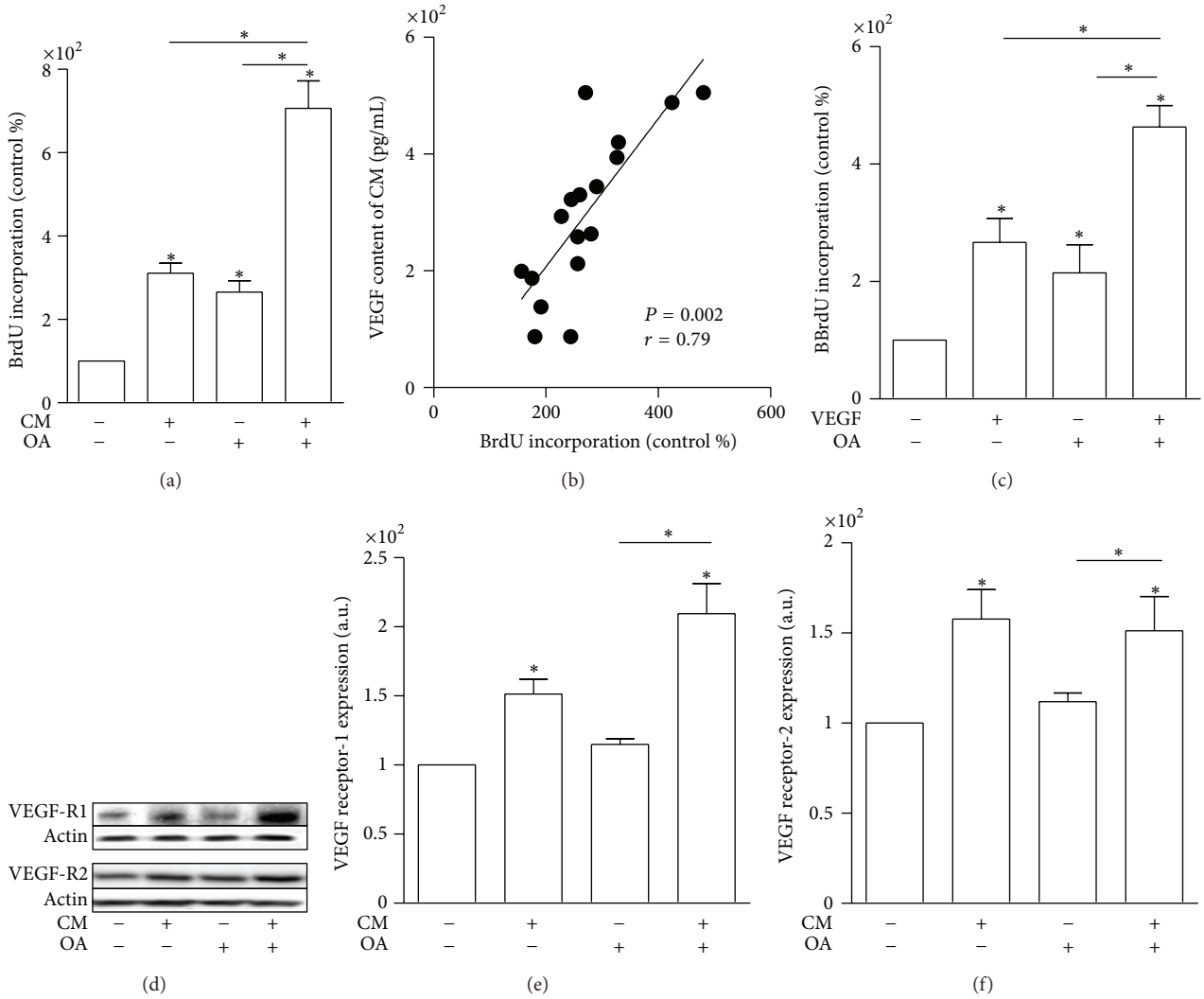

Figure 1: CM-induced proliferation is mediated by VEGF. (a) VSMC were serum starved for 24 h and subsequently incubated with CM, 100 μmol/L OA, or the combination of CM and OA in the presence of BrdU for 18 h. Incorporation of BrdU into DNA was measured. Data are expressed relative to control, taken as 100%. Data are mean values ± SEM of three independent experiments. $^*P < 0.05$ compared to control or designated treatment. (b) The proliferative effect of CM correlates significantly with its VEGF content as measured by ELISA. Statistical result of linear regression analysis is indicated in the graph. (c) VSMC were serum starved for 24 h and subsequently incubated with 250 pg/mL VEGF, 100 μmol/L OA, or the combination of VEGF and OA in the presence of BrdU for 18 h. Incorporation of BrdU into DNA was measured. Data are expressed relative to control, taken as 100%. Data are mean values ± SEM of three independent experiments. $^*P < 0.05$ compared to control or designated treatment. (d–f) VSMC were treated with CM, OA, and their combination for 24 h. Total cell lysates were resolved by SDS-PAGE and immunoblotted with specific VEGF-R1 and -2 antibodies. A representative blot is shown. Data are mean values ± SEM of three independent experiments. All data were normalized to the level of actin expression and are expressed relative to the control.

2.4. Culture of Human Vascular Smooth Muscle Cells (VSMC). Primary human coronary artery VSMC were obtained from PromoCell (Heidelberg, Germany). VSMC from four different donors (Caucasian, male, 23, 31, and 40 years old; female, 56 years old) were used as subconfluent cells of passage 3. Cells were characterized as VSMC by morphologic criteria and by immunostaining with smooth muscle α-actin.

2.5. In Vitro Analysis of VSMC Proliferation. To monitor DNA synthesis, VSMC were seeded in 96-well culture dishes and allowed to attach for 24 h, followed by serum starvation for an additional 24 h period. Cells were then stimulated for 18 h as outlined above in the presence of BrdU (10 μM). 10.000 VSMC per 15 mm^2 well were incubated with the CM of 35.000 adipocytes. The BrdU ELISA Kit was used to determine proliferation according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer, Mannheim, Germany).

2.6. Immunoblotting. VSMC were treated as indicated and lysed in a buffer containing 50 mM HEPES, pH 7.4, 1%

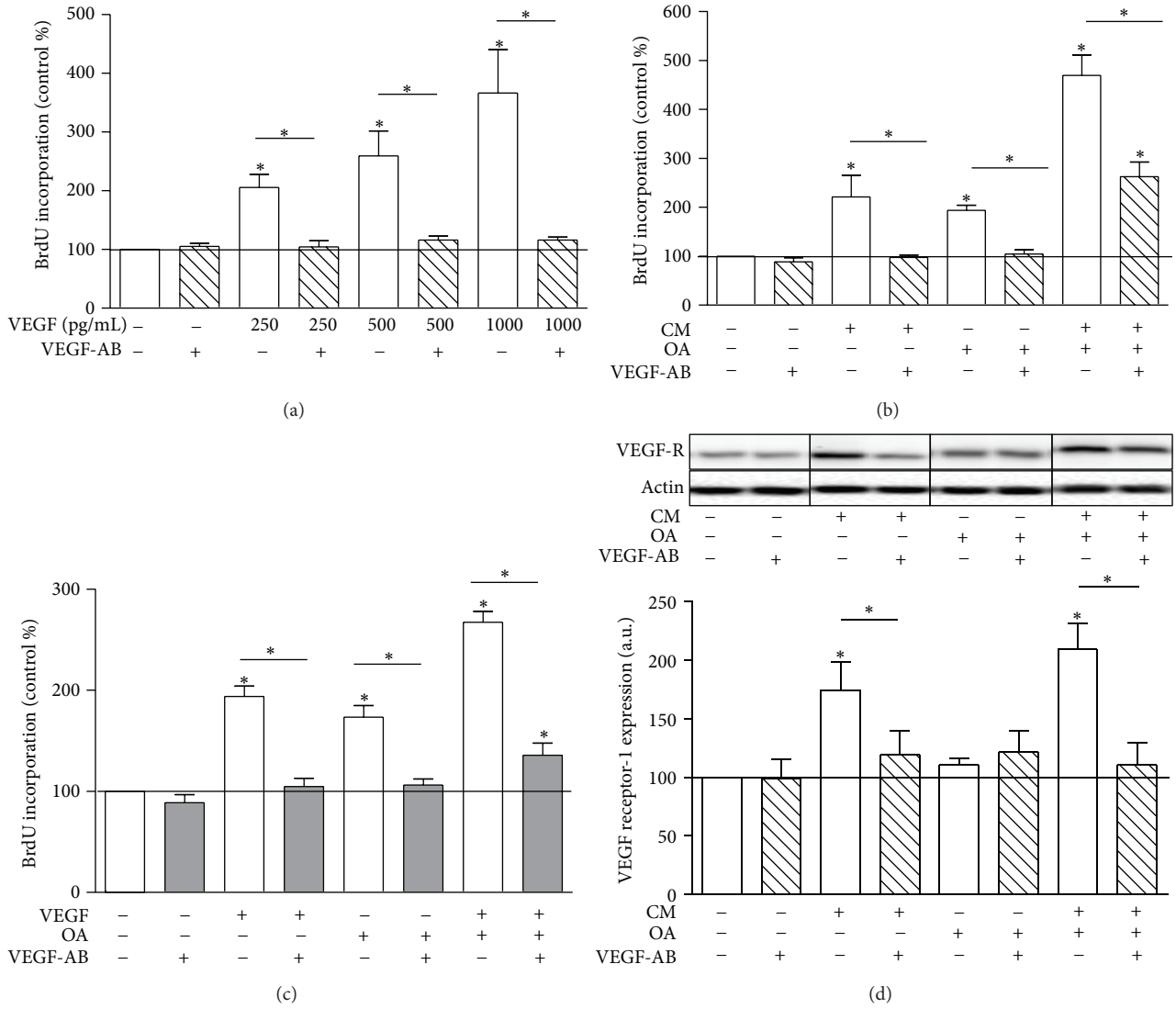

Figure 2: VEGF neutralization prevents CM- and OA-induced proliferation. Cells were treated with 250, 500, and 1000 pg/mL VEGF (a), CM, OA and CMOA (b and d), and accordingly VEGF, OA, and VEGFOA (c) as described in the legend to Figure 1 in the presence or absence of a specific neutralizing VEGF antibody for 24 h. (a–c) BrdU incorporation into DNA was determined as described in the legend to Figure 1. Data are expressed relative to control. (d) Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific VEGF-R1 antibody. For VEGF-R1, lanes were excised from a single western blot and displayed in the present order. Data are mean values ± SEM of three independent experiments. All data were normalized to the level of actin expression and are expressed relative to the control. $^{*}P < 0.05$ compared to untreated control or designated data.

TritonX100, complete protease inhibitor, and PhosStop phosphatase inhibitor cocktail. After incubation for 2 h at 4°C, the suspension was centrifuged at 10.000 ×g for 15 min. Thereafter, 5 μg protein of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvinylidene fluorid filters in a semidry blotting apparatus [16]. Filters were blocked with Tris-buffered saline, containing 0.1% Tween and 5% nonfat dry milk, and subsequently incubated overnight with a 1 : 1000 dilution of the appropriate antibodies. After washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, USA). Signals were visualized and evaluated on a VersaDoc work station (Bio-Rad Laboratories, Munich, Germany).

2.7. Presentation of Data and Statistics. Data are expressed as mean ± SEM. One-way ANOVA (post hoc test: Bonferroni's multiple comparison test) was used to determine statistical significance. All statistical analyses were done using Prism (GraphPad, La Jolla, USA) considering a P value of less than

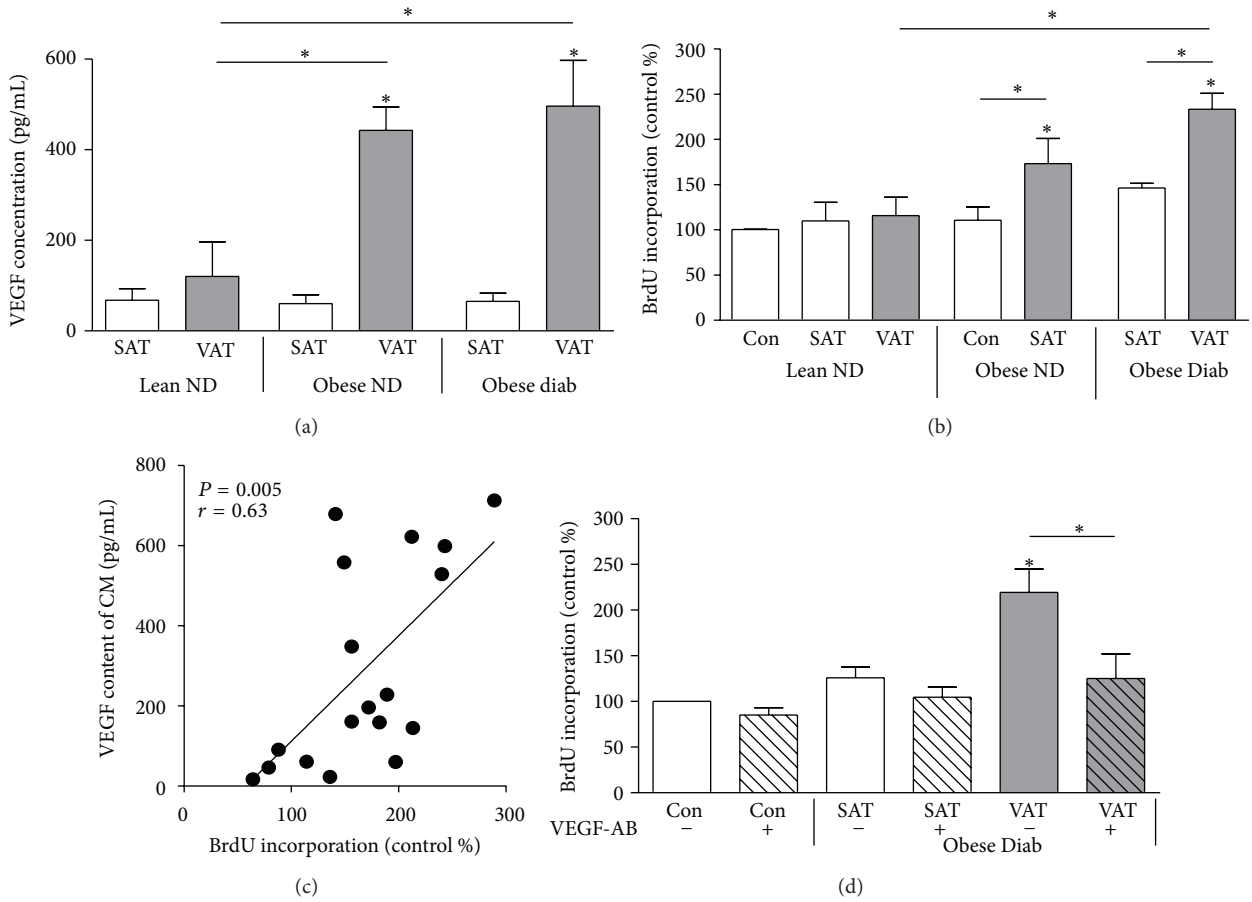

Figure 3: VEGF release is higher from VAT of obese subjects correlating with induction of VSMC proliferation. (a) VEGF content of CM from VAT and SAT was measured in duplicates by ELISA. (b and d) VSMC were treated with CM from paired SAT and VAT of lean nondiabetic (ND), obese ND, and obese type 2 diabetes (Diab) patients for 24 h. For (d), VSMC were treated in the presence or absence of a VEGF-neutralizing antibody. VSMC proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, which was set as 100%. Data are presented as mean ± SEM from five independent experiments. For (d), all data were normalized to the level of actin expression and are expressed relative to the control $^*P < 0.05$ compared to control or designated data. For (d), lanes were excised from a single western blot and displayed in the presented order. (c) The proliferative effect of CM from VAT correlates significantly with its VEGF content as measured by ELISA. Statistical result of linear regression analysis is indicated in the graph.

0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

3. Results

3.1. VEGF Content of CM Correlates with CM-Induced Proliferation. We have previously shown that CM from *in vitro* differentiated human adipocytes induces proliferation of human coronary artery VSMC with a large majority of the CMs tested inducing a prominent proliferation of more than 2-fold compared to control [10]. While CM and OA induce similarly strong proliferation of VSMC, their combination increased the proliferation in a synergistic way (Figure 1(a)), as reported previously [10]. The proliferative effect of CM negatively correlated with its adiponectin concentrations, but no correlation was found with IL-6 in CM [10]. Correlating CM content of growth inducing factors to CM-induced proliferation, we found that CM-induced proliferation strongly correlated with the VEGF content of the respective CM (Figure 1(b)). When replacing CM by VEGF at the average concentration found in the tested CM (250 pg/mL human recombinant VEGF), the proliferative effect of CM could be mimicked (Figure 1(c)) in accordance with previous data [10]. Treatment with CM, OA, or the combination of CMOA induced VEGF secretion of VSMC (control 178 ± 20 pg/mL, CM-treated 507 ± 20 pg/mL, OA-treated 414 ± 52 pg/mL, and CMOA-treated cells 1109 ± 69 pg/mL, $n = 4$) as previously described [10]. These data suggest that VSMC significantly contribute to proliferation by releasing VEGF for autocrine/paracrine stimulation. CM and the combination of CMOA also increased the expression of VEGF-R1 and -2, whereas OA had no effect on its expression (Figures 1(d)–1(f)).

3.2. VEGF Is an Important Factor for CM- and OA-Induced VSMC Proliferation. In order to assess the importance of

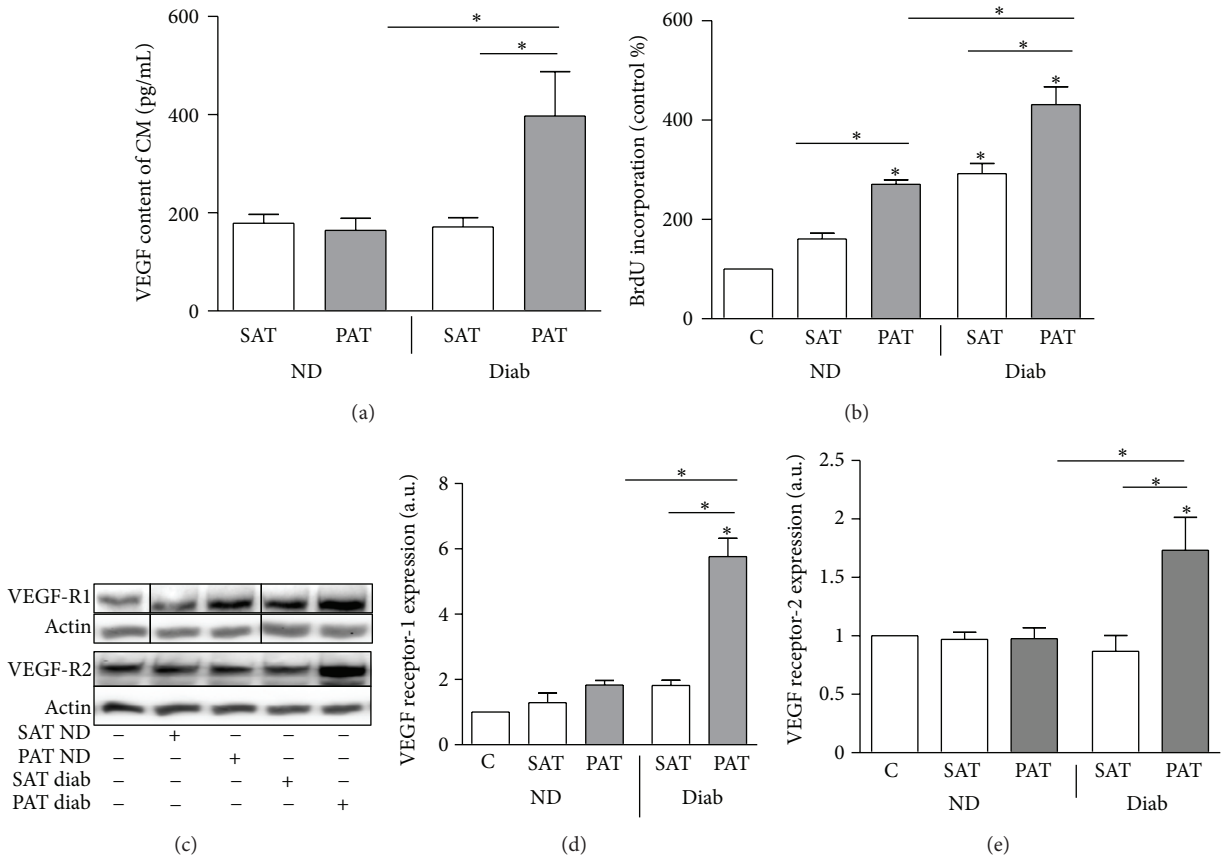

Figure 4: VEGF release is higher from PAT of type 2 diabetic patients correlating with VSMC proliferation and VEGF-R1/2 expression. (a) VEGF content of CM from PAT and SAT was measured in duplicates by ELISA. (b) VSMC were treated with CM from paired SAT and PAT of patients with type 2 diabetes (Diab) and nondiabetics controls (ND) for 24 h. The proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, which was set as 100%. Data are presented as mean ± SEM from five independent experiments. (c–e) Total cell lysates were resolved by SDS-PAGE and immunoblotted with specific VEGF-R1 and 2 antibodies. Data are mean values ± SEM of three to five independent experiments. All data were normalized to the level of actin expression and are expressed relative to the control. $^{*}P < 0.05$ compared to control or designated data.

VEGF in CM-induced proliferation, VEGF was neutralized with a specific antibody. To establish effective VEGF neutralization, VEGF was applied at concentrations ranging from 250 pg/mL to 1 μg/mL, which mimics both the average VEGF concentration in CM and spans to the highest VEGF release by VSMC observed after CMOA treatment (Figure 2(a)). Neutralizing VEGF prevented the CM-, VEGF-, and OA-induced proliferation completely (Figures 2(b) and 2(c)), underlining our hypothesis that CM and OA increase VSMC proliferation via VEGF. VEGF blocking significantly reduced the proliferative effect of the combination CMOA, which is still elevated compared to untreated control (Figure 2(b)), illustrating that under this condition VEGF is not the only factor involved in the proliferative effect. Furthermore, the induction of VEGF-R1 by CM and the combination of CM and OA was also completely prevented by VEGF blocking (Figure 2(d)).

3.3. VEGF Release Is Higher from VAT of Obese Subjects Compared to SAT and VAT from Lean Controls Correlating with VSMC Proliferation. VEGF release from VAT was significantly higher compared to SAT in both obese subjects without type 2 diabetes and with type 2 diabetes (Figure 3(a)). On the other hand, VEGF release was low and comparable from VAT and SAT of lean subjects. Accordingly, VSMC proliferation was induced by CM from VAT of obese subjects, but not by CM from SAT, VAT from lean patients, and SAT from obese patients (Figure 3(b)). Similar to CM derived from human adipocytes (see Figure 1(b)), the VEGF content of CM from VAT correlated significantly with the proliferative effect of the respective CM (Figure 3(c)). However, VEGF content of CM from SAT was not correlated to proliferation (data not shown). Neutralization of VEGF prevented proliferation induced by CM from VAT of obese subjects with type 2 diabetes (Figure 3(d)).

3.4. PAT of Type 2 Diabetic Patients Releases Increased Amounts of VEGF and Induces Significant Proliferation of VSMC. The release of VEGF was comparable from SAT and PAT of the nondiabetic subjects and from SAT of patients

with type 2 diabetes (Figure 4(a)) while its release by PAT of type 2 diabetic patients was significantly increased. Accordingly, CM from PAT of type 2 diabetic patients induced the strongest proliferative effect on VSMC (Figure 4(b)). In both groups of patients, CM from PAT induced a stronger proliferation as compared to CM from SAT. Furthermore, CM from PAT of patients with type 2 diabetes induced a 2- to 4-fold increase of VEGF-R1 and -2 expression in VSMC (Figures 4(c) and 4(d)). In contrast, PAT of nondiabetics exerted a comparable effect to the respective SAT. Our findings suggest that VEGF may be an important adipokine produced in PAT especially from patients with type 2 diabetes that might directly induce proliferation and expression of VEGF-R1 in VSMC.

4. Discussion

Obesity is strongly related to the development of cardiovascular diseases, and adipokines have been suggested to be a molecular link in this relationship [4, 17]. This study was designed to elucidate mechanisms on how the secretory output from adipose tissue is related to VSMC proliferation. We could show in a previous study that CM of *in vitro* differentiated adipocytes induces proliferation of VSMC in negative correlation to the adiponectin content of CM [10]. Searching for an active component of CM being responsible for VSMC proliferation, we found VEGF in CM to be significantly correlated with proliferation. VEGF is traditionally known as an endothelial cell-specific growth factor, which modulates vascular disease by inducing endothelial proliferation mainly through the VEGF-R2 [18]. However, an increase of VEGF and VEGF-Rs could be observed in other injured cells of the arterial wall like monocytes and VSMC [19]. Vascular inflammation and the proliferation of endothelial cells as well as VSMC are enhanced through angiotensin II-induced VEGF release and expression of VEGF-R [20–23].

Here, we report that CM and OA as well as their combination induce release of VEGF also by VSMC, which in turn might autostimulate proliferation. This effect is similar to hypoxia-induced proliferation of VSMC, where an autocrine proliferative action of VEGF has been described [24]. In addition, it has been described that proinflammatory stimulation with angiotensin II and IL-1beta and activation of JNK induce VEGF release by VSMC [25, 26]. It might be speculated that proinflammatory adipokines present in CM are, therefore, responsible for the induction of VEGF. Furthermore, VEGF might autostimulate its release which might be worth to study in the future. In addition to stimulating the release of VEGF from VSMC, CM induced VEGF-R1 and -2 expression which mediate proliferation and migration of VSMC [27]. Our data suggest that both VEGF-Rs are regulated by VEGF itself, as neutralizing VEGF prevented CM-induced expression of these receptors in parallel to proliferation. In fact, a regulation of VEGF-R2 by VEGF has already been proposed [28]. Interestingly, OA stimulates VEGF secretion but not the expression of VEGF-Rs. As oleic acid has complex effects on VSMC, it can only be speculated that it directly or indirectly affects VEGF-R expression by still unknown mechanisms.

VEGF is a proinflammatory factor [29, 30], and we previously demonstrated that CM activates NF-κB signaling that is essential for CM-induced proliferation [10]. VEGF is not primarily activating NF-κB at concentrations measured and used in this study [29]. However, CM contains various adipokines including MCP-1 and chemerin [12, 31] that have been described to strongly activate NF-κB. Activated NF-κB could then in turn lead to increased VEGF expression and release as described in the literature [32]. Thereby, proinflammatory factors in CM may contribute to proliferation by inducing VEGF via NF-κB. Blocking VEGF with a specific neutralizing VEGF-antibody reduced the CM, OA, and VEGF-induced proliferation of VSMC completely. In contrast, the strong proliferative effect of the combination of CM and OA was not totally prevented, illustrating that VEGF is not the only important factor for the synergism of CMOA. As CM contains various adipokines such as IL-6, IL-8, or MCP-1 [12], it is possible that some of these factors induce proliferation in addition to VEGF and that these factors might also be involved in the synergistic effects of CM and OA. Several adipokines such as leptin and resistin affected VSMC proliferation and function [33, 34] but are not probable to play a role in this scenario. Leptin release by adipocytes differentiated *in vitro* is too low to explain CM-induced proliferation [35], and resistin is only secreted from mouse but not from human adipocytes [12].

We show here that VEGF release is higher from VAT of obese subjects irrespective of their metabolic status. Previous studies have shown that VEGF expression is already increased along with increased inflammatory adipokines in VAT of children compared to SAT [36]. In adults, VEGF expression in obese patients is higher in VAT as compared to SAT [37]. Increased VEGF expression might be associated with higher inflammation in VAT as VEGF expression, and secretion is stimulated by inflammatory cytokines such as IL-6 [38]. Recently, it has also been suggested that hypoxia can induce VEGF expression in expanded adipose tissue [39]. VEGF in adipose tissue is recently discussed both as a "bad" and a "good" guy. One important function of VEGF within adipose tissue is to stimulate angiogenesis, which is crucial for increasing blood capillaries in expanding adipose tissue by stimulating endothelial cell growth. Here, VEGF is described as beneficial for preventing adipose tissue hypoxia and thus inflammation. In fact, several groups have demonstrated that inducible overexpression of VEGF in adipose tissue prevents obesity-induced hypoxia and inflammation in adipose tissue in parallel to improved metabolic phenotype [40–42]. Conversely, adipose-specific ablation of VEGF decreases vascularization and increases inflammation together with deteriorated metabolic control in high-fat diet-treated mice [42]. In the context of existing obesity, increased VEGF expression is rather seen as another stimulator of proinflammatory adipocytes further deteriorating adipose tissue function. Here, inducible ablation of VEGF in adipose tissue protects from obesity and induces brown adipose tissue within white adipose tissue in mice [43]. Taken together with another similar study using different mouse models [41], it has been suggested that the effects of VEGF within adipose are context dependent. While high VEGF expression

in already expanded adipose tissue deteriorates metabolic control, increased angiogenic activity by VEGF induction during expansion of adipose tissue is beneficial to maintain normal metabolic function. As no detailed studies on VEGF expression of human adipose tissue during obesity development exist, further work is needed to expand this view to the human situation.

It has been proposed that PAT is involved in a paracrine crosstalk with cells of the vascular wall and that adipokines are major players in mechanisms linking PAT to inflammation and vascular dysfunction [44, 45]. Here, we demonstrate that PAT from patients with type 2 diabetes is characterized by a significantly higher VEGF release as compared to SAT and also compared to PAT from nondiabetics. Higher VEGF release is paralleled by a stronger induction of VEGF-R1 and -2. The use of paired biopsies from human SAT and PAT further strengthens the notion of VEGF release from PAT for a potential paracrine crosstalk. The VEGF content of CM from SAT and PAT from patients without diabetes as well as SAT from type 2 diabetic subjects was similar but did not have the same effect on VSMC proliferation. It should be noted that the use of adipose tissue explants is a more complex setting than using CM from adipocytes as the secretory output from adipose tissue explants also contains factors derived from other cell types present in adipose tissue as preadipocytes and macrophages. Further work will be needed to elucidate the differences in adipokine expression and secretion between PAT and other fat depots in health and disease in order to identify other putative factors responsible for PAT-induced vascular dysfunction.

A limitation of the study is that the small amounts of CM that could be prepared from the surgical biopsies from PAT disallowed experiments assessing the impact of VEGF neutralizing antibodies. In this respect, it should be noted that the secretory profile of human adipocytes and adipose tissue biopsies is complex, as illustrated for example, by disease and depot-specific differences in the adipokines released [15, 46]. The fact that VEGF content and the potential of PAT-derived conditioned media to induce VSMC proliferation do not seem to fully correlate suggests that a contribution of other adipokines cannot be excluded. For example, activin A, which is released in substantial amounts from epicardial adipose tissue, which is highly related to PAT [15, 47], has also been linked to VSMC proliferation [48]. Notably, activin A release is elevated in epicardial adipose tissue from patients with type 2 diabetes and may thus explain higher proliferation of VSMC treated with CM from PAT of patients with type 2 diabetes and PAT of patients without diabetes compared to SAT of patients without diabetes despite similar VEGF content in these CMs. Differently from PAT-induced VSMC proliferation, VAT-induced proliferation could be explained by higher VEGF content in CM from VAT of obese patients with type 2 diabetes and completely prevented by VEGF neutralization. Therefore, further studies remain required to further detail the impact of PAT-derived adipokines on VSMC proliferation.

In conclusion, our results demonstrate that VEGF is mediating CM-induced proliferation of VSMC. As an adipokine and growth factor that is released in high amounts from VAT of obese patients and PAT of patients with type 2 diabetes compared to SAT of lean and nondiabetic controls, VEGF might be a link between adipose tissue inflammation and abnormal VSMC proliferation.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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References

[1] N. Ouchi, J. L. Parker, J. J. Lugus, and K. Walsh, "Adipokines in inflammation and metabolic disease," *Nature Reviews Immunology*, vol. 11, no. 2, pp. 85–97, 2011.

[2] S. Lehr, S. Hartwig, and H. Sell, "Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders," *Proteomics*, vol. 6, no. 1-2, pp. 91–101, 2012.

[3] H. Sell and J. Eckel, "Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 13, no. 4, pp. 366–370, 2010.

[4] A. Taube, R. Schlich, H. Sell, K. Eckardt, and J. Eckel, "Inflammation and metabolic dysfunction: links to cardiovascular diseases," *American Journal of Physiology*, vol. 302, pp. H2148–H2165, 2012.

[5] H. Ruan and H. F. Lodish, "Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-α," *Cytokine and Growth Factor Reviews*, vol. 14, no. 5, pp. 447–455, 2003.

[6] F. B. Hu, W. C. Willett, T. Li, M. J. Stampfer, G. A. Colditz, and J. E. Manson, "Adiposity as compared with physical activity in predicting mortality among women," *The New England Journal of Medicine*, vol. 351, no. 26, pp. 2694–2703, 2004.

[7] R. M. van Dam, W. C. Willett, J. E. Manson, and F. B. Hu, "The relationship between overweight in adolescence and premature death in women," *Annals of Internal Medicine*, vol. 145, no. 2, pp. 91–97, 2006.

[8] P. Mathieu, P. Poirier, P. Pibarot, I. Lemieux, and J.-P. Després, "Visceral obesity the link among inflammation, hypertension, and cardiovascular disease," *Hypertension*, vol. 53, no. 4, pp. 577–584, 2009.

[9] G. Iacobellis and A. M. Sharma, "Epicardial adipose tissue as new cardio-metabolic risk marker and potential therapeutic target in the metabolic syndrome," *Current Pharmaceutical Design*, vol. 13, no. 21, pp. 2180–2184, 2007.

[10] D. Lamers, R. Schlich, S. Greulich, S. Sasson, H. Sell, and J. Eckel, "Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth

muscle cells," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 5, pp. 1177–1188, 2011.

[11] H. Hauner, T. Petruschke, M. Russ, K. Rohrig, and J. Eckel, "Effects of tumour necrosis factor alpha (TNFα) on glucose transport and lipid metabolism of newly differentiated human fat cells in cell culture," *Diabetologia*, vol. 38, no. 7, pp. 764–771, 1995.

[12] D. Dietze-Schroeder, H. Sell, M. Uhlig, M. Koenen, and J. Eckel, "Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors," *Diabetes*, vol. 54, no. 7, pp. 2003–2011, 2005.

[13] H. Sell, K. Eckardt, A. Taube et al., "Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility," *American Journal of Physiology*, vol. 294, no. 6, pp. E1070–E1077, 2008.

[14] S. Greulich, D. H. de Wiza, S. Preilowski et al., "Secretory products of guinea pig epicardial fat induce insulin resistance and impair primary adult rat cardiomyocyte function," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 11, pp. 2399–2410, 2010.

[15] S. Greulich, B. Maxhera, G. Vandenplas et al., "Secretory products from epicardial adipose tissue of patients with type 2 diabetes mellitus induce cardiomyocyte dysfunction," *Circulation*, vol. 126, pp. 2324–2334, 2012.

[16] A. Wichelhaus, M. Russ, S. Petersen, and J. Eckel, "G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats," *American Journal of Physiology*, vol. 267, no. 2, pp. H548–H555, 1994.

[17] P. Mathieu, I. Lemieux, and J.-P. Després, "Obesity, inflammation, and cardiovascular risk," *Clinical Pharmacology and Therapeutics*, vol. 87, no. 4, pp. 407–416, 2010.

[18] M. Shibuya, "Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis," *Journal of Biochemistry and Molecular Biology*, vol. 39, no. 5, pp. 469–478, 2006.

[19] Q. Zhao, K. Egashira, K.-I. Hiasa et al., "Essential role of vascular endothelial growth factor and Flt-1 signals in neointimal formation after periadventitial injury," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 12, pp. 2284–2289, 2004.

[20] Q. Zhao, M. Ishibashi, K.-I. Hiasa, C. Tan, A. Takeshita, and K. Egashira, "Essential role of vascular endothelial growth factor in angiotensin II-induced vascular inflammation and remodeling," *Hypertension*, vol. 44, no. 3, pp. 264–270, 2004.

[21] H. Kitayama, Y. Maeshima, Y. Takazawa et al., "Regulation of angiogenic factors in angiotensin II infusion model in association with tubulointerstitial injuries," *American Journal of Hypertension*, vol. 19, no. 7, pp. 718–727, 2006.

[22] D. Herr, M. Rodewald, H. M. Fraser et al., "Regulation of endothelial proliferation by the renin-angiotensin system in human umbilical vein endothelial cells," *Reproduction*, vol. 136, no. 1, pp. 125–130, 2008.

[23] P. Pan, H. Fu, L. Zhang et al., "Angiotensin II upregulates the expression of placental growth factor in human vascular endothelial cells and smooth muscle cells," *BMC Cell Biology*, vol. 11, article 36, 2010.

[24] M. Osada-Oka, T. Ikeda, S. Akiba, and T. Sato, "Hypoxia stimulates the autocrine regulation of migration of vascular smooth muscle cells via HIF-1α-dependent expression of thrombospondin-1," *Journal of Cellular Biochemistry*, vol. 104, no. 5, pp. 1918–1926, 2008.

[25] Y. D. Jung, W. Liu, N. Reinmuth et al., "Vascular endothelial growth factor is upregulated by interleukin-1β in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway," *Angiogenesis*, vol. 4, no. 2, pp. 155–162, 2001.

[26] S.-I. Miura, Y. Matsuo, and K. Saku, "Jun N-terminal kinase inhibitor blocks angiogenesis by blocking VEGF secretion and an MMP pathway," *Journal of Atherosclerosis and Thrombosis*, vol. 15, no. 2, pp. 69–74, 2008.

[27] S. Banerjee, S. Mehta, I. Haque et al., "VEGF-A165 induces human aortic smooth muscle cell migration by activating neuropilin-1-VEGFR1-PI3K axis," *Biochemistry*, vol. 47, no. 11, pp. 3345–3351, 2008.

[28] X.-H. Yang, X.-Y. Man, S.-Q. Cai, Y.-G. Yao, Z.-Y. Bu, and M. Zheng, "Expression of VEGFR-2 on HaCaT cells is regulated by VEGF and plays an active role in mediating VEGF induced effects," *Biochemical and Biophysical Research Communications*, vol. 349, no. 1, pp. 31–38, 2006.

[29] T. Marumo, V. B. Schini-Kerth, and R. Busse, "Vascular endothelial growth factor activates nuclear factor-κB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells," *Diabetes*, vol. 48, no. 5, pp. 1131–1137, 1999.

[30] I. Kim, S.-O. Moon, S. H. Kim, H. J. Kim, Y. S. Koh, and G. Y. Koh, "Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-κB activation in endothelial cells," *Journal of Biological Chemistry*, vol. 276, no. 10, pp. 7614–7620, 2001.

[31] H. Sell, J. Laurencikiene, A. Taube et al., "Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells," *Diabetes*, vol. 58, no. 12, pp. 2731–2740, 2009.

[32] S. Wang, Z. Liu, L. Wang, and X. Zhang, "NF-kappaB signaling pathway, inflammation and colorectal cancer," *Cellular & Molecular Immunology*, vol. 6, pp. 327–334, 2009.

[33] P. Calabro, I. Samudio, J. T. Willerson, and E. T. H. Yeh, "Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways," *Circulation*, vol. 110, no. 21, pp. 3335–3340, 2004.

[34] L. Li, J.-C. Mamputu, N. Wiernsperger, and G. Renier, "Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin," *Diabetes*, vol. 54, no. 7, pp. 2227–2234, 2005.

[35] S. Famulla, D. Lamers, S. Hartwig et al., "Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells," *International Journal of Obesity*, vol. 35, no. 6, pp. 762–772, 2011.

[36] C. S. Tam, L. K. Heilbronn, C. Henegar et al., "An early inflammatory gene profile in visceral adipose tissue in children," *International Journal of Pediatric Obesity*, vol. 6, no. 2, pp. e360–e363, 2011.

[37] S. Ledoux, I. Queguiner, S. Msika et al., "Angiogenesis associated with visceral and subcutaneous adipose tissue in severe human obesity," *Diabetes*, vol. 57, no. 12, pp. 3247–3257, 2008.

[38] G. Rega, C. Kaun, S. Demyanets et al., "Vascular endothelial growth factor is induced by the inflammatory cytokines interleukin- 6 and oncostatin m in human adipose tissue in vitro and in murine adipose tissue in vivo," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 7, pp. 1587–1595, 2007.

[39] A. M. Fusaru, C. G. Pisoschi, A. Bold et al., "Hypoxia induced VEGF synthesis in visceral adipose depots of obese diabetic patients," *Romanian Journal of Morphology and Embryology*, vol. 53, pp. 903–909, 2013.

[40] I. Elias, S. Franckhauser, T. Ferre et al., "Adipose tissue overexpression of vascular endothelial growth factor protects against diet-induced obesity and insulin resistance," *Diabetes*, vol. 61, pp. 1801–1813, 2012.

[41] K. Sun, I. W. Asterholm, C. M. Kusminski et al., "Dichotomous effects of VEGF-A on adipose tissue dysfunction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 15, pp. 5874–5879, 2012.

[42] H. K. Sung, K. O. Doh, J. E. Son et al., "Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis," *Cell Metabolism*, vol. 17, pp. 61–72, 2013.

[43] X. Lu, Y. Ji, L. Zhang et al., "Resistance to obesity by repression of VEGF gene expression through induction of brown-like adipocyte differentiation," *Endocrinology*, vol. 153, pp. 3123–3132, 2012.

[44] D. M. Ouwens, H. Sell, S. Greulich, and J. Eckel, "The role of epicardial and perivascular adipose tissue in the pathophysiology of cardiovascular disease," *Journal of Cellular and Molecular Medicine*, vol. 14, no. 9, pp. 2223–2234, 2010.

[45] R. I. Meijer, E. H. Serne, Y. M. Smulders, V. W. M. Van Hinsbergh, J. S. Yudkin, and E. C. Eringa, "Perivascular adipose tissue and its role in type 2 diabetes and cardiovascular disease," *Current Diabetes Reports*, vol. 11, no. 3, pp. 211–217, 2011.

[46] S. Lehr, S. Hartwig, D. Lamers et al., "Identification and validation of novel adipokines released from primary human adipocytes," *Molecular & Cellular Proteomics*, vol. 11, no. 1, Article ID M111.010504, 2012.

[47] N. Venteclef, V. Guglielmi, E. Balse et al., "Human epicardial adipose tissue induces fibrosis of the atrial myocardiumthrough the secretion of adipo-fibrokines," *European Heart Journal*. In press.

[48] S. H. Cho, Z. Yao, S.-W. Wang et al., "Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells," *Journal of Immunology*, vol. 170, no. 8, pp. 4045–4052, 2003.

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Research Article

High Sugar Intake and Development of Skeletal Muscle Insulin Resistance and Inflammation in Mice: A Protective Role for PPAR-δ Agonism

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Peroxisome Proliferator Activated Receptor (PPAR)-δ agonists may serve for treating metabolic diseases. However, the effects of PPAR-δ agonism within the skeletal muscle, which plays a key role in whole-body glucose metabolism, remain unclear. This study aimed to investigate the signaling pathways activated in the gastrocnemius muscle by chronic administration of the selective PPAR-δ agonist, GW0742 (1 mg/kg/day for 16 weeks), in male C57Bl6/J mice treated for 30 weeks with high-fructose corn syrup (HFCS), the major sweetener in foods and soft-drinks (15% wt/vol in drinking water). Mice fed with the HFCS diet exhibited hyperlipidemia, hyperinsulinemia, hyperleptinemia, and hypoadiponectinemia. In the gastrocnemius muscle, HFCS impaired insulin and AMP-activated protein kinase signaling pathways and reduced GLUT-4 and GLUT-5 expression and membrane translocation. GW0742 administration induced PPAR-δ upregulation and improvement in glucose and lipid metabolism. Diet-induced activation of nuclear factor-κB and expression of inducible-nitric-oxide-synthase and intercellular-adhesion-molecule-1 were attenuated by drug treatment. These effects were accompanied by reduction in the serum concentration of interleukin-6 and increase in muscular expression of fibroblast growth factor-21. Overall, here we show that PPAR-δ activation protects the skeletal muscle against the metabolic abnormalities caused by chronic HFCS exposure by affecting multiple levels of the insulin and inflammatory cascades.

1. Introduction

In healthy humans, skeletal muscle accounts for ~70–80% of the insulin-stimulated glucose uptake, being the major site of glucose disposal and, thus, exerting a key role in regulating whole body glucose homeostasis. Accordingly, understanding changes that occur to this tissue during obesity and diabetes development is crucial to elucidate the underlying causes of insulin resistance and to reveal new targets for its treatment. Insulin resistance in skeletal muscle has long been recognized as a characteristic feature of type 2 diabetes and plays a major role in the pathogenesis of the disease [1]. Although several epidemiological data have shown that the consumption of added sugars as ingredients in processed or prepared foods and caloric beverages has dramatically increased over the last decades, most of the experimental studies investigating the development of insulin resistance in the skeletal muscle have been based on genetic manipulation or use of high fat diets [2–4]. In contrast, the molecular mechanisms underlying the detrimental effects of sugar, mainly those on skeletal muscle, are not completely understood. In the present study, we used a previously developed mouse model of chronic exposure to high-fructose corn syrup (HFCS) [5] to investigate the deleterious effects of high sugar intake on skeletal muscle. HFCS syrup, used as an ingredient in processed or prepared foods and caloric

beverages, is synthesized by refining corn starch, contains 55% fructose and 42% glucose and to date accounts for over 40% of all added caloric sweeteners [6].

The Peroxisome Proliferator Activated Receptor (PPAR) superfamily of transcription factors, that includes the isoforms PPAR-α, PPAR-δ, and PPAR-γ, has been widely shown to exert crucial roles in energy homeostasis regulation and glucose metabolism. PPAR isoforms display tissue-specific expression and gene-regulatory profiles. PPAR-δ, one of the most promising pharmacological target implicated in obesity-associated insulin resistance [7], is highly expressed in skeletal muscle, at 10- and 50-folds higher levels compared with PPAR-α and PPAR-γ, respectively [8]. However, its potential effects in affecting skeletal muscle glucose intake and insulin sensitivity are only now being elucidated. Schuler et al. [9] showed that mice in which PPAR-δ is selectively ablated in skeletal myocytes exhibit fiber-type switching, obesity, and type 2 diabetes. Besides, a very recent paper has suggested that the improvement of glucose homeostasis by angiotensin receptor blockers in hypertensive patients involves a selective PPAR-δ activation in the skeletal muscle [10]. So far, the exact molecular mechanisms underlying the observed PPAR-δ-induced changes have not yet been determined. Hence, the present study aimed to determine whether chronic administration of the selective PPAR-δ agonist GW0742 in HFCS-fed mice may ameliorate the impairment of signaling pathways triggered in skeletal muscles by chronic high sugar intake. Several studies have revealed that a large number of muscle-derived secretory cytokines (collectively termed myokines) can act locally in an autocrine/paracrine manner, linking skeletal muscle to regulation of physiological processes in other tissues. We have, thus, also investigated whether PPAR-δ agonism may affect the serum levels of the well-known myokine interleukin-6 (IL-6) and the muscular expression of a member of the fibroblast growth factor (FGF) superfamily, FGF-21, a recently identified myokine involved in the interorgan communication [11]. To further extend our investigation on the ability of PPAR-δ agonism to modulate inflammatory pathways involved in local insulin resistance pathogenesis, the effects of PPAR-δ activation on nuclear translocation of the transcription nuclear factor-kappaB (NF-κB) and expression of its target genes have also been studied.

2. Materials and Methods

2.1. Animals and Diets. Four-week-old male C57Bl6/J mice (Harlan-Italy; Udine, Italy) were housed in a controlled environment at 25 ± 2°C with alternating 12-h light and dark cycles. They were provided with a Piccioni pellet diet (n. 48, Gessate Milanese, Italy) and water *ad libitum*. All the animals were fed with a normal pellet diet for 1 week prior to the experiment. The animals were then allocated to two dietary regimens, chow diet and normal drinking water (control) or a chow diet and 15% (wt/vol) HFCS solution in drinking water (HFCS) for 30 weeks. All diets contained a standard mineral and vitamin mixture. The concentration of HFCS solution as well as the period of dietary manipulation was chosen according to previous animal studies investigating the metabolic effects of long-term (6-7 months) access to HFCS. Body mass, intake of water, and food were recorded weekly. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/92), and the experiment was approved by the Turin University Ethics Committee.

2.2. Drug Administration. After the initial period of 14 weeks of dietary manipulation, each of the two diet groups (control and HFCS diet) was further subdivided to obtain four different treatment groups: chow diet and normal drinking water (control, $n = 10$), chow diet supplemented with GW0742 (1 mg/kg/day) and normal drinking water (control + GW, $n = 6$), chow diet and 15% (wt/vol) HFCS solution in drinking water (HFCS, $n = 10$), and chow diet supplemented with GW0742 (1 mg/kg/day) and 15% (wt/vol) HFCS solution in drinking water (HFCS + GW, $n = 10$). The drug was daily administered with the food for the last 16 weeks, and the mice were allowed to continue to feed on their respective diets until the end of the study. GW0742 is a highly potent and selective PPAR-δ agonist (murine EC_{50}: 28 nM for PPAR-δ; 8,900 nM for PPAR-α; >10,000 nM for PPAR-γ), with an acceptable pharmacokinetic profile and activity *in vivo* [12]. The dose and the kinetics of administration were chosen based on those we have previously shown to improve glucose tolerance and insulin sensitivity *in vivo* [5].

2.3. Oral Glucose Tolerance Test (OGTT). One day before the mice were due to be killed, the OGTT was performed after a fasting period of 6 h by administering glucose (2 g/kg) by oral gavage. Once before administration and 15, 30, 60, 90, 120, and 150 min afterward, blood was obtained from the saphenous vein, and glucose concentration was measured with a conventional Glucometer (Accu-Check Compact kit, Roche Diagnostics Gmbh, Mannheim, Germany).

2.4. Blood Biochemical Analysis. After 16 weeks from the start of the drug treatment (i.e., after 30 weeks of dietary manipulation), the mice were anaesthetised with i.p. injection (30 mg/kg) of Zoletil 100 (Laboratories Virbac, France) and killed by aortic exsanguination. Blood samples were collected, and plasma was isolated. Glycemia was measured using the Accu-Check Compact kit. The serum lipid profile was determined by measuring the content of triglycerides, total cholesterol, high-density lipoprotein (HDL), and low density lipoprotein (LDL) by standard enzymatic procedures using reagent kits (Hospitex Diagnostics, Florence, Italy). Plasma leptin and adiponectin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Leptin and Adiponectin Mouse ELISA Kits, Abcam, Cambridge UK). The gastrocnemius muscle and epididymal fat were isolated, weighed, rapidly freeze-clamped with liquid nitrogen, and stored at −80°C.

2.5. Tissue Extracts. Gastrocnemius extracts were prepared using the Meldrum method [13] with modification. Briefly, gastrocnemius were homogenised at 10%(w/v) in a Potter-Elvehjem homogenizer (Wheaton, NJ, USA) using a

homogenisation buffer containing 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, and 2.5 μg/mL leupeptin. Homogenates were centrifuged at 4000 RPM at 4°C for 5 min. Supernatants were removed and centrifuged at 14000 RPM at 4°C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were resuspended in an extraction buffer and centrifuged at 14000 RPM for 20 minutes at 4°C. The supernatants thus obtained, containing nuclear proteins, were carefully removed. The amount of proteins contained in the cytosolic and nuclear fraction was determined using a BCA protein assay following the manufacturers' instructions. Samples were stored at −80°C until use.

2.6. Skeletal Muscle Triglyceride Level. Skeletal muscle triglycerides were extracted from gastrocnemius homogenates and assayed using reagent kits according to the manufacturer's instructions (Triglyceride Quantification Kit, Abnova Corporation, Aachen, Germany).

2.7. Western Blot Analysis. About 60 μg of total proteins was loaded for western blot experiments. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldene difluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-PPAR-δ, dilution 1 : 500; rabbit antitotal GSK-3β, dilution 1 : 200; goat anti-pGSK-3β Ser^9, dilution 1 : 200; rabbit antitotal Akt, dilution 1 : 1000; mouse anti-pAkt Ser^{473}, dilution 1 : 1000; goat anti-ICAM-1, dilution 1 : 500; rabbit antitotal IRS-1, dilution 1 : 200; goat anti-pIRS-1 Ser^{307}, dilution 1 : 200; rabbit anti-GLUT-4, dilution 1 : 2000; rabbit anti-GLUT-5, dilution 1 : 100; rabbit anti-iNOS, dilution 1 : 200; rabbit anti-NF-κB dilution 1 : 1000; rabbit antitotal AMPK, dilution 1 : 1000; rabbit anti-pAMPK Thr^{172}, dilution 1 : 1000; rabbit antitotal ACC, dilution 1 : 500; rabbit anti-pACC Ser^{79}, dilution 1 : 1000; rabbit anti-CPT-1, dilution 1 : 200). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1 : 10000) and developed using the ECL detection system. The immunoreactive bands were visualised by autoradiography and the density of the bands was evaluated densitometrically using Gel-Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with tubulin monoclonal antibody (dilution 1 : 5000) and subsequently with an anti-mouse antibody (dilution 1 : 10000) to assess gel-loading homogeneity.

2.8. Immunohistochemistry. Immunohistochemical staining was performed on 10-μm acetone fixed cryostatic sections of gastrocnemius. Nonspecific binding sites were blocked for 1 h with 3% BSA in PBS. For immunodetection of glucose transporter type-4 (GLUT-4), sections were incubated overnight with rabbit anti-GLUT-4 antibody (Abcam), dilution 1 : 200, and for 1 hour with goat anti-rabbit IgG-HRP conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). For detection of glucose transporter type 5 (GLUT-5), sections were incubated overnight with rabbit anti-GLUT-5 antibody (Abcam), dilution 1 : 50, for 1 hour with swine anti-rabbit IgG-biotinylated secondary antibody (Dako, Glostrup, Denmark), and for 1 hour with Streptavidin HRP conjugate (Southern Biotech, Birmingham, AL, USA). The specific staining was detected with diaminobenzidine (DAB, Sigma-Aldrich), and sections were visualized with Olympus-Bx4I microscope connected by a photographic attachment (Carl Zeiss, Oberkochen, Germany). For each antibody, a negative control was included in which the primary antibody was replaced with a nonimmune isotypic control antibody.

2.9. Determination of IL-6 and FGF-21. Serum levels of IL-6 and FGF-21 levels in gastrocnemius homogenates were measured by ELISA according to the manufacturer's instructions (Mouse FGF-21 and IL-6 ELISA kits, R&D Systems, Abingdon, UK).

2.10. Materials. Unless otherwise stated, all compounds were purchased from the Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). The BCA Protein Assay kit and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Rockford, IL, USA), and PVDF was from the Millipore Corporation (Bedford, Massachusetts, USA). Antibodies were from Cell-Signaling Technology (Beverly, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Abcam (Cambridge, CB, UK). The anti-mouse, anti-rabbit, and anti-goat horseradish peroxidase-linked antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Luminol ECL was from PerkinElmer (Waltham Massachusetts, USA).

2.11. Statistical Analysis. All values in both the text and figures are expressed as mean ± SD for n observations. One-way analysis of variance with Dunnett's post-hoc test was performed using the GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, California, USA), and P values below 0.05 were considered as significant.

3. Results

3.1. Effects of HFCS Diet and GW0742 Administration on Body Mass Change, Food Intake, and Blood Parameters. After 30 weeks of dietary manipulation, mice on HFCS group had significantly higher body mass than the control group, with an increase in body mass over 30% (36.00 ± 1.41 g versus 32.25 ± 1.67 g; $P < 0.01$), whereas GW0742 administration induced a slight but not significant reduction in body mass (35.00 ± 2.83 g). Epididymal fat mass was increased by HFCS manipulation in comparison to control diet (4.22 ± 0.35% body mass versus 3.66 ± 0.29% body mass; $P < 0.01$), and values were reduced to the control level by drug administration (3.86 ± 0.33% body mass). In contrast, neither dietary manipulation nor drug treatment affected gastrocnemius mass (% body mass: 0.97 ± 0.19 control, 0.92 ± 0.09 HFCS, and 0.92 ± 0.06 HFCS + GW). As we previously reported [5], the HFCS diet caused a significant increase in serum triglycerides, total cholesterol, and LDL concentrations and a marked decrease in the HDL levels. GW0742 administration

reverted the deleterious effects of HFCS diet on the serum lipid profile. Interestingly, the HFCS diet significantly affected insulin sensitivity. A significant increase in serum insulin levels was observed in mice of HFCS group compared to control mice (2.26±0.51 μg/L versus 1.27±0.07 μg/L), and this increase was almost completely abolished by GW0742 (1.51 ± 0.46 μg/L). Fasting glucose concentrations were elevated in serum of HFCS animals in comparison to control animals (103.4 ± 12.0 mg/dL versus 78.7 ± 0.3 mg/dL) and reduced to control levels by GW0742 (81.3 ± 10.9 mg/dL). Moreover, HFCS mice showed a significant impairment in glucose tolerance to exogenously administered glucose. Although all the groups reached a glycemic peak at 15 min postglucose challenge (control: 223.1 ± 12.0 mg/dL; HFCS: 212.7 ± 7.3 mg/dL; HFCS + GW: 198.8 ± 10.3 mg/dL), glycemic levels at 30–60 min post glucose challenge in HFCS-fed mice were higher than those recorded in the control group (30 min: 182.9 ± 8.3 mg/dL versus 129.4 ± 8.0 mg/dL, $P < 0.05$; 60 min: 130.7 ± 10.3 mg/dL versus 96.8 ± 4.0 mg/dL, $P < 0.05$). GW0742 significantly ($P < 0.05$) improved glucose tolerance in HFCS-fed mice, showing significantly reduced glucose concentrations at both 30 and 60 min postglucose challenge (89.7 ± 5.2 mg/dL and 86.7 ± 4.3 mg/dL, resp.).

Data on the effects of dietary manipulation and chronic GW0742 treatment on serum levels of IL-6, adiponectin, and leptin are reported in Table 1. The experimental diet caused a more than fourfold increase in serum IL-6 concentrations, whereas GW0742 administration significantly decreased the IL-6 levels. Adiponectin and leptin showed different patterns: in fact, the level of adiponectin in HFCS mice was lower than in control mice, while that of leptin increased versus controls. When GW0742 was administered to HFCS mice there was a significant increase ($P < 0.01$) in the adiponectin levels associated with a significant decrease ($P < 0.01$) in leptin serum concentration.

It must be noted that in control mice GW0742 had no significant effect on any of the previously described metabolic parameters.

3.2. Effect of GW0742 on Skeletal Muscle PPAR-δ Expression. As shown in Figure 1, HFCS diet did not affect PPAR-δ protein level expression in the mouse gastrocnemius. In contrast, daily administration of GW0742 resulted in a twofold increase in expression of its pharmacological target, with maximum effect in the presence of dietary manipulation.

3.3. The Effects of HFCS Diet on Insulin Signal Transduction Were Reverted by GW0742 Administration. The HFCS diet did not alter the protein expression of IRS-1, Akt, or GSK-3β compared to the control group. However, HFCS caused a marked increase in Ser^{307} phosphorylation of IRS-1 in parallel with reduced Ser^{473} phosphorylation of Akt (Figures 2(a) and 2(b)). Ser^{9} phosphorylation of GSK-3β, a downstream target of Akt, was also reduced in the presence of HFCS (Figure 2(c)), suggestive of impaired insulin signaling downstream of IRS-1. Most notably, GW0742 significantly attenuated all the effects of HFCS on IRS-1, Akt, and GSK-3β phosphorylation, measured at the steady state.

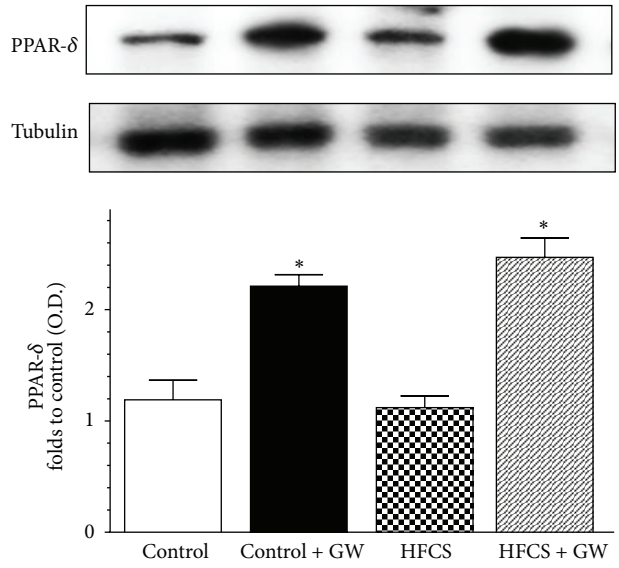

Figure 1: Effects of dietary manipulation and GW0742 treatment on PPAR-δ expression in the mouse gastrocnemius. Protein expression was measured by western blot analysis in gastrocnemius homogenates of mice fed with a standard (control) or HFCS diet (HFCS) in the absence or presence of GW0742 treatment (1 mg/kg/day) (control + GW; HFCS + GW). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin, and normalized using the related control band. Data are means ± SD of three separate experiments. $^{*}P < 0.01$ versus HFCS.

3.4. Effects of HFCS Diet and GW0742 Treatment on GLUT-4 and GLUT-5 Expression and Translocation. In comparison to control animals, GLUT-4 expression was reduced in the gastrocnemius of HFCS mice, without reaching a statistical significance, and increased following drug treatment (Figure 3(a)). Similarly, the HFCS diet markedly reduced GLUT-5 expression, whereas GW0742 administration resulted in a significant increase in GLUT-5 expression (Figure 3(c)). Notably, GW0742 treatment not only increased carriers expression levels but also induced a significant membrane translocation, thus increasing muscle glucose uptake (Figures 3(b) and 3(d)).

3.5. Effects of HFCS Diet and GW0742 Treatment on Skeletal Muscle Triglyceride Content and AMPK/ACC and CPT-1 Signaling Pathway. The triglyceride content was doubled in the gastrocnemius of HFCS mice in comparison with control animals, whereas skeletal muscle triglyceride accumulation was significantly reduced by GW0742 administration (Figure 4).

Changes in the phosphorylation/activation of the AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) system, whose central role in the regulation of cellular lipid homeostasis is well known, were evaluated by immunoblotting experiments on gastrocnemius homogenates. As reported in Figure 5, there was no significant effect of HFCS feeding or drug treatment on total AMPK and ACC protein content. In contrast, chronic HFCS exposure markedly reduced Thr^{172} phosphorylation of AMPK and

Table 1: Effects of chronic *in vivo* treatment with GW0742 on mouse blood parameters after 30 weeks of dietary manipulation.

	Control ($n = 10$)	Control + GW0742 ($n = 6$)	HFCS ($n = 10$)	HFCS + GW0742 ($n = 10$)
IL-6 (pg/mL)	20.80 ± 2.11	18.90 ± 4.22	91.55 ± 16.47*	28.80 ± 7.99§
Adiponectin (μg/mL)	4.53 ± 0.23	4.75 ± 0.46	1.93 ± 0.08*	3.69 ± 0.43*§
Leptin (pg/mL)	206.65 ± 18.77	198.07 ± 22.17	446.67 ± 52.61*	266.12 ± 41.21*§

HFCS: high-fructose corn syrup; IL-6: interleukin-6.
Data are means ± S.D.
*$P < 0.01$ versus Control.
§$P < 0.01$ versus HFCS.

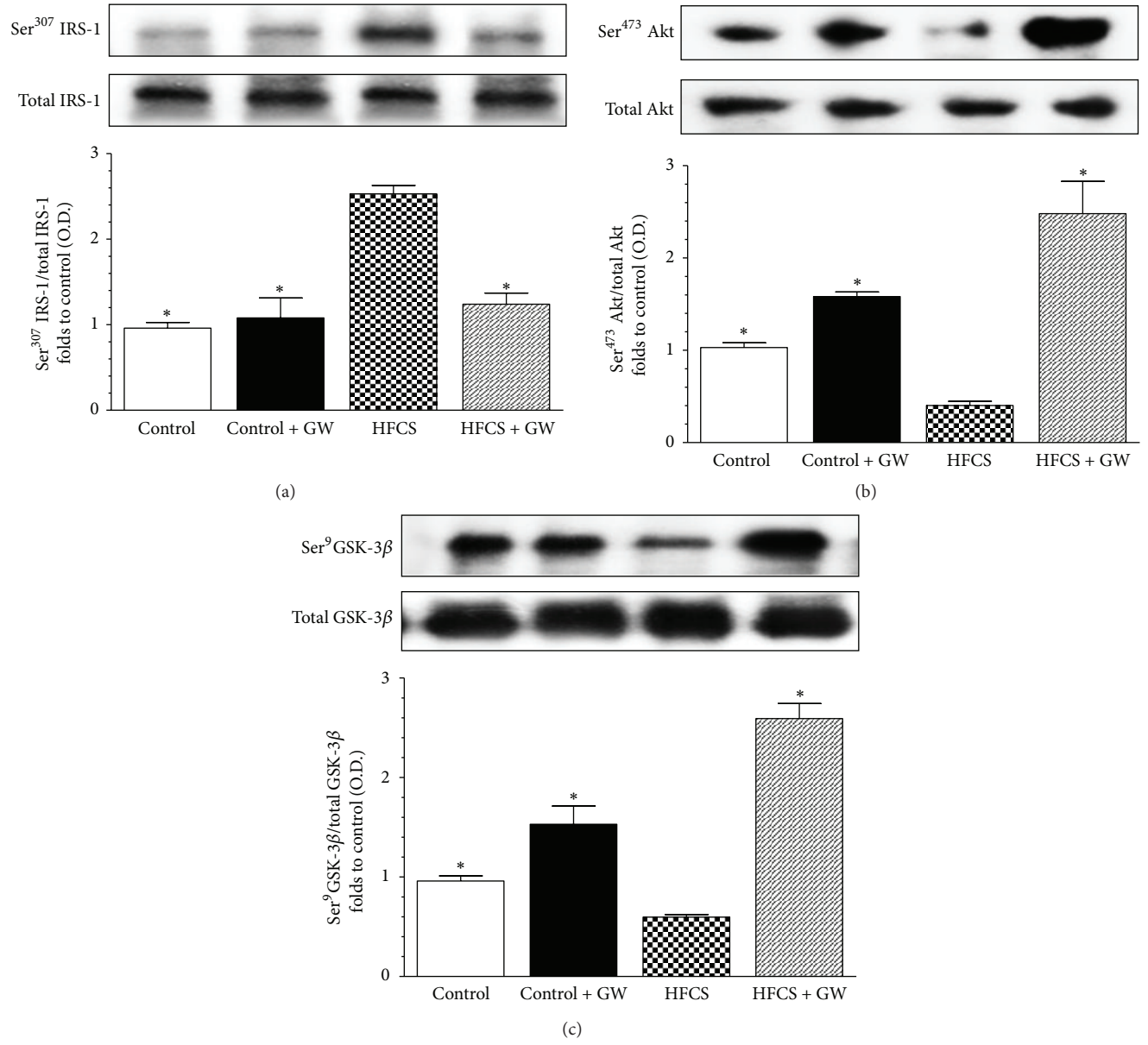

Figure 2: Effects of GW0742 treatment on insulin signal transduction in the gastrocnemius of mice fed with HFCS diet. Total IRS-1 protein expression and Ser^{307} phosphorylation (a), total Akt protein expression and Ser^{473} phosphorylation (b), and total GSK-3β protein expression and Ser^{9} phosphorylation (c) were analyzed by western blot on the gastrocnemius homogenates obtained from mice fed with a standard (control) or HFCS diet (HFCS) for 30 weeks and treated with GW0742 (1 mg/kg/day) added during the last 16 weeks (control + GW; HFCS + GW). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin contents, and normalized using the related control band. The data are means ± SD of three separate experiments. *$P < 0.01$ versus HFCS.

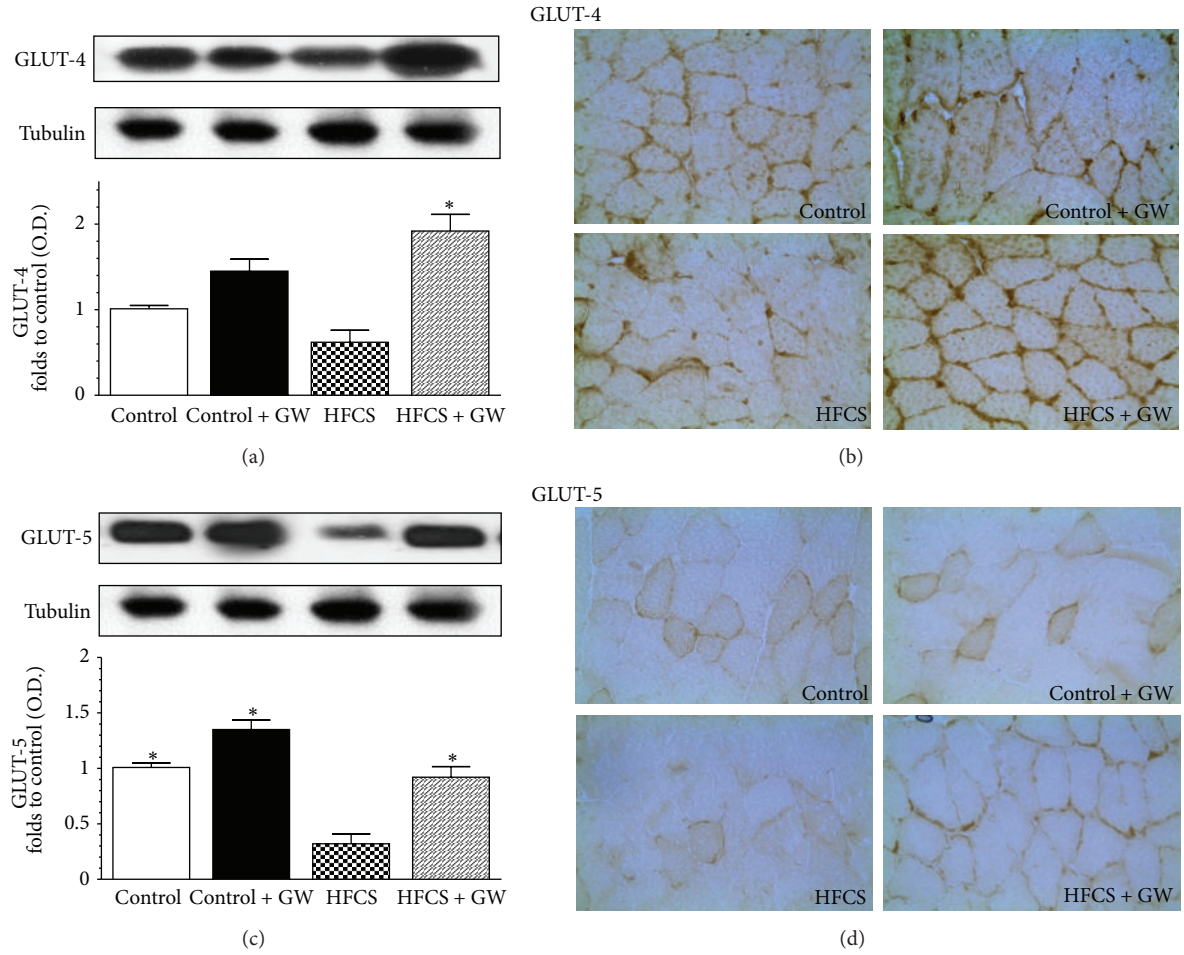

Figure 3: Effects of two dietary regimens either normal (control) or a diet enriched with 15% HFCS solution (HFCS) on GLUT-4 and GLUT-5 expression (resp., (a) and (c)) and membrane translocation (resp., (b) and (d), original magnification: 400x) in the gastrocnemius of mice treated with GW0742 (1 mg/kg/day, control + GW; HFCS + GW). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin, and normalized using the related control band. Data are means ± SD of three separate experiments. $^{*}P < 0.01$ versus HFCS.

its substrate Ser^{79} ACC. Interestingly, GW0742 treatment led to a robust increase in phosphorylation of both AMPK and ACC in HFCS-fed mice (Figures 5(a) and 5(b)). The effects of PPAR-δ agonism on fatty acid oxidation in skeletal muscle cells are further indicated by the significant increase in the protein expression of carnitine palmitoyl transferase-1 (CPT-1) elicited by GW0742 in HFCS-fed mice (Figure 5(c)).

3.6. Effects of HFCS Diet and GW0742 Treatment on NF-κB Activation, ICAM-1, and iNOS Expression. To investigate the muscle inflammatory response induced by HFCS-diet and the intracellular signaling pathway(s) that might be involved in the protective mechanisms evoked by GW0742, we firstly evaluated the effects on NF-κB activation. When compared to control mice, HFCS mice developed significant increase in the nuclear translocation of the p65 NF-κB subunit in the mouse gastrocnemius, indicating the activation of this transcriptional factor (Figure 6(a)). Treatment with GW0742 resulted in a significant reduction in nuclear translocation of p65 and, hence, in the activation of NF-κB. The intercellular adhesion molecule-1 (ICAM-1), whose role in the recruitment of neutrophils is widely accepted, was slightly detected in the gastrocnemius of control animals, whereas its expression was dramatically increased by HFCS diet (Figure 6(b)). As shown in Figure 6(c), also the expression of the inducible nitric oxide synthase (iNOS) was increased in the presence of dietary manipulation. Interestingly, these changes were suppressed by GW0742 administration, as evidenced by densitometric analysis of the related autoradiograms.

3.7. Effects of HFCS Diet and GW0742 Treatment on Skeletal Muscle FGF-21 Production. The beneficial effects of PPAR-δ activation were associated with a dramatic increase of FGF-21 production in the mouse gastrocnemius. As shown in Figure 7, in comparison to control mice (46.45 ± 1.13 pg/mg protein), HFCS diet did not affect the production of this

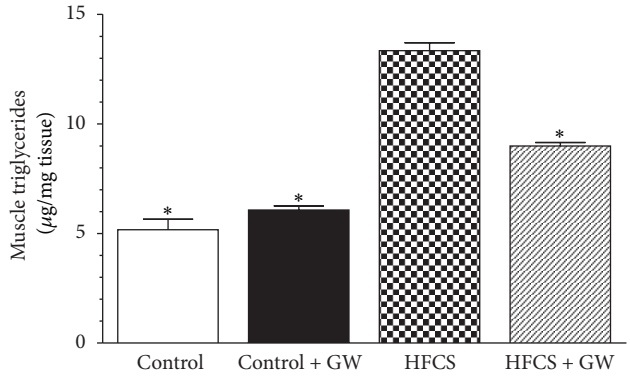

Figure 4: Triglyceride content in gastrocnemius of mice fed with a standard diet (control) or a HFCS diet (HFCS) for 30 weeks and treated with GW0742 (1 mg/kg/day) added during the last 16 weeks (control + GW; HFCS + GW). Data are means ± SD of eight animals/group. $^{*}P < 0.01$ versus HFCS.

myokine (49.38 ± 4.12 pg/mg protein), whereas GW0742 treatment induced a twofold increase (87.08 ± 6.37 pg/mg protein).

4. Discussion

In agreement with previous observations [14, 15], the present study shows that chronic exposure to the most widely used added sugar HFCS caused a significant increase in body mass associated with increases in serum levels of triglycerides, LDL-cholesterol, glucose, insulin, IL-6, leptin, and hypoadiponectinemia. Using the same experimental protocol here reported, we have recently demonstrated that the molecular mechanism underlying the deleterious effects of HFCS involves the hepatic upregulation of fructokinase, the main fructose-metabolizing enzyme, which may account for the increase in serum levels of free fatty acids and hyperuricemia [5]. We also reported that the documented increase in serum uric acid level significantly contributed to the development of chronic kidney injury. Interestingly, we demonstrated that chronic administration of the PPAR-δ ligand GW0742 exerted beneficial effects by preventing the upregulation of fructokinase in the liver and activation of the inflammatory signaling complex NLRP3 inflammasome in the kidney. Despite these preliminary data, so far, there are no studies on the effects of long-term exposure to HFCS, the major sweetener added to beverages and food, on skeletal muscle, which is a major site of postprandial glucose disposal and is therefore one of the insulin-sensitive tissues most likely to manifest early signs of insulin resistance. Here, we investigated (i) the mechanisms underlying metabolic disturbances in skeletal muscle of mice exposed for 30 weeks to high intake of HFCS and (ii) the local effects evoked by PPAR-δ chronic activation. The sugar was added to the drink water at a concentration that covers 10% of the daily caloric intake, corresponding to the average energy intake in the form of ingested sweeteners in the western diet. We documented the HFCS-induced alteration in the insulin signal transduction pathway, as shown by the impaired phosphorylation of IRS-1 protein as well as of the downstream key insulin signaling molecules, Akt, and GSK-3β, an Akt substrate [16]. This is in keeping with previous studies showing that IRS-1 serine phosphorylation can interfere with subsequent Akt and GSK-3β phosphorylation, disrupting insulin signal transduction [17]. In our experimental model, oral administration of the selective PPAR-δ agonist GW0742 was associated with a significant improvement of the defective insulin signaling in the skeletal muscle, which may account at least in part for the changes in serum lipid profile and insulin sensitivity. As the inhibition of GSK-3β evokes an increase in the glycogen synthesis [18, 19], we may speculate that GW0742 administration modulates muscle glycogen storage through inactivation of GSK-3β. Although no direct evidence of drug treatment on glucose storage has been reported in our study, we documented the effects of chronic PPAR-δ activation on glucose transporters expression and distribution. Specifically, we found that PPAR-δ activation by GW0742 evoked an increase in expression of GLUT-4, the most abundant glucose transporter isoform in skeletal muscle [20], and its translocation from intracellular compartments to the plasma membrane, thus facilitating glucose transport. Similarly, GLUT-5, the fructose carrier with low capacity to transport glucose [21], was scarcely detectable in the gastrocnemius muscle of HFCS-fed mice, and its expression and membrane translocation were enhanced by chronic PPAR-δ activation. Because fructose is highly present in the HFCS diet and contributes to carbohydrate metabolism in muscle [22], the increase in GLUT-5 may favor a better utilization of fructose supplied in the diet. In any case, the reduction in GLUT-4 and GLUT-5 expression and translocation detected in the HFCS group could represent an adaptation to the chronic exposure to a sugar-enriched diet, while the increases in these carrier isoforms in the presence of the selective PPAR-δ agonist GW0742 may account, at least in part, for the improvement in insulin-induced muscle glucose uptake. Recent data have shown that Akt inhibitors or dominant-negative Akt constructs regulate the translocation, targeting, and fusion of GLUT-4-containing vesicles [23, 24]. Similarly, IRS-1 phosphorylation has been reported to affect GLUT-4 translocation and subsequent glucose uptake in mouse skeletal myocytes [25]. We may, thus, speculate that the beneficial effects of the PPAR-δ ligand GW0742 are secondary to activation of the IRS-1/Akt/GSK-3β pathway. This is also supported by previous findings showing that the chronic administration of GW0742 in rats results in phosphoinositide-dependent kinase phosphorylation and, hence, increased Akt phosphorylation [26]. Another enzyme complex which could be involved in PPAR-δ metabolic effects within the skeletal muscle is AMPK, a serine/threonine protein kinase that has emerged as a key player in both lipid and glucose metabolism in skeletal muscles [27, 28]. In mouse C2C12 myotubes, AMPK activation potentiated insulin action by reducing IRS-1 serine phosphorylation [29], while reduced muscle AMPK activity has been reported to aggravate muscle insulin resistance following a high-fat diet over 30 weeks [30]. In agreement with these observations, we found that GW0742 increased phosphorylation

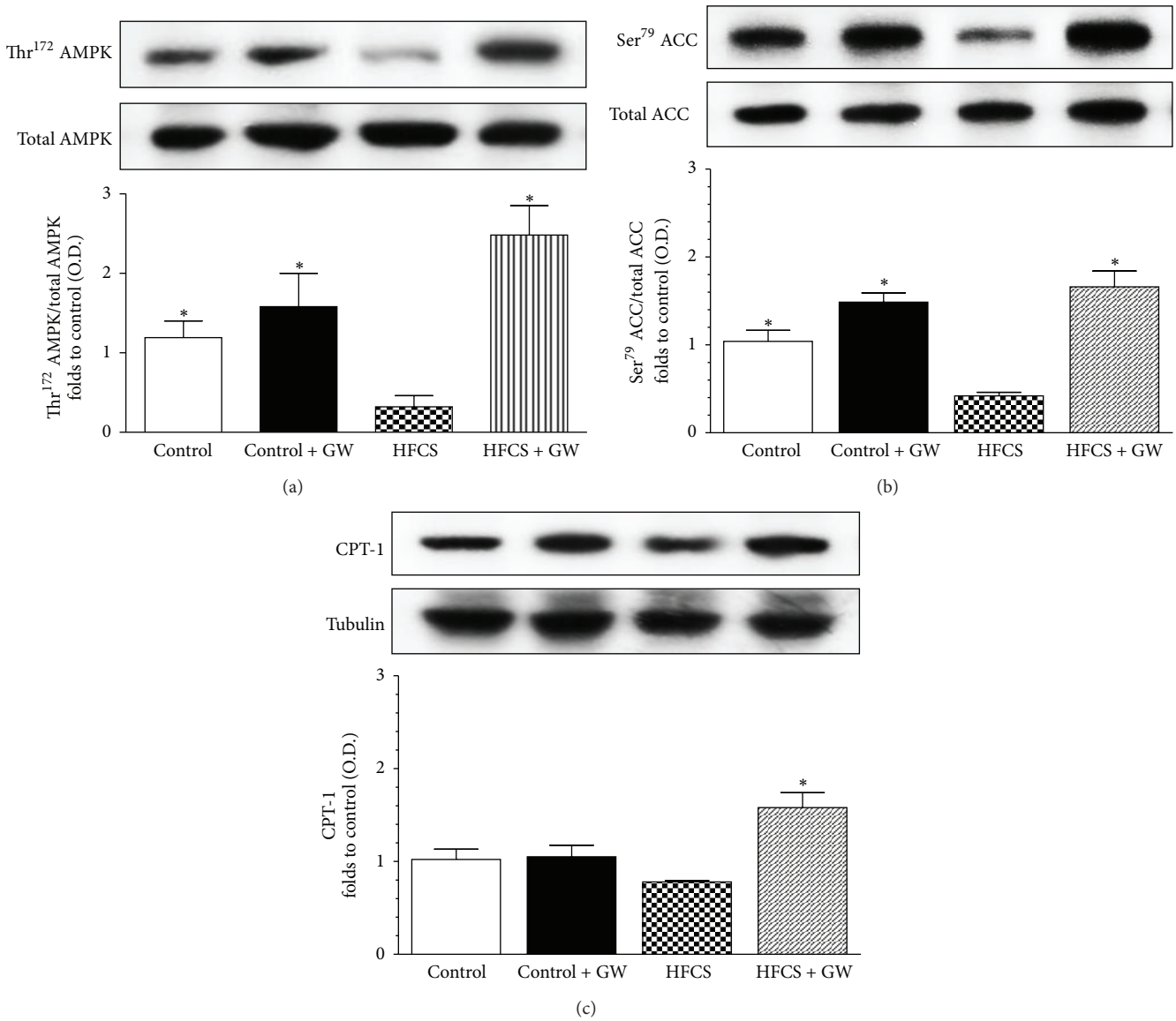

Figure 5: Effects of GW0742 treatment on AMPK/ACC phosphorylation and CPT-1 expression in the gastrocnemius of mice fed with HFCS diet. Total AMPK protein expression and Thr^{172} phosphorylation (a), total ACC protein expression and Ser^{79} phosphorylation (b), and CPT-1 expression (c) were analyzed by western blot on the gastrocnemius homogenates obtained from mice fed with a standard (control) or HFCS diet (HFCS) for 30 weeks and treated with GW0742 (1 mg/kg/day) added during the last 16 weeks (control + GW; HFCS + GW). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin contents, and normalized using the related control band. The data are means ± SD of three separate experiments. $^{*}P < 0.01$ versus HFCS.

of Thr^{172} regulatory site on AMPK which, in turn, causes ACC phosphorylation. When ACC is inactive (phosphorylated form), a fall in malonyl-coenzyme A occurs, which disinhibits CPT-1 and increases mitochondrial import and oxidation of long-chain fatty acids [31]. Our data demonstrate that chronic exposure to GW0742 administration increased skeletal muscle CPT-1 expression, thus shunting toward fatty acid oxidation, and this effect was associated with reduced triglyceride accumulation. These findings suggest that the beneficial effects evoked by the PPAR-δ agonist GW0742 are at least partially dependent on AMPK activation. The direct involvement of PPAR-δ agonism in mediating improvements in muscle insulin sensitivity and lipid metabolism is also confirmed by results showing that PPAR-δ is expressed in the gastrocnemius of both control and HFCS-fed mice and, more importantly, that its expression is substantially increased by chronic administration of GW0742, thus suggesting that the observed drug effects are due to a ligand-dependent PPAR-δ activation. This is consistent with previous works showing that PPAR-δ activation is involved in regulating myogenesis [32–35]. In search of the mechanism(s) underlying the protective action of GW0742, we investigated whether PPAR-δ activation may affect the local inflammatory response associated with muscular metabolic injury. Although PPAR-δ has

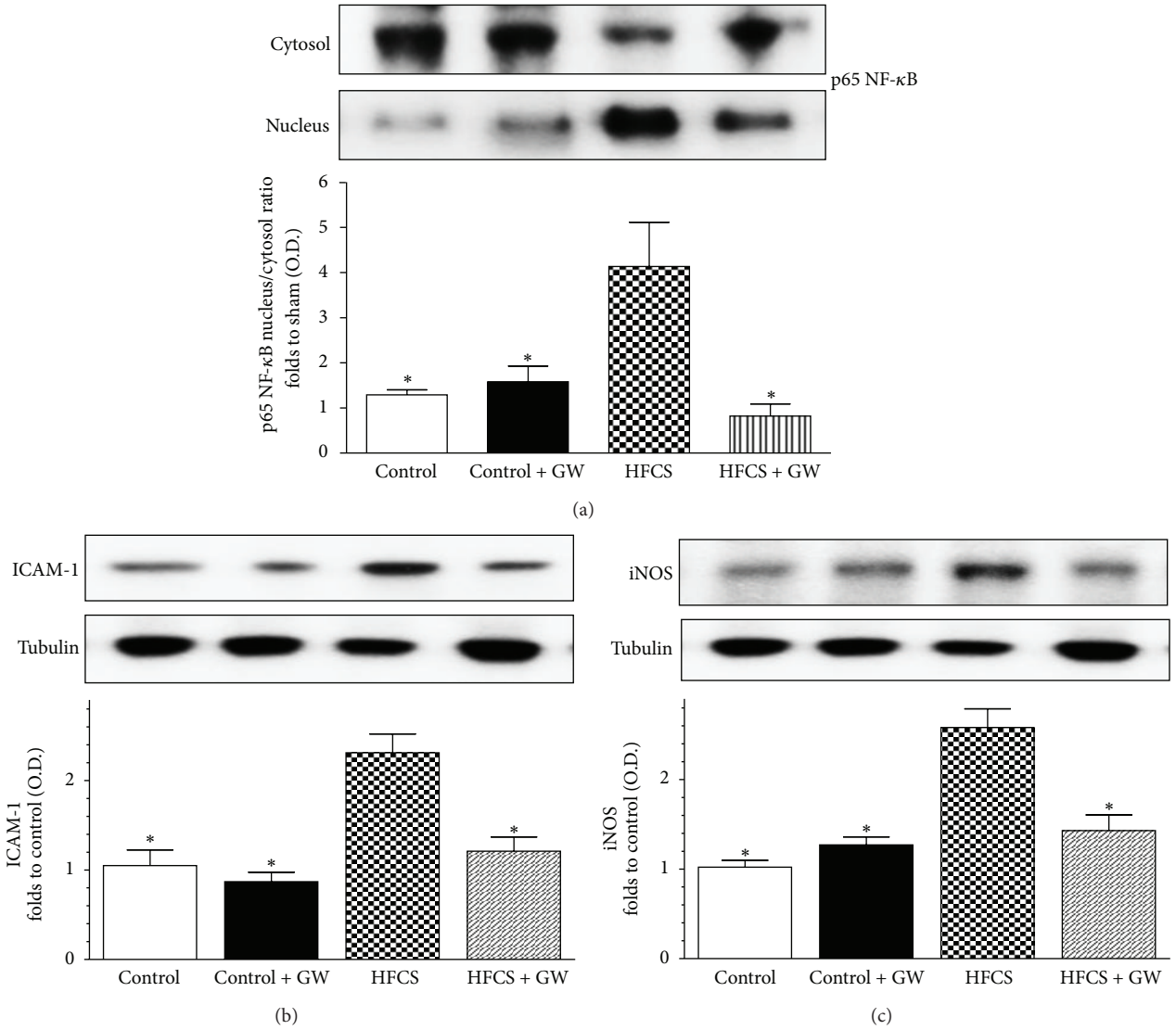

Figure 6: Effects of dietary manipulation and GW0742 treatment on NF-κB p65 translocation (a), ICAM-1 (b) and iNOS (c) protein expression in the mouse gastrocnemius. Protein expression was measured by Western blot analysis in gastrocnemius homogenates of mice fed with a standard (control) or HFCS diet (HFCS) in the absence or presence of GW0742 treatment (1 mg/kg/day) (control + GW; HFCS + GW). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin contents, and normalized using the related control band. NF-κB p65 subunit levels in cytosolic and nuclear fractions are expressed as nucleus/cytosol ratio normalized using the related control band. Data are means ± SD of three separate experiments. $^*P < 0.01$ versus HFCS.

been implicated in the regulation of systemic inflammatory responses associated with metabolic dysregulation [7], so far, reports on anti-inflammatory effects of PPAR activation in skeletal muscle are rather scarce. Here, we show for the first time that in the skeletal muscle GW0742 reverts the diet-induced increase in the nuclear translocation of NF-κB p65, a transcriptional factor that plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators of local and systemic inflammation (such as cytokines, chemokines, and cell adhesion molecules). This observation is in agreement with previous studies reporting that PPAR-δ activation induces the physical interaction between PPAR-δ and the p65 subunit of NF-κB and reduced the LPS-induced degradation of the inhibitory protein "Inhibitor of kappa B," thus preventing NF-κB activation [36, 37]. The reduction of NF-κB activation by GW0742 treatment may account for the observed reduction in the serum levels of IL-6, which is known to be mostly released from skeletal muscle, and in the local expression of the NF-κB-dependent proteins iNOS and ICAM-1, whose role in the development of insulin resistance has been recently documented [38, 39]. As several studies have reported an association between GSK-3β and NF-κB activity [40], we might speculate that PPAR-δ activation phosphorylates, and hence, activates the Akt pathway, which in turn phosphorylates, and hence, inhibits GSK-3β, presumably resulting

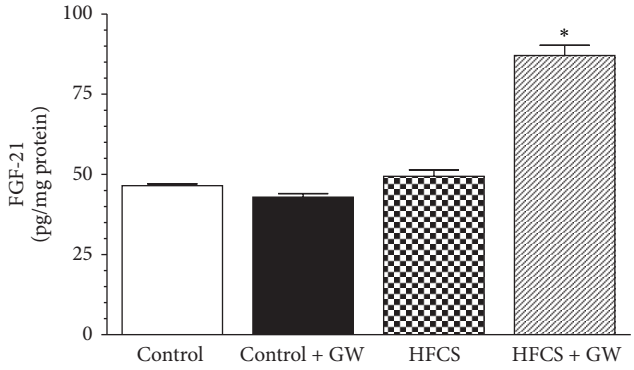

Figure 7: Fibroblast growth factor-21 (FGF-21) levels were analyzed by ELISA in gastrocnemius homogenates of mice fed with a standard (control) or HFCS diet in the absence or presence of GW0742 (1 mg/kg/day). Data are means ± SD of five animals/group. $^{*}P < 0.01$ versus HFCS.

in the inhibition of NF-κB and, in turn, NF-κB-dependent proinflammatory gene transcription. Although this cross talk among different signaling pathways by pharmacological PPAR-δ modulation is intriguing, it must be stressed, however, that the lack of direct experimental evidence of a causal relationship between the improved local and systemic insulin sensitivity and the treatment-induced reduction of NF-κB activation in our experimental model limit the interpretation of the molecular mechanism(s) underlying our findings. One of the most recently identified mediators that facilitates organ cross talk and the related control of impaired glucose homeostasis in metabolic diseases is a member of the FGF family, FGF-21. FGF-21 has been previously shown to lower blood glucose levels in several diabetic rodent and monkey models [41, 42], to regulate lypolisis in white adipose tissue [43, 44] and substrate utilization in the liver [45]. FGF-21 was also shown to directly enhance skeletal muscle glucose uptake [46]. The metabolic effects of FGF-21 on glucose metabolism involve selective regulation by different PPAR isoforms. Liver-derived FGF-21 is stimulated by PPAR-α ligands [47]. Conversely, PPAR-γ activation with feeding promotes FGF-21 production in adipocytes but not in liver [48, 49]. To our knowledge, this is the first paper that demonstrates a correlation between PPAR-δ activation and increased FGF-21 levels in skeletal muscle, thus adding an original piece of evidence to the complex mechanisms by which PPAR-δ can regulate several biological functions. Our observation is also in keeping with a previous study showing that circulating FGF-21 levels consistently increase in human subjects in response to pharmacological activation of PPAR-δ [50]. Although a previous investigation has revealed that PPAR-γ directly regulates expression of the FGF-21 gene through elements located within the 500-bp upstream region of the gene [49], to date, a consensus sequence of PPAR-δ binding site in the promoter region of the FGF-21 gene has not yet been identified. Besides *in vitro* experiments with FGF-21, small interfering RNA are warranted to clarify whether GW0742 beneficial effects are related to the direct modulation of FGF-21 biological functions. The recent findings on FGF-21 ability to prevent insulin resistance in human myoblasts by inhibiting NF-κB activation [51] may also suggest that the muscle-derived FGF-21 acts in an autocrine fashion to amplify PPAR-δ inhibitory effects on the expression of NF-κB-dependent inflammatory genes. This further supports the existence of multiple anti-inflammatory pathways involved in the beneficial effects evoked by PPAR-δ activation.

In conclusion, we have shown that feeding mice with a HFCS diet for 30 weeks evoked skeletal muscle insulin resistance and lipid accumulation which were associated with activation of inflammatory pathways. All these effects were attenuated by selective PPAR-δ activation. In addition, this study, showing a strong induction of FGF-21 in the skeletal muscle after chronic administration of the PPAR-δ ligand GW0742, enhances our knowledge of the mechanisms of action of PPAR-δ agonism and provides further insights into the role of FGF-21 as mediator of the tissue cross-talk that underlines the integrated control of the metabolic inflammation.

Authors' Contribution

Elisa Benetti and Raffaella Mastrocola contributed equally to this work. Massimo Collino and Marco A. Minetto are shared senior authors of this study.

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References

[1] V. T. Samuel and G. I. Shulman, "Mechanisms for insulin resistance: common threads and missing links," *Cell*, vol. 148, no. 5, pp. 852–871, 2012.

[2] J. Hoeks, J. de Wilde, M. F. M. Hulshof et al., "High fat diet-induced changes in mouse muscle mitochondrial phospholipids do not impair mitochondrial respiration despite insulin resistance," *PLoS ONE*, vol. 6, no. 11, Article ID e27274, 2011.

[3] A. Vijayakumar, Y. Wu, H. Sun et al., "Targeted loss of ghr signaling in mouse skeletal muscle protects against high-fat diet-induced metabolic deterioration," *Diabetes*, vol. 61, no. 1, pp. 94–103, 2012.

[4] P. M. Badin, I. K. Vila, K. Louche et al., "High-fat diet-mediated lipotoxicity and insulin resistance is related to impaired lipase expression in mouse skeletal muscle," *Endocrinology*, vol. 154, no. 4, pp. 1444–1453, 2013.

[5] M. Collino, E. Benetti, M. Rogazzo et al., "Reversal of the deleterious effects of chronic dietary HFCS-55 intake by PPAR-δ agonism correlates with impaired NLRP3 inflammasome activation," *Biochemical Pharmacology*, vol. 85, no. 2, pp. 257–264, 2013.

[6] M. Collino, "High dietary fructose intake: sweet or bitter life?" *World Journal of Diabetes*, vol. 2, pp. 77–81, 2011.

[7] E. Benetti, N. S. A. Patel, and M. Collino, "The role of PPARβ/δ in the management of metabolic syndrome and its associated cardiovascular complications," *Endocrine, Metabolic and Immune Disorders—Drug Targets*, vol. 11, no. 4, pp. 273–284, 2011.

[8] G. D. Barish, V. A. Narkar, and R. M. Evans, "PPARδ: a dagger in the heart of the metabolic syndrome," *Journal of Clinical Investigation*, vol. 116, no. 3, pp. 590–597, 2006.

[9] M. Schuler, F. Ali, C. Chambon et al., "PGC1α expression is controlled in skeletal muscles by PPARβ, whose ablation results in fiber-type switching, obesity, and type 2 diabetes," *Cell Metabolism*, vol. 4, no. 5, pp. 407–414, 2006.

[10] L. Li, Z. Luo, H. Yu et al., "Telmisartan improves insulin resistance of skeletal muscle through peroxisome proliferator-activated receptor-δ activation," *Diabetes*, vol. 62, no. 3, pp. 762–774, 2013.

[11] D. Cuevas-Ramos, C. A. Aguilar-Salinas, and F. J. Gomez-Perez, "Metabolic actions of fibroblast growth factor 21," *Current Opinion in Pediatrics*, vol. 24, no. 4, pp. 523–529, 2012.

[12] T. L. Graham, C. Mookherjee, K. E. Suckling, C. N. A. Palmer, and L. Patel, "The PPARδ agonist GW0742X reduces atherosclerosis in LDLR$^{-/-}$ mice," *Atherosclerosis*, vol. 181, no. 1, pp. 29–37, 2005.

[13] D. R. Meldrum, R. Shenkar, B. C. Sheridan, B. S. Cain, E. Abraham, and A. H. Harken, "Hemorrhage activates myocardial NFκB and increases TNF-α in the heart," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 10, pp. 2849–2854, 1997.

[14] K. S. Collison, S. M. Saleh, R. H. Bakheet et al., "Diabetes of the liver: the link between nonalcoholic fatty liver disease and HFCS-55," *Obesity*, vol. 17, no. 11, pp. 2003–2013, 2009.

[15] H. R. Light, E. Tsanzi, J. Gigliotti, K. Morgan, and J. C. Tou, "The type of caloric sweetener added to water influences weight gain, fat mass, and reproduction in growing Sprague-Dawley female rats," *Experimental Biology and Medicine*, vol. 234, no. 6, pp. 651–661, 2009.

[16] P. Cohen and M. Goedert, "GSK3 inhibitors: development and therapeutic potential," *Nature Reviews Drug Discovery*, vol. 3, no. 6, pp. 479–487, 2004.

[17] C. M. Taniguchi, B. Emanuelli, and C. R. Kahn, "Critical nodes in signalling pathways: insights into insulin action," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 2, pp. 85–96, 2006.

[18] D. A. E. Cross, D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings, "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B," *Nature*, vol. 378, no. 6559, pp. 785–789, 1995.

[19] J. N. Nielsen and J. F. P. Wojtaszewski, "Regulation of glycogen synthase activity and phosphorylation by exercise," *Proceedings of the Nutrition Society*, vol. 63, no. 2, pp. 233–237, 2004.

[20] K. Bouzakri, A. Zachrisson, L. Al-Khalili et al., "siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle," *Cell Metabolism*, vol. 4, no. 1, pp. 89–96, 2006.

[21] G. W. Gould and G. D. Holman, "The glucose transporter family: structure, function and tissue-specific expression," *Biochemical Journal*, vol. 295, part 2, pp. 329–341, 1993.

[22] J. R. Zierath, L. A. Nolte, E. Wahlstrom et al., "Carrier-mediated fructose uptake significantly contributes to carbohydrate metabolism in human skeletal muscle," *Biochemical Journal*, vol. 311, part 2, pp. 517–521, 1995.

[23] E. Gonzalez and T. E. McGraw, "Insulin signaling diverges into Akt-dependent and -independent signals to regulate the recruitment/docking and the fusion of GLUT4 vesicles to the plasma membrane," *Molecular Biology of the Cell*, vol. 17, no. 10, pp. 4484–4493, 2006.

[24] D. Leto and A. R. Saltiel, "Regulation of glucose transport by insulin: traffic control of GLUT4," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 6, pp. 383–396, 2012.

[25] X. Zeng, C. Zhang, M. Tong et al., "Knockdown of NYGGF4 increases glucose transport in C2C12 mice skeletal myocytes by activation IRS-1/PI3K/AKT insulin pathway," *Journal of Bioenergetics and Biomembranes*, vol. 44, no. 3, pp. 351–355, 2012.

[26] T. Yue, S. S. Nerurkar, W. Bao et al., "In vivo activation of peroxisome proliferator-activated receptor-δ protects the heart from ischemia/reperfusion injury in Zucker fatty rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 325, no. 2, pp. 466–474, 2008.

[27] B. F. Holmes, E. J. Kurth-Kraczek, and W. W. Winder, "Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle," *Journal of Applied Physiology*, vol. 87, no. 5, pp. 1990–1995, 1999.

[28] N. Musi and L. J. Goodyear, "AMP-activated protein kinase and muscle glucose uptake," *Acta Physiologica Scandinavica*, vol. 178, no. 4, pp. 337–345, 2003.

[29] S. N. Jakobsen, D. G. Hardie, N. Morrice, and H. E. Tornqvist, "5′-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside," *The Journal of Biological Chemistry*, vol. 276, no. 50, pp. 46912–46916, 2001.

[30] N. Fujii, R. C. Ho, Y. Manabe et al., "Ablation of AMP-activated protein kinase α 2 activity exacerbates insulin resistance induced by high-fat feeding of mice," *Diabetes*, vol. 57, no. 11, pp. 2958–2966, 2008.

[31] N. B. Ruderman, A. K. Saha, and E. W. Kraegen, "Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity," *Endocrinology*, vol. 144, no. 12, pp. 5166–5171, 2003.

[32] S. Luquet, J. Lopez-Soriano, D. Holst et al., "Peroxisome proliferator-activated receptor δ controls muscle development and oxidative capability," *FASEB Journal*, vol. 17, no. 15, pp. 2299–2301, 2003.

[33] T. Tanaka, J. Yamamoto, S. Iwasaki et al., "Activation of peroxisome proliferator-activated receptor δ induces fatty acid β-oxidation in skeletal muscle and attenuates metabolic syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 26, pp. 15924–15929, 2003.

[34] Y. Wang, C. Zhang, R. T. Yu et al., "Regulation of muscle fiber type and running endurance by PPARδ," *PLoS Biology*, vol. 2, no. 10, article e294, 2004.

[35] S. Bonala, S. Lokireddy, H. Arigela et al., "Peroxisome proliferator-activated receptor β/δ induces myogenesis by modulating myostatin activity," *The Journal of Biological Chemistry*, vol. 287, no. 16, pp. 12935–12951, 2012.

[36] A. Planavila, R. Rodríguez-Calvo, M. Jové et al., "Peroxisome proliferator-activated receptor β/δ activation inhibits hypertrophy in neonatal rat cardiomyocytes," *Cardiovascular Research*, vol. 65, no. 4, pp. 832–841, 2005.

[37] G. Ding, L. Cheng, Q. Qin, S. Frontin, and Q. Yang, "PPARδ modulates lipopolysaccharide-induced TNFα inflammation signaling in cultured cardiomyocytes," *Journal of Molecular and Cellular Cardiology*, vol. 40, no. 6, pp. 821–828, 2006.

[38] A. F. Rubio-Guerra, H. Vargas-Robles, A. M. Serrano, G. Vargas-Ayala, L. Rodriguez-Lopez, and B. A. Escalante-Acosta, "Correlation between the levels of circulating adhesion

molecules and atherosclerosis in hypertensive type-2 diabetic patients," *Clinical and Experimental Hypertension*, vol. 32, no. 5, pp. 308–310, 2010.

[39] E. R. Ropelle, J. R. Pauli, D. E. Cintra et al., "Targeted disruption of inducible nitric oxide synthase protects against aging, S-nitrosation, and insulin resistance in muscle of male mice," *Diabetes*, vol. 62, no. 2, pp. 466–470, 2013.

[40] K. P. Hoeflich, J. Luo, E. A. Rubie, M. Tsao, O. Jin, and J. R. Woodgett, "Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation," *Nature*, vol. 406, no. 6791, pp. 86–90, 2000.

[41] A. Kharitonenkov, T. L. Shiyanova, A. Koester et al., "FGF-21 as a novel metabolic regulator," *Journal of Clinical Investigation*, vol. 115, no. 6, pp. 1627–1635, 2005.

[42] A. Kharitonenkov, V. J. Wroblewski, A. Koester et al., "The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21," *Endocrinology*, vol. 148, no. 2, pp. 774–781, 2007.

[43] P. Arner, A. Pettersson, P. J. Mitchell, J. D. Dunbar, A. Kharitonenkov, and M. Rydén, "FGF21 attenuates lipolysis in human adipocytes—a possible link to improved insulin sensitivity," *FEBS Letters*, vol. 582, no. 12, pp. 1725–1730, 2008.

[44] X. Li, H. Ge, J. Weiszmann et al., "Inhibition of lipolysis may contribute to the acute regulation of plasma FFA and glucose by FGF21 in ob/ob mice," *FEBS Letters*, vol. 583, no. 19, pp. 3230–3234, 2009.

[45] M. K. Badman, P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier, and E. Maratos-Flier, "Hepatic fibroblast growth factor 21 is regulated by PPARα and is a key mediator of hepatic lipid metabolism in ketotic states," *Cell Metabolism*, vol. 5, no. 6, pp. 426–437, 2007.

[46] F. L. Mashili, R. L. Austin, A. S. Deshmukh et al., "Direct effects of FGF21 on glucose uptake in human skeletal muscle: implications for type 2 diabetes and obesity," *Diabetes/Metabolism Research and Reviews*, vol. 27, no. 3, pp. 286–297, 2011.

[47] T. Inagaki, P. Dutchak, G. Zhao et al., "Endocrine regulation of the fasting response by PPARα-mediated induction of fibroblast growth factor 21," *Cell Metabolism*, vol. 5, no. 6, pp. 415–425, 2007.

[48] E. S. Muise, B. Azzolina, D. W. Kuo et al., "Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor γ and altered metabolic states," *Molecular Pharmacology*, vol. 74, no. 2, pp. 403–412, 2008.

[49] H. Wang, L. Qiang, and S. R. Farmer, "Identification of a domain within peroxisome proliferator-activated receptor γ regulating expression of a group of genes containing fibroblast growth factor 21 that are selectively repressed by SIRT1 in adipocytes," *Molecular and Cellular Biology*, vol. 28, no. 1, pp. 188–200, 2008.

[50] C. Christodoulides, P. Dyson, D. Sprecher, K. Tsintzas, and F. Karpe, "Circulating fibroblast growth factor 21 is induced by peroxisome proliferator-activated receptor agonists but not ketosis in man," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 9, pp. 3594–3601, 2009.

[51] M. S. Lee, S. Choi, E. S. Ha et al., "Fibroblast growth factor-21 protects human skeletal muscle myotubes from palmitate-induced insulin resistance by inhibiting stress kinase and NF-κB," *Metabolism: Clinical and Experimental*, vol. 61, no. 8, pp. 1142–1151, 2012.

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Review Article

Visfatin/Nampt: An Adipokine with Cardiovascular Impact

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Adipose tissue is acknowledged as an endocrine organ that releases bioactive factors termed adipokines. Visfatin was initially identified as a novel adipokine with insulin-mimetic properties in mice. This adipokine was identical to two previously described molecules, namely, pre-B cell colony-enhancing factor (PBEF) and the enzyme nicotinamide phosphoribosyltransferase (Nampt). Enhanced circulating visfatin/Nampt levels have been reported in metabolic diseases, such as obesity and type 2 diabetes. Moreover, visfatin/Nampt circulating levels correlate with markers of systemic inflammation. In cardiovascular diseases, visfatin/Nampt was initially proposed as a clinical marker of atherosclerosis, endothelial dysfunction, and vascular damage, with a potential prognostic value. Nevertheless, beyond being a surrogate clinical marker, visfatin/Nampt is an active player promoting vascular inflammation, and atherosclerosis. Visfatin/Nampt effects on cytokine and chemokine secretion, macrophage survival, leukocyte recruitment by endothelial cells, vascular smooth muscle inflammation and plaque destabilization make of this adipokine an active factor in the development and progression of atherosclerosis. Further research is required to fully understand the mechanisms mediating the cellular actions of this adipokine and to better characterize the factors regulating visfatin/Nampt expression and release in all these pathologic scenarios. Only then, we will be able to conclude whether visfatin/Nampt is a therapeutical target in cardiometabolic diseases.

1. Introduction

The adipose tissue (AT) is no longer considered a triglyceride-storing depot but a real endocrine organ that synthesizes and secretes a wide range of diverse bioactive factors, called adipokines. These adipokines can act locally within the adipose tissue, but can also trigger an effect on distant organs or tissues through their release to the systemic circulation. Adipokines comprise cytokines and chemokines, such as tumor necrosis factor- (TNF-) α, interleukins (IL), or monocyte chemotactic protein-1 (MCP-) 1, vasoactive and coagulation factors, such as angiotensinogen or plasminogen activator inhibitor-1, (PAI-1), and proteins more specifically secreted by the adipose tissue, such as leptin or adiponectin. In the last years, the number of the adipokines has notably increased with novel adipokines such as visfatin/Nampt, resistin, apelin, or dipeptidyl peptidase-4 (DPP-4) among many others [1–4].

Adipokines exhibit a wide range of functions including the regulation of food intake and body weight homeostasis, insulin sensitivity, reproduction, immunity, inflammation, or vascular homeostasis [4, 5]. In obesity and type 2 diabetes mellitus, there is an imbalanced adipokine production that has been locally associated with AT inflammation, and the onset of insulin resistance, but also with chronic systemic inflammation, cardiovascular disease, and endothelial dysfunction [6]. In the context of metabolic diseases, adipokines are not only key mediators in the complex crosstalk between AT with other insulin-sensitive organs such as liver, skeletal muscle, and AT itself, but also have an impact on the cardiovascular system. Therefore, in the last years, there is a growing interest in the potential role of adipokines as biomarkers of low-grade inflammation and metabolic-related cardiovascular complications. In this review, we will specifically focus on the adipokine visfatin/Nampt and its impact on the cardiovascular (CV) system.

2. Visfatin/Nampt: An Adipokine and Beyond

In 2005, Fukuhara et al. firstly described visfatin as an adipokine exhibiting insulin mimetic properties in mice [2]. However, the expectation initially arisen by the novel adipokine was soon blunted when the authors had to retract their paper due to the lack of reproducibility of the hypoglycemic properties [5]. Visfatin was found identical to pre-B cell colony-enhancing factor (PBEF), a previously described cytokine promoting maturation on early B-lineage precursor cells [6].

The term visfatin refers to visceral fat, since it was initially suggested that visfatin was mainly produced in visceral fat compared to subcutaneous fat in both mice and humans [2]. Nevertheless, other groups have later reported similar visfatin levels in human subcutaneous and visceral fat tissue [7, 8]. Importantly, visfatin is also found in other fat depots such as perivascular and epicardial fat [9, 10], where it might exert a paracrine cardiovascular effect as will be further discussed. Within the adipose tissue, visfatin is not only synthesized and released by adipocytes but also by inflammatory cells, like activated macrophages, whose infiltration in AT is markedly increased in relation with obesity [11].

Visfatin displays intrinsic enzymatic activity as a nicotinamide phosphoribosyltransferase (Nampt), as reported in 2002 by Rongvaux et al. [12]. In mammals, intracellular Nampt catalyzes the rate-limiting step in the salvage pathway leading to the synthesis of nicotinamide adenine dinucleotide (NAD^+), an essential coenzyme in multiple cellular redox reactions [13, 14]. More specifically, Nampt synthesizes nicotinamide mononucleotide (NMN) and inorganic pyrophosphate by condensing nicotinamide and 5-pyrophosphoribosyl-1-pyrophosphate (PRPP). In a second step, NMN is transformed into NAD by nicotinamide mononucleotide adenylyltransferase (Nmnat) [14].

In mammals, two isoforms of Nampt have been described. Intracellular Nampt (iNampt), which plays a central role in maintaining the activity of NAD-dependent enzymes, is implicated in the regulation of cellular metabolism in response to nutrient availability, maturation and cell survival [14–17]. On the contrary, the other isoform, extracellular Nampt (eNampt), is secreted by different cell types [18]. In this review, we will mainly focus on eNampt as this isoform may represent a mediator of interorgan crosstalk.

In the current literature, we find indistinctly the terms visfatin/Nampt/PBEF to refer to this adipokine. According to the HUGO Gene Nomenclature Committee (HGNC) and the Mouse Genomic Nomenclature Committee (MGNC) the official nomenclature of the gene and the protein is Nampt [19]. However, since the Nampt activity is not always mediating visfatin/Nampt effects, we will employ visfatin/Nampt throughout this review.

In the last years, visfatin/Nampt has indeed arisen as a multifaceted and ubiquitously expressed molecule that exerts multiple biological actions beyond the adipose tissue [20, 21]. Indeed, among adipocytes, visfatin/Nampt is expressed in a wide range of cell types from the immune system, chondrocytes, and amniotic epithelium cells among others [11, 22, 23]. In 1994, Samal et al. initially provided qualitative evidence for visfatin expression in lysates from human heart, pancreas, liver, and skeletal muscle at mRNA levels [6]. More recently, visfatin protein expression has been reported in human myoblasts [24] and human hepatocytes, from which visfatin is actively secreted [25]. Moreover, visfatin expression in liver and skeletal muscle has been further confirmed by other studies [26, 27], raising the possibility that these organs could be potential sources of visfatin plasma levels and thus contributing to metabolic interorgan crosstalk.

3. Circulating Visfatin/Nampt as a Biomarker of Inflammation, and CV Disease

3.1. Obesity, Type 2 Diabetes and the Metabolic Syndrome. Obesity and type 2 diabetes represent two independent risk factors for inflammation-related atherothrombotic diseases. In the context of metabolic diseases, most studies have reported elevated circulating levels of visfatin/Nampt in different clinical conditions, such as obesity, type 2 diabetes mellitus, and the metabolic syndrome which represent independent risk factors for inflammation-related atherothrombotic diseases [28, 29]. Nevertheless, as reviewed by others, there are conflicting results in different reports studying the variation of visfatin/Nampt levels in these disease states, where circulating visfatin/Nampt levels have been found unmodified or even lower compared to healthy controls [29, 30]. However, in the last years, several studies have established positive associations between enhanced circulating visfatin/Nampt levels and atherogenic inflammatory diseases, therefore supporting a role for visfatin as a potential biomarker of cardiovascular complications associated to metabolic disorders.

In the last years, visfatin/Nampt has been proposed as a marker of endothelial dysfunction, an initial and crucial step in the progression of the atherosclerotic process [31]. In type 2 diabetic patients, it has been reported a strong correlation between visfatin/Nampt levels and impaired vascular endothelial function determined as brachial artery flow-mediated dilation (FMD) and creatinine clearance [32]. Although in this latter work, no association was found between inflammatory markers, such as high-sensitivity C-reactive protein (hsCRP) and fibrinogen, neither with atherosclerosis, as evaluated by assessing intima-media thickness (IMT), and so the authors concluded that visfatin/Nampt is more likely not a surrogate marker of inflammation nor atherosclerosis. Additionally, Uslu et al. have found that type 2 diabetic patients display enhanced circulating visfatin levels which positively correlate with enhanced levels of the marker of endothelial dysfunction homocystein. Surprisingly, this work could not find a positive correlation between visfatin levels in type 2 diabetic patients and asymmetric dimethylarginine (ADMA), the major endogenous inhibitor of the endothelial nitric oxide synthase (eNOS) [33]. Homocysteine has been proposed as an intermediate factor in the relationship between endothelial dysfunction and renal function [34].

In both patients with the metabolic syndrome and type 2 diabetes, it has been suggested that enhanced visfatin/Nampt

levels are associated with advanced carotid atherosclerosis, estimated as the intima-media thickness (IMT) in this artery [35, 38]. Indeed, Kadoglou et al. have proposed visfatin/Nampt circulating levels as a marker of advanced carotid atherosclerosis for type 2 diabetic patients. In morbid obese patients, epicardial fat thickness as assessed by echocardiography was related to enhanced visfatin/Nampt and plasminogen activator inhibitor-1 (PAI-1) levels as well as visceral obesity [36].

On the contrary, some authors claim that high visfatin/Nampt levels, instead of depicting changes in the atherosclerotic process are more likely reflecting changes in the systemic inflammation in patients with renal and cardiovascular disease. Thus, in patients undergoing hemodialysis, visfatin/Nampt levels are associated with high-sensitivity C-reactive protein (hsCRP), considered one of the most powerful predictors of atherosclerosis and vascular death, but not with other parameters of atherosclerosis such as ADMA levels, aortic pulse wave, brachial pressure index in the ankle, or the percentage of calcification in the abdominal aortic wall [45]. In this line, Visfatin/Nampt expression is upregulated in circulating blood monocytes from obese type 2 diabetic patients compared to nondiabetic obese patients [50], indicating that enhanced visfatin/Nampt expression may be related to type 2 diabetes rather than obesity. On the contrary, Oki et al. [51] have reported that serum visfatin/Nampt levels positively correlate with inflammatory markers, independently of the insulin resistance state. Hence, the significance of visfatin/Nampt levels variations in metabolic diseases remains to be more accurately addressed. However, it seems clear that visfatin/Nampt levels are positively associated to a series of inflammatory conditions, independently of other potential metabolic implications. Thus, serum visfatin/Nampt levels have been positively correlated with circulating inflammatory markers, such as IL-6, CRP, and MCP-1 [40, 45, 51].

3.2. Chronic Kidney Disease. Diabetes is the main risk factor for the development and progression of chronic kidney disease (CKD) [52, 53]. In patients with CKD, visfatin/Nampt levels positively correlate with soluble markers of endothelial dysfunction such as vascular, intercellular, and melanoma cells adhesion molecule-1 (VCAM-1, ICAM-1, and MCAM-1, resp.) [43, 44]. The relation between visfatin/Nampt levels and endothelial function in CKD is not merely descriptive, but may also have a functional impact, since visfatin/Nampt levels negatively correlate with endothelial function estimated as flow-mediated dilation (FMD) in brachial artery or glomerular filtration rate (GFR) [41, 42]. Indeed, the improvement of endothelial function after kidney transplantation correlates with a reduction in circulating visfatin/Nampt levels [46].

3.3. Polycystic Ovary Syndrome. Visfatin/Nampt has been recently proposed as a candidate in the pathogenesis of endothelial dysfunction in polycystic ovary syndrome (PCOS), the main androgen excess disorder in women [39]. PCOS is characterized by obesity, insulin resistance, and endothelial dysfunction. It has been previously proposed that PCOS is associated with a dysfunctional secretion pattern of adipokines. In this regard, enhanced visfatin/Nampt levels and expression in AT have been previously reported in PCOS [8, 54]. Interestingly, enhanced visfatin/Nampt levels have been associated with reduced brachial artery flow-mediated vasodilatation in patients with PCOS and significantly predicted impaired endothelial function [39]. Similar to what described for type 2 diabetic patients by Takebayashi et al. visfatin/Nampt levels did not correlate with inflammatory markers as hsCRP or carotid IMT.

3.4. Preeclampsia. Preeclampsia is a hypertensive disorder in pregnancy and is associated with increased cardiovascular disease (CVD) risk later in life and is a major cause of maternal and fetal mortality and morbidity in pregnancy [55]. Preeclampsia shares cardiovascular risk factors with the metabolic syndrome such as subclinical inflammation, insulin resistance, and obesity. Since visfatin/Nampt levels are enhanced in all these pathological conditions, several groups have aimed to determine if visfatin/Nampt may contribute to preeclampsia. Thus, different groups have reported enhanced maternal visfatin/Nampt serum levels in preeclamptic patients compared to their matched pregnant controls [37, 56, 57]. Furthermore, Fasshauer et al. also found positive correlations between visfatin/Nampt serum concentrations and age, blood pressure, creatinine, free fatty acids (FFA), and the inflammatory markers IL-6 and CRP [37]. On the contrary, other groups suggest that decreased expression of visfatin, as a proangiogenic factor, may be associated with the pathogenesis of preeclampsia. Thus, Hu et al. have reported that maternal plasma visfatin/Nampt levels were downregulated in women with mild preeclampsia and to a higher extent in women with severe preeclampsia [58]. In this line, it has been recently reported by Kim et al. that in placental biopsies visfatin/Nampt expression is reduced compared to the visfatin/Nampt levels in placentas from normotensive women [59]. Reduced placental perfusion is an initial event in preeclampsia, initiating a sequence of events leading to altered vascular function and hypertension. Thus, decreased placental perfusion-induced endothelial dysfunction has been suggested as the cause leading to progressive vasoconstriction, hyperresponsiveness, and impaired relaxation of uterine arcuate arteries [60]. However, it has been recently demonstrated that impaired placental perfusion is not the mechanism responsible for visfatin/Nampt induction in preeclampsia [56]. Ognjanovic et al. have demonstrated that visfatin/Nampt is also locally expressed in foetal membranes and secreted from amniotic epithelium cells in the human placenta and that its secretion is increased in response to proinflammatory stimuli such as lipopolysaccharide (LPS) and IL-1β [23]. On the other hand, exogenous administration of visfatin/Nampt to human foetal membranes leads to an increase in inflammatory cytokines such as IL-1β, TNF-α, and IL-6 [23, 61]. Visfatin/Nampt also prevented actinomycin D-induced apoptosis, therefore suggesting a protective role for visfatin/Nampt in preventing apoptosis induced by chronic distension, labor, or infection in the placenta [61]. Thus, whether enhanced visfatin/Nampt

levels is a deleterious factor promoting endothelial dysfunction in placenta and leading to preeclampsia or, if on the contrary, is a beneficial factor preventing apoptosis under inflammatory conditions and promoting angiogenesis in the placenta, or if it is just a biomarker, is still an open question [62].

3.5. Acute Coronary Syndromes. Enhanced circulating visfatin/Nampt levels have been proposed to correlate with the development of atherosclerotic plaques, and thus visfatin/Nampt has been proposed as a marker of atherosclerosis by several groups [35, 40]. It has been described that in coronary artery diseases (CADs), and more specifically in acute coronary syndrome, circulating inflammatory markers such as IL-6 and MCP-1 positively correlate with visfatin/Nampt levels [40]. In patients with coronary artery disease (CAD) and acute myocardial infarction, a positive association between visfatin/Nampt expression and unstable atherosclerotic lesions has been established [63]. Interestingly, another positive correlation has been established between visfatin/Nampt expression in both pericardiac and periaortic fat and coronary artery atherosclerosis [64], which underpins that not only circulating but also perivascular visfatin/Nampt may exert an important paracrine effect promoting the development of atherosclerotic lesions. A higher expression of visfatin/Nampt has been found not only in the smooth muscle within atherosclerotic plaques [64] but also in foam cells of unstable plaques from patients that suffered an acute myocardial infarction [63]. Therefore, it has been proposed that visfatin/Nampt localization within the lesions may be related to atherosclerotic plaque destabilization [63]. In this line, Yu et al. have recently described that visfatin/Nampt levels are upregulated in the circulation of patients suffering a ST-segment elevation myocardial infarction (STEMI), the most acute form of MI. Furthermore, the authors found enhanced visfatin/Nampt expression in macrophages present in the coronary rupture plaques. These results support the hypothesis that leukocytes-derived visfatin/Nampt may play a role in the pathogenesis of coronary plaques rupture. Importantly, this group has previously proposed that enhanced circulating visfatin/Nampt levels associate with the occlusion of infarct-related artery (IRA) and circulating hsCRP levels [48] and associate with the degree of myocardial damage [49]. In the light of the bulk of evidence presented herein, visfatin/Nampt arises as a relevant molecule promoting plaque destabilization and rupture in different types of acute coronary syndromes.

3.6. Cerebrovascular Diseases. Visfatin/Nampt has also been proposed to play a role in cerebrovascular diseases [65]. Thus, Lu et al. have demonstrated that plasma visfatin/Nampt was increased in a Chinese population of patients with ischemic stroke and correlated with hsCRP levels in these patients [47]. In this line, it has been recently proposed that aging decreases intracellular visfatin/Nampt expression in the murine brain, in parallel to increased visfatin/Nampt circulating plasma levels, and may contribute to endothelial dysfunction in the brain [66].

3.7. Non-metabolic Chronic Inflammatory Diseases. In the context of other non-metabolic chronic inflammatory diseases characterized by systemic inflammation enhanced visfatin/Nampt levels have been additionally reported. Thus, visfatin/Nampt circulating levels are enhanced in osteoarthritis [67, 68], Crohn's disease, and ulcerative colitis [20, 69]. Moreover, in patients with acute lung injury (ALI), visfatin/Nampt is currently considered a biomarker of this disease [70]. Visfatin/Nampt also seems to play a role in several types of infections like sepsis [71] or intrauterine infection (chorioamnionitis) [61, 72]. Additionally, visfatin/Nampt also may have a crucial role in autoimmune inflammatory diseases since enhanced visfatin/Nampt levels have been reported in psoriasis [53], rheumatoid arthritis (RA) [43], and inflammatory bowel disease (IBD). Interestingly, visfatin/Nampt has been identified as a novel "universal marker of chronic inflammation" whose RNA is upregulated in the mononuclear cells from peripheral blood from patients suffering any of these three chronic inflammatory diseases, which allows to discriminate patients with chronic inflammation and healthy controls mRNA expression levels [73].

In RA, it has been proposed that visfatin/Nampt can be a potent mediator of inflammation [67]. However, Senolt et al. provided evidence for a positive correlation between the levels of serum visfatin/Nampt and total number of B cells in RA, more than activity of the disease [74]. Indeed both visfatin/Nampt levels and B cell number were reduced after treatment with rituximab.

In patients with psoriasis, it has been speculated that visfatin enhanced levels may lead to atherosclerosis and vascular complications as frequent comorbidities found in this disease [75]. Analogously, it has been suggested that the proinflammatory and matrix-degrading activities of visfatin reported in the context of RA [67] may contribute to the enhanced risk for CVD in these patients [76]. However, the relation between enhanced visfatin levels and CVD in these inflammatory diseases needs to be further explored.

In the light of all these studies, we can conclude that visfatin/Nampt is upregulated and may play a role in both atherosclerosis and endothelial dysfunction (Table 1). But beyond being a marker for CVD, growing evidence supports a role for visfatin/Nampt as direct factor triggering vascular injury. Here, we will try to summarize the most relevant previous and recent evidences about visfatin/Nampt direct actions on the cardiovascular system (Figure 1). More specifically, we will focus on visfatin/Nampt effect on proliferation, and angiogenesis of vascular cells, visfatin/Nampt inflammatory effects, extracellular matrix degradation and finally apoptosis/cell survival.

4. Direct Cardiovascular Actions of Visfatin/Nampt

4.1. Cell Proliferation and Angiogenesis. Proliferation of vascular smooth muscle cell (VSMC) is a hallmark of the development of atherosclerotic lesions. Importantly, perivascular visfatin/Nampt can act as a growth factor in vascular smooth muscle cells, promoting cell proliferation in rat aortic

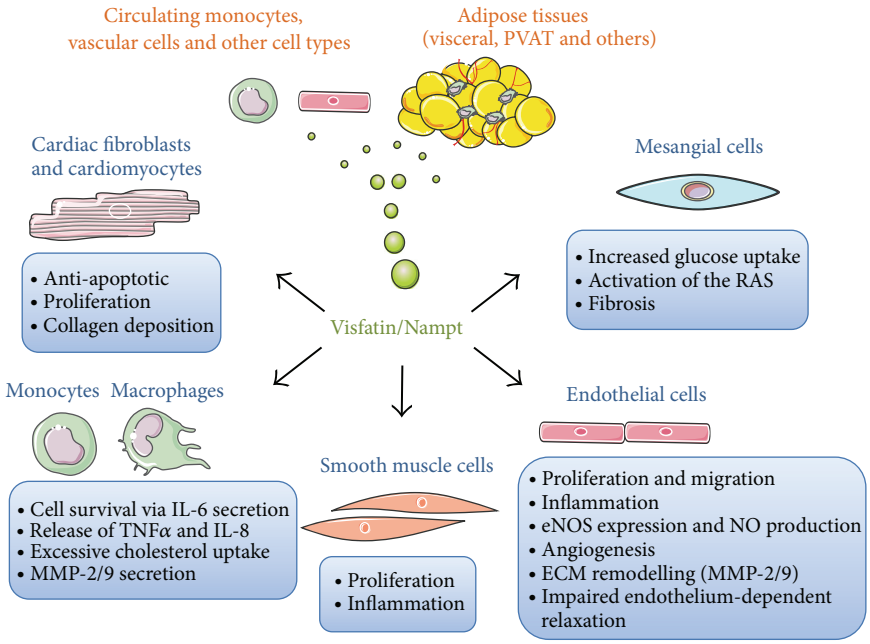

Figure 1: Diagram summarizing the main reported direct actions of visfatin/Nampt in cells in the cardiovascular system, namely, cardiac fibroblast and cardiomyocytes, mesangial cells, monocytes and macrophages, smooth muscle cells, and endothelial cells. eNOS: endothelial nitric oxide synthase, IL-8: interleukin-8, MMP-2/9: matrix metalloproteinase-2/9, NO: nitric oxide, RAS: renin-angiotensin system, and TNF-α: tumor necrosis factor-α.

Table 1: Summary of the main correlations reported between visfatin circulating levels and CV disease in human subjects.

Clinical condition	Main correlations found	References
↔TD2	+Endothelial dysfunction (FMD)	[32]
↑TD2	+Homocystein, ±ADMA	[33]
↑TD2	+Carotid IMT	[35]
↑Morbid obesity	+Epicardial fat thickness, +PAI-1	[36]
↑Preeclampsia	+CRP, +IL-6, +blood pressure, and +free fatty acids	[37]
↑Metabolic syndrome	+IL-6, +MCP-1, and +IMT	[38]
↑Metabolic syndrome	+Blood pressure	[29]
↑PCOS	+Endothelial dysfunction (FMD), ±hsCRP, and ±IMT	[39]
↑CAD	+MCP-1, +IL-6	[40]
↑CKD	−GFR, +TD2, and +endothelial dysfunction (FMD)	[41]
↑CKD	+GFR, +IL-6, +CRP, and +sVCAM-1	[42]
↑CKD	+sVCAM-1, +sICAM-1, and +MCAM	[43, 44]
↑Hemodialysis	±Atherosclerosis, +hsCRP	[45]
↑Renal transplantation	+Endothelial function (FMD)	[46]
↑Ischemic stroke	+hsCRP, −LDLc	[47]
↑STEMI	+Occlusion IRA, +hsCRP, and +myocardial damage	[48, 49]

↔: no change in circulating visfatin levels. ↑: enhanced circulating visfatin levels. ↓: reduced circulating visfatin levels. +: positive correlation reported, −: negative correlation reported, ±: no significant correlation reported. CAD: coronary artery disease, FMD: flow-mediated dilation, GFR: glomerular filtration rate, hsCRP: high-sensitivity C-reactive protein, IL: interleukin, IMT: intima-media thickness, IRA: infarct-related artery, LDLc: low-density lipoprotein-associated cholesterol, MCAM: melanoma cell adhesion molecule, MCP-1: monocyte chemotactic protein-1, PAI-1: plasminogen activator inhibitor-1, PCOS: polycystic ovary syndrome, sICAM: soluble intercellular adhesion molecule, sVCAM: soluble vascular cell adhesion molecule, and TD2: type 2 diabetes mellitus.

smooth muscle cells though Nampt enzymatic activity [10]. Visfatin/Nampt emerges as a player in the development and progression of atherosclerotic lesions by directly promoting smooth muscle cell proliferation.

Aberrant angiogenesis is now considered a feature of the atherogenic process in both coronary and carotid diseases [77]. In this line, it has also been described that visfatin/Nampt can promote endothelial cell proliferation, migration, and capillary tube formation in a concentration-dependent manner in HUVEC [78–81]. These proliferative effects of visfatin/Nampt seem to be mediated, or at least partially mediated, by a master molecule in endothelial proliferation and neovessel formation: vascular endothelial cell growth factor (VEGF) [78]. Thus, visfatin/Nampt upregulates

VEGF synthesis and secretion as well as the expression of the VEGF receptor 2, which has been proposed to mediate the angiogenic actions of VEGF [78, 82]. Besides VEGF, visfatin/Nampt upregulates the production of other pro-angiogenic soluble factors, such as fibroblast growth factor-2 (FGF-2), MCP-1, and IL-6, in endothelial cells [82–84]. Indeed, both MCP-1 and FGF-2 have also been identified as mediators of visfatin/Nampt-induced angiogenesis [82, 83]. Beyond *in vitro* studies, the angiogenic activities of visfatin/Nampt have been demonstrated in *ex vivo* and *in vivo* approaches [78, 79]. Thus, visfatin/Nampt is able to induce the formation of functional neovessels in chick chorioallantoic membrane and mouse Matrigel plug [79]. In this line, it has been described that the injection delivery of a plasmid containing visfatin/Nampt improved limb perfusion in a mouse undergoing unilateral hindlimb ischemia [80].

Furthermore, visfatin/Nampt enhances the expression, protein levels and activity of matrix metalloproteinases, MMP-2/9, which are enzymes promoting angiogenesis through the degradation of the extracellular matrix, while it decreases the levels of their tissue inhibitors, TIMP-1 and -2, respectively [78]. The impact of visfatin on matrix remodelling will be discussed in more detail below.

The forementioned proangiogenic properties of visfatin/Nampt make of this adipokine a potential therapeutic candidate in diseases where neovascularisation is necessary to overcome restricted blood flow supply such as ischemic stroke or macrovascular peripheral limb ischemia. Very recently, Kim et al. have proposed that visfatin/Nampt proangiogenic effects are mediated by the induction of thromboxane synthase (TSA) with the subsequent TXA_2 release by endothelial cells (HMECs and HUVECs) [85]. The authors more specifically demonstrate that visfatin/Nampt upregulation of IL-8 via TXA_2 is the responsible mechanism for visfatin/Nampt-induced angiogenesis [85].

It has been recently proposed that visfatin/Nampt exerts a neuroprotective role for photothrombosis-induced ischemia with both *in vitro* and *in vivo* approaches/models [86]. Thus, heterozygous visfatin/Nampt knockout mice ($Pbef^{+/-}$) display larger size of ischemic lesions than wild-type mice [86]. On the other hand, visfatin/Nampt may contribute to exacerbated angiogenesis leading to ischemic heart disease, diabetes, or atherosclerosis and may therefore arise as a novel pharmacological target for treating such conditions.

Visfatin/Nampt proangiogenic actions also promote tumor growth. Thus, circulating visfatin/Nampt levels are enhanced in several malignancies such as endometrial [87], gastric, or colorectal cancers [88, 89]. Inhibition of NAD enzymatic activity is an emerging therapeutic strategy for cancer treatment [25, 90]. In this context, two Nampt inhibitors, APO866 (or FK866) and CSH-828, are being used in clinical trials as NAD-depleting anticancer agents [91, 92].

Visfatin/Nampt proliferative effects are not restricted to the vascular wall, since visfatin/Nampt also promotes proliferation in rat cardiac fibroblasts. The proliferation of cardiac fibroblasts together with an excessive accumulation of extracellular matrix represents the basis of myocardial fibrosis. *In vitro* stimulation of cardiac fibroblasts by visfatin/Nampt requires the activation of Akt/PKB and the MAPKs p38 and JNK, but not ERK 1/2 [93]. Pillai et al. have described that cardiac-specific overexpressing Nampt transgenic mice show increased cardiac fibrosis. Moreover, both recombinant and adenoviral Nampt delivery increased proliferation *in vitro* in rat cardiomyocytes, which was inhibited by Nampt-blocking antibody [94]. Taking into account that visfatin/Nampt is expressed in periadventitial and apical epicardial adipose tissues [64], and with the recent evidence that it is also secreted by rat cardiomyocytes [94], not only circulating visfatin/Nampt but also visfatin/Nampt locally produced in the CV system could play a detrimental role in promoting myocardial fibrosis and remodeling.

4.2. Inflammation. Growing scientific evidence supports that visfatin/Nampt can directly promote vascular inflammation by activating different cell types including endothelial cells and vascular smooth muscle cells. Moreover, visfatin/Nampt can also contribute to vascular inflammation through its immunomodulatory properties on immune cells [69]. Thus, visfatin/Nampt can exert direct actions on monocytes. Hence, visfatin/Nampt promotes the synthesis and release of pro-inflammatory cytokines, such as tumor necrosis factor-(TNF-) α and IL-8, by peripheral mononuclear cells [63]. Additionally, visfatin/Nampt promotes macrophage survival [95], which may help perpetuating vascular inflammation.

In cultured human vascular smooth muscle cells, our group demonstrated for the first time that visfatin/Nampt could directly exert inflammatory effects. Thus, exogenous administration of visfatin/Nampt activates ERK 1/2 and NF-κB, resulting in enhanced expression of the inducible nitric oxide synthase (iNOS) [96]. iNOS is a pro-inflammatory enzyme contributing to dysregulated NO production and subsequent peroxynitrite formation. Thus, iNOS induction plays a key role in endothelial dysfunction and vascular injury in diabetes-related vascular complications [104].

Several reports suggest that visfatin/Nampt can additionally promote endothelial activation. In HUVEC, visfatin/Nampt activates the inflammation-related transcription factor NF-κB [82, 84, 97] and promotes the expression of cell adhesion molecules, such as ICAM-1, VCAM-1, or E-selectin [84, 97], as key molecules implicated in leukocyte recruitment and early proatherosclerotic events [105]. Visfatin/Nampt further upregulates the release of several cytokines and chemokines by endothelial cells, including IL-6, IL-8, or MCP-1 and its putative receptor CCR2 [84, 98], and thus promotes the adhesion of human THP-1 monocytes to endothelial cells [98]. The MAPK ERK 1/2 and p38, as well as PI3K and the intracellular generation of reactive oxygen species, have been proposed as responsible molecules in endothelial cell inflammation induced by visfatin/Nampt [97, 98].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a superoxide anion-generating and proinflammatory enzyme closely associated to endothelial dysfunction [106]. Boini et al. have proposed that visfatin/Nampt induces the activation of NADPH oxidase, increasing superoxide anion production resulting in the disruption of microtubular networks in GECs and increased glomerular permeability

Table 2: Direct vascular proinflammatory actions of visfatin and their proposed underlying mechanisms.

Target cell type	Cellular actions	Mechanism of action	References
Smooth muscle cells	ERK 1/2-NF-κB activation iNOS induction	Nampt activity, insulin receptor independent	[96]
Endothelial cells	NF-κB activation	Insulin receptor independent	[82, 84, 97]
	IL-6, IL-8 release	N.D.	[84, 98]
	MCP-1 release	N.D.	[82, 98]
	CCR2 expression	N.D.	[82]
	ICAM-1, VCAM-1, and E-selectin induction	Insulin receptor independent	[84, 97]
	MMP-2 and MMP-9 activation	N.D.	[99]
	NADPH oxidase activation	Nampt activity (HUVEC)	[100]
		Lipid rafts (BCAEC and GEC)	[101, 102]
Monocytes	Binding to endothelial cells	N.D.	[84]
	MMP-9 activation	Insulin receptor independent	[63]
Macrophages	Cell survival	STAT3/IL-6 release Nampt- and insulin receptor-independent	[95]
	Lipid accumulation	SR-A, CD36 activation	[103]
Peripheral blood mononuclear cells	Cytokine release (IL-8, TNF-α)	Insulin receptor	[63]

N.D.: not determined. BCAEC: bovine coronary artery endothelial cells, CCR2: chemokine receptor type 2, CD36: cluster of differentiation 36, ERK 1/2: extracellular signal-regulated kinase 1/2, GEC: glomerular endotelial cells, HUVEC: human umbilical vein endothelial cells, ICAM-1: intercellular adhesion molecule-1, IL: interleukin, MCP-1: monocyte chemotactic protein-1, MMP: matrix metalloproteinase, NF-κB: nuclear factor-κB, iNOS: inducible nitric oxide synthase, SR-A: scavenger receptor-A, STAT3: signal transducer and activator of transcription 3, TNF-α: tumor necrosis factor-α, and VCAM-1: vascular cell adhesion molecule-1.

[101]. Analogously, visfatin/Nampt triggers NADPH oxidase subunits assembly and activation through lipid rafts in bovine coronary artery endothelial cells [107], thereby contributing to endothelial dysfunction in coronary circulation. Alternative to NADPH activation, it has been suggested that visfatin/Nampt deleterious effect on diabetic nephropathy may be due in part to the activation of intrarenal renin-angiotensin system [108]. Inappropriate activation of the renin-angiotensin system is known to be implicated in CV diseases related to CKD. Thus, it has been reported that visfatin/Nampt leads to activation of the renin-angiotensin system by upregulating the expression levels of renin, angiotensinogen and angiotensin I and II in a dose-dependent manner in cultured rat mesangial cells [108].

In another clinical condition such as myocardial infarction, and more specifically in STEMI, visfatin/Nampt circulating levels and intracellular expression in macrophages and monocytes are enhanced [109]. Thus, Chiu et al. conclude that male patients with STEMI show increased visfatin/Nampt expression in leukocytes, which may aggravate the development of instability of atherosclerotic plaques [109]. Interestingly, Zhou et al. have recently reported that visfatin/Nampt promotes lipid accumulation mainly through excessive cholesterol uptake in RAW264.7 macrophages and in peritoneal macrophages isolated from ApoE knockout mice and accelerates the process of atherosclerosis mainly through modulating the expression of the macrophage scavenger receptor class A (SR-A) and CD36 [103] (Table 2).

4.3. Extracellular Matrix. Adya et al. reported that on one hand visfatin/Nampt upregulated the expression and activity of the matrix metalloproteinases (MMP)-2/9 and downregulated the expression of the inhibitors of these MMPs such as TIMP-1 and -2, in monocytes and endothelial cells [78]. MMP-2 and -9 are pivotal enzymes in the degradation of extracellular matrix (ECM), thus facilitating atherosclerotic plaque vulnerability [110].

Cardiac fibrosis is the consequence of excessive accumulation of ECM. As we have previously mentioned, visfatin/Nampt may also contribute to cardiac fibrosis. Thus, visfatin/Nampt not only promotes proliferation in rat cardiac fibroblasts but also upregulates the mRNA expression and protein levels of procollagen I and II in this cell type, leading to enhanced types I and III collagen release [93].

Visfatin/Nampt has also been proposed to promote renal fibrosis, a typical feature of CKD. In the context of CKD, visfatin/Nampt promoted fibrosis in rat mesangial cells by upregulating the synthesis of key profibrotic molecules such as transforming growth factor-β1 (TGF-β1), PAI-1 and type I collagen, thus increasing the risk for CVD [111]. These results provide evidence for visfatin/Nampt as a novel factor promoting cardiac and renal fibrosis whether directly upregulating procollagen and MMPs secretion or indirectly contributing to fibrosis by upregulating the secretion of other classic fibrosis mediators such as TGF-β or PAI-1.

4.4. Cell Survival/Apoptosis. There are conflicting results regarding the role of visfatin/Nampt in the regulation of cell survival and apoptosis. On one hand, intracellular visfatin/Nampt plays a central role in maintaining the activity of NAD-dependent enzymes regulating cellular metabolism [14–17]. On the other hand, extracellular visfatin/Nampt

exerts antiapoptotic effects and promotes cell survival in several cardiovascular cells. Thus, it has been demonstrated that visfatin/Nampt attenuates cell apoptosis induced by hydrogen peroxide in human endothelial cells [99], rat VSMC [10], and in both rat cardiac fibroblasts [93] and cardiomyocytes [94].

Several works have suggested that visfatin/Nampt could exert direct cardioprotective effects. In cultured murine cardiomyocytes undergoing hypoxia and reoxygenation, visfatin/Nampt administered at the time of reoxygenation, triggered delayed cell death, due at least in part to a delayed opening of the mitochondrial permeability transition pore (mPTP) by oxidative stress. The mPTP is a nonspecific mitochondrial channel, whose opening in the first minutes of reperfusion is a critical determinant of cardiomyocyte death [112]. Cardiac specific Nampt overexpression in mice has been proposed to prevent myocardial injury in response to myocardial ischemia and reperfusion [113]. Intravenous administration of visfatin/Nampt at the time of reperfusion reduced the infarct size in a mouse model of ischemia-reperfusion [114]. On the contrary, Pillai et al. have reported that both exogenous visfatin/Nampt and visfatin/Nampt overexpressions promote cardiac hypertrophy and adverse ventricular remodeling [94].

On the other hand, intracellular Nampt activity is acknowledged to play a central role in cell maturation and survival in smooth muscle cells *in vitro* [16, 17]. Van der Veer et al. have demonstrated that iNampt is crucial in order to induce maturation and prevent premature senescence in human VSMC [16, 17].

Zhang et al. have reported that visfatin/Nampt knockout heterozygous mice ($Pbef^{+/-}$) subjected to photothrombosis, a cerebral ischemia model, displayed more severe brain damage and neuronal degeneration compared to wild-type mice [86]. In a model for cerebral ischemia in rats, Nampt overexpression in brain prevented neuronal death [115]. Therefore, visfatin/Nampt may represent an interesting pharmacological tool to treat both cardiac and cerebral damage derived from ischemia-reperfusion.

Importantly, visfatin/Nampt promotes macrophage survival through a mechanism involving the release of IL-6 [95]. Moreover, visfatin/Nampt also inhibits neutrophile apoptosis in clinical and experimental sepsis [71]. Thus, visfatin/Nampt can help to perpetuating cell-mediated inflammation at the sites of elevated concentrations of this adipocytokine.

4.5. Vascular Tone. Impairment of endothelial vasodilatory responses is one of the earliest markers of vascular disease [116]. To date, there are only scarce and conflicting reports analyzing the direct actions of visfatin/Nampt vascular tone regulation.

In human umbilical vein and coronary artery endothelial cells (HUVEC; HCAEC), extracellular visfatin/Nampt can enhance endothelial nitric oxide synthase (eNOS) expression and activity resulting in enhanced NO production [80]. This *in vitro* study does not support a role for visfatin/Nampt as a biomarker of endothelial dysfunction in clinical conditions such as CKD or type 2 diabetes as previously summarized. Hence, this effect on eNOS and NO production was not considered beneficial by the authors, who associated them to endothelial cell proliferation and angiogenesis considered as two deleterious proatherosclerotic events [80]. Analogously, it has been reported that visfatin, addition to an organ bath triggers the relaxation of noradrenaline (NA) precontracted rat aortic rings [117].

On the contrary, Xia et al. have shown that visfatin/Nampt inhibits the vasorelaxant response to BK in bovine coronary arteries through NADPH oxidase activation by membrane raft clustering [102]. In this line, our group has provided the first evidence for visfatin/Nampt as an active player in endothelial dysfunction in humans. Thus, we propose that besides a novel biomarker in this clinical condition, visfatin/Nampt is an active agent promoting endothelial dysfunction. We have demonstrated that visfatin/Nampt impairs endothelium-dependent relaxations in both rat and human mesenteric microvessels [100]. Importantly, the impairment of endothelium-dependent relaxation exerted by visfatin/Nampt was mediated by NADPH oxidase activation and required Nampt enzymatic activity. Visfatin/Nampt directly enhanced NADPH oxidase activity as determined in HUVEC and rat microvessels. On the contrary, both the Nampt inhibitors, APO866 or the NADPH inhibitor apocynin, prevented visfatin/Nampt-induced impaired endothelial vasorelaxation [100]. In opposition to the relaxation exerted by visfatin/Nampt in noradrenaline precontracted aortic rings described by Yamawaki et al. we did not observe any influence of visfatin/Nampt on NA-induced vasoconstriction in rat mesenteric microvessels [117]. Our results are in line with the activation of NADPH oxidase by visfatin/Nampt in bovine coronary artery endothelial cells reported by Xia et al. Therefore, more in-depth studies using both *in vivo* and *in vitro* approaches are needed to fully understand the capacity of visfatin/Nampt to impair vasodilation.

5. Mechanisms of Action of Visfatin/Nampt in the CV System

5.1. The Insulin Receptor. Visfatin/Nampt was initially proposed by Fukuhara et al. as a potential beneficial tool able to bind and activate the insulin receptor triggering glucose-lowering properties [2]. However, two years later, the authors retracted their paper, and the role of insulin receptor in visfatin/Nampt-mediated actions is still a matter of controversy [5]. In this line, the glucose-lowering actions of visfatin/Nampt have been hardly reproduced. However, in the context of CV diseases it has been proposed that the insulin receptor mediates some of the *in vitro* effects reported for visfatin/Nampt on IL-8 and TNF-α secretion of by human peripheral blood mononuclear cells [63], the upregulation of MMP-9 in THP-1 monocytes [63], or enhanced glucose uptake by rat mesangial cells [118]. On the contrary, a role for insulin receptor has been discarded in a wide range of cell types, including vascular cells and macrophages [10, 95, 97].

5.2. Nampt Activity. Nampt enzymatic activity has been proposed as an alternative mechanism for visfatin/Nampt actions in the CV system. Thus, the pharmacological inhibition of iNampt by APO866 decreased cell survival and promoted

senescence in VSMC [17]. On the contrary, eNampt activity has been proposed to exert proatherogenic effects in smooth muscle cells. Wang et al. have reported that visfatin/Nampt from PVAT can promote smooth muscle cells proliferation through an eNampt-dependent mechanism [10].

Moreover, blockade of the insulin receptor did not affect the reported inflammatory effect of visfatin/Nampt in smooth muscle cells while APO866, the inhibitor of Nampt, completely prevented visfatin/Nampt-induced inflammatory signaling [96]. Additionally, we demonstrated that visfatin/Nampt impaired endothelium-dependent relaxation in rat and human mesenteric microvessels through its intrinsic eNampt activity [100]. Thus, in the presence of the Nampt inhibitor APO866, visfatin/Nampt deleterious effects on the endothelium were prevented. This proinflammatory profile of eNampt activity is further supported by the observation that inflammatory cytokine secretion by leukocytes is regulated by eNampt activity [119].

On the other hand, it has been proposed that eNampt may enhance intracellular NAD^+ levels conferring a higher resistance of cardiomyocytes to oxidative stress in ischemia-reperfusion [113]. On the contrary, a deleterious role has also been proposed for eNampt activity in myocardial infarction. Thus, Montecucco et al. have very recently shown that treatment with of the Nampt inhibitor APO866 reduced myocardial infarct size, neutrophil infiltration, and reactive oxygen species (ROS) generation within infarcted hearts *in vivo* in a mouse model of ischemia and reperfusion [120]. Moreover, exogenous administration of APO866 prevented CXCL2-induced neutrophil recruitment and thereby reduced neutrophil-mediated tissue injury in mice *in vitro* [120]. Thus, the pharmacological inhibition of Nampt emerges as an effective therapeutic tool to reduce smooth muscle cell proliferation, inflammation, endothelial dysfunction, and oxidative stress-mediated tissue damage in myocardial infarction.

In this line, some of the vascular and renal actions of visfatin/Nampt have also been attributed to the product of Nampt activity, NMN [10, 96, 100, 115]. NMN, the product of Nampt, exerted growth-factor-like activity in rat smooth muscle cells, through the activation of MAPKs ERK 1/2 and p38 [10]. Analogously, our group demonstrated that NMN, the product of Nampt is able to reproduce the activation of the ERK 1/2-NF-κB signaling pathway induced by visfatin/Nampt, leading to iNOS upregulation in human VSMC [96]. Moreover, NMN was able to both impair endothelium-dependent relaxation in rat and human microvessels. Analogously, NMN upregulated NADPH oxidase activity in rat microvessels and HUVEC mimicking the effects exerted by visfatin/Nampt. However, NMN does not always reproduce visfatin/Nampt actions [95], indicating that visfatin/Nampt may have other additional mechanisms of action.

Interestingly, it has been recently proposed that visfatin/Nampt can induce prostaglandin E(2) (PGE(2)) synthesis in chondrocytes by both Nampt activity and insulin receptor activation [121]. Whether this dual mechanism of action can be extrapolated to some of the detrimental effects of visfatin/Nampt in the CV system needs to be further explored.

5.3. Other Mechanisms. As stated before, PBEF was first identified as an immunomodulatory cytokine able to synergize with interleukin 7 (IL-7) and stem cell factor (SCF) to promote pre-B cell colony formation [6]. A role for insulin receptor in visfatin/Nampt-mediated cytokine release has been discarded [69]. Analogously, the upregulation of the CAMs induced by visfatin/Nampt human endothelial cells did not depend on insulin receptor activation [84, 97]. In this regard, Li et al. reported that visfatin/Nampt-induced survival of macrophages under endoplasmic reticulum stress was not mediated by Nampt activity nor through the insulin receptor [95]. Therefore, Li and other authors have proposed that the effects of visfatin/Nampt on monocytes and endothelial cells activation may be mediated by a yet unidentified receptor. Interestingly, Xia et al. have proposed that visfatin/Nampt may act as a ligand of inflammatory or death receptor [102]. Thus, they propose that visfatin/Nampt effects on the impairment of endothelium-dependent vasodilation in bovine coronary arteries may be mediated through a death receptor, which leads to the formation of the signalling platforms called membrane rafts (MRs) [102]. This opinion is supported by the observation that visfatin/Nampt enhanced acid sphingomyelinase (ASMase) activity. ASMase is an enzyme that promotes the clustering of membrane rafts (MR) resulting in MR-associated transmembrane signaling. This ASMase is translocated onto the plasma membrane via membrane proximal lysosome trafficking and fusion upon stimulation of death receptors [122]. Visfatin/Nampt pharmacological or genetic silencing prevented MR clustering and consequent formation of the MR signalling platforms or signalosomes in coronary arterial ECs. Both the pharmacological inhibition of ASMase with amitriptyline and genetic silencing with siRNA almost completely abolished visfatin/Nampt effects on endothelial injury. Moreover, the authors claim that the death receptor agonist FasL mimicked the effect exerted by visfatin/Nampt on endothelium-dependent vasodilation [102]. However, this hypothesis does not match with the proposed antiapoptotic effects of visfatin/Nampt in cardiomyocytes. Furthermore, it has been recently reported that visfatin/Nampt has a protective effect on oxygen peroxide-induced myocardial apoptosis, not by inhibiting the death receptor-dependent apoptotic pathways, but most likely acting on p53-mediated, mitochondria-dependent apoptotic signaling and via involvement of the AMPK signaling pathway in H9c2 cardiomyocytes [123].

6. Sources of Visfatin/Nampt with Potential CV Impact

Visceral adipose tissue was initially proposed as the main source of circulating visfatin/Nampt in humans [2]. Visfatin/Nampt plasma levels were proposed to correlate with the amount of visceral fat in humans as determined by computerized tomography [2]. Nevertheless, further evidence has demonstrated that visfatin/Nampt is expressed in similar levels in human subcutaneous AT [7, 8]. As previously mentioned, visfatin/Nampt is found in perivascular and epicardial fat and can therefore act in a paracrine manner on the CV

system [9, 10]. Indeed, epicardial AT explants virtually release the same levels as upper abdominal SAT [9]. Epicardial fat (EF) is now considered a true kind of visceral adipose tissue (VAT) depot surrounding the heart. The impact of epicardial fat remains relatively unstudied and is now under intensive investigation in order to explore its contribution to CVD due to its ability to synthesize and release several inflammatory adipokines. Indeed, epicardial fat has been proposed as a marker of visceral adiposity [124] and as an indicator of cardiovascular risk [125].

As we have previously mentioned, macrophages represent a major source of visfatin/Nampt in AT [11]. In line with the proposed inflammatory properties of visfatin/Nampt, other circulating cytokine-producing cell types have been described as sources of visfatin/Nampt, namely, activated lymphocytes, monocytes, or neutrophiles [6, 63, 71].

Therefore, it is not striking that visfatin/Nampt expression has been reported in macrophages from atherosclerotic plaques [35, 63, 109]. However, besides being expressed in macrophages and foam cells, visfatin/Nampt is also found in different cell types present in the vascular wall. Thus, visfatin expression has been reported in both coronary and aortic VSMCs [64]. A single report by Lovren et al. has proposed the human endothelial cells (HUVECs and HCAECs) as an additional source for visfatin/Nampt within the vascular wall [80]. In this line, visfatin mRNA expression has been reported in endothelial cells [126].

These observations suggest that, besides cells of circulating origin, other cell types from the atherosclerotic lesion can represent an additional source of this adipocytokine, which may then reach high local concentrations within the vascular wall.

Cardiomyocytes have very recently been discovered as a visfatin/Nampt-secreting cell type [94]. Indeed, visfatin/Nampt secretion by cardiomyocytes is enhanced in response to stress and has been proposed to induce cardiac hypertrophy and fibrosis through the activation of JNK1, p38, and ERK. These results suggest that visfatin/Nampt is a positive regulator of cardiac hypertrophy and adverse ventricular remodeling [94]. Therefore, although VAT may be the main source contributing to circulating levels of visfatin/Nampt, we cannot ignore the impact of visfatin/Nampt from other adipose depots such as PVAT or EF that can act in a paracrine manner in the CV system. Moreover, visfatin/Nampt from activated immune cells, prone to be recruited into the vascular wall, or locally synthesized visfatin/Nampt by cardiomyocytes or vascular cells may also contribute in an autocrine manner to the reported impact of visfatin/Nampt in the CV system.

7. Conclusions

In 2005, visfatin/Nampt was identified as a novel adipocytokine with suggested beneficial effects in the context of metabolic disorders, as an insulin-mimetic with glucose-lowering properties. The retraction of Fukuhara and colleagues combined with growing evidence supporting a role for visfatin/Nampt in metabolic diseases suddenly shifted the role of this adipokine from friend to foe. However, in light of the recent advances in the field, to the question "visfatin/Nampt: friend or foe?", the answer should be "depending on the scenario."

As discussed in this review, there is growing clinical evidence supporting a role for visfatin as a biomarker or even a predictor of inflammation, and endothelial injury in several metabolic diseases.

Importantly, *in vitro* and *ex vivo* approaches have now provided evidence that visfatin/Nampt may exert direct deleterious actions on the cardiovascular system, including cell proliferation, monocyte/macrophage activation and recruitment, vascular inflammation and remodeling, all of which leading to the development of atherosclerotic lesions. In this context, the pharmacological inhibition of the cardiovascular actions of visfatin might represent a novel therapeutic approach to prevent and treat cardiometabolic complications. In the same line, the inhibition of visfatin proangiogenic actions might also be useful in treating but pathologies implying excessive neovascularization. Indeed, there are currently ongoing clinical trials at an early phase in this direction. In this context, the pharmacological inhibition of the cardiovascular actions on the visfatin/Nampt might represent a novel therapeutic approach to prevent and treat cardiometabolic complications.

On the other hand, beneficial actions have also been proposed for visfatin/Nampt, since the administration of visfatin/Nampt has shown beneficial effects in ischemia-related clinical conditions, such as stroke, peripheral limb ischemia, or myocardial ischemia-reperfusion, where it may become a beneficial pharmacological tool.

Although visfatin has emerged in the last years as a promising pharmacological target in the context of cardiovascular complications, further research is still required to understand the impact of visfatin in different scenarios and clinical conditions and to evaluate the real value of visfatin as a therapeutical target in the cardiovascular system.

Conflict of Interests

The authors declare they have no conflict of interests to disclose.

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References

[1] J. Boucher, B. Masri, D. Daviaud et al., "Apelin, a newly identified adipokine up-regulated by insulin and obesity," *Endocrinology*, vol. 146, no. 4, pp. 1764–1771, 2005.

[2] A. Fukuhara, M. Matsuda, M. Nishizawa et al., "Visfatin: a protein secreted by visceral fat that Mimics the effects of insulin," *Science*, vol. 307, no. 5708, pp. 426–430, 2005.

[3] D. Lamers, S. Famulla, N. Wronkowitz et al., "Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome," *Diabetes*, vol. 60, no. 7, pp. 1917–1925, 2011.

[4] C. M. Steppan, S. T. Bailey, S. Bhat et al., "The hormone resistin links obesity to diabetes," *Nature*, vol. 409, no. 6818, pp. 307–312, 2001.

[5] A. Fukuhara, M. Matsuda, M. Nishizawa et al., "Retraction," *Science*, vol. 318, no. 5850, p. 565, 2007.

[6] B. Samal, Y. Sun, G. Stearns, C. Xie, S. Suggs, and I. McNiece, "Cloning and characterization of the cDNA encoding a novel human pre-B- cell colony-enhancing factor," *Molecular and Cellular Biology*, vol. 14, no. 2, pp. 1431–1437, 1994.

[7] J. Berndt, N. Klöting, S. Kralisch et al., "Plasma visfatin concentrations and fat depot-specific mRNA expression in humans," *Diabetes*, vol. 54, no. 10, pp. 2911–2916, 2005.

[8] B. K. Tan, J. Chen, J. E. Digby, S. D. Keay, C. R. Kennedy, and H. S. Randeva, "Increased visfatin messenger ribonucleic acid and protein levels in adipose tissue and adipocytes in women with polycystic ovary syndrome: parallel increase in plasma visfatin," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 12, pp. 5022–5028, 2006.

[9] K. H. Cheng, C.S. Chu, K.T. Lee et al., "Adipocytokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease," *International Journal of Obesity*, vol. 32, no. 2, pp. 268–274, 2008.

[10] P. Wang, T. Xu, Y. Guan, D. Su, G. Fan, and C. Miao, "Perivascular adipose tissue-derived visfatin is a vascular smooth muscle cell growth factor: role of nicotinamide mononucleotide," *Cardiovascular Research*, vol. 81, no. 2, pp. 370–380, 2009.

[11] C. A. Curat, V. Wegner, C. Sengenès et al., "Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin," *Diabetologia*, vol. 49, no. 4, pp. 744–747, 2006.

[12] A. Rongvaux, R. J. Shea, M. H. Mulks et al., "Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis," *European Journal of Immunology*, vol. 32, no. 11, pp. 3225–3234, 2002.

[13] L. Formentini, F. Moroni, and A. Chiarugi, "Detection and pharmacological modulation of nicotinamide mononucleotide (NMN) in vitro and in vivo," *Biochemical Pharmacology*, vol. 77, no. 10, pp. 1612–1620, 2009.

[14] J. R. Revollo, A. A. Grimm, and S. Imai, "The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells," *Journal of Biological Chemistry*, vol. 279, no. 49, pp. 50754–50763, 2004.

[15] C. Ho, E. van der Veer, O. Akawi, and J. G. Pickering, "SIRT1 markedly extends replicative lifespan if the NAD+ salvage pathway is enhanced," *FEBS Letters*, vol. 583, no. 18, pp. 3081–3085, 2009.

[16] E. van der Veer, Z. Nong, C. O'Neil, B. Urquhart, D. Freeman, and J. G. Pickering, "Pre-B-cell colony-enhancing factor regulates NAD+-dependent protein deacetylase activity and promotes vascular smooth muscle cell maturation," *Circulation Research*, vol. 97, no. 1, pp. 25–34, 2005.

[17] E. van der Veer, C. Ho, C. O'Neil et al., "Extension of human cell lifespan by nicotinamide phosphoribosyltransferase," *Journal of Biological Chemistry*, vol. 282, no. 15, pp. 10841–10845, 2007.

[18] J. R. Revollo, A. Korner, K. F. Mills et al., "Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme," *Cell Metabolism*, vol. 6, no. 5, pp. 363–375, 2007.

[19] S. Imai, "Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases," *Current Pharmaceutical Design*, vol. 15, no. 1, pp. 20–28, 2009.

[20] S. Pilz, H. Mangge, B. Obermayer-Pietsch, and W. März, "Visfatin/pre-B-cell colony-enhancing factor: a protein with various suggested functions," *Journal of Endocrinological Investigation*, vol. 30, no. 2, pp. 138–144, 2007.

[21] J. M. Stephens and A. J. Vidal-Puig, "An update on visfatin/pre-B cell colony-enhancing factor, an ubiquitously expressed, illusive cytokine that is regulated in obesity," *Current Opinion in Lipidology*, vol. 17, no. 2, pp. 128–131, 2006.

[22] M. Gosset, F. Berenbaum, C. Salvat et al., "Crucial role of visfatin/pre-B cell colony-enhancing factor in matrix degradation and prostaglandin E2 synthesis in chondrocytes: possible influence on osteoarthritis," *Arthritis and Rheumatism*, vol. 58, no. 5, pp. 1399–1409, 2008.

[23] S. Ognjanovic, T. L. Ku, and G. D. Bryant-Greenwood, "Pre-B-cell colony-enhancing factor is a secreted cytokine-like protein from the human amniotic epithelium," *American Journal of Obstetrics and Gynecology*, vol. 193, no. 1, pp. 273–282, 2005.

[24] S. R. Costford, S. Bajpeyi, M. Pasarica et al., "Skeletal muscle NAMPT is induced by exercise in humans," *American Journal of Physiology*, vol. 298, no. 1, pp. E117–E126, 2010.

[25] A. Garten, S. Petzold, A. Barnikol-Oettler et al., "Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes," *Biochemical and Biophysical Research Communications*, vol. 391, no. 1, pp. 376–381, 2010.

[26] L. Frydelund-Larsen, T. Akerstrom, S. Nielsen, P. Keller, C. Keller, and B. K. Pedersen, "Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise," *American Journal of Physiology*, vol. 292, no. 1, pp. E24–E31, 2007.

[27] M. Kukla, M. Ciupińska-Kajor, M. Kajor et al., "Liver visfatin expression in morbidly obese patients with nonalcoholic fatty liver disease undergoing bariatric surgery," *Polish Journal of Pathology*, vol. 61, no. 3, pp. 147–153, 2010.

[28] M. P. Chen, F. M. Chung, D. M. Chang et al., "Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 1, pp. 295–299, 2006.

[29] T. D. Filippatos, C. S. Derdemezis, I. F. Gazi et al., "Increased plasma visfatin levels in subjects with the metabolic syndrome," *European Journal of Clinical Investigation*, vol. 38, no. 1, pp. 71–72, 2008.

[30] G. Sommer, A. Garten, S. Petzold et al., "Visfatin/PBEF/Nampt: structure, regulation and potential function of a novel adipokine," *Clinical Science*, vol. 115, no. 1, pp. 13–23, 2008.

[31] P. M. Vanhoutte, "Endothelial dysfunction: the first step toward coronary arteriosclerosis," *Circulation Journal*, vol. 73, no. 4, pp. 595–601, 2009.

[32] K. Takebayashi, M. Suetsugu, S. Wakabayashi, Y. Aso, and T. Inukai, "Association between plasma visfatin and vascular endothelial function in patients with type 2 diabetes mellitus," *Metabolism*, vol. 56, no. 4, pp. 451–458, 2007.

[33] S. Uslu, N. Kebapci, M. Kara et al., "Relationship between adipocytokines and cardiovascular risk factors in patients with type 2 diabetes mellitus," *Experimental and Therapeutic Medicine*, vol. 4, no. 1, pp. 113–120, 2012.

[34] F. Stam, C. van Guldener, C. G. Schalkwijk et al., "Impaired renal function is associated with markers of endothelial dysfunction and increased inflammatory activity," *Nephrology Dialysis Transplantation*, vol. 18, no. 5, pp. 892–898, 2003.

[35] N. P. E. Kadoglou, N. Sailer, A. Moumtzouoglou et al., "Visfatin (Nampt) and ghrelin as novel markers of carotid atherosclerosis in patients with type 2 diabetes," *Experimental and Clinical Endocrinology and Diabetes*, vol. 118, no. 2, pp. 75–80, 2010.

[36] A. E. Malavazos, F. Ermetici, E. Cereda et al., "Epicardial fat thickness: relationship with plasma visfatin and plasminogen activator inhibitor-1 levels in visceral obesity," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 18, no. 8, pp. 523–530, 2008.

[37] M. Fasshauer, T. Waldeyer, J. Seeger et al., "Serum levels of the adipokine visfatin are increased in pre-eclampsia," *Clinical Endocrinology*, vol. 69, no. 1, pp. 69–73, 2008.

[38] H. Tan, M. Zhong, H. Gong, S. Wang, Y. Zhang, and W. Zhang, "Increased serum visfatin in patients with metabolic syndrome and carotid atherosclerosis," *Clinical Endocrinology*, vol. 69, no. 6, pp. 878–884, 2008.

[39] C. E. Pepene, "Evidence for visfatin as an independent predictor of endothelial dysfunction in polycystic ovary syndrome," *Clinical Endocrinology*, vol. 76, no. 1, pp. 119–125, 2012.

[40] S. W. Liu, S. B. Qiao, J. S. Yuan, and D. Q. Liu, "Association of plasma visfatin levels with inflammation, atherosclerosis and acute coronary syndromes (ACS) in humans," *Clinical Endocrinology*, vol. 71, no. 2, pp. 202–207, 2009.

[41] M. I. Yilmaz, M. Saglam, J. J. Carrero et al., "Serum visfatin concentration and endothelial dysfunction in chronic kidney disease," *Nephrology Dialysis Transplantation*, vol. 23, no. 3, pp. 959–965, 2008.

[42] J. Axelsson, A. Witasp, J. J. Carrero et al., "Circulating levels of Visfatin/pre-B-cell colony-enhancing factor 1 in relation to genotype, GFR, body composition, and survival in patients with CKD," *American Journal of Kidney Diseases*, vol. 49, no. 2, pp. 237–244, 2007.

[43] J. Malyszko, J. S. Malyszko, and M. Mysliwiec, "Visfatin, a new adipocytokine, is predominantly related to inflammation/endothelial damage in kidney allograft recipients," *Transplantation Proceedings*, vol. 41, no. 1, pp. 150–153, 2009.

[44] J. Malyszko, J. S. Malyszko, K. Pawlak, and M. Mysliwiec, "Visfatin and apelin, new adipocytokines, and their relation to endothelial function in patients with chronic renal failure," *Advances in Medical Sciences*, vol. 53, pp. 32–36, 2008.

[45] A. Kato, M. Odamaki, J. Ishida, and A. Hishida, "Relationship between serum Pre-B cell colony-enhancing factor/visfatin and atherosclerotic parameters in chronic hemodialysis patients," *American Journal of Nephrology*, vol. 29, no. 1, pp. 31–35, 2008.

[46] M. I. Yilmaz, M. Saglam, J. J. Carrero et al., "Normalization of endothelial dysfunction following renal transplantation is accompanied by a reduction of circulating visfatin/NAMPT. A novel marker of endothelial damage?" *Clinical Transplantation*, vol. 23, no. 2, pp. 241–248, 2009.

[47] L. F. Lu, S. S. Yang, C. P. Wang et al., "Elevated Visfatin/Pre-B-cell colony-enhancing factor plasma concentration in ischemic stroke," *Journal of Stroke and Cerebrovascular Diseases*, vol. 18, no. 5, pp. 354–359, 2009.

[48] T. H. Yu, L. F. Lu, W. C. Hung et al., "Circulating visfatin level at admission is associated with occlusion of the infarct-related artery in patients with acute ST-segment elevation myocardial infarction," *Acta Cardiologica Sinica*, vol. 27, no. 2, pp. 77–85, 2011.

[49] L. F. Lu, C. P. Wang, T. H. Yu et al., "Interpretation of elevated plasma visfatin concentrations in patients with ST-elevation myocardial infarction," *Cytokine*, vol. 57, no. 1, pp. 74–80, 2012.

[50] M. Laudes, F. Oberhauser, D. M. Schulte et al., "Visfatin/PBEF/Nampt and resistin expressions in circulating blood monocytes are differentially related to obesity and type 2 diabetes in humans," *Hormone and Metabolic Research*, vol. 42, no. 4, pp. 268–273, 2010.

[51] K. Oki, K. Yamane, N. Kamei, H. Nojima, and N. Kohno, "Circulating visfatin level is correlated with inflammation, but not with insulin resistance," *Clinical Endocrinology*, vol. 67, no. 5, pp. 796–800, 2007.

[52] F. L. Brancati, P. K. Whelton, B. L. Randall, J. D. Neaton, J. Stamler, and M. J. Klag, "Risk of end-stage renal disease in diabetes mellitus: a prospective cohort study of men screened for MRFIT," *Journal of the American Medical Association*, vol. 278, no. 23, pp. 2069–2074, 1997.

[53] L. L. Humphrey, D. J. Ballard, P. P. Frohnert, C.P. Chu, M. O'Fallon, and P. J. Palumbo, "Chronic renal failure in non-insulin-dependent diabetes mellitus. A population-based study in Rochester, Minnesota," *Annals of Internal Medicine*, vol. 111, no. 10, pp. 788–796, 1989.

[54] I. Kowalska, M. Straczkowski, A. Nikolajuk et al., "Serum visfatin in relation to insulin resistance and markers of hyperandrogenism in lean and obese women with polycystic ovary syndrome," *Human Reproduction*, vol. 22, no. 7, pp. 1824–1829, 2007.

[55] E. A. Steegers, P. von Dadelszen, J. J. Duvekot, and R. Pijnenborg, "Pre-eclampsia," *The Lancet*, vol. 376, no. 9741, pp. 631–644, 2010.

[56] A. F. Ferreira, J. C. Rezende, C. O. R. de Cassia et al., "Maternal serum visfatin at 11–13 weeks' gestation in preeclampsia," *Journal of Human Hypertension*, vol. 27, no. 4, pp. 261–264, 2013.

[57] E. Zulfikaroglu, F. Isman, A. PayaslI, S. KIlIc, M. Kucur, and N. DanIsman, "Plasma visfatin levels in preeclamptic and normal pregnancies," *Archives of Gynecology and Obstetrics*, vol. 281, no. 6, pp. 995–998, 2010.

[58] W. Hu, Z. Wang, H. Wang, H. Huang, and M. Dong, "Serum visfatin levels in late pregnancy and pre-eclampsia," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 87, no. 4, pp. 413–418, 2008.

[59] S. C. Kim, M. J. Park, B. S. Joo et al., "Decreased expressions of vascular endothelial growth factor and visfatin in the placental bed of pregnancies complicated by preeclampsia," *Journal of Obstetrics and Gynaecology Research*, vol. 38, no. 4, pp. 665–673, 2012.

[60] J. M. Roberts and D. W. Cooper, "Pathogenesis and genetics of pre-eclampsia," *The Lancet*, vol. 357, no. 9249, pp. 53–56, 2001.

[61] S. Ognjanovic and G. D. Bryant-Greenwood, "Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes," *American Journal of Obstetrics and Gynecology*, vol. 187, no. 4, pp. 1051–1058, 2002.

[62] T. D. Filippatos, H. S. Randeva, C. S. Derdemezis et al., "Visfatin/PBEF and atherosclerosis-related diseases," *Current Vascular Pharmacology*, vol. 8, no. 1, pp. 12–28, 2010.

[63] T. B. Dahl, A. Yndestad, M. Skjelland et al., "Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization," *Circulation*, vol. 115, no. 8, pp. 972–980, 2007.

[64] S. G. Spiroglou, C. G. Kostopoulos, J. N. Varakis, and H. H. Papadaki, "Adipokines in periaortic and epicardial adipose

tissue: differential expression and relation to atherosclerosis," *Journal of Atherosclerosis and Thrombosis*, vol. 17, no. 2, pp. 115–130, 2010.

[65] P. Wang, P. M. Vanhoutte, and C. Y. Miao, "Visfatin and cardiocerebro-vascular disease," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 1, pp. 1–9, 2011.

[66] L. Y. Liu, F. Wang, X. Y. Zhang et al., "Nicotinamide phosphoribosyltransferase may be involved in age-related brain diseases," *PLoS One*, vol. 7, no. 10, Article ID e44933, 2012.

[67] F. Brentano, O. Schorr, C. Ospelt et al., "Pre-B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities," *Arthritis and Rheumatism*, vol. 56, no. 9, pp. 2829–2839, 2007.

[68] M. A. Nowell, P. J. Richards, C. A. Fielding et al., "Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 7, pp. 2084–2095, 2006.

[69] A. R. Moschen, A. Kaser, B. Enrich et al., "Visfatin, an adipocytokine with proinflammatory and immunomodulating properties," *Journal of Immunology*, vol. 178, no. 3, pp. 1748–1758, 2007.

[70] S. Q. Ye, B. A. Simon, J. P. Maloney et al., "Pre-B-cell colony-enhancing factor as a potential novel biomarker in acute lung injury," *American Journal of Respiratory and Critical Care Medicine*, vol. 171, no. 4, pp. 361–370, 2005.

[71] S. H. Jia, Y. Li, J. Parodo et al., "Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis," *Journal of Clinical Investigation*, vol. 113, no. 9, pp. 1318–1327, 2004.

[72] S. Ognjanovic, S. Bao, S. Y. Yamamoto, J. Garibay-Tupas, B. Samal, and G. D. Bryant-Greenwood, "Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes," *Journal of Molecular Endocrinology*, vol. 26, no. 2, pp. 107–117, 2001.

[73] B. Mesko, S. Poliska, A. Szegedi et al., "Peripheral blood gene expression patterns discriminate among chronic inflammatory diseases and healthy controls and identify novel targets," *BMC Medical Genomics*, vol. 3, p. 15, 2010.

[74] L. Senolt, O. Krystufkova, H. Hulejova et al., "The level of serum visfatin (PBEF) is associated with total number of B cells in patients with rheumatoid arthritis and decreases following B cell depletion therapy," *Cytokine*, vol. 55, no. 1, pp. 116–121, 2011.

[75] S. Gerdes, S. Osadtschy, M. Rostami-Yazdi et al., "Leptin, adiponectin, visfatin and retinol-binding protein-4-mediators of comorbidities in patients with psoriasis?" *Experimental Dermatology*, vol. 21, no. 1, pp. 43–47, 2012.

[76] M. Scotece, J. Conde, R. Gomez et al., "Role of adipokines in atherosclerosis: interferences with cardiovascular complications in rheumatic diseases," *Mediators of Inflammation*, vol. 2012, Article ID 125458, 2012.

[77] K. S. Moulton, "Angiogenesis in atherosclerosis: gathering evidence beyond speculation," *Current Opinion in Lipidology*, vol. 17, no. 5, pp. 548–555, 2006.

[78] R. Adya, B. K. Tan, A. Punn, J. Chen, and H. S. Randeva, "Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis," *Cardiovascular Research*, vol. 78, no. 2, pp. 356–365, 2008.

[79] S. R. Kim, S. K. Bae, K. S. Choi et al., "Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2," *Biochemical and Biophysical Research Communications*, vol. 357, no. 1, pp. 150–156, 2007.

[80] F. Lovren, Y. Pan, P. C. Shukla et al., "Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis," *American Journal of Physiology*, vol. 296, no. 6, pp. E1440–E1449, 2009.

[81] J. Xiao, Z. Xiao, Z. Liu et al., "Involvement of dimethylarginine dimethylaminohydrolase-2 in visfatin-enhanced angiogenic function of endothelial cells," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 3, pp. 242–249, 2009.

[82] R. Adya, B. K. Tan, J. Chen, and H. S. Randeva, "Pre-B cell colony enhancing factor (PBEF)/visfatin induces secretion of MCP-1 in human endothelial cells: role in visfatin-induced angiogenesis," *Atherosclerosis*, vol. 205, no. 1, pp. 113–119, 2009.

[83] Y. Bae, M. Bae, S. Kim, J. H. Lee, H. Wee, and S. Bae, "Upregulation of fibroblast growth factor-2 by visfatin that promotes endothelial angiogenesis," *Biochemical and Biophysical Research Communications*, vol. 379, no. 2, pp. 206–211, 2009.

[84] W. J. Lee, C. S. Wu, H. Lin et al., "Visfatin-induced expression of inflammatory mediators in human endothelial cells through the NF-kappaB pathway," *International Journal of Obesity*, vol. 33, no. 4, pp. 465–472, 2009.

[85] S. R. Kim, Y. H. Jung, H. J. Park et al., "Upregulation of thromboxane synthase mediates visfatin-induced interleukin-8 expression and angiogenic activity in endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 418, no. 4, pp. 662–668, 2012.

[86] W. Zhang, Y. Xie, T. Wang et al., "Neuronal protective role of PBEF in a mouse model of cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 30, no. 12, pp. 1962–1971, 2010.

[87] W. Tian, Y. Zhu, Y. Wang et al., "Visfatin, a potential biomarker and prognostic factor for endometrial cancer," *Gynecologic Oncology*, vol. 129, no. 3, pp. 505–512, 2013.

[88] M. S. Fazeli, H. Dashti, S. Akbarzadeh et al., "Circulating levels of novel adipocytokines in patients with colorectal cancer," *Cytokine*, vol. 62, no. 1, pp. 81–85, 2013.

[89] T. E. Nakajima, Y. Yamada, T. Hamano et al., "Adipocytokine levels in gastric cancer patients: resistin and visfatin as biomarkers of gastric cancer," *Journal of Gastroenterology*, vol. 44, no. 7, pp. 685–690, 2009.

[90] F. Montecucco, M. Cea, I. Bauer et al., "Nicotinamide phosphoribosyltransferase (Nampt) inhibitors as therapeutics: rationales, controversies, clinical experience," *Current Drug Targets*, vol. 14, no. 6, pp. 637–643, 2013.

[91] A. Pogrebniak, I. Schemainda, K. Azzam et al., "Chemopotentiating effects of a novel NAD biosynthesis inhibitor, FK866, in combination with antineoplastic agents," *European Journal of Medical Research*, vol. 11, no. 8, pp. 313–321, 2006.

[92] A. Ravaud, T. Cerny, C. Terret et al., "Phase I study and pharmacokinetic of CHS-828, a guanidino-containing compound, administered orally as a single dose every 3 weeks in solid tumours: an ECSG/EORTC study," *European Journal of Cancer*, vol. 41, no. 5, pp. 702–707, 2005.

[93] X. Y. Yu, S. B. Qiao, H. S. Guan et al., "Effects of visfatin on proliferation and collagen synthesis in rat cardiac fibroblasts," *Hormone and Metabolic Research*, vol. 42, no. 7, pp. 507–513, 2010.

[94] V. B. Pillai, N. R. Sundaresan, G. Kim et al., "Nampt secreted from cardiomyocytes promotes development of cardiac hypertrophy and adverse ventricular remodeling," *American Journal of Physiology*, vol. 304, no. 3, pp. 415–426, 2013.

[95] Y. Li, Y. Zhang, B. Dorweiler et al., "Extracellular nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism," *Journal of Biological Chemistry*, vol. 283, no. 50, pp. 34833–34843, 2008.

[96] T. Romacho, V. Azcutia, M. Vázquez-Bella et al., "Extracellular PBEF/NAMPT/visfatin activates pro-inflammatory signalling in human vascular smooth muscle cells through nicotinamide phosphoribosyltransferase activity," *Diabetologia*, vol. 52, no. 11, pp. 2455–2463, 2009.

[97] S. R. Kim, Y. H. Bae, S. K. Bae et al., "Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells," *Biochimica et Biophysica Acta*, vol. 1783, no. 5, pp. 886–895, 2008.

[98] S. W. Liu, S. B. Qiao, J. S. Yuan, and D. Q. Liu, "Visfatin stimulates production of monocyte chemotactic protein-1 and interleukin-6 in human vein umbilical endothelial cells," *Hormone and Metabolic Research*, vol. 41, no. 4, pp. 281–286, 2009.

[99] R. Adya, B. K. Tan, J. Chen, and H. S. Randeva, "Nuclear factor-kappaB induction by visfatin in human vascular endothelial cells," *Diabetes Care*, vol. 31, no. 4, pp. 758–760, 2008.

[100] S. Vallejo, T. Romacho, J. Angulo et al., "Visfatin impairs endothelium-dependent relaxation in rat and human mesenteric microvessels through nicotinamide phosphoribosyltransferase activity," *PLoS One*, vol. 6, no. 11, Article ID e27299, 2011.

[101] K. M. Boini, C. Zhang, M. Xia et al., "Visfatin-induced lipid raft redox signaling platforms and dysfunction in glomerular endothelial cells," *Biochimica et Biophysica Acta*, vol. 1801, no. 12, pp. 1294–1304, 2010.

[102] M. Xia, C. Zhang, K. M. Boini, A. M. Thacker, and P. Li, "Membrane raftlysosome redox signalling platforms in coronary endothelial dysfunction induced by adipokine visfatin," *Cardiovascular Research*, vol. 89, no. 2, pp. 401–409, 2011.

[103] F. Zhou, Y. Pan, Z. Huang et al., "Visfatin induces cholesterol accumulation in macrophages through up-regulation of scavenger receptor-A and CD36," *Cell Stress and Chaperones*, 2013.

[104] P. Pacher, I. G. Obrosova, J. G. Mabley, and C. Szabó, "Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies," *Current Medicinal Chemistry*, vol. 12, no. 3, pp. 267–275, 2005.

[105] E. Galkina and K. Ley, "Vascular adhesion molecules in atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, pp. 2292–2301, 2007.

[106] R. M. Touyz and A. M. Briones, "Reactive oxygen species and vascular biology: implications in human hypertension," *Hypertension Research*, vol. 34, no. 1, pp. 5–14, 2011.

[107] M. Xia, C. Zhang, K. M. Boini, A. M. Thacker, and P. Li, "Membrane raftlysosome redox signalling platforms in coronary endothelial dysfunction induced by adipokine visfatin," *Cardiovascular Research*, vol. 89, no. 2, pp. 401–409, 2011.

[108] Q. Huang, Y. Guo, H. Zeng, W. Xie, H. Yan, and H. Ding, "Visfatin stimulates a cellular renin-angiotensin system in cultured rat mesangial cells," *Endocrine Research*, vol. 36, no. 3, pp. 93–100, 2011.

[109] C. A. Chiu, T. H. Yu, W. C. Hung et al., "Increased expression of visfatin in monocytes and macrophages in male acute myocardial infarction patients," *Mediators of Inflammation*, vol. 2012, Article ID 469852, 7 pages, 2012.

[110] E. Oviedo-Orta, A. Bermudez-Fajardo, S. Karanam, U. Benbow, and A. C. Newby, "Comparison of MMP-2 and MMP-9 secretion from T helper 0, 1 and 2 lymphocytes alone and in coculture with macrophages," *Immunology*, vol. 124, no. 1, pp. 42–50, 2008.

[111] H. K. Song, M. H. Lee, B. K. Kim et al., "Visfatin: a new player in mesangial cell physiology and diabetic nephropathy," *American Journal of Physiology*, vol. 295, no. 5, pp. 1485–1494, 2008.

[112] D. J. Hausenloy and D. M. Yellon, "The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 4, pp. 339–341, 2003.

[113] C. P. Hsu, S. Oka, D. Shao, N. Hariharan, and J. Sadoshima, "Nicotinamide phosphoribosyltransferase regulates cell survival through nad+ synthesis in cardiac myocytes," *Circulation Research*, vol. 105, no. 5, pp. 481–491, 2009.

[114] S. Y. Lim, S. M. Davidson, A. J. Paramanathan, C. C. T. Smith, D. M. Yellon, and D. J. Hausenloy, "The novel adipocytokine visfatin exerts direct cardioprotective effects," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 4, pp. 1395–1403, 2008.

[115] P. Wang, T. Xu, Y. Guan et al., "Nicotinamide phosphoribosyltransferase protects against ischemic stroke through SIRT1-dependent adenosine monophosphate-activated kinase pathway," *Annals of Neurology*, vol. 69, no. 2, pp. 360–374, 2011.

[116] C. Rask-Madsen and G. L. King, "Mechanisms of disease: endothelial dysfunction in insulin resistance and diabetes," *Nature Clinical Practice Endocrinology & Metabolism*, vol. 3, no. 1, pp. 46–56, 2007.

[117] H. Yamawaki, N. Hara, M. Okada, and Y. Hara, "Visfatin causes endothelium-dependent relaxation in isolated blood vessels," *Biochemical and Biophysical Research Communications*, vol. 383, no. 4, pp. 503–508, 2009.

[118] H. K. Song, M. H. Lee, B. K. Kim et al., "Visfatin: a new player in mesangial cell physiology and diabetic nephropathy," *American Journal of Physiology*, vol. 295, no. 5, pp. 1485–1494, 2008.

[119] N. Busso, M. Karababa, M. Nobile et al., "Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD," *PLoS One*, vol. 3, no. 5, Article ID e2267, 2008.

[120] F. Montecucco, I. Bauer, V. Braunersreuther et al., "Inhibition of nicotinamide phosphoribosyltransferase reduces neutrophil-mediated injury in myocardial infarction," *Antioxidants & Redox Signaling*, vol. 18, no. 6, pp. 630–641, 2013.

[121] C. Jacques, M. Holzenberger, Z. Mladenovic et al., "Proinflammatory actions of visfatin/nicotinamide phosphoribosyltransferase (Nampt) involve regulation of insulin signaling pathway and Nampt enzymatic activity," *Journal of Biological Chemistry*, vol. 287, no. 18, pp. 15100–15108, 2012.

[122] R. M. Touyz, "Lipid rafts take center stage in endothelial cell redox signaling by death receptors," *Hypertension*, vol. 47, no. 1, pp. 16–18, 2006.

[123] J. Xiao, B. Sun, M. Li et al., "A novel adipocytokine visfatin protects against H_2O_2-induced myocardial apoptosis: a missing link between obesity and cardiovascular disease," *Journal of Cellular Physiology*, vol. 228, no. 3, pp. 495–501, 2012.

[124] G. Iacobellis, F. Assael, M. C. Ribaudo et al., "Epicardial fat from echocardiography: a new method for visceral adipose tissue prediction," *Obesity Research*, vol. 11, no. 2, pp. 304–310, 2003.

[125] G. Iacobellis, M. C. Ribaudo, F. Assael et al., "Echocardiographic epicardial adipose tissue is related to anthropometric and clinical parameters of metabolic syndrome: a new indicator of cardiovascular risk," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 11, pp. 5163–5168, 2003.

[126] M. R. Williams, N. Kataoka, Y. Sakurai, C. M. Powers, S. G. Eskin, and L. V. McIntire, "Gene expression of endothelial cells due to interleukin-1 beta stimulation and neutrophil transmigration," *Endothelium*, vol. 15, no. 1-2, pp. 73–84, 2008.

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Review Article

The NLRP3 Inflammasome as a Novel Player of the Intercellular Crosstalk in Metabolic Disorders

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The combination of obesity and type 2 diabetes is a serious health problem, which is projected to afflict 300 million people worldwide by 2020. Both clinical and translational laboratory studies have demonstrated that chronic inflammation is associated with obesity and obesity-related conditions such as insulin resistance. However, the precise etiopathogenetic mechanisms linking obesity to diabetes remain to be elucidated, and the pathways that mediate this phenomenon are not fully characterized. One of the most recently identified signaling pathways, whose activation seems to affect many metabolic disorders, is the "inflammasome," a multiprotein complex composed of NLRP3 (nucleotide-binding domain and leucine-rich repeat protein 3), ASC (apoptosis-associated speck-like protein containing a CARD), and procaspase-1. NLRP3 inflammasome activation leads to the processing and secretion of the proinflammatory cytokines interleukin- (IL-) 1β and IL-18. The goal of this paper is to review new insights on the effects of the NLRP3 inflammasome activation in the complex mechanisms of crosstalk between different organs, for a better understanding of the role of chronic inflammation in metabolic disease pathogenesis. We will provide here a perspective on the current research on NLRP3 inflammasome, which may represent an innovative therapeutic target to reverse the detrimental metabolic consequences of the metabolic inflammation.

1. The NLRP3 Inflammasome: An Overview

The inflammasomes are signaling platforms, which are assembled in response to pathogen-associated and damage-associated molecular pattern molecules and environmental irritants. Currently, inflammasomes are distinguished into two families: the NOD-like receptor (NLR) family and the pyrin and HIN200 (haematopoietic interferon-inducible nuclear antigens with 200 amino-acid repeats) domain-containing protein (PYHIN) family. The NLR family consists of NLRP1, NLRP2, NLRP3, NLRP6, NLRC4, and NLRP12. The PYHIN family consists of AIM2 and IFI16. Each inflammasome is induced by numerous different exogenous and endogenous signals. This review will focus on the NLRP3 inflammasome. The NLRP3 inflammasome is a multiprotein, large cytoplasmic complex (>700 kDa), composed of a specific member of the NOD-like receptor protein (NLRP) subfamily, the adaptor protein named apoptosis-associated speck-like protein containing a CARD (ASC), and procaspase-1, which are preferentially expressed in adipose tissue macrophages (ATMs) [1]. Unlike the typical signaling cascades downstream of many innate receptors such as other NLRP members, the NLRP3 inflammasome is a proteolytic caspase-1-activating platform. The activation of NLRP3 leads to oligomerization and recruitment of ASC. NLRP3 contains an N-terminal pyrin domain (PYD), which is used to physically interact with the PYD domain of ASC, thus facilitating the subsequent recruitment and activation of procaspase-1. Caspase-1 is then autocatalytically cleaved to its active form (Figure 1). Caspase-1 does not play a major role in apoptosis. Instead, once activated, caspase-1, as far as we are currently aware, cleaves the proforms of two potent proinflammatory cytokines interleukin- (IL-) 1β and IL-18 in the cytoplasm. This has two main effects; firstly it activates the two cytokines and secondly in this mature form these cytokines can be released from the cell. The active form of caspase-1 also has the ability to induce the release of IL-1α and HMGB-1

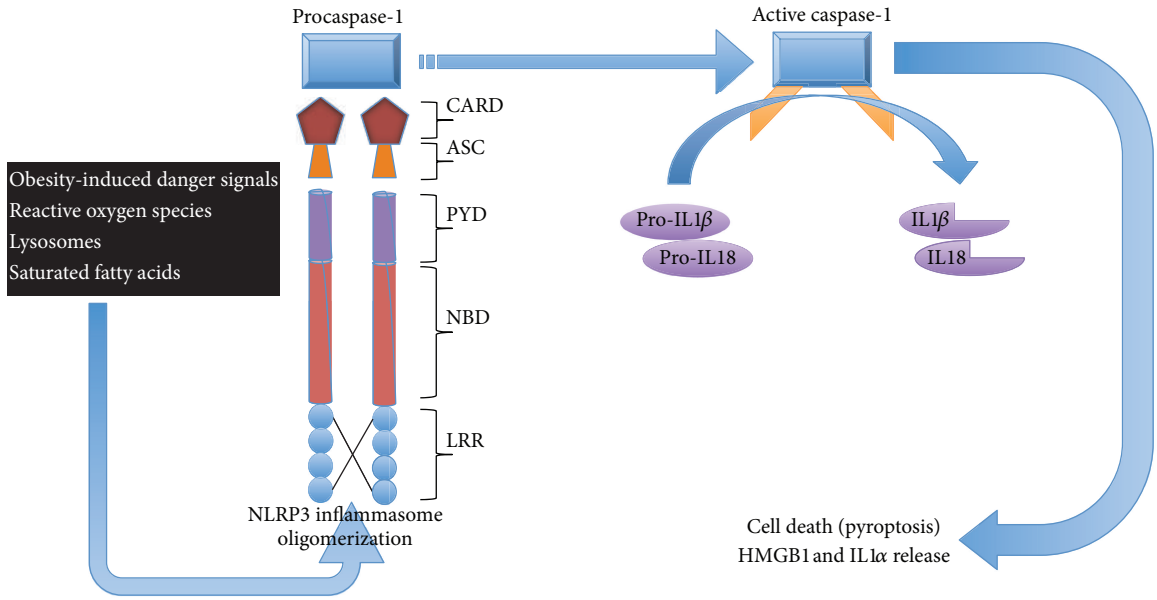

Figure 1: The release of obesity-related danger signals such as reactive oxygen species, lysosomes, and other obesity-induced danger signals resulting in the oligomerization of NLRP3 in adipose tissue. The NLRP3 inflammasome is made up of carboxy terminal leucine-rich repeats (LRRs), a nucleotide-binding domain (NBD), and an N-terminal pyrin domain (PYD). The resulting oligomerization causes the recruitment of procaspase-1 via homotypic binding of caspase activation and recruitment domain (CARD) or through the PYD by means of the adapter apoptosis-associated speck-like protein containing a CARD (ASC). Caspase-1 is therefore activated and initiates the cleavage of prointerleukin (IL)1β and pro-IL18 to form the active cytokines IL1β and IL18. The activation of caspase-1 also results in pyroptosis (a form of lytic cell death during inflammation) and the release of high mobility group box 1 (HMGB1) and IL1α.

(high mobility group box 1), as well as initiate a lytic form of cell death called pyroptosis [2–4] (Figure 1). The primary role of the inflammasome and its products seems to be as part of the body's innate immune system, in that they can be triggered to assist in the defense against invading pathogens. Indeed much of the data published on the inflammasome/caspase 1 is on its role in the body's response to microbial molecules (bacterial, fungal, or viral) with conserved molecular structures known as "pathogen associated molecular patterns" (PAMPs) [5, 6]. In addition to PAMPs, the NLRP3 inflammasome is also proficient in sensing stress to endogenous (nonmicrobial) danger signals ("danger associated molecular patterns," DAMPs) from damaged cells. DAMPs can include molecules such as reactive oxygen species (ROS), adenosine triphosphate (ATP), hypotonic stress, uric acid crystals, or noxious exogenous factors such as environmental insults, asbestos, and UV radiation [7].

There are a number of potential mechanisms for the assembly of the NLRP3 inflammasome, as described earlier. According to one hypothesis, mitochondria are the principal source of reactive oxygen species (ROS) required for inflammasome activation; several recent studies have implicated ROS produced by mitochondria, rather than phagosomes, in NLRP3 activation exerting an indirect effect on pathways of metabolism [8, 9]. A second mechanism involves the disruption of lysosomal membrane integrity by crystalline materials and peptide aggregates [10, 11]. Upon uptake of such substances, lysosomal rupture leads to the leakage of lysosomal proteases, specifically cathepsins B and L, into the cytosol where they could possibly mediate NLRP3 inflammasome activation by an as-yet-undefined cleavage event. In addition, type-2 diabetic patients and mice fed a high-fat diet demonstrate IL-1β production following inflammasome activation from obesity-induced danger signals [12]. Mice have also been shown to become glucose intolerant following activation of the inflammasome in hematopoietic cells by the saturated fatty acid palmitate [13]. Very recently, Vajjhala and colleagues [14] have shed light on the molecular details of the complex mechanisms of NLRP3 inflammasome assembly and activation, identifying multiple binding sites on the PYD domain of the adaptor protein ASC which allow self-association and interaction with binding partners.

2. The NLRP3 Inflammasome in Obesity and Type 2 Diabetes

Several *in vitro*, *in vivo* studies and clinical trials provide evidence that supports a causative role of IL-1β in the pathogenesis of type 2 diabetes [15], and elevation in circulating levels of IL-1β predicts type 2 diabetes when combined with serum IL-6 levels [16]. Prolonged IL-1β treatment has been demonstrated to reduce the insulin-induced glucose uptake in murine adipocytes [17]. In contrast, addition of the IL-1β receptor antagonist to adipocytes resulted in increased insulin sensitivity as reflected by increased levels of phosphorylated AKT in response to insulin. Similarly, IL-1β can inhibit the insulin-stimulated glycogen synthesis in rat hepatocytes [18]. These results were confirmed by showing

that IL-1β knockout mice were more insulin sensitive as compared to wild-type control animals [19]. In humans, elevated plasma levels of IL-1β have been found to be predictive of type 2 diabetes [16], and clinical studies have suggested that treatment with the IL-1β receptor antagonist anakinra has beneficial effects in type 2 diabetic patients [20]. A number of recent landmark studies have pointed out a key role for an excessive NLRP3 inflammasome activation in the IL-1β-related development of type 2 diabetes. The association between the NLRP3 inflammasome and both insulin resistance and obesity has been suggested by animal studies showing that genetic ablation of NLRP3 improved insulin sensitivity and glucose homeostasis [21]. Specifically, adipocytes isolated from NLRP3-deficient mice showed an increase in insulin sensitivity as determined by phosphorylation of Akt. In line with the rise in insulin sensitivity, IL-1β production of adipose tissue isolated from NLRP3 knockout mice was significantly reduced as compared to white adipose tissue from wild-type animals. Other studies [12, 13] have shown that improvement in insulin sensitivity (increased phosphorylation of the insulin receptor subtrate-1 and Akt) can also be detected in liver and muscle of NLRP3 knockout mice on a high-fat diet for 12 weeks. This effect was associated with a significant reduction in the tissue mRNA expression of inflammatory cytokines compared to wild-type control [13]. Ablation of the NLRP3 in mice has been also reported to protect from obesity-associated macrophage activation in adipose tissue, reducing M1-like macrophage gene expression (tumor necrosis factor-α, chemokine ligand 20, and chemokine ligand 11) and increasing the expression of M2-like cytokines (interleukin-10). This effect was associated with an increase in the number of M2 macrophages in NLRP3-deficient obese mice, without affecting the M1 macrophage frequency [12]. To confirm the clinical relevance of these data generated from mouse models, the same authors have demonstrated that weight loss reduced NLRP3 expression in abdominal subcutaneous adipose tissue in obese patients with type 2 diabetes, which was accompanied by improved glucose homeostasis [12]. Furthermore, strong correlations between the expression of NLRP3 inflammasome-related genes and insulin resistance have been recently reported in obese male subjects with impaired glucose tolerance [22]. Additionally, type 2 diabetic patients showed elevated levels of NLRP3, ASC, IL-1β, and IL-18 mRNA and protein expression in monocyte-derived macrophages, compared with those in healthy control subjects. Besides, the cleavage of caspase-1 and release of mature IL-1β were significantly elevated in monocyte-derived macrophages from type 2 diabetic patients compared with controls [23]. Inflammatory cytokines are known to contribute crucially to the development of insulin resistance by activating different kinases that disrupt insulin signaling. The endoplasmatic reticulum (ER) is an extensive membrane network which has been recently demonstrated to be involved in the transduction of cytokines effects into activation of different kinases. The early steps of insulin biosynthesis occur in the ER of pancreatic β cells, thus further suggesting the key role of ER load and folding activity in the insulin biosynthesis [24]. A major role of the ER is to ensure the synthesis and folding of membrane and secreted proteins, and any disturbance in this function (e.g., excessive protein synthesis or accumulation of unfolded or misfolded proteins in the ER lumen) leads to an "ER stress" response, also known as the unfolded protein response (UPR). The recent literature suggests that ER stress may act directly as a negative modulator of the insulin biosynthesis and insulin signaling pathways but also indirectly by promoting lipid accumulation [25, 26]. ER stress also plays a role in the dysregulation of adipokine secretion by adipose tissue, frequently observed in obesity and insulin resistance [27, 28], and CD14+ monocytes isolated from diabetic patients showed evidence of ER stress, which may underlie the functional defects in these cells [29]. Interestingly, ER stress has been recently demonstrated to activate the NLRP3 inflammasome, resulting in the subsequent release of IL-1β by human macrophages, with an activation mechanism similar to that of other known NLRP3 activators, requiring ROS generation and potassium efflux [30]. The thioredoxin-interacting protein (TXNIP), a critical node in the development of ER stress leading to programmed cell death of pancreatic β cells, activates the NLRP3 inflammasome, causing procaspase-1 cleavage and IL-1β secretion in human monocytic cells [31]. The role of ER stress in promoting NLRP3 inflammasome activation is consistent with the subcellular localization of NLRP3. In resting cells, NLRP3 is associated with ER membranes, and then upon activation NLRP3 is redistribute to the perinuclear space where it colocalizes with endoplasmic reticulum and mitochondria organelle clusters [9].

3. The NLRP3 Inflammasome and the Organ Crosstalk in the Metabolic Inflammation

NLRP3 inflammasome plays a substantial role in sensing obesity-associated inducers of caspase-1 activation and therefore regulates the magnitude of the inflammation and its downstream effects on insulin signaling in different organs, as reported here later.

3.1. Immune Effector Cells. NLRP3 expression is detected mainly in the cytosol of granulocytes, monocytes, dendritic cells, T and B cells, and osteoblasts [32]. Thus, most of the first studies characterizing the role of NLRP3 signaling have been conducted in cells of the immune system. Several studies on innate immune cells have demonstrated that the myeloid-derived NLRP3 inflammasome complex may contribute to promote inflammatory cytokine production and insulin resistance through reduction of insulin signaling. *In vitro* experiments have shown that elevated concentration of saturated fatty acids (SFAs), caused by a high-fat diet, may activate the NLRP3 inflammasome in macrophages through a newly identified AMP-activated protein kinase and unc-51-like kinase-1 autophagy signaling cascade [13]. Besides, both *ex vivo* and *in vivo* exposure of bone marrow derived dendritic cells to dietary SFA resulted in increased NLRP3 inflammasome activation and reduced adipocyte insulin sensitivity. More specifically, dietary SFA may act as a primer of the NLRP3 inflammasome protein complex enhancing

NLRP3, caspase-1, and pro-IL-1β mRNA expression. A second signal is then required to induce maturation of IL-1β from inactive pro-IL-1β. This second step can be triggered by exposure to ATP, ROS, or ceramide [33]. Overall, these data suggest that exposure to dietary SFA represents the key metabolic stressor relevant to both priming and processing of IL-1β in both adipocytes and innate immune cells. However, it must be stressed that the high expression of NLRP3 in primary adipocyte fractions of enzymatically digested adipose tissue may be attributable in large part to lipid-laden macrophages that contaminate enriched adipocyte fractions, as also suggested by immunofluorescence and qRT-PCR data, showing that NLRP3 is highly expressed in adipose tissue macrophages with low expression in adipocytes [12]. Besides, standard isolation procedures for isolating primary adipose cells often involve collagenase digestion, which have been shown to be a potent inducer of cytokine gene transcription and protein secretion [34]. These findings highlight a new model of organ crosstalk, in which leukocyte and macrophage recruitment in key insulin target tissues, such as liver, adipose, and muscle, may promote insulin resistance by enhancing inflammasome activation. This is in keeping with recent studies showing that IL-1β's role in regulating the endocrine function of adipose tissue is mediated by its own ability to evoke local macrophage recruitment and lipid accumulation in an autocrine/paracrine manner [35]. As in diabetic patients pancreas, adipose tissue, liver, and kidney, with infiltrated macrophages, are major sites of origin of inflammation, it might be intriguing to investigate the specific contribution of NLRP3 inflammasome activation in these different insulin target tissues and to identify the specific inducers that selectivity participate in the mechanism of tissue NLRP3 inflammasome activation.

3.2. Pancreas. Pancreatic islets of type 2 diabetic patients have amyloid deposition and increased production of proinflammatory cytokines and chemokines. The unique, primary component of islet amyloid deposits is the islet amyloid polypeptide (IAPP; also known as amylin). Mice overexpressing IAPP produce higher amounts of IL-1β [36], and exposure to high levels of IL-1β has been demonstrated to induce beta cell death in cell culture, interfering with signaling to NF-κB through IKKβ or the IκBα super-repressor [37]. In keeping with these results, neutralizing IL-1β on isolated beta cells using IL-1 receptor antagonist significantly improves β-cell survival [38]. However, the precise mechanism(s) by which IL-1β affects pancreatic β-cell failure is still debated. Zhou et al. [39] were the first to identify a possible signaling pathway involved in NLRP3 inflammasome activation under conditions of metabolic stress. They showed that thioredoxin-interacting protein (TXNIP), also known as vitamin D3 upregulated protein 1 (VDUP1), is an upstream and highly selective activating ligand for NLRP3, with no effect on the activity of other inflammasomes (e.g., NLRC4 and AIM2). TXNIP-dependent NLRP3 inflammasome activation drives IL-1β secretion from pancreatic islets in response to chronic elevated glucose, thus suggesting, for the first time, that NLRP3, activated under conditions of metabolic stress, mediates IL-1β-driven islet failure. Other authors have identified oligomers of IAPP, as a key trigger for NLRP3 inflammasome activation and the following processing of IL-1β [40]. Obesity-induced pancreatic β-cell death is regulated, at least in part, by the NLRP3 inflammasome, as demonstrated in NLRP3-deficient mice in late-stage obesity, where the ablation of NLRP3 is associated with reduced cell death and increase in pancreatic islet size and local insulin levels [41].

3.3. Adipose Tissue. As noted by Vandanmagsar et al., the NLRP3 inflammasome is activated in adipose tissue in mouse models of obesity and attenuated by calorie restriction. NLRP3 inflammasome levels also correlate with glycaemia in type 2 diabetes patients after weight loss interventions [12]. Besides, mice deficient in inflammasome components are protected from body-weight gain and adipocyte hypertrophy, induced by chronic exposure to a high-fat diet [1]. NLRP3 inflammasome components have been reported to be abundantly represented in adipocytes of patients with metabolic syndrome, mainly in adipocytes from samples of visceral adipose tissue. In contrast, the inflammasome in subcutaneous adipose tissue adipocytes did not seem to be grossly influenced by the presence of the metabolic syndrome [42]. Interestingly, caspase-1 activation in adipose tissue of obese animals takes place partly independent of macrophage infiltration. Partial depletion of macrophages from adipose tissue of obese animals decreased the expression of the macrophage marker CD68, with no significant alteration in the expression of caspase-1, thus suggesting that the effects of the NLRP3-ASC-caspase-1 protein complex on adipose tissue are not only exerted though infiltrating macrophages. This observation was also confirmed in *in vitro* experiments showing caspase-1 activation in adipocytes in settings free of inflammatory cells [43] and increased insulin sensitivity in adipocytes lacking of caspase-1 or the inflammasome component NLRP3 [21]. In addition, adipocyte upregulation of IL-1β expression and secretion in response to inflammatory stimuli has been shown to induce hepatic insulin resistance, thus suggesting a further intriguing role for NLRP3 inflammasome activation in the dysfunctional communication between adipocytes and hepatocytes [35, 43]. Overall these data reveal a novel metabolic function of the NLRP3 inflammasome in adipose tissue, suggesting that its pharmacological modulation in obese and/or patients with type 2 diabetes may restore the metabolic function of adipose tissue and subsequently improve insulin sensitivity.

3.4. Liver. The involvement of the inflammasome in nonalcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) is slowly being elucidated. The presence of NLRP3 inflammasome and/or inflammasome activation has been shown in sinusoidal endothelial cells [44], stellate cells [45], and hepatocytes [46]. Recently, inflammasome activation has been associated with NASH, and long-term high-fat diet administration resulted in reduced hepatic steatosis in NLRP3 knockout mice [12]. Selective deficiency in IL-1β in liver parenchymal cells, but not in bone-marrow-derived cells, protected mice from diet-induced steatohepatitis and fibrosis [47]. Increased mRNA expression of NLRP3 inflammasome components was found in human livers

of NASH patients [46] where NLRP3 levels were decreased after weight loss. These observations suggest that inflammasome activation by different cell types may contribute to different aspects of steatohepatitis. In contrast, to date, there are no data suggesting a potential role of NLRP3 inflammasome activation on impaired glycogen synthesis and/or augmented glycogenolysis.

3.5. Gut. There is evidence that the inflammasome components are important in the maintenance of the integrity of the intestinal epithelium and the defense against pathogenic organisms that can invade the gastrointestinal tract. For instance, mice lacking the inflammasome components NLRP3 and caspase-1 are hypersusceptible to gastrointestinal inflammation induced by Citrobacter rodentium, an enteric bacterial pathogen of the mouse intestinal tract that triggers inflammatory responses resembling those of humans infected with enteropathogenic and enterohemorrhagic *Escherichia coli*. The increased host susceptibility to *C. rodentium* is due to the failure to produce normal levels of IL-1β and IL-18 in the presence of NLRP3 and *Caspase-1* deficiency [48]. NLRP3-deficient mice had been reported to show increased susceptibility to dextran-sulfate-sodium- (DSS-) induced colitis with increased mortality and weight loss in three different studies [49–51]. However, other authors did not show a negative regulatory effect of NLRP3 on colitis, showing that NLRP3-null mice or mice pretreated with the caspase-1 inhibitor pralnacasan had less severe colitis when treated with DSS, which was related to decreased IL-1β secretion of DSS-exposed NLRP3-deficient macrophages *in vitro* [52, 53]. This discrepancy could be due not only to differences in protocols but also to baseline differences in the gut microbiota that might account for the dissimilar phenotypes. The crucial role of inflammasome components in the impairments of the gut microbiota composition is also suggested by recent studies demonstrating that NLRP3 inflammasome regulates the gastrointestinal microbiome and can thereby affect host susceptibility to diseases beyond the gastrointestinal tract, including obesity and diabetes. In particular, modulation of the intestinal microbiota through multiple inflammasome components has been recently demonstrated to be a critical determinant of NAFLD/NASH progression as well as multiple other aspects of metabolic syndrome such as weight gain and glucose homeostasis [54]. Inflammasome-deficiency-associated changes in the configuration of the gut microbiota are associated with exacerbated hepatic steatosis and inflammation through influx of TLR4 and TLR9 agonists into the portal circulation, leading to enhanced hepatic tumour-necrosis factor- (TNF-) α expression that drives NASH progression [54].

3.6. Kidney. Little is known of the role of the NLRP3 inflammasome complex in the development of renal metabolic damage. In humans, IL-18 and caspase-1 are expressed in renal tubular epithelium, and patients with chronic kidney disease or the nephrotic syndrome exhibit elevated levels of IL-18 [55–57]. In a cohort of renal biopsies from patients with nondiabetic kidney disease, levels of mRNA encoding NLRP3 correlate with renal function [58], strongly suggesting that NLRP3 contributes to the pathogenesis of chronic kidney disease. This is supported by experimental data showing that inflammasome-regulated cytokines such as IL-1β and IL-18 are implicated in animal models of chronic kidney disease, including glomerulonephritis and renal ischemic injury [59]. In an animal study aimed to evaluate the renal consequences of the chronic administration of high-fructose corn syrup (HFCS-55), the major sweetener in foods and soft-drinks, we have recently demonstrated that HFCS-55 feeding caused a significant increase in body weight and more importantly dyslipidemia, hyperinsulinemia, and an increase in insulin resistance due to impaired insulin signaling [60]. Most notably, the HFCS-55 diet evoked upregulation of renal NLRP3 expression, resulting in activation of caspase-1 and the subsequent cleavage of pro-IL1β to the biologically active secreted form IL-1β. These effects were due, at least in part, to the marked hyperuricemia afforded by the dietary manipulation, as also confirmed by a previous study demonstrating that increased levels of uric acid directly activate the NLRP3 inflammasome [61]. Similarly, rats fed with fructose, which is known to raise uric acid levels, showed a significant increase in renal protein levels of NLRP3 [62]. However, one important question that remains to be answered regards the specific cell types involved in renal NLRP3 activation. As several studies have shown that monocyte/macrophage recruitment to the kidney significantly contributes to the renal injury, we cannot rule out that the increased NLRP3 activation in the kidney is due to an increase in the infiltrating macrophages or, more likely, to a crosstalk between macrophages and tubular/glomerular cells.

3.7. Skeletal Muscle. Although it has been recently proposed that sarcopenia (loss of muscle mass) and myosteatosis (fat infiltration in skeletal muscle) exert a key role in triggering insulin resistance in obese patients, so far the potential role of NLRP3 complex activation in muscle activity and muscle production of inflammatory mediators has not yet been investigated. However, there is evidence that components of the inflammasome complex are upregulated in dysferlin-deficient human muscle, thus suggesting that skeletal muscle cells can actively participate in inflammasome activation [63]. This is a crucial point as recent studies have demonstrated that skeletal muscle cells produce and release cytokines (myokines) that act in an autocrine, paracrine, and/or endocrine manner to modulate metabolic and inflammatory process. For example, it has been demonstrated very recently that muscular expression of PGC-1 alpha stimulates the secretion of a newly identified myokine, irisin, which improves glucose homeostasis and causes weight loss [64]. However, the interactions between local NLRP3 expression/activity and myokines production as well as the effects of these interactions on muscle structure, function, and insulin-sensitivity in animals and humans have never been investigated. Interestingly, both IL-1β and IL-18 seem to exert a crucial role also in the initiation and progression of the idiopathic inflammatory myopathies, a heterogeneous group of chronic disorders with predominant inflammation in muscle tissue, including dermatomyositis, polymyositis, and myositis [65–67]. Studies elucidating the detailed involvement of muscular

inflammasome protein complex may thus provide promising targets for new therapies for this heterogeneous group of inflammatory muscle diseases.

4. NLRP3 Activation and Cardiovascular Complications of Metabolic Disorders

Individuals with obesity and insulin resistance have an increased burden of cardiovascular disease (CVD). In the Kuopio Ischemic Heart Disease study, Lakka et al. [68] reported a 4.26-fold relative risk for mortality due to heart disease and a 1.77 relative risk for all-cause mortality in obese, insulin-resistant patients. Similarly, in the Botnia study the risk for coronary heart disease (CHD) and stroke was shown to be increased threefold and the risk for cardiovascular mortality was increased six fold [69]. The Hoorn Study examined 615 men and 749 women aged 50 to 75 years without diabetes or a history of CVD at baseline and reported that the development of insulin resistance and/or obesity was associated with about a twofold increase in age-adjusted risk of fatal CVD in men and nonfatal CVD in women [70]. The pathophysiological mechanism by which metabolic disorders increase cardiovascular risk remains under debate. Studies published during the past decade have convincingly demonstrated a pathophysiological role for the inflammatory response in the development of both insulin resistance and related CVD. The finding a little over a decade ago that the secretion of IL-1β and IL-18 was increased in an ischemia/reperfusion (I/R) model of suprafused human atrial myocardium [71] provided the first clear link between inflammasome activation and CVD development. Experimental studies in mice with genetic deletion of caspase-1 have identified caspase-1 inhibition as a potential target for pharmacological intervention in the setting of CVD [72–74]. A recent report described formation of the inflammasome in a mouse model of myocardial I/R, mainly in cardiac fibroblasts and infiltrating cells, and reported that ASC knockout mice were protected, with a significant decline in cardiac infiltration of phagocytes, inflammatory cytokine levels, infarct size, and myocardial fibrosis and dysfunction [75]. The inflammasome was also detected in cardiomyocytes bordering the infarct zone during the infarct process, and prevention of inflammasome activation limited infarct size and cardiac enlargement after acute myocardial I/R injury in the mouse [76]. NLRP3 deficiency protects mice also from renal I/R injury [77, 78]. Both studies showed that the absence of NLRP3 protected kidneys against renal I/R injury to a greater extent than the absence of ASC, suggesting that NLRP3 may play an additional role in renal I/R injury independently of ASC and caspase-1. Overall, the ability of members of the NLRP3 inflammasome protein complex to target molecular and cellular pathways involved in both metabolic and cardiovascular diseases suggest that selective pharmacological modulation of NLRP3 inflammasome has the potential to exert synergistic effects in the control of metabolic disorders and its cardiovascular complications. Thus, this unique therapeutic strategy could decrease the burden of cardiovascular morbidity and mortality in the presence of obesity and insulin resistance, although, to date, there are no clinical data to support this concept.

5. Conclusions

In conclusion, NLRP3 inflammasome is a novel protein complex that integrates multiple exogenous and endogenous danger signals into the immediate secretion of IL-1β and IL-18. Most recent data suggest that activation of the NLRP3 inflammasome complex contributes to the pathophysiological mechanisms that explain the development of visceral obesity and insulin resistance. Thanks to its wide distribution in different tissues and organs, the NLRP3 inflammasome protein complex may represent a crucial signaling pathway that facilitates organ crosstalk and local injury in tissues target of metabolic damage. A better understanding of this novel pathway could help to clarify the crucial role of the molecular mechanisms of interorgan crosstalk during obesity and insulin resistance development. Studies using animal models and human biopsies will be useful to determine the spatial and temporal expression of inflammasome components inside the organs and to correlate these findings with disease activity or prognosis. Gene polymorphism studies on suitable patient cohorts could help to determine the functional significance of the protein expression data. Finally, the identification of selective pharmacological tools able to affect expression and/or activity of this novel pathway could represent the ultimate proof of significance of the inflammasome-caspase-1-IL-1β/18 axis in the development of metabolic inflammation. The effects evoked by these novel pharmacological tools should be compared with effects obtained by targeting selective cytokine receptor activities in order to better elucidate the potential crucial role of NLRP3 inflammasome protein complex in mediating inflammatory diseases. This approach may not only offer a potentially fruitful area of research, but it will also hopefully lead to novel and specific therapies for obesity-related conditions such as insulin resistance and its associated cardiovascular complications.

References

[1] R. Stienstra, J. A. van Diepen, C. J. Tack et al., "Inflammasome is a central player in the induction of obesity and insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 37, pp. 15324–15329, 2011.

[2] J. P.-Y. Ting, S. B. Willingham, and D. T. Bergstralh, "NLRs at the intersection of cell death and immunity," *Nature Reviews Immunology*, vol. 8, no. 5, pp. 372–379, 2008.

[3] G. Yeretssian, K. Labbé, and M. Saleh, "Molecular regulation of inflammation and cell death," *Cytokine*, vol. 43, no. 3, pp. 380–390, 2008.

[4] M. K. Atianand, V. A. Rathinam, and K. A. Fitzgerald, "SnapShot: inflammasomes," *Cell*, vol. 153, pp. 272–272.e1, 2013.

[5] L. Franchi, R. Muñoz-Planillo, T. Reimer, T. Eigenbrod, and G. Núñez, "Inflammasomes as microbial sensors," *European Journal of Immunology*, vol. 40, no. 3, pp. 611–615, 2010.

[6] T.-D. Kanneganti, "Central roles of NLRs and inflammasomes in viral infection," *Nature Reviews Immunology*, vol. 10, no. 10, pp. 688–698, 2010.

[7] G. Y. Chen and G. Nuñez, "Sterile inflammation: sensing and reacting to damage," *Nature Reviews Immunology*, vol. 10, no. 12, pp. 826–837, 2010.

[8] K. Nakahira, J. A. Haspel, V. A. K. Rathinam et al., "Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome," *Nature Immunology*, vol. 12, no. 3, pp. 222–230, 2011.

[9] R. Zhou, A. S. Yazdi, P. Menu, and J. Tschopp, "A role for mitochondria in NLRP3 inflammasome activation," *Nature*, vol. 469, pp. 221–225, 2011.

[10] A. Halle, V. Hornung, G. C. Petzold et al., "The NALP3 inflammasome is involved in the innate immune response to amyloid-β," *Nature Immunology*, vol. 9, no. 8, pp. 857–865, 2008.

[11] V. Hornung, F. Bauernfeind, A. Halle et al., "Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization," *Nature Immunology*, vol. 9, no. 8, pp. 847–856, 2008.

[12] B. Vandanmagsar, Y.-H. Youm, A. Ravussin et al., "The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance," *Nature Medicine*, vol. 17, no. 2, pp. 179–188, 2011.

[13] H. Wen, D. Gris, Y. Lei et al., "Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling," *Nature Immunology*, vol. 12, no. 5, pp. 408–415, 2011.

[14] P. R. Vajjhala, R. E. Mirams, and J. M. Hill, "Multiple binding sites on the pyrin domain of ASC protein allow self-association and interaction with NLRP3 protein," *The Journal of Biological Chemistry*, vol. 287, pp. 41732–41743, 2012.

[15] C. A. Dinarello, M. Y. Donath, and T. Mandrup-Poulsen, "Role of IL-1β in type 2 diabetes," *Current Opinion in Endocrinology, Diabetes and Obesity*, vol. 17, no. 4, pp. 314–321, 2010.

[16] J. Spranger, A. Kroke, M. Möhlig et al., "Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study," *Diabetes*, vol. 52, no. 3, pp. 812–817, 2003.

[17] J. Jager, T. Grémeaux, M. Cormont, Y. Le Marchand-Brustel, and J.-F. Tanti, "Interleukin-1β-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression," *Endocrinology*, vol. 148, no. 1, pp. 241–251, 2007.

[18] T. Kanemaki, H. Kitade, M. Kaibori et al., "Interleukin 1β and interleukin 6, but not tumor necrosis factor α, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes," *Hepatology*, vol. 27, no. 5, pp. 1296–1303, 1998.

[19] P. A. Cleary, T. J. Orchard, S. Genuth et al., "The effect of intensive glycemic treatment on coronary artery calcification in type 1 diabetic participants of the diabetes control and complications trial/epidemiology of diabetes interventions and complications (DCCT/EDIC) study," *Diabetes*, vol. 55, no. 12, pp. 3556–3565, 2006.

[20] C. M. Larsen, M. Faulenbach, A. Vaag et al., "Interleukin-1-receptor antagonist in type 2 diabetes mellitus," *The New England Journal of Medicine*, vol. 356, no. 15, pp. 1517–1526, 2007.

[21] R. Stienstra, L. A. B. Joosten, T. Koenen et al., "The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity," *Cell Metabolism*, vol. 12, no. 6, pp. 593–605, 2010.

[22] G. H. Goossens, E. E. Blaak, R. Theunissen et al., "Expression of NLRP3 inflammasome and T cell population markers in adipose tissue are associated with insulin resistance and impaired glucose metabolism in humans," *Molecular Immunology*, vol. 50, no. 3, pp. 142–149, 2012.

[23] H. M. Lee, J. J. Kim, H. J. Kim, M. Shong, B. J. Ku, and E. K. Jo, "Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes," *Diabetes*, vol. 62, pp. 194–204, 2013.

[24] H. P. Harding and D. Ron, "Endoplasmic reticulum stress and the development of diabetes: a review," *Diabetes*, vol. 51, supplement 3, pp. S455–S461, 2002.

[25] M. Cnop, F. Foufelle, and L. A. Velloso, "Endoplasmic reticulum stress, obesity and diabetes," *Trends in Molecular Medicine*, vol. 18, no. 1, pp. 59–68, 2012.

[26] M. Flamment, E. Hajduch, P. Ferre, and F. Foufelle, "New insights into ER stress-induced insulin resistance," *Trends in Endocrinology & Metabolism*, vol. 23, pp. 381–390, 2012.

[27] L. Xu, G. A. Spinas, and M. Niessen, "ER stress in adipocytes inhibits insulin signaling, represses lipolysis, and alters the secretion of adipokines without inhibiting glucose transport," *Hormone and Metabolic Research*, vol. 42, no. 9, pp. 643–651, 2010.

[28] J. Deng, S. Liu, L. Zou, C. Xu, B. Geng, and G. Xu, "Lipolysis response to endoplasmic reticulum stress in adipose cells," *The Journal of Biological Chemistry*, vol. 287, no. 9, pp. 6240–6249, 2012.

[29] T. Komura, Y. Sakai, M. Honda, T. Takamura, K. Matsushima, and S. Kaneko, "CD14+ monocytes are vulnerable and functionally impaired under endoplasmic reticulum stress in patients with type 2 diabetes," *Diabetes*, vol. 59, no. 3, pp. 634–643, 2010.

[30] P. Menu, A. Mayor, R. Zhou et al., "ER stress activates the NLRP3 inflammasome via an UPR-independent pathway," *Cell Death and Disease*, vol. 3, no. 1, article e261, 2012.

[31] A. G. Lerner, J. P. Upton, P. V. Praveen et al., "IRE1alpha induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress," *Cell Metabolism*, vol. 16, pp. 250–264, 2012.

[32] J. A. Kummer, R. Broekhuizen, H. Everett et al., "Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response," *Journal of Histochemistry and Cytochemistry*, vol. 55, no. 5, pp. 443–452, 2007.

[33] C. M. Reynolds, F. C. McGillicuddy, K. A. Harford, O. M. Finucane, K. H. Mills, and H. M. Roche, "Dietary saturated fatty acids prime the NLRP3 inflammasome via TLR4 in dendritic cells-implications for diet-induced insulin resistance," *Molecular Nutrition & Food Research*, vol. 56, pp. 1212–1222, 2012.

[34] H. Ruan, M. J. Zarnowski, S. W. Cushman, and H. F. Lodish, "Standard isolation of primary adipose cells from mouse epididymal fat pads induces inflammatory mediators and down-regulates adipocyte genes," *The Journal of Biological Chemistry*, vol. 278, no. 48, pp. 47585–47593, 2003.

[35] O. Nov, H. Shapiro, H. Ovadia et al., "Interleukin-1beta regulates fat-liver crosstalk in obesity by auto-paracrine modulation of adipose tissue inflammation and expandability," *PLoS One*, vol. 8, Article ID e53626, 2013.

[36] T. Mandrup-Poulsen, "IAPP boosts islet macrophage IL-1 in type 2 diabetes," *Nature Immunology*, vol. 11, no. 10, pp. 881–883, 2010.

[37] J. J. Collier, S. J. Burke, M. E. Eisenhauer et al., "Pancreatic β-cell death in response to pro-inflammatory cytokines is distinct

from genuine apoptosis," *PLoS ONE*, vol. 6, no. 7, Article ID e22485, 2011.

[38] A. Ardestani, N. S. Sauter, F. Paroni et al., "Neutralizing interleukin-1β(IL-1β) induces β-cell survival by maintaining PDX1 protein nuclear localization," *The Journal of Biological Chemistry*, vol. 286, no. 19, pp. 17144–17155, 2011.

[39] R. Zhou, A. Tardivel, B. Thorens, I. Choi, and J. Tschopp, "Thioredoxin-interacting protein links oxidative stress to inflammasome activation," *Nature Immunology*, vol. 11, no. 2, pp. 136–140, 2010.

[40] S. L. Masters, A. Dunne, S. L. Subramanian et al., "Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β 2 in type 2 diabetes," *Nature Immunology*, vol. 11, no. 10, pp. 897–904, 2010.

[41] Y.-H. Youm, A. Adijiang, B. Vandanmagsar, D. Burk, A. Ravussin, and V. D. Dixit, "Elimination of the NLRP3-ASC inflammasome protects against chronic obesity-induced pancreatic damage," *Endocrinology*, vol. 152, no. 11, pp. 4039–4045, 2011.

[42] S. Madec, C. Rossi, M. Chiarugi et al., "Adipocyte P2X7 receptors expression: a role in modulating inflammatory response in subjects with metabolic syndrome?" *Atherosclerosis*, vol. 219, no. 2, pp. 552–558, 2011.

[43] O. Nov, A. Kohl, E. C. Lewis et al., "Interleukin-1β may mediate insulin resistance in liver-derived cells in response to adipocyte inflammation," *Endocrinology*, vol. 151, no. 9, pp. 4247–4256, 2010.

[44] A. B. Imaeda, A. Watanabe, M. A. Sohail et al., "Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome," *The Journal of Clinical Investigation*, vol. 119, no. 2, pp. 305–314, 2009.

[45] A. Watanabe, M. A. Sohail, D. A. Gomes et al., "Inflammasome-mediated regulation of hepatic stellate cells," *American Journal of Physiology*, vol. 296, no. 6, pp. G1248–G1257, 2009.

[46] T. Csak, M. Ganz, J. Pespisa, K. Kodys, A. Dolganiuc, and G. Szabo, "Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells," *Hepatology*, vol. 54, no. 1, pp. 133–144, 2011.

[47] Y. Kamari, A. Shaish, E. Vax et al., "Lack of interleukin-1α or interleukin-1β inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice," *Journal of Hepatology*, vol. 55, no. 5, pp. 1086–1094, 2011.

[48] Z. Liu, M. H. Zaki, P. Vogel et al., "Role of inflammasomes in host defense against Citrobacter rodentium infection," *The Journal of Biological Chemistry*, vol. 287, pp. 16955–16964, 2012.

[49] I. C. Allen, E. M. Tekippe, R.-M. T. Woodford et al., "The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer," *Journal of Experimental Medicine*, vol. 207, no. 5, pp. 1045–1056, 2010.

[50] M. H. Zaki, K. L. Boyd, P. Vogel, M. B. Kastan, M. Lamkanfi, and T.-D. Kanneganti, "The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis," *Immunity*, vol. 32, no. 3, pp. 379–391, 2010.

[51] S. A. Hirota, J. Ng, A. Lueng et al., "NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis," *Inflammatory Bowel Diseases*, vol. 17, no. 6, pp. 1359–1372, 2011.

[52] C. Bauer, F. Loher, M. Dauer et al., "The ICE inhibitor pralnacasan prevents DSS-induced colitis in C57BL/6 mice and suppresses IP-10 mRNA but not TNF-α mRNA expression," *Digestive Diseases and Sciences*, vol. 52, no. 7, pp. 1642–1652, 2007.

[53] C. Bauer, P. Duewell, C. Mayer et al., "Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome," *Gut*, vol. 59, no. 9, pp. 1192–1199, 2010.

[54] J. Henao-Mejia, E. Elinav, C. Jin et al., "Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity," *Nature*, vol. 482, no. 7384, pp. 179–185, 2012.

[55] K. Matsumoto and K. Kanmatsuse, "Elevated interleukin-18 levels in the urine of nephrotic patients," *Nephron*, vol. 88, no. 4, pp. 334–339, 2001.

[56] G. Lonnemann, D. Novick, M. Rubinstein, and C. A. Dinarello, "Interleukin-18, interleukin-18 binding protein and impaired production of interferon-γ in chronic renal failure," *Clinical Nephrology*, vol. 60, no. 5, pp. 327–334, 2003.

[57] S. Gauer, O. Sichler, N. Obermüller et al., "IL-18 is expressed in the intercalated cell of human kidney," *Kidney International*, vol. 72, no. 9, pp. 1081–1087, 2007.

[58] A. Vilaysane, J. Chun, M. E. Seamone et al., "The NLRP3 inflammasome promotes renal inflammation and contributes to CKD," *Journal of the American Society of Nephrology*, vol. 21, no. 10, pp. 1732–1744, 2010.

[59] H.-J. Anders and D. A. Muruve, "The inflammasomes in kidney disease," *Journal of the American Society of Nephrology*, vol. 22, no. 6, pp. 1007–1018, 2011.

[60] M. Collino, E. Benetti, M. Rogazzo et al., "Reversal of the deleterious effects of chronic dietary HFCS-55 intake by PPAR-delta agonism correlates with impaired NLRP3 inflammasome activation," *Biochemical Pharmacology*, vol. 85, pp. 257–264, 2013.

[61] C. Jin and R. A. Flavell, "Molecular mechanism of NLRP3 inflammasome activation," *Journal of Clinical Immunology*, vol. 30, no. 5, pp. 628–631, 2010.

[62] Q.-H. Hu, X. Zhang, Y. Pan, Y.-C. Li, and L.-D. Kong, "Allopurinol, quercetin and rutin ameliorate renal NLRP3 inflammasome activation and lipid accumulation in fructose-fed rats," *Biochemical Pharmacology*, vol. 84, pp. 113–125, 2012.

[63] R. Rawat, T. V. Cohen, B. Ampong et al., "Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle," *American Journal of Pathology*, vol. 176, no. 6, pp. 2891–2900, 2010.

[64] P. Boström, J. Wu, M. P. Jedrychowski et al., "A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis," *Nature*, vol. 481, no. 7382, pp. 463–468, 2012.

[65] I. Lundberg, A. K. Kratz, H. Alexanderson, and M. Patarroyo, "Decreased expression of interleukin-1alpha, interleukin-1beta, and cell adhesion molecules in muscle tissue following corticosteroid treatment in patients with polymyositis and dermatomyositis," *Arthritis & Rheumatism*, vol. 43, pp. 336–348, 2000.

[66] M. Tucci, C. Quatraro, F. Dammacco, and F. Silvestris, "Interleukin-18 overexpression as a hallmark of the activity of autoimmune inflammatory myopathies," *Clinical and Experimental Immunology*, vol. 146, no. 1, pp. 21–31, 2006.

[67] J. Schmidt, K. Barthel, A. Wrede, M. Salajegheh, M. Bähr, and M. C. Dalakas, "Interrelation of inflammation and APP in sIBM: IL-1β induces accumulation of β-amyloid in skeletal muscle," *Brain*, vol. 131, no. 5, pp. 1228–1240, 2008.

[68] H.-M. Lakka, D. E. Laaksonen, T. A. Lakka et al., "The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men," *The Journal of the American Medical Association*, vol. 288, no. 21, pp. 2709–2716, 2002.

[69] B. Isomaa, P. Almgren, T. Tuomi et al., "Cardiovascular morbidity and mortality associated with the metabolic syndrome," *Diabetes Care*, vol. 24, no. 4, pp. 683–689, 2001.

[70] A. A. W. A. van der Heijden, M. M. Ortegon, L. W. Niessen, G. Nijpels, and J. M. Dekker, "Prediction of coronary heart disease risk in a general, pre-diabetic, and diabetic population during 10 years of follow-up: accuracy of the Framingham, SCORE, and UKPDS risk functions—The Hoorn Study," *Diabetes Care*, vol. 32, no. 11, pp. 2094–2098, 2009.

[71] B. J. Pomerantz, L. L. Reznikov, A. H. Harken, and C. A. Dinarello, "Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1β," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 5, pp. 2871–2876, 2001.

[72] S. Frantz, A. Ducharme, D. Sawyer et al., "Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 6, pp. 685–694, 2003.

[73] F. M. Syed, H. S. Hahn, A. Odley et al., "Proapoptotic effects of caspase-1/interleukin-converting enzyme dominate in myocardial ischemia," *Circulation Research*, vol. 96, no. 10, pp. 1103–1109, 2005.

[74] S. Merkle, S. Frantz, M. P. Schön et al., "A role for caspase-1 in heart failure," *Circulation Research*, vol. 100, no. 5, pp. 645–653, 2007.

[75] M. Kawaguchi, M. Takahashi, T. Hata et al., "Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury," *Circulation*, vol. 123, no. 6, pp. 594–604, 2011.

[76] E. Mezzaroma, S. Toldo, D. Farkas et al., "The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 49, pp. 19725–19730, 2011.

[77] S. S. Iyer, W. P. Pulskens, J. J. Sadler et al., "Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 48, pp. 20388–20393, 2009.

[78] A. A. Shigeoka, J. L. Mueller, A. Kambo et al., "An inflammasome-independent role for epithelial-expressed Nlrp3 in renal ischemia-reperfusion injury," *The Journal of Immunology*, vol. 185, pp. 6277–6285, 2010.

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Research Article

Nitric Oxide Is a Mediator of Antiproliferative Effects Induced by Proinflammatory Cytokines on Pancreatic Beta Cells

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Nitric oxide (NO) is involved in several biological processes. In type 1 diabetes mellitus (T1DM), proinflammatory cytokines activate an inducible isoform of NOS (iNOS) in β cells, thus increasing NO levels and inducing apoptosis. The aim of the current study is to determine the role of NO (1) in the antiproliferative effect of proinflammatory cytokines IL-1β, IFN-γ, and TNF-α on cultured islet β cells and (2) during the insulitis stage prior to diabetes onset using the Biobreeding (BB) rat strain as T1DM model. Our results indicate that NO donors exert an antiproliferative effect on β cell obtained from cultured pancreatic islets, similar to that induced by proinflammatory cytokines. This cytokine-induced antiproliferative effect can be reversed by L-NMMA, a general NOS inhibitor, and is independent of guanylate cyclase pathway. Assays using NOS isoform specific inhibitors suggest that the NO implicated in the antiproliferative effect of proinflammatory cytokines is produced by inducible NOS, although not in an exclusive way. In BB rats, early treatment with L-NMMA improves the initial stage of insulitis. We conclude that NO is an important mediator of antiproliferative effect induced by proinflammatory cytokines on cultured β cell and is implicated in β-cell proliferation impairment observed early from initial stage of insulitis.

1. Introduction

Type 1 diabetes mellitus (T1DM) is characterized by a loss of beta cell mass due to an autoimmune process. In a phase prior to the onset, immune cells infiltrate the islets creating an inflammatory microenvironment responsible for the β-cell-specific toxicity. Proinflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ), secreted by activated lymphocytes and macrophages during insulitis, induce NFkB mediated iNOS expression which has a key role in β-cell apoptosis in the early T1DM stage [1].

Nitric oxide is a ubiquitous molecule which acts as messenger in diverse biological processes. It is produced in tissues by the enzyme nitric oxide synthase (NOS) by oxidation of the amino acid L-arginine. There are three different tissue-specific NOS isoforms: Ca^{2+}/calmodulin-independent and inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). These last two isoforms also exhibit constitutive expression and Ca^{2+}/calmodulin-dependent activity.

Expression of NOS isoforms has been studied in pancreatic islets, and the nNOS form has been observed in β-cell cells, located, mainly, in insulin secretory granules. It exhibits cytochrome C reductase activity in addition to NO

production activity, and a balance between both functions is essential for a normal insulin secretion in response to glucose stimulation. In contrast, eNOS expression has not been found in rat islets under basal conditions [2, 3].

Several mechanisms have been proposed in explaining NO-mediated β-cell cell apoptosis. Prooxidant agents formed from NO induce damages in DNA which activate p53 which induces proapoptotic expression of genes such as BAX, FAS, NOXA, and PUMA. A p53-independent mechanism has been described in which NO acts by inhibiting the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b (SERCA2b) protein and, subsequently, by inducing the endoplasmic reticulum stress mechanisms [4, 5]. The importance of the mitochondrial pathway has also been highlighted in NO-mediated β-cell cell apoptosis [6, 7]. Paradoxically, an antiapoptotic effect of low concentration of NO has been reported in several systems, including β cells [8–12].

In addition to apoptosis, cell proliferation has been described as a NO-regulated process. Although certain activating effects have been reported in physiological systems [13, 14], the main role of NO in cellular proliferation is inhibitory. In the subventricular zone, NO induces inhibition of stem cell proliferation by a nitrosylation process [15]. Other proposed mechanism for NO antiproliferative action is a G1-S inhibition mediated by an induction of the cell cycle inhibitor p21 [16] or cyclins inhibition [17]. Very few studies have examined the role of NO in proliferation in β cells. Recently, NO-mediated neogenesis stimulation has been observed in an alloxan-induced murine model of diabetes [18].

A proinflammatory cytokine-mediated inhibition of cultured β-cell proliferation in addition to the apoptotic effect has been observed by our group. As described previously, NO production is one of the most important events mediated by proinflammatory cytokines. Therefore, the aim of this study was to assess the importance of NO in the cytokine-mediated antiproliferative effect on cultured β cells and to determine the role(s) of different NO synthase isoforms present in pancreatic islets.

2. Methods

2.1. Animals. All animal procedures were performed with the approval of the Cádiz University School of Medicine (Cádiz, Spain) Committee for the Ethical Use and Care of Experimental Animals.

Bio-Breeding (BB) and Wistar rats were kept under conventional conditions in an environment-controlled room (20-21°C, 12 h light-dark cycle) with water and standard laboratory rat chow available *ad libitum* and their weight was daily recovered. Blood extracted from the tail vein was used in BB rats for weekly random glucose measurements using an automatic glucose monitor (Accu-Chek Optimum, Roche Diagnostic, Basel, Switzerland).

2.2. Isolation and Culture of Rat Islets. Pancreatic islets were isolated from adult male Wistar rats as described previously by McDaniel et al. [19]. Isolated islets were cultured in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Gibco Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Pen-Strep, Bio-Whittaker Europe, Verviers, Belgium), and 10% fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA, USA). Glucose concentration used was 5.5 mM. Proinflammatory cytokines (PeproTech EC Ltd, London, UK) used in the experiments were recombinant human IL-1β (50 U/mL), recombinant rat IFN-γ (1000 U/mL), and recombinant rat TNF-α (1000 U/mL). These concentrations were selected as being appropriate based on the results of previous published studies [20, 21].

2.3. Culture Treatment. Pancreatic islet cultures were treated with different drugs related to NO metabolism. NO donors, S-nitroso-N-acetyl-DL-penicillamine (SNAP), and diethylenetriamine/nitric oxide adduct (DETA-NO), obtained from Sigma-Aldrich (St. Louis, MO, USA) show different NO release rates. DETA-NO is a member of the NONOates family and has a half-life ($t_{1/2}$) of 20 h at 37°C, pH 7.4. It generates 2 moles of NO following simple first-order kinetics. The $t_{1/2}$ of SNAP is approximately 6 h at 37°C and pH 6–8 although its biological activity is highly influenced by the molecular environment of the parent thiol. Inhibitors of NOS 7-nitroindazole (7-Ni), N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride (1400 W), and N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-NIO), purchased from Sigma-Aldrich (St. Louis, MO, USA) were used to inhibit the activity of neuronal, inducible, and endothelial isoforms of NOS, respectively.

2.4. Proliferation Assays in Cultured Islets. Proliferating β cells were detected using 5-bromo-2′-deoxyuridine (BrdU) 5 μmol/L label (Sigma-Aldrich, St. Louis, MO, USA) which was added to the cultures from the start of the assay together with the drugs being tested, when appropriate. Following the appropriate culture duration, the islets were recovered and incubated for 15 min with trypsin-EDTA (0.25% trypsin, 1 mM EDTA) in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco Invitrogen, Carlsbad, CA, USA) at 37°C, and the islets were gently dispersed. After washing with PBS, cells were cytospin on poly-L-lysine-coated slides and fixed in 4% methanol-free formaldehyde. Slides were immuno-stained using monoclonal mouse anti-BrdU (Dako Cytomation, Denmark) and polyclonal guinea pig anti-insulin (Sigma-Aldrich, St. Louis, MO, USA) antibodies, according to the manufacturer's instructions. Cells were permeabilized by incubation for 30 min with 0.1% Triton-X100 in PBS and washed twice with 100 mM glycine buffer containing 0.1% Triton-X100 and 3% bovine serum albumin. Cells were then treated with HCl (2N) in PBS for 30 min, neutralized with borax/borate buffer (0.1 M, pH 8.9) for 30 min, washed, and incubated overnight at 4°C with anti-BrdU and anti-insulin antibodies. Stained cells were revealed using anti-mouse IgG antibody (alexa-546 conjugated) and anti-guinea pig IgG (alexa-488 conjugated) antibody (Molecular Probes Inc, Eugene, OR, USA). Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). To determine

the proliferating fraction, total and insulin-positive/BrdU-positive cells were analyzed using a fluorescence microscope in randomized conditions, by a single investigator. For statistical purposes, the percentage of positive cells was calculated for each incubation condition.

2.5. Immunofluorescence Staining of Cultured Pancreatic Islets. 48 h cultured islets in presence and absence of cytokines were processed for histology study by histogel and paraffin inclusion. 10 μm sections were stained using a polyclonal rabbit anti-iNOS (ab15323, Abcam, Cambridge, UK) and a polyclonal guinea pig anti-insulin (Sigma-Aldrich, St. Louis, MO) and examined using a microscope equipped with a digital camera and the image analysis CellDsoftware (Olympus, Hamburg, Germany).

2.6. Treatment Protocol. Randomly grouped BB rats daily received an intraperitoneal injection of vehicle (0.1% DMSO diluted in injection water) alone or containing L-NMMA at a dose of 30 mg/kg, from 4 to 9 weeks of age. Animals were sacrificed at 4 (before treatment), 7, and 9 weeks of age.

2.7. IPGTT Assay. Wistar, BB, and thymectomized BB rats were fasted overnight (16–18 h) and a blood sample was collected from the tail vein (fasting or 0 min sample). Then, an intraperitoneal injection of 40% solution of glucose was administered (2 g/kg), followed by blood sampling at 15, 30, 60, and 120 min after the glucose administration. Glycemia was measured with an automatic glucose monitor (Accu-Chek Optimum, Roche Diagnostic, Basel, Switzerland). Blood samples obtained from fasting and 15 min after glucose administration were also used to plasma extraction and insulin quantification by ELISA technique (Insulin rat ultrasensitive kit. ALPCO, Salem, MA, USA).

2.8. Quantification of β-Cell Mass. 7 and 9 weeks of age BB rat pancreas were resected, weighed, fixed in Bouin's solution for 6 h, and postfixed in formalin for 12 h. Then, they were dehydrated, paraffin embedded, and longitudinal 10 μm microtome sections were obtained. Insulin was stained in obtained pancreas by immunohistochemical techniques using a mouse anti-rat insulin monoclonal antibody and a peroxidase conjugated goat anti-mouse IgG antibody and revealed with DAB kit (Sigma Aldrich, St. Louis, MO, USA). Insulin-positive areas were quantified in two complete sections of each pancreas using a microscope equipped with a digital camera and the image analysis Cell D software (Olympus, Hamburg, Germany). The investigators were blinded with respect to the provenance of the samples. β-cell mass values were calculated by multiplying the total insulin-positive area/total pancreatic area ratio by the total pancreas weight.

2.9. Western Blotting. Equivalent numbers of islets under the different experimental conditions described previously were lysed in lysis buffer (125 mM Tris-HCl buffer pH 6–8, 2% SDS, 1 mM DTT and containing protease and phosphatase inhibitors). Protein (40 μg) was loaded and electrophoresed on 8% SDS-PAGE. Proteins were transfer-blotted on to polyvinylidene fluoride (PDFV) membranes and then incubated in blocking buffer (5% nonfat milk in 10 mM Tris-HCl, 1.15 M NaCl and 0.1% Tween-20) for 1 h at room temperature. The blots were then incubated with rabbit polyclonal antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz CA, USA) and iNOS (Transduction Laboratories BD, NJ, USA) overnight according to the manufacturer's instructions, followed by incubation with peroxidase conjugated anti-rabbit IgG for 1 h at room temperature. Stained bands were revealed using Immun-Star Western C kit (Bio-Rad, Hercules CA, USA) and quantified by Quantity One software Version Upgrade (Bio-Rad, Hercules, CA, USA). β-tubulin expression was used as loading control and eNOS expression was calculated as ratio of eNOS: β-tubulin densities.

2.10. Histological Examination of β-Cell Infiltration. Histological examination of 7 and 9 weeks of age rat pancreatic islets was performed in Harris' H&E stained pancreas sections using ×20 objective lens. The severity of insulitis was graded as a function of the mononuclear cell infiltration of the pancreatic islets: 0 = no infiltrate; 1 = periductular infiltrate; 2 = periislet infiltrate; 3 = intraislet infiltrate; 4 = intraislet infiltrate associated with β-beta cell destruction. Thirty islets were examined in each pancreas and the mean score was calculated by dividing the total score by the number of islets examined.

2.11. Statistical Analysis. Results are presented as means ± SEM of measurements performed in at least 3 animals. Statistical comparisons were performed by Mann-Whitney test. All P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effect of NO Donors on β-Cell Proliferation in Cultured Pancreatic Islets. To determine the role of NO donors on β-cell proliferation, pancreatic islets were treated with proinflammatory cytokines or different concentrations of NO donors SNAP and DETA, alone or in combination with the caspase-3 inhibitor z-VAD-fmk. β-cell proliferation measured by BrdU incorporation showed that the NO donors, DETA-NO (Figure 1(a)) and SNAP (Figure 1(b)) exert an antiproliferative action on β cells in a dose-dependent manner. This antiproliferative effect was similar to that obtained by proinflammatory cytokines. This effect of NO donors was not modified by addition of z-VAD-fmk to the cultures.

3.2. Role of NO in Antiproliferative Effect of Proinflammatory Cytokines on Pancreatic β Cells. To determine the contribution of NO to the antiproliferative effect of proinflammatory cytokines on pancreatic β cells, pancreatic islets were cultured over a 48 h period and treated with proinflammatory cytokines, alone or in the presence of L-NMMA (an inhibitor of nitric oxide synthase). Inhibition of β-cell proliferation induced by proinflammatory cytokines was completely abolished by L-NMMA treatment (Figure 2).

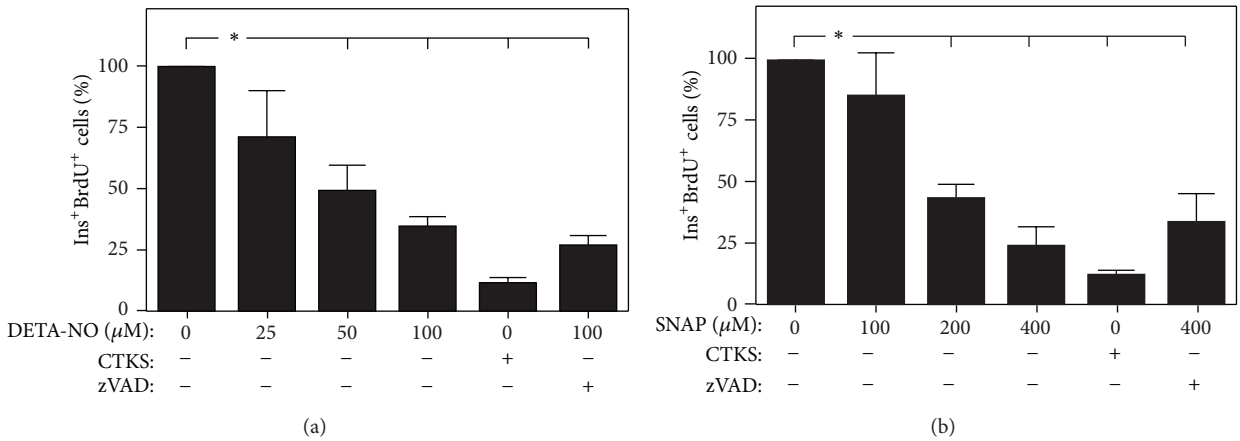

Figure 1: Effect of NO donors in cultured beta cell proliferation. Rat islets were cultured for 48 h and treated with NO donors DETA-NO (a) and SNAP (b) at increasing concentrations, alone or in combination with zVADfmk (100 μM), a caspase inhibitor, and with the combination of proinflammatory cytokines IL-1β (50 U/mL) + IFN-γ (1000 U/mL) + TNF-α (1000 U/mL) (CTKS). Cultures were stained with BrdU over the course of the culture period. Bar graphs are percentage means ± SEM of accumulated BrdU/insulin-positive cells over the culture period relative to insulin-positive cells. Values are the means of 4 experiments. $^{*}P < 0.05$ treated versus control.

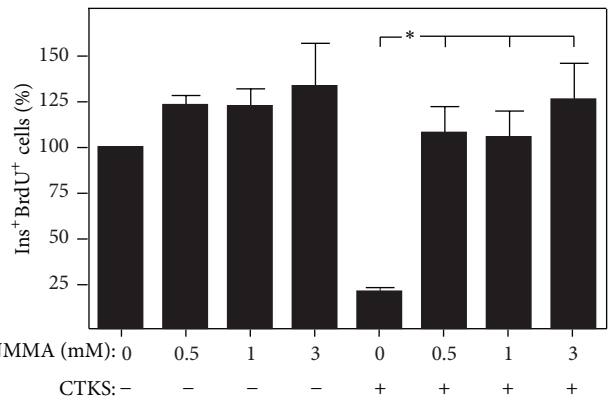

Figure 2: Effect of NO inhibition on islet β-cell proliferation. Rat islets were cultured for 48 h in the presence of cytokines IL-1β (50 U/mL) + IFN-γ (1000 U/mL) + TNF-α (1000 U/mL) (CTKS) alone or in combination with NOS inhibitor L-NMMA and stained with BrdU over the course of the culture period. Results are presented as percentage means ± SEM of combined BrdU/insulin positive cells relative to insulin-positive cells in a minimum of 5 experiments. $^{*}P < 0.05$ treated versus control.

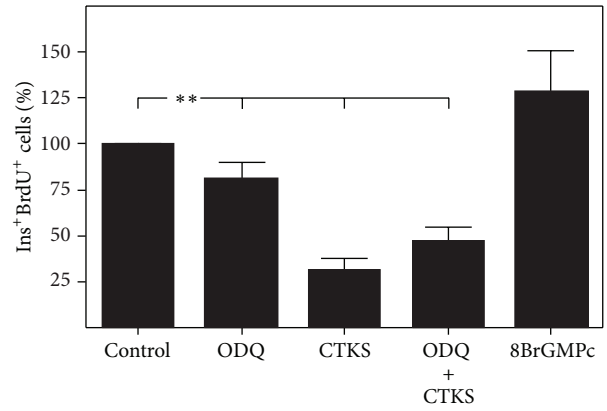

Figure 3: Effect of cGMP pathway alterations in β-cell proliferation in response to proinflammatory cytokines. Rat islets were cultured for 48 h in the presence of cGMP pathway activator 8BrGMPc (100 μM) and a mixture of cytokines IL-1β (50 U/mL) + IFN-γ (1000 U/mL) + TNF-α (1000 U/mL) (CTKS) alone or in combination with ODQ (10 μM), an inhibitor of guanylate cyclase. Results are presented as percentage means ± SEM of combined BrdU/insulin-positive cells relative to insulin positive cells in a minimum of 5 experiments. $^{**}P < 0.01$ treated versus control.

3.3. Role of Guanylate Cyclase in the NO Effect on β-Cell Proliferation. Guanylate cyclase is the enzyme through which NO exerts the greater part of its effect. To assess the implication of the guanylate cyclase pathway in the NO effect on β-cell proliferation, cultures of pancreatic islet were treated for 48 h with 8Br-cGMP (a guanylate cyclase analogue) and its inhibitor ODQ, alone or in combination with proinflammatory cytokines. As shown in Figure 3, the cGMP analogue exerts no effect on β-cell proliferation, and the antiproliferative effect of cytokines is not altered by the cGMP inhibitor ODQ.

3.4. Involvement of Different Isoforms of NOS in Proinflammatory Cytokine-Induced Decrease on β-Cell Proliferation. To study the role of constitutive and inducible NOS isoforms in the antiproliferative action of cytokines, cultured pancreatic islets were treated with proinflammatory cytokines in addition to 1400 W and 7-Ni, inhibitors of inducible and constitutive isoforms of nitric oxide synthase, respectively; the resultant β-cell proliferation was quantified. The antiproliferative effect of proinflammatory cytokines underwent no modification when islet cultures were incubated with the constitutive NOS inhibitor (Figure 4(e)). Related

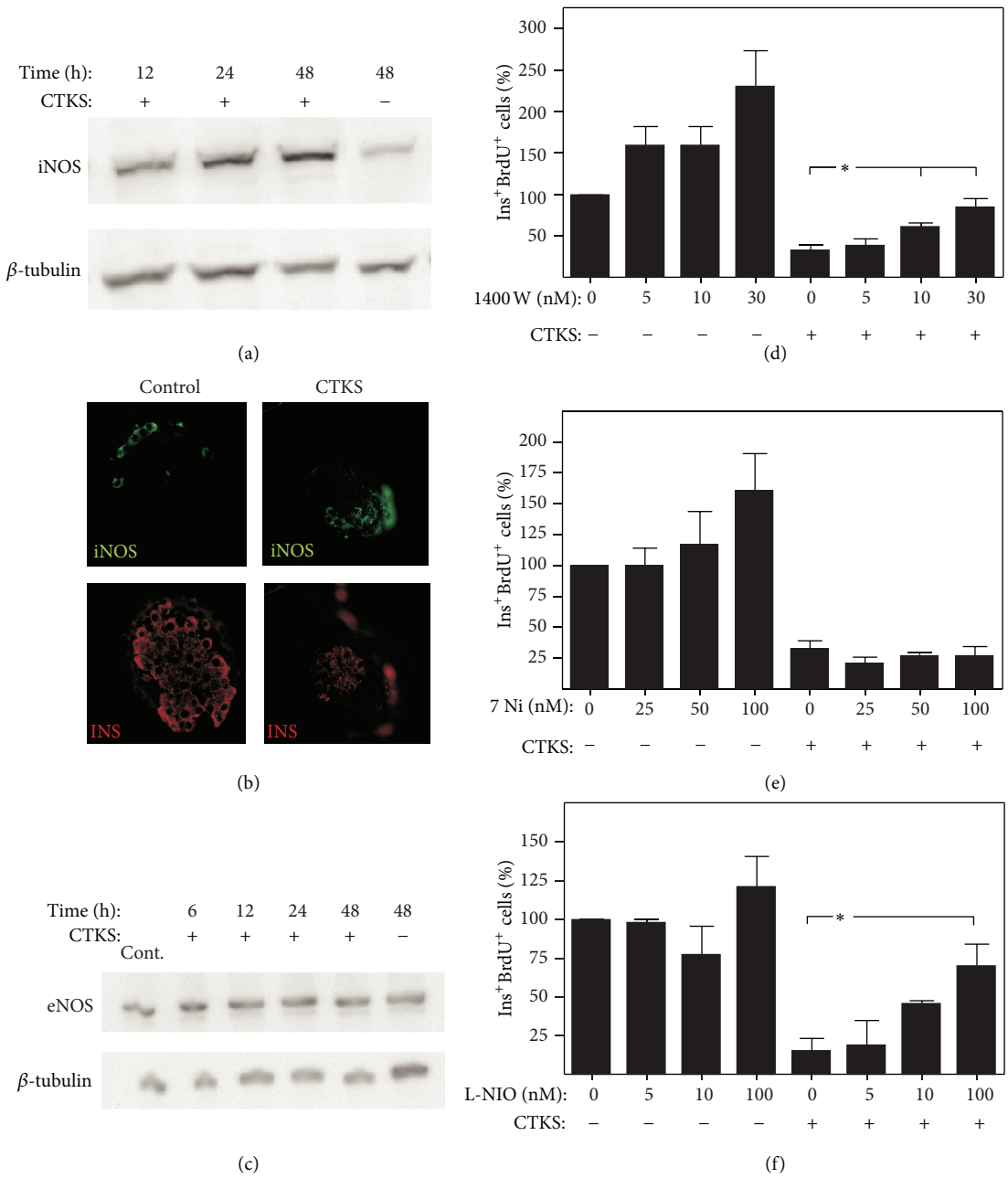

Figure 4: Role of different NOS isoforms in cytokine-induced antiproliferation in cultured β cells. (a) and (c) iNOS and eNOS expression was assessed using western blot analysis in pancreatic islets cultured over 48 h under basal conditions and treated with a mixture of cytokines IL-1β (50 U/mL) + IFN-γ (1000 U/mL) + TNF-α (1000 U/mL) (CTKS) over 6, 12, 24, and 48 h. A representative image is shown (Cont: positive brain control in eNOS Western blot). (b) Representative image of iNOS (green) and insulin (red) immunostaining of control and cytokines treated (CTKS) pancreatic islets cultured over 48 h. (d), (e), and (f) rat islets cultured over 48 h in the presence of 7 Ni (e), 1400 W (d), and L-NIO (f) alone or with the mixture of cytokines (CTKS). Results are presented as percentage means ± SEM of combined BrdU/insulin-positive cells relative to insulin-positive cells in a minimum of 5 experiments. $^*P < 0.05$ treated versus control.

to iNOS, cytokine-dependent induction of protein was observed from 24 h of culture (Figure 4(a)). iNOS expression study by immunohistochemical techniques in control cultured pancreatic islets showed a scarce stain with peripheral profile. However, cultured islet in presence of cytokines showed an increased iNOS expression localized internally in the islet (Figure 4(b)). The specific inhibitor 1400 W partially reverted cytokine-induced decreased proliferation in a dose-dependent manner (Figure 4(d)). Endothelial isoform of NOS expression was initially examined in pancreatic islets cultured under control condition, or after stimulation with proinflammatory cytokines. As shown in Figure 4(c), eNOS

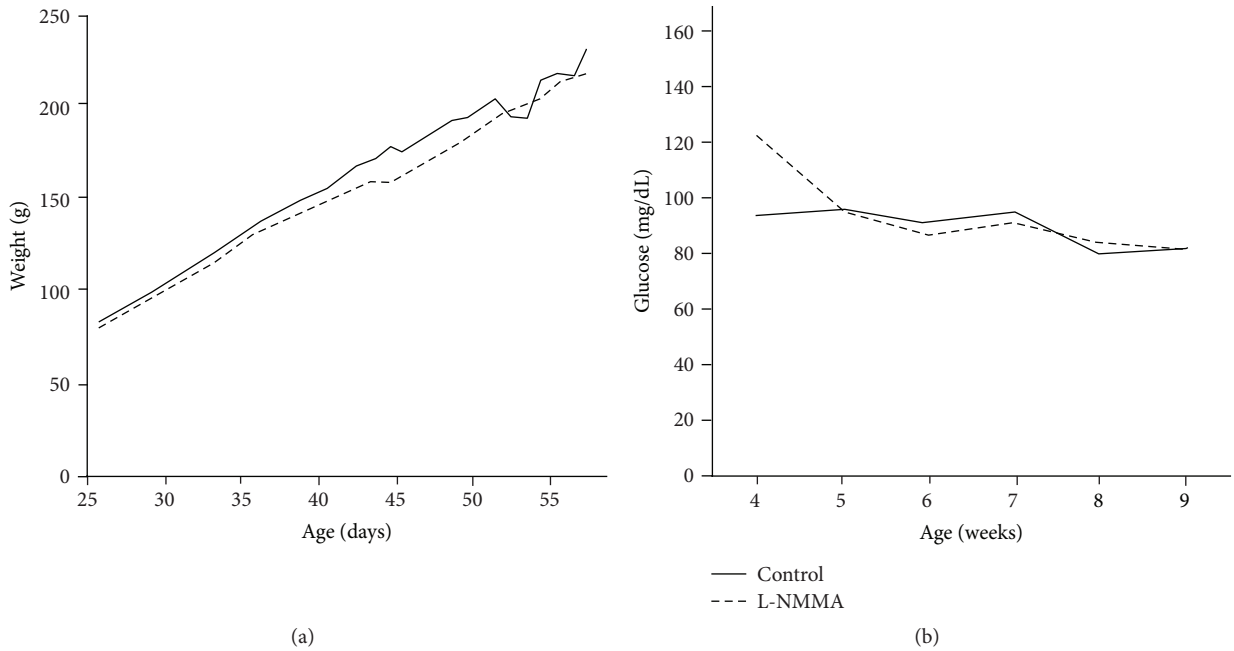

Figure 5: (a) Body weights were daily recorded in untreated (solid line) and L-NMMA-treated (dotted line) BB rats between 4 and 9 weeks of age. (b) Random blood glucose levels were weekly determined in untreated (solid line) and L-NMMA-treated (dotted line) BB rats between 4 and 9 weeks of age. The results are presented as means of weight (g) and glycemia (mg/mL) in a $n = 5$ animals.

isoform was expressed in pancreatic islets and was not altered by exposure to proinflammatory cytokines. To study the role of eNOS on the antiproliferative effect of cytokines, L-NIO (an inhibitor with higher affinity for eNOS than the other isoforms) was added to cultures in addition to cytokines, and β-cell proliferation was quantified. A partial inhibition of the antiproliferative effect of proinflammatory cytokines was observed in treated cultures (Figure 4(f)) mediated by L-NIO.

3.5. Effect of Early Treatment with L-NMMA on BB Rats Weight and Glucose Homeostasis during Insulitis Stage. Weight and random glycemia monitoring along treatment period show no differences in those parameters between control and L-NMMA-treated animals. Glucose homeostasis was studied by IPGTT and insulin determination before and 15 min after intraperitoneal glucose administration. As shown in Figure 5, IPGTT displays a normal curve at studied times in L-NMMA treated as in control animals (Figure 6(a)). In addition, insulin quantification also shows no changes between control and treated animals at none of studied times (Figure 6(b)).

3.6. Effect of Early Treatment with L-NMMA on β-Cell Mass in BB Rats during Insulitis Stage. BB rats treated with L-NMMA from 4 to 7 or 9 weeks of age were sacrificed and pancreas was removed from each rat to quantify beta cell mass by immunohistological techniques. In Figure 7, a beta cell mass loss undergone by BB rats between 7 and 9 weeks of age can be observed. L-NMMA reverts this effect at both studied times.

3.7. Effect of Early Treatment with L-NMMA on Infiltration Levels during Insulitis Stage. To test the effect of L-NMMA treatment on infiltration levels, H&E-stained sections obtained from treated and control BB rats were evaluated and infiltration scores were calculated. No changes were observed in infiltration level in response to L-NMMA at none of studied times (Figure 8).

4. Discussion

Currently, NO is considered an important mediator of cell signaling via its classical guanylate cyclase pathway or by exerting posttranslational modifications on an increasing number of proteins and hence regulating their activity. In the present study, we analyzed the role of NO in the previously described antiproliferative action of proinflammatory cytokines on pancreatic β cells. Different NO donors have been tested with respect to apoptosis induction in β cells. Loss of membrane integrity and cytochrome C release induced by DETA-NO and SNAP in insulin producing cell lines has been reported [22, 23]. In our system, NO donors SNAP and DETA-NO induced a decrease in cultured β-cell proliferation at concentrations up to 50 μM for DETA-NO and up to 400 μM for SNAP. This effect is dose dependent and similar to that obtained with proinflammatory cytokines (IL-1β + TNF-α + IFN-γ) treatment (Figure 1). The antiproliferative effect observed in response to NO donors is not dependent on the biological $t_{1/2}$ and suggests that NO exerts its biological action in the first hours after exposure. To discard the possibility that the decrease in proliferation was due to a cytokine-induced apoptosis in proliferating β cell, we

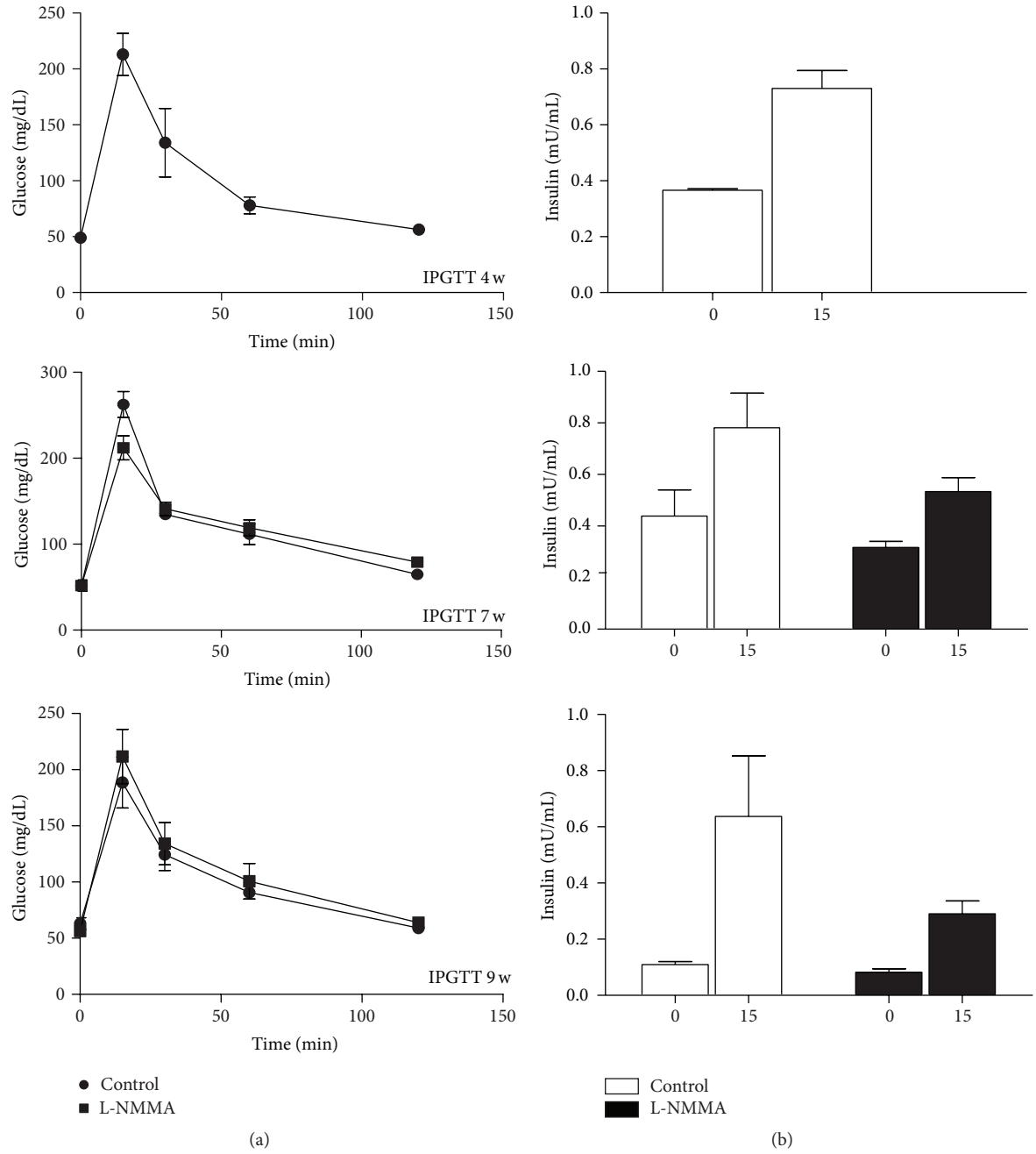

Figure 6: (a) IPGTT was performed in BB rats treated with vehicle (circle) or L-NMMA (square) at 4, 7, and 9 weeks of age. The results are presented as means ± SEM of glycemia (mg/mL) at the stated times after glucose injection in an $n = 5$ animals. (b) plasma insulin was quantified in blood samples obtained from BB rats treated with vehicle (white bars) or L-NMMA (black bars) at 4, 7, and 9 weeks of age. The results are presented as means ± SEM of insulin concentration (mU/mL) at fasting and 15 min after glucose injection in an $n = 5$ animals.

treated pancreatic β-cell cultures with the caspase-3 inhibitor zVADfmk, in addition to the NO donors. β-cell apoptosis inhibition induced by zVADfmk exerts no effect on the antiproliferative effect of NO donors suggesting that the two processes are unrelated.

The mechanism by which NO induces β-cell apoptosis has been extensively studied. Although studies in neonatal rat islets indicated that cytokine-induced NO production regulates the expression of up to 42 proteins, a considerable number of proteins are regulated in a NO-independent manner [24]. Thus, the cytokine-induced apoptosis in mouse β cells is not caused by the inducible isoform of NOS alone [25]. Endoplasmic reticulum stress has been described as a possible mechanism by which NO induces apoptosis [26]. Its presence regulates unfolded protein response (UPR) signaling pathway that determines apoptotic response to reticulum

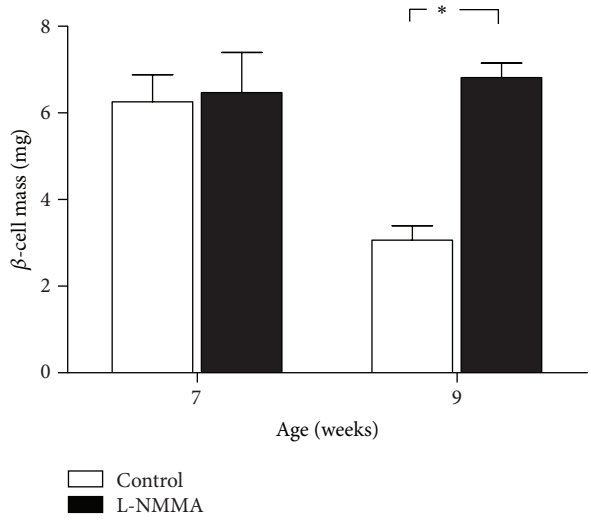

Figure 7: β-cell mass was determined in pancreatic sections from BB rats treated with vehicle (white bars) or L-NMMA (black bars) at 7 and 9 weeks of age. β-cell mass is presented in the bar graph as means ± SEM of values calculated as the ratio of insulin-positive area/total pancreatic area multiplied by the total pancreatic weight. Values are obtained from a mean of 5 animals. $^{*}P < 0.05$ treated versus control.

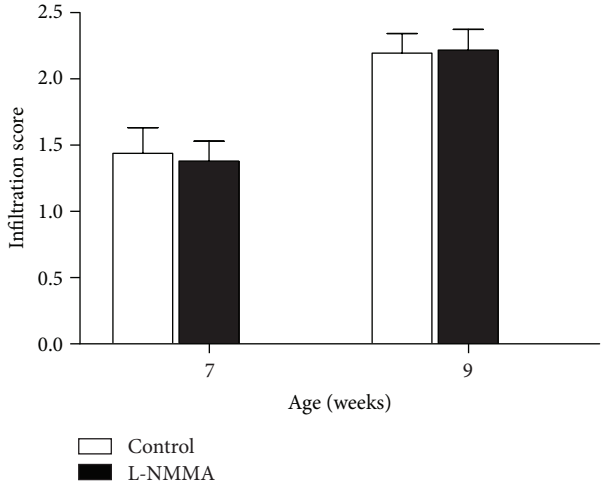

Figure 8: Infiltration scores were determined in Harris' H&E stained pancreatic sections of BB rats between 7 and 9 weeks of age treated with vehicle (white bars) and L-NMMA (black bars). Results are expressed as the means ± SEM of infiltration scores derived from a mean of 5 animals.

stress [27]. In addition to cytokines, there is evidence of NO participation in mitochondrial dysfunction and cell death occurring in PC12 cells in response to amyloid β [28].

Results from our study suggest that, in addition to apoptosis, NO can be implicated in the antiproliferative effect of proinflammatory cytokines on pancreatic β cell since treatment with NOS inhibitor L-NMMA is capable of reverting this cytokine-induced effect (Figure 2). Very few studies have investigated the possible mechanisms underlying the effect of NO on cellular proliferation. In pulmonary microvascular smooth muscle cells, exogenous NO upregulates p21, a protein which mediates cell cycle arrest [16]. Further, NO is capable of inhibiting ERK 1/2 and AKT activation in breast cancer cell lines and pancreatic β cells [29, 30]. These results are in accord with those observed by our group; that is, proinflammatory cytokines induced an inhibition of ERK 1/2 in islet cultures [31] and in early stages of insulitis [32]. However, NO has also been described as an inducer of proliferation, when acting at low concentrations. For example, in mesangial cells, the NO donor SNAP increases proliferation at concentrations of up to 200 μM and inhibits proliferation at higher concentrations, in a process implying AKT signaling and cox-2 protein [33].

Activation of NO-sensitive guanylyl cyclase by NO binding to the enzyme's prosthetic heme group has been described as one of the most important NO-mediated cellular effects [34]. Nevertheless, results observed in our system suggest that this pathway is not involved in the NO-mediated antiproliferative effect observed in β cells from islets treated with proinflammatory cytokines (Figure 4). These results are in accord with those previously reported in tumor cell lines in which the proliferative effect of low NO concentration is mediated by the cGMP pathway, but the antiproliferative effect induced by high concentrations of NO is independent of this pathway [14]. A proposed cGMP-independent mechanism of NO action is its direct interaction with protein and, as such, performing posttranslational modifications such as nitration or s-nitrosylation. For example, s-nitrosylation of EGF receptor has been reported in human neuroblastoma NB69 as a mechanism of NO-mediated antiproliferative effect [35].

The role of NOS isoforms in NO production in response to proinflammatory cytokines has not been well defined. Our results show that iNOS, the expression of which is induced in our system after 24 h culture in the presence of proinflammatory cytokines, is implicated while constitutive isoform is not implicated in cytokine-induced antiproliferative response in β cells (Figure 4). Of note is that although iNOS inhibitor 1400 W inhibited the cytokine-mediated antiproliferative effect, its effect was only partial, and was unlike the total reversion by L-NMMA (a general NOS inhibitor). It is also interesting the effect of 1400 W increasing beta cell proliferation in absence of cytokines which could be due to a certain level of islet cells iNOS activation induced by islet isolation process. This partial effect of 1400 W suggests that other NOS isoforms are participating in antiproliferative response to cytokines. Endothelial NOS determination by western blotting was performed in cultured rat islets in the presence of proinflammatory cytokines and, contrary to the results reported by Lajoix et al. [2], eNOS expression was observed. This expression is maintained in culture and was not affected by the presence of cytokines (Figure 4(b)) and could be attributed to endothelial cells in the profuse vascular network present in pancreatic islets. Once the presence of eNOS in islets is confirmed, its implication in β-cell antiproliferative response to cytokines was tested in islet cultures treated with L-NIO [36] (an inhibitor whose primary

target is eNOS) alone or in combination with cytokines. L-NIO induced a partial reversion of cytokine-induced β-cell antiproliferation in a similar manner to that induced by the iNOS inhibitor 1400 W. These results suggest participation of both NO isoforms in the antiproliferative response induced by proinflammatory cytokines, possibly by eNOS producing NO in a paracrine manner. In this sense, it has recently reported that inflammatory environment induces changes in eNOS which may adopt functional features of iNOS in a Ca^{2+}-independent way [35].

To assess NO involvement in the pathophysiology of T1DM, we treated Biobreeding rats, an animal model of autoimmune diabetes, with the general NOS inhibitor L-NMMA at early stages of the insulitis process. Previous observations from our group indicated a halting of β-cell proliferation between 4 and 7 weeks of age and which can be reversed by administering neutralizing antibody against IFN-γ [37]. Treatment with L-NMMA of BB rats from 5 weeks of age induced a reversal of β-cell proliferation loss observed in untreated animals (Figure 5). In addition, a relative recovery of β-cell area with a regional pattern in treated animals at 7 weeks of age was observed. The effects, either on proliferation of β cell or on β-cell area, were very much more evident in the head than in the tail of the pancreas. A similar regional pattern of response was reported in earlier studies using a malnutrition model [38]. A plausible explanation for this regional effect could be a different vascularization in the head area compared to that in the tail which precluded equivalent drug availability in all areas of the pancreas volume. Further, a different response to environmental factors due to a different islet composition could underlie this observed effect [39]. This issue warrants further investigation.

In conclusion, T1DM prevention strategies focus, essentially, on early stages of the disease before clinical onset when there is enough β-cell mass in the pancreas to maintain its metabolic function. Our present study highlights the importance of NO as a mediator of the observed impaired β-cell proliferative response from the initial stages of insulitis. This impairment in proliferative capacity precludes an appropriate regenerative response of β cells to autoimmune damage. Our data also highlight the property of L-NMMA to maintain β-cell proliferative capacity in early insulitis stage and, as such, confirms that nitric oxide might be an important therapeutic target in the strategies of T1DM prevention.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

Authors' Contribution

Manuel Aguilar-Diosdado and Carmen Segundo have participated equally in the study and in the preparation of this paper.

Acknowledgments

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References

[1] D. L. Eizirik, M. Flodström, A. E. Karlsen, and N. Welsh, "The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells," *Diabetologia*, vol. 39, no. 8, pp. 875–890, 1996.

[2] A.-D. Lajoix, H. Reggio, T. Chardès et al., "A neuronal isoform of nitric oxide synthase expressed in pancreatic β-cells controls insulin secretion," *Diabetes*, vol. 50, no. 6, pp. 1311–1323, 2001.

[3] E. Gurgul-Convey, K. Hanzelka, and S. Lenzen, "Is there a role for neuronal nitric oxide synthase (nNOS) in cytokine toxicity to pancreatic beta cells?" *Nitric Oxide*, vol. 27, pp. 235–241, 2012.

[4] A. K. Cardozo, F. Ortis, J. Storling et al., "Cytokines downregulate the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b and deplete endoplasmic reticulum Ca^{2+}, leading to induction of endoplasmic reticulum stress in pancreatic β-cells," *Diabetes*, vol. 54, no. 2, pp. 452–461, 2005.

[5] D. L. Eizirik, A. K. Cardozo, and M. Cnop, "The role for endoplasmic reticulum stress in diabetes mellitus," *Endocrine Reviews*, vol. 29, no. 1, pp. 42–61, 2008.

[6] C. Holohan, E. Szegezdi, T. Ritter, T. O'Brien, and A. Samali, "Cytokine-induced β-cell apoptosis is NO-dependent, mitochondria-mediated and inhibited by BCL-XL: apoptosis," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 2, pp. 591–606, 2008.

[7] I. Mehmeti, E. Gurgul-Convey, S. Lenzen, and S. Lortz, "Induction of the intrinsic apoptosis pathway in insulin-secreting cells is dependent on oxidative damage of mitochondria but independent of caspase-12 activation," *Biochimica et Biophysica Acta*, vol. 1813, no. 10, pp. 1827–1835, 2011.

[8] J. R. Tejedo, R. Ramírez, G. M. Cahuana, P. Rincón, F. Sobrino, and F. J. Bedoya, "Evidence for involvement of c-Src in the anti-apoptotic action of nitric oxide in serum-deprived RINm5F cells," *Cellular Signalling*, vol. 13, no. 11, pp. 809–817, 2001.

[9] M. Sata, M. Kakoki, D. Nagata et al., "Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism," *Hypertension*, vol. 36, no. 1, pp. 83–88, 2000.

[10] P. R. Dash, J. E. Cartwright, P. N. Baker, A. P. Johnstone, and G. S. J. Whitley, "Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation," *Experimental Cell Research*, vol. 287, no. 2, pp. 314–324, 2003.

[11] M. Ozaki, S. Kawashima, T. Hirase et al., "Overexpression of endothelial nitric oxide synthase in endothelial cells is protective against ischemia-reperfusion injury in mouse skeletal muscle," *American Journal of Pathology*, vol. 160, no. 4, pp. 1335–1344, 2002.

[12] Y.-G. Kwon, J.-K. Min, K.-M. Kim, D.-J. Lee, T. R. Billiar, and Y.-M. Kim, "Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production," *Journal of Biological Chemistry*, vol. 276, no. 14, pp. 10627–10633, 2001.

[13] A. Hajri, E. Metzger, F. Vallat et al., "Role of nitric oxide in pancreatic tumour growth: in vivo and in vitro studies," *British Journal of Cancer*, vol. 78, no. 7, pp. 841–849, 1998.

[14] A. Bal-Price, J. Gartlon, and G. C. Brown, "Nitric oxide stimulates PC12 cell proliferation via cGMP and inhibits at higher concentrations mainly via energy depletion," *Nitric Oxide*, vol. 14, no. 3, pp. 238–246, 2006.

[15] A. Torroglosa, M. Murillo-Carretero, C. Romero-Grimaldi, E. R. Matarredona, A. Campos-Caro, and C. Estrada, "Nitric oxide decreases subventricular zone stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway," *Stem Cells*, vol. 25, no. 1, pp. 88–97, 2007.

[16] W. H. Stotz, D. Li, and R. A. Johns, "Exogenous nitric oxide upregulates p21waf1/cip1 in pulmonary microvascular smooth muscle cells," *Journal of Vascular Research*, vol. 41, no. 3, pp. 211–219, 2004.

[17] S. Pervin, R. Singh, and G. Chaudhuri, "Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 6, pp. 3583–3588, 2001.

[18] A. Vasilijevic, B. Buzadzic, A. Korac, V. Petrovic, A. Jankovic, and B. Korac, "Beneficial effects of L-arginine nitric oxide-producing pathway in rats treated with alloxan," *Journal of Physiology*, vol. 584, pp. 921–933, 2007.

[19] M. L. McDaniel, J. R. Colca, N. Kotagal, and P. E. Lacy, "A subcellular fractionation approach for studying insulin release mechanisms and calcium metabolism in islets of Langerhans," *Methods in Enzymology*, vol. 98, pp. 182–200, 1983.

[20] D. L. Eizirik, S. Sandler, N. Welsh et al., "Cytokines suppress human islet function irrespective of their effects on nitric oxide generation," *Journal of Clinical Investigation*, vol. 93, no. 5, pp. 1968–1974, 1994.

[21] A. Hoorens and D. Pipeleers, "Nicotinamide protects human beta cells against chemically-induced necrosis, but not against cytokine-induced apoptosis," *Diabetologia*, vol. 42, no. 1, pp. 55–59, 1999.

[22] D. Mokhtari, J. W. Myers, and N. Welsh, "The MAPK kinase kinase-1 is essential for stress-induced pancreatic islet cell death," *Endocrinology*, vol. 149, no. 6, pp. 3046–3053, 2008.

[23] J. Tejedo, J. C. Bernabé, R. Ramírez, F. Sobrino, and F. J. Bedoya, "NO induces a cGMP-independent release of cytochrome c from mitochondria which precedes caspase 3 activation in insulin producing RINm5F cells," *FEBS Letters*, vol. 459, no. 2, pp. 238–243, 1999.

[24] N. E. John, H. U. Andersen, S. J. Fey et al., "Cytokine- or chemically derived nitric oxide alters the expression of proteins detected by two-dimensional gel electrophoresis in neonatal rat islets of Langerhans," *Diabetes*, vol. 49, no. 11, pp. 1819–1829, 2000.

[25] D. Liu, D. Pavlovic, M.-C. Chen, M. Flodström, S. Sandler, and D. L. Eizirik, "Cytokines induce apoptosis in β-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS(−/−)," *Diabetes*, vol. 49, no. 7, pp. 1116–1122, 2000.

[26] S. Oyadomari, K. Takeda, M. Takiguchi et al., "Nitric oxide-induced apoptosis in pancreatic β cells is mediated by the endoplasmic reticulum stress pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 19, pp. 10845–10850, 2001.

[27] J. Y. Chan, G. J. Cooney, T. J. Biden, and D. R. Laybutt, "Differential regulation of adaptive and apoptotic unfolded protein response signalling by cytokine-induced nitric oxide production in mouse pancreatic beta cells," *Diabetologia*, vol. 54, no. 7, pp. 1766–1776, 2011.

[28] U. Keil, A. Bonert, C. A. Marques et al., "Amyloid β-induced changes in nitric oxide production and mitochondrial activity lead to apoptosis," *Journal of Biological Chemistry*, vol. 279, no. 48, pp. 50310–50320, 2004.

[29] S. Pervin, R. Singh, W. A. Freije, and G. Chaudhuri, "MKP-1-induced dephosphorylation of extracellular signal-regulated kinase is essential for triggering nitric oxide-induced apoptosis in human breast cancer cell lines: implications in breast cancer," *Cancer Research*, vol. 63, no. 24, pp. 8853–8860, 2003.

[30] J. Størling, J. Binzer, A. K. Andersson et al., "Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt," *Diabetologia*, vol. 48, no. 10, pp. 2039–2050, 2005.

[31] M. Blandino-Rosano, G. Perez-Arana, J. M. Mellado-Gil, C. Segundo, and M. Aguilar-Diosdado, "Anti-proliferative effect of pro-inflammatory cytokines in cultured β cells is associated with extracellular signal-regulated kinase 1/2 pathway inhibition: protective role of glucagon-like peptide-1," *Journal of Molecular Endocrinology*, vol. 41, no. 1-2, pp. 35–44, 2008.

[32] G. Pérez-Arana, M. Blandino-Rosano, A. Prada-Oliveira, M. Aguilar-Diosdado, and C. Segundo, "Decrease in β-cell proliferation precedes apoptosis during diabetes development in bio-breeding/worcester rat: beneficial role of exendin-4," *Endocrinology*, vol. 151, no. 6, pp. 2538–2546, 2010.

[33] L. S. Meei, F. C. Kuo, J. S. Yen, W. L. Wan, Y. L.-S. Shoei, and H. L. Shing, "Activation of phosphoinositide 3-kinase in response to inflammation and nitric oxide leads to the up-regulation of cyclooxygenase-2 expression and subsequent cell proliferation in mesangial cells," *Cellular Signalling*, vol. 17, no. 8, pp. 975–984, 2005.

[34] L. J. McDonald and F. Murad, "Nitric oxide and cGMP signaling," *Advances in Pharmacology*, vol. 34, pp. 263–275, 1995.

[35] M. Murillo-Carretero, A. Torroglosa, C. Castro, A. Villalobo, and C. Estrada, "S-Nitrosylation of the epidermal growth factor receptor: a regulatory mechanism of receptor tyrosine kinase activity," *Free Radical Biology and Medicine*, vol. 46, no. 4, pp. 471–479, 2009.

[36] R. Greco, A. S. Mangione, D. Amantea, G. Bagetta, G. Nappi, and C. Tassorelli, "IkappaB-alpha expression following transient focal cerebral ischemia is modulated by nitric oxide," *Brain Research*, vol. 1372, pp. 145–151, 2011.

[37] A.-M. Martin, M. N. Maxson, J. Leif, J. P. Mordes, D. L. Greiner, and E. P. Blankenhorn, "Diabetes-prone and diabetes-resistant BB rats share a common major diabetes susceptibility locus, iddm4: additional evidence for a 'universal autoimmunity locus' on rat chromosome 4," *Diabetes*, vol. 48, no. 11, pp. 2138–2144, 1999.

[38] I. Avril, B. Blondeau, B. Duchene, P. Czernichow, and B. Bréant, "Decreased β-cell proliferation impairs the adaptation to pregnancy in rats malnourished during perinatal life," *Journal of Endocrinology*, vol. 174, no. 2, pp. 215–223, 2002.

[39] L. Orci, "Macro- and micro-domains in the endocrine pancreas," *Diabetes*, vol. 31, no. 6, pp. 538–568, 1982.

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Review Article

Influence of Gut Microbiota on Subclinical Inflammation and Insulin Resistance

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Obesity is the main condition that is correlated with the appearance of insulin resistance, which is the major link among its comorbidities, such as type 2 diabetes, nonalcoholic fatty liver disease, cardiovascular and neurodegenerative diseases, and several types of cancer. Obesity affects a large number of individuals worldwide; it degrades human health and quality of life. Here, we review the role of the gut microbiota in the pathophysiology of obesity and type 2 diabetes, which is promoted by a bacterial diversity shift mediated by overnutrition. Whole bacteria, their products, and metabolites undergo increased translocation through the gut epithelium to the circulation due to degraded tight junctions and the consequent increase in intestinal permeability that culminates in inflammation and insulin resistance. Several strategies focusing on modulation of the gut microbiota (antibiotics, probiotics, and prebiotics) are being experimentally employed in metabolic derangement in order to reduce intestinal permeability, increase the production of short chain fatty acids and anorectic gut hormones, and promote insulin sensitivity to counteract the inflammatory status and insulin resistance found in obese individuals.

1. Introduction

Insulin resistance is the main outcome caused by nutrient overload, lipids, infections, and sepsis-induced inflammation that affects insulin-sensitive tissues, such as the liver, muscle, adipose tissue, and hypothalamus, and which also promotes defects in cell signaling pathways and homeostasis [1]. The ingestion of an unbalanced diet and low physical activity observed in recent years in the global population are the main drivers of the epidemic rates of obesity reached in the past few decades [2]. A prospective study evaluated more than 9 million people worldwide over the last three decades and observed that, globally, the average body mass index (BMI) increased by 0.4-0.5 kg/m^2 per decade; moreover, subregion trends showed that the average BMI increased by 1.4 kg/m^2 in men and 1.9 kg/m^2 in women, per decade [3]. However, not only developed countries such as the United States are affected by this epidemic of obesity, but also countries under development, such as Brazil and other countries, are also affected in a similar way [4].

The World Health Organization has observed that more than 1.4 billion adults are overweight and, of these, at least 200 million men and 300 million women are clinically obese [5]. Some studies have shown that the increased rate of obesity has slowed down over the last five years, trended by some specific population groups, in eastern Europe, South America, and even in some specific population groups in the United States. However, the prevalence of obesity remains high and the health costs associated with obese individuals are huge, ranging from 2 to 7% of the health budgets in high income countries, which is followed by the low income nations, while the mortality among obese people is increased [3, 6, 7].

Obesity is characterized by chronic subclinical inflammation that affects insulin activity in its metabolically sensitive tissues, notably the liver, muscle, and adipose tissue, and drives a metabolic disorder that culminates in the deregulation of glucose homeostasis [8]. Since the observation that obese adipose tissue shows increased expression of the proinflammatory cytokine TNF-α [9], extensive research

efforts have been made regarding inflammation in metabolic tissues, trying to determine what primes the insulin resistance phenomenon, the consequences of obesity, how to improve the molecular knowledge of insulin activity attenuation mediated by obesity, and how it can be managed in order to improve patient quality of life. Attempts have also been made to reduce or prevent the incidence of obesity and its comorbidities.

Here, we will focus on reviewing the latest contributions to the literature on the influence of the intestine on the pathogenesis of insulin resistance and the consequences of insulin resistance on the liver, muscle, adipose tissue, and hypothalamus, as well as the mechanisms by which the gut microbiota influences systemic insulin resistance.

2. Intestinal Participation in Insulin Resistance

For several decades, it has been known that the mammalians intestine harbors a great number of bacteria (~10^{14} bacteria) that even surpasses the total number of cells that comprise mammalian tissues, systems, and, ultimately, the entire body [10]. Furthermore, it is estimated that the gut microbiota contains, at least, 100-fold more genes than the mammalian genome. These bacteria can live in a symbiotic way but, in certain cases, promote disease [11]. However, over the last ten years, this community that lives within the mammalian body has been gaining the status of a microbial organ that contributes to homeostasis and impacts directly on energy metabolism and insulin sensitivity [12, 13].

3. The Obese Microbiome Has Increased Energy Harvesting Ability

One of the first and foremost important observations on the role of the gut microbiota in insulin sensitivity and body weight management was observed when *germ-free* mice that were infected with the gut microbiota content of a conventionally raised mice showed an increase (by about 60%) in body fat content. Moreover, the occurrence of insulin resistance and glucose intolerance was seen within 14 days, even with a reduction in food intake (standard chow), providing novel evidence that the bacteria community, in some way, controlled energy metabolism [14]. Additionally, it was described that the activity of a lipoprotein lipase (LPL) suppressor, known as fasting-induced adipocyte factor (Fiaf) or angiopoietin-like protein 4 (ANGPTL4), controlled the fat storage abnormalities observed in conventionalized *germ-free* mice, where the gut microbiota induced selective suppression of this protein in intestinal cells and promoted an increase in LPL activity. This resulted in increased triglyceride storage in adipocytes, which was prevented upon the conventionalization of Fiaf$^{-/-}$ *germ-free* mice [14].

It was also shown that the resistance of *germ-free* mice against diet-induced obesity relied on increased liver and skeletal muscle AMPK activity and its downstream targets. This induced the activation of fatty acid oxidation and increased energy expenditure, which regulated body weight gain, since Fiaf expression in the gut epithelium was highly suppressed in *germ-free* mice fed a high-fat diet, keeping the body weight almost unaltered, a feature that was not maintained in Fiaf$^{-/-}$ *germ-free* mice [15].

The great increase in interest in the relationship between the gut microbiota and mammalian metabolism has led to the utilization of very elegant and novel molecular techniques based on microbial DNA sequencing [16, 17]. This interest has also brought new insight into the epidemic obesity rates throughout the world and the consequent incidence of type 2 diabetes, comorbidities, and cancer.

Metagenomic analyses of human volunteers showed that almost all bacteria present in the distal gut and feces belong to two main bacterial phyla, *Bacteroidetes* and *Firmicutes* [18]. The predominance of these phyla is also seen in lean mice, with a balance among the bacterial phyla, but in genetically obese *ob/ob* mice, this balance is broken. In obese mice, a great increase in bacteria from the phylum *Firmicutes* and a comparable decrease in the prevalence of *Bacteroidetes* were observed, indicating gut microbiota alteration driven by obesity, predisposing for or associated with this metabolic condition [19]. Moreover, it was demonstrated that microbiota transplantation from *ob/ob* mice to *germ-free* recipients induced a greater increase in body fat content when compared with *germ-free* mice that were the recipients of lean mice gut microbiota, indicating that this difference in the intestinal flora induced the obese phenotype [20]. Additionally, it was shown that the microbiota in the feces of lean and obese humans was different in a similar way as that observed in mice [20]. All this information led to the hypothesis that the gut microbiota from *ob/ob* genetically obese mice is capable of harvesting more energy from the diet [21, 22], probably by the presence and/or increased prevalence of bacteria that produce enzymes that are more efficient in degrading the nutrients available in the diet.

The relationship between the phyla *Bacteroidetes* and *Firmicutes* is the main focus of discussion when obesity is studied, with a considerable amount of data showing an increase in *Firmicutes* prevalence and a reduction in *Bacteroidetes* [19, 20, 23–26]. However, this issue has not yet been completely addressed, as we can find several studies in the literature that maintain the opposite, where the prevalence of *Firmicutes* is decreased in overweight and obese individuals, as well as in obese mice, with an accompanying increase in *Bacteroidetes* prevalence [27–30]. Additionally, the presence of nonsignificant prevalent bacteria phyla in the gut of lean mice, such as *Verrucomicrobia*, has not been well explored until now, with very few observations on increases in pathological conditions (obesity and cancer) and no mechanistic evaluations [27, 31]. The differences observed among gut microbiota human studies still have to be addressed, evaluating the ethnics and feeding behavior of the studied population as well as the standardization of the methods used. In animal studies, the probable cause of the opposite outcomes may be a factor of different mouse strains used. The issue of the "healthy" bacterial group profile is a very important point in gut microbiota research and needs to be further explored. Thus, no proposed treatment attempting

to induce the proliferation of certain bacterial phyla has been suggested until now.

4. Obesity and High-Fat Diet Increase Circulating Endotoxin Levels and Activation of the Inflammatory Response

A structural particularity of bacteria splits them into Gram positive and Gram negative, based on the cell wall structure. Each of the two most prevalent phyla belongs to one of these groups; that is, *Firmicutes* are Gram positive and *Bacteroidetes* are Gram negative bacteria. The latter group harbor lipopolysaccharides (LPS) in their cell wall, which is a large molecule formed by a lipid and a polysaccharide that elicits a strong immune response, promoting inflammation to protect the organism from bacterial infection [32]. LPS is a potent activator of pathogen-associated molecular pattern (PAMP) responses, primarily via toll-like receptor 4 (TLR4), which activates an extensive cell signaling pathway that induces the inflammatory response and cytokine expression and secretion [33] (Figure 1).

Several studies have shown that circulating levels of LPS are elevated in obese mice, rats (genetically or induced by diet), and humans. In rodents, this is directly related to increased intestinal permeability [34–36]. This phenomenon occurs due to reduced expression and activity of tight junction proteins, such as zonula occludens-1 (ZO-1) and occludin, that create, together with gut epithelial cells, a barrier that separates the intestinal lumen and its bacterial population and products from peritoneal tissues. This degradation of tight junction function leads to the leakage of bacterial products, such as LPS, and bacterial translocation, which have recently been described as key factors in both human and mice insulin resistance and inflammation [26, 35, 37–39] (Figure 1). Recent evidence also indicates that LPS can be transported along with chylomicrons into the circulation instead of depending on epithelial injury to reach insulin sensitive organs; the inhibition of chylomicron synthesis blocks endotoxin uptake [40].

LPS is a very specific TLR4 ligand with great affinity. Its cell signaling pathway, composed of a diverse number of proteins, culminates with the inflammatory response mediated by LPS contact with cells [41–43]. Beyond the high specificity of TLR4 in identifying LPS from Gram negative bacteria, which can reach insulin sensitive tissues through the circulation from the gut and drive the inflammatory response to protect the host from bacterial infection, TLR4 has a direct role in insulin resistance mediated by obesity. LPS from the gut can react with free-fatty acids (FFA), mostly the saturated type, that are increased in the circulation of obese individuals due to an increase in adipose tissue-mediated lipolysis, *de novo* liver lipogenesis and ectopic lipid accumulation [44]. TLRs have been related to the gut microbiota, whereas single TLR-deficient mice, such as $TLR2^{-/-}$ [26] and $TLR5^{-/-}$ [45] mice, show a different microbiotic profile when compared to their control littermates. This immune modulation of the intestinal flora can induce symptoms of metabolic syndrome, such as increased body weight, blood glucose, and insulin resistance. The $TLR4^{-/-}$ mice do not present an altered prevalence of gut microbiota bacteria as seen in the TLR-deficient mice cited above [46]. In obesity and conditions related to intestinal permeability, TLR4 is known to be involved in the inflammatory response that culminates in insulin resistance and metabolic derangement, as those responses are attenuated by the inhibition of this protein activity [27, 34, 47–49], such as in TLR4 loss-of-function C3H/HeJ mice [50], in $CD14^{-/-}$ mice [51], and in $TLR4^{-/-}$ mice [52] (Figure 1). Recent investigations have shown that fatty acids do not activate TLR4 directly, questioning the real influence of this receptor in lipid-induced insulin resistance caused by obesity [53, 54]. Nevertheless, a hepatic protein has been identified, called fetuin-A (FetA), which is a major carrier of FFAs in the circulation [55], that acts as an endogenous ligand of TLR4, thus activating its signaling pathway, promoting insulin resistance, which was blocked in the absence of FetA, and attenuating insulin resistance induced by FFA [56]. There is evidence that shows increased FetA expression in HepG2 cells, a liver cell lineage, after treatment with thapsigargin, an ER stress inducer, in a time- and dose-dependent manner, which is blocked by pretreating the cells with 4-phenylbutyrate, a compound that inhibits ER stress [57]. These results were similar in diet-induced obese mice, which exhibited ER stress, attenuated by 4-phenylbutyrate treatment and accompanied by a reduction in FetA expression and improved insulin resistance [57]. Additionally, it was demonstrated that in obese individuals and rodents, circulating levels of FetA are increased and correlate with body weight [58, 59] and that body weight loss brings the FetA circulating levels back to normal in children [59]. As well, $FetA^{-/-}$ mice are protected from obesity and insulin resistance induced by aging [60, 61].

Other PAMPs, as well as damage-associated molecular patterns (DAMPs), such as the inflammasome seem to be related to intestinal epithelial integrity. The activation of DAMPs and PAMPs in the gut is necessary for the maintenance of barrier function, while nucleotide-binding oligomerization domain protein-like receptor 3 (NLRP3) and NLRP6-deficient mice show increased intestinal permeability and increased risk of colitis [28, 62], allowing the occurrence of dysbiosis and insulin resistance and a greater possibility of nonalcoholic steatohepatitis (NASH). The inflammasome is a group of protein complexes that recognizes a wide range of bacterial, damage and stress signals; it results in caspase-1 activation and subsequent proinflammatory cytokine secretion and cell death [63]. Mice deficient in inflammasome proteins show altered gut microbiota when compared to wild-type littermates, with an increased prevalence of bacteria from the *Prevotellaceae* family, which is part of the *Bacteroidetes* phylum, and higher translocation of bacterial products from the gut to the circulation. In particular, TLR4 and TLR9 agonists induced inflammation and insulin resistance; this feature is transmissible to newborn and adult mice that come in direct contact with inflammasome-deficient animals [28, 62]. In addition to gut microbiota modulation, inflammasome proteins are activated in macrophages by LPS, which enter into the circulation due

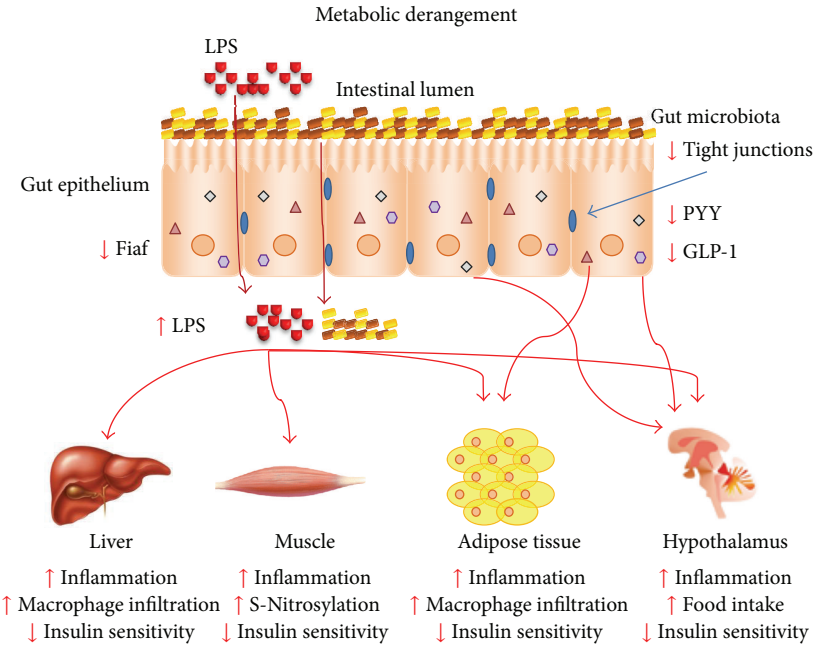

Figure 1: The gut microbiota is modulated by metabolic derangement, such as nutrition overload and obesity, which promote a cluster of metabolic disease-associated processes that culminate in bacterial products and whole bacteria translocation to the circulation through increased intestinal permeability caused by a reduction in tight junction expression. This triggers an immune response, inflammation, and immune cell infiltration of liver and adipose tissue. It induces insulin resistance in various tissues by diverse mechanisms and food intake deregulation in the hypothalamus promoted by the insulin and leptin resistance and also inhibited expression of gut-secreted anorectic hormones, such as GLP-1 and PYY. Additionally, there is a reduction in the intestinal Fiaf expression mediated by bacteria that deregulate the fat storage and lipid metabolism favoring the obese phenotype.

to increased intestinal permeability under obese conditions. The macrophage inflammatory activity is regulated by the TLR-induced activation of nuclear factor κB (NF-κB) [64, 65].

In addition to the bacterial immune response mediated by TLR4, viral infections also activate pattern recognition receptors (PRRs) and trigger a specific cellular signaling pathway that terminates with the activation of c-Jun N-terminal kinase (JNK), inhibitor of nuclear factor-κB kinase subunit β (IKKβ), NF-κB, and transcription of proinflammatory cytokines, which are mediators of insulin resistance. The double-stranded RNA-activated protein kinase (PKR) is one of the molecules responsible for protection from viral infections, as it identifies double-stranded RNA (dsRNA) viruses and activates the innate immune response against these pathogens [66, 67]. PKR is also related to obesity and insulin resistance, as its phosphorylation is increased in high-fat diet fed mice, leading to activation of JNK [68] and IKKβ [69], and culminating in serine phosphorylation of IRS-1 and insulin resistance [70]; all these inflammatory features are absent or attenuated in PKR$^{-/-}$ mice [71, 72]. It has been described that PKR is also activated by bacterial products, such as LPS [73, 74], in a dsRNA-independent way, possibly by the action of a cellular protein designated PKR-activating protein (PACT), which possesses dsRNA binding domains and interacts with other molecules that bind to PKR [75] to trigger the same signaling pathway as viral infection [76]. This could integrate the PKR signaling pathway with gut microbiota metabolic effects on insulin resistance, but has not yet been investigated (Figure 1).

The activation of inflammatory pathways by gut-derived LPS, mainly via TLR4, leads to increased expression of inducible nitric oxide synthase (iNOS) [77, 78]. In obesity, an increase in iNOS expression is also observed in insulin sensitive tissues, which promotes a phenomenon known as S-nitrosation/S-nitrosylation, where nitric oxide (NO) reacts with cysteine residues to form S-nitrosothiol adducts, thereby modulating protein function [79, 80]. Thus, LPS induce S-nitrosation/S-nitrosylation of the insulin signaling pathway (IR, IRS-1, and Akt), inducing insulin resistance in the liver, muscle, and adipose tissue in a more particular way than serine phosphorylation of IRS-1 [81–83]. The targeted disruption of iNOS and its pharmacological inhibition attenuates the S-nitrosation/S-nitrosylation of insulin signaling proteins and inflammation and consequently improves insulin sensitivity [84–87] (Figure 1). Besides gut-derived LPS, other bacteria metabolites could induce S-nitrosation/S-nitrosylation of diverse proteins, modulating their activity and promoting biological effects in several tissues. Therefore, this field needs more attention and further studies.

5. Metabolic Role of Short-Chain Fatty Acids Derived from Gut Microbiota

The gut microbiota has an impact on mammalian physiology (mostly metabolic and immune functions) through several

mechanisms. Some of them are discussed previously, but the gut bacteria can also interact with the host system through their metabolites, mainly short-chain fatty acids (SCFA), which are their principal end-products and are represented for the most part by acetate, propionate, and butyrate, which have physiological effects in different tissues [88]. Gut microbiota fermentation, due to anaerobic bacteria, degrades polysaccharides that are not cleaved by mammalian enzymes, in other words, nondigestible carbohydrates, to produce short-chain fatty acids and other subproducts in the cecum and colon [89]. Moreover, SCFA diversity demonstrates metabolic cooperation among the bacterial community, as no bacterial genus can hydrolyze all kinds of nutrients nor produce all metabolites found in the gut lumen [90], indicating that most of the physiological interactions of the gut microbiota with the host are not dependent on just one particular bacterial type, but the entire community.

As metabolites, SCFA have diverse effects on various cells. They are taken up by the host through passive diffusion and via mono-carboxylic acid transporters, such as monocarboxylate transporter 1 (MCT1) [91]. Primarily, they work as an energy source for colonic epithelial cells, which derive 60%–70% of their fuel influx from SCFA. Butyrate has a particular importance as an energy source for those cells, as almost 65% is cleared by the mucosa and more than 70% of oxygen consumption in isolated colonocytes is due to butyrate oxidation [89]. Butyrate may also play a critical role in cell growth and differentiation [92, 93]. Acetate is likely to be used as a cholesterol or fatty acid precursor, and propionate is a gluconeogenic substrate [94, 95]. The relative levels of the specific enzymes for SCFA degradation (acetyl-CoA, propionyl-CoA, and butyryl-CoA) in different tissues are the determinants of SCFA metabolization [96]. Other molecules with metabolic regulatory functions can be released by gut bacteria, such as conjugated linoleic acids (CLA) [97, 98], which are lipid metabolites, or bile acids [99] and gases, such as methane and H_2S [100, 101], but they have minor roles in mammal physiology when compared to SCFA.

SCFA also act as anti-inflammatory molecules, as acetate, propionate, and butyrate are capable of inhibiting NF-κB activation in the host immune cells by binding to G-protein-coupled receptor, 43 and 41 (GPR43 and GPR41), thereby blocking inflammatory responses and suppressing TNF-α and IL-6 release; butyrate also reduces IL-12 and increases IL-10 expression [102–105]. GPR43 seems to have great importance in mediating acetate-induced anti-inflammatory stimuli since the deletion of this gene promotes an increased inflammatory response in dextran sodium sulfate- (DSS-) induced colitis, which is attenuated by the administration of acetate in mice that express GPR43 [106]. Moreover, $GPR43^{-/-}$ immune cells are hyperactive and promote an increased response to chemoattractants, such as C5a and proinflammatory cytokines [106].

Acetate and butyrate are also important in epithelial barrier function maintenance as they stimulate the production and secretion of mucus, by goblet cells, which protect the epithelium through increased expression of mucin [107]. Butyrate increases MUC-2 expression by 23-fold in a goblet cell line *in vitro*, demonstrating the importance of this function in modulating intestinal permeability [108]. Butyrate also affects tight junction protein expression, that is, zonulin and occludin, and even low concentrations of this SCFA seem to reduce intestinal permeability [109, 110]. Even with this potent mucus releasing effect by butyrate, it seems that acetate has more pronounced effects in epithelial protection, as activation of GPR43 by acetate protects mice from a lethal infection with an *E. coli* strain [111]. Moreover, the inhibition of GPR strongly attenuates the effects of acetate in terms of epithelial survival and integrity [112].

The products of gut microbiota fermentation, such as acetate and butyrate, are able to increase fatty acid oxidation and energy expenditure. There is evidence that acetate intake by humans promotes a reduction in body weight, circulating cholesterol, and triglyceride levels [113]. The administration of acetate or an increase in gut production mediated by the gut microbiota modulates activated 5′-AMP-activated protein kinase (AMPK), which inhibits acetyl-CoA carboxylase (ACC), thereby promoting fatty acid oxidation and energy expenditure [114] and leading to increased insulin sensitivity and reduced glucose intolerance in diabetic rats and in high-fat diet fed mice [27, 115]. Butyrate administration also increases AMPK activation in muscle, culminating in increased energy expenditure, as observed by an increase in brown adipose tissue mass and UCP1 expression [116].

It has been shown in cell culture experiments that short-chain fatty acids, in particular propionate and butyrate, can modulate the expression of Fiaf in intestinal cells, which was proposed as a gut microbiota mediator of fat storage [14]. Intestinal cell culture lines were exposed to short-chain fatty acids, which promoted an increase in Fiaf expression via PPARγ in colon cells. This was not reproduced in adipocyte cell lines, leading to the inhibition of LPL, adipogenesis and a proposed increase in fat storage mechanism control and metabolic improvement [117–119].

The activity of gut hormones in the control of appetite has been shown [120], and it is likely that the gut microbiota should interfere with the expression and activity of these hormones. Gut bacteria seem to modulate the secretion of glucagon-like peptide-1 (GLP-1), which is produced by L-cells in the colon, and peptide YY (PYY) [121], produced by ileum and colon cells, as well as leptin production by adipose tissue cells via GPR41 [122]. All of these have anorectic effects in the hypothalamus and thus promote satiety. This modulation seems to be, at least in part, mediated by the SCFA produced by fermentative bacteria. Evidence of SCFA and satiety show that an acetate infusion induces an increase in the circulating levels of GLP-1 and PYY in hyperinsulinemic overweight women [123]. Propionate reduces food intake in animal feeding studies [124–126], whereas supplementation of a dairy beverage fermented by propionic acid bacteria producer also increases satiety in humans [127]. In a similar way to propionate, butyrate induces satiety, upregulating anorectic neuropeptide expression, such as PYY and proglucagon in rat epithelial cells [126, 128]. It is possible that these SCFA mechanisms of appetite regulation are mediated by GPR41 and dependent on the intestinal transit rate, as a deficiency in this receptor is associated with reduced PYY expression and

faster intestinal transit with reduced energy harvesting from the diet [129]. Most of the observations on SCFA-regulated appetite are descriptive, and the mechanisms controlled by these gut microbiota-derived molecules are not yet known.

6. Gut Microbiota Modulation

In the last decade, a great body of evidence and knowledge about the gut microbiota and its interaction with the host, immunity, and metabolism has provided new insight regarding the influence of this forgotten "organ" on the most prevalent metabolic disease, obesity. By several mechanisms, gut bacteria influence the chronic low grade inflammation that culminates in insulin resistance and the increase in fat deposition and body weight gain, characteristic of obese individuals. With the acknowledgement of these obesity and inflammation induction mechanisms, several strategies to block or attenuate them are being developed and tested, in order to benefit obese and type 2 diabetic patients.

6.1. Antibiotic Therapy. It has been shown that the use of broad spectrum antibiotic therapy greatly modifies the gut microbiota profile in mice, improving the metabolic derangement induced by genetic obesity and/or high-fat diet feeding, but the prevalence of surviving bacteria and the benefits for the host have not been determined, as the concept of a "healthy" gut microbiota is still under investigation. The main mechanism suggested by antibiotic administration is a reduction in circulating LPS levels, which attenuates inflammation and improves the insulin resistance induced by obesity in the liver, muscle, and adipose tissue [27, 35, 130, 131]. Additionally, intestinal permeability may be reduced after the evaluation of tight junction protein expression in epithelial cells of antibiotic-treated animals, showing increased expression and function [35]. This improvement in insulin resistance was also observed in high-fat diet fed mice even in absence of a difference in body weight, which was achieved by submitting a group of animals to pair-feeding in comparison to the antibiotic-treated animals, which presented lower circulating LPS levels and TLR4 activation. This probably led to reduced intestinal permeability, leading to a reduction in JNK and IKKβ activation and reduced serine phosphorylation of IRS-1 in the liver, muscle, and adipose tissue, altogether seen as increased activation of the insulin signaling pathway, and to inhibition of macrophage infiltration into adipose tissue. Moreover, antibiotic treatment increases the portal acetate levels, which activates AMPK, fatty acid oxidation, and, possibly, energy expenditure [27]. However, even with this striking metabolic improvement in antibiotic therapy experiments, it seems that translating this strategy to humans is not the best option, as there are complex issues such as antibiotic resistance in chronic administration panels and evidence that indicates a relationship between chronic low-dose antibiotic therapy and body weight gain [132, 133] (Figure 2).

6.2. Probiotics. Probiotics are defined as live microorganisms that confer unspecified health benefits to the host [134]. Evidence from metagenomic profiles indicates that the obese phenotype shows an increased prevalence of *Firmicutes* [20, 26] in the gut microbiota profile, inferring a negative correlation with metabolism and insulin sensitivity. However, apart from these inconclusive observations on the "healthy" gut microbiota, the most commonly used probiotics are *Lactobacillus*, which belong to *Firmicutes* and *Bifidobacterium* [135]. *Lactobacillus* strain administration leads to several metabolic benefits in both rodents and humans, that is, a reduction in adipocyte cell size and body fat in high-fat diet fed mice [136], a reduction in fat mass and BMI, the promotion of insulin sensitivity [137, 138], and restriction of excessive body weight gain in the first years of life of young children [139]. Although the mechanism by which *Lactobacillus* control excessive adiposity has not been described, changes in fat storage genes expression, such as Fiaf, have been proposed to mediate this probiotic effect [140]. Data from *Bifidobacterium* administration show that the production of acetate mediates gut epithelial integrity and barrier function, protecting animals from a lethal bacterial infection [111], but no mechanism as to how acetate induces this effect has been proposed. It is tempting to suggest that acetate binding to GPR43 and its activation mediate the beneficial effects of treatment with *Bifidobacterium*.

A probiotic compound that has been well studied and appears in a great number of articles, named VSL#3, composed of a blend of probiotic bacteria, modulates the gut microbiota profile [141] and shows interesting effects, such as the promotion of epithelial integrity by modulation of tight junctions [142], a reduction in inflammatory status in a chronic colitis animal model [141], protection against DSS-induced colitis, and near reversal of atherosclerotic lesions in $ApoE^{-/-}$ mice, which develop atherosclerosis spontaneously [143]. In a clinical trial, VSL#3 promoted reduced intestinal permeability and improved immune activity [144]. Bacterial translocation to mesenteric adipose tissue, which seems to precede type 2 diabetes onset, is prevented by probiotic treatment. Apparently, this mechanism is mediated by acetate production and increased gut epithelial integrity [39]. It has also been shown that probiotic treatment-induced gut microbiota modulation can suppress high-fat-diet induced NF-κB activation and inflammation-induced insulin resistance [145]. Probiotics can also mimic commensal bacteria and modulate protective epithelial mucus production, reduce bacterial adhesion, increase tight junction expression, enhance epithelial and immune cell survival, induce defensins, and stimulate IgA production as well as stimulate TLRs to promote gut homeostasis [146–148] (Figure 2).

6.3. Prebiotics. Prebiotics are defined as a nonviable food component that confers a health benefit on the host associated with gut microbiota modulation [149]. The most common prebiotics used in gut microbiota modulation studies are the inulins, fructooligosaccharides, various types of galactooligosaccharides, and resistant starches. Prebiotics act by modulation of the gut microbiota profile and serve as a substrate for the production of metabolically active metabolites, in particular, SCFA, that are, acetate, propionate,

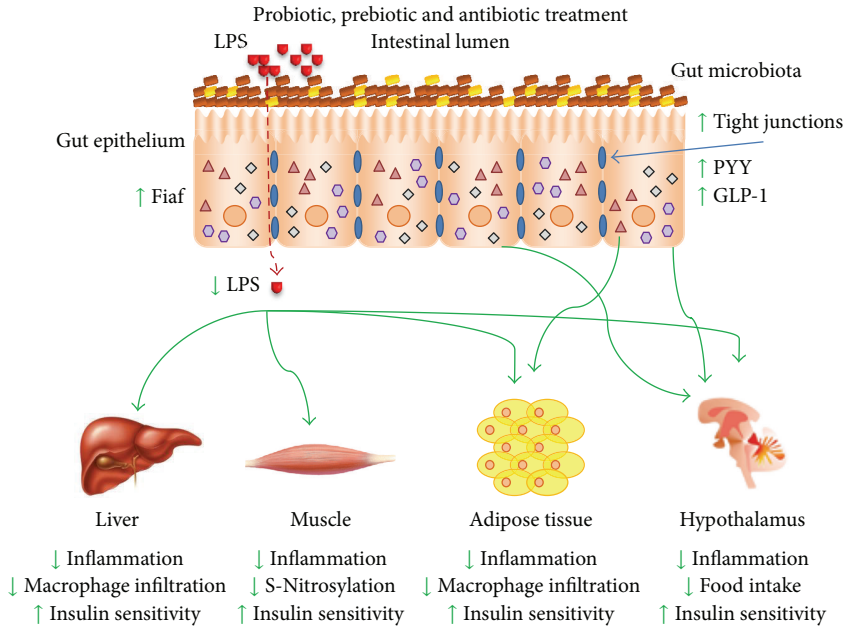

Figure 2: The advent of products (antibiotics, probiotics, and prebiotics) capable of modulating the gut microbiota profile, their products and metabolites (i.e., LPS, short-chain fatty acids), promotes a shift on the bacterial community prevalence, which favored the increase in tight junctions expression and function, reducing intestinal permeability and bacterial products circulation levels. Thus, the LPS circulating levels and inflammatory status in insulin-sensitive tissues are reduced, as well as muscle S-nitrosylation and liver and adipose tissue macrophage infiltration, promoting increased insulin sensitivity and the whole body metabolism. GLP-1 and PYY circulating levels are increased after treatment with gut microbiota modulators which together with the improvement in insulin sensitivity in the hypothalamus promoted reduction in food intake by satiety mechanisms and in conjunction with the increased Fiaf expression, contributed to reduce body weight.

and butyrate [150, 151]. Several studies have related prebiotic treatment to a reduction in ectopic lipid accumulation such as steatosis, reduced fat storage in white adipose tissue, in systemic inflammation, and insulin resistance in high-fat diet fed and genetically obese models [152–154], and also reduced endotoxemia [155]. In clinical experiments, beneficial effects of prebiotic administration were observed, such as a reduction in BMI, waist circumference, fat mass, and insulin resistance [156–158]. The food intake regulation is another important feature of gut microbiota modulation by prebiotics, which induce gut hormone production, such as GLP-1 and PYY, that signal via anorectic pathways in the hypothalamus, and a reduction in ghrelin expression, a gastric orexigenic peptide, thereby reducing food intake [159, 160]. Even fibers that do not change the gut microbiota profile, in a similar way to inulin-type fructans, induce food intake reduction by increasing circulating levels of GLP-1 and PYY [161–165], evidencing the important role of SCFA derived from prebiotic fermentation. Data from a 12-week prebiotic treatment in obese subjects showed a modulated gut microbiota profile, increased PYY, and decreased ghrelin circulating levels, while a single dose of prebiotics (inulin) also decreased plasma ghrelin and increased GLP-1 [166, 167]. Prebiotics also induce the production and secretion of GLP-2 by L cells; this hormone has relevant activity on gut barrier function and reduces gut permeability in obese animals [168] (Figure 2).

6.4. Bariatric Surgery. Recent data on obese subjects who have undergone gastric bypass surgery indicate that six months after the procedure, the gut microbiota profile was changed and bacteria diversity was reduced in comparison with the subjects that were obese and did not undergo the surgical procedure [169]. In addition to the changes promoted by gastric bypass, physiologically and anatomically, a number of factors could contribute to this gut profile alteration throughout this six months, primarily food intake behavior and weight loss, placing the direct influence of bariatric surgery in doubt. An early evaluation of the gut microbiota after the procedure is not viable due to the antibiotic treatment indicated for surgical recovery. Nevertheless, a recent study brought new light to this issue, where mice that underwent gastric bypass surgery presented a distinct gut microbiota profile when compared with the sham-operated mice one week after of the procedure, when no body weight difference was detected. Furthermore, the authors placed a group of mice under diet restriction to mimic the weight loss achieved by the gastric bypass group for 10–12 weeks and found that the microbiota profile was different as well, indicating that, indeed, the bariatric surgery modifies the bacterial composition of the gut [170]. In addition, they showed that gut microbiota transplantation from a sham-operated animal to *germ-free* mice increased adiposity as well as circulating leptin levels concomitantly with reduced food intake, as was previously demonstrated [14]. However,

in the recipients of gut microbiota from mice that underwent gastric bypass, none of those parameters were altered when compared to *germ-free* mice. There was also a reduction in body weight, suggesting that the gastric bypass-associated gut microbiota may either reduce energy harvesting from the diet or produce signals to regulate energy expenditure and/or lipid metabolism in a Fiaf-independent manner since colonization of *germ-free* mice with either sham or gastric bypass gut microbiota inhibits Fiaf expression in intestinal cells, but this is still unknown [170]. Based on the studies cited previously, gastric bypass seems to promote gut microbiota modulation that induces beneficial effects in obese humans and rodents.

7. Conclusions

In summary, the literature presents incontestable data that the gut microbiota and its modulation by nutrients and excessive energy intake heavily influence glucose metabolism, lipid storage, inflammation, and insulin activity in a negative way, as observed in obesity and diabetes. Furthermore, there are intense investigations going on in this field, highlighting several strategies to modulate the gut microbiota profile to a "healthier" status in an attempt to increase insulin sensitivity by blockage of the insulin resistance inductors mediated by the interaction between bacteria and the intestinal environment of the host.

Some issues have been raised from these studies and have not been fully explored until now, such as PKR activity modulated by gut microbiota. Besides bacterial products, viruses are also found in metagenomic profiles, and viral signaling could contribute to the increased inflammation promoted by intestinal products in obesity. In a similar way, the nitrosative contribution from gut microbiota has not yet been raised as having an impact on the metabolic derangement mediated by obesity and microbial products. Also, it is unknown if attempts to ameliorate the metabolic status by gut microbiota modulation will regulate both phenomena. SCFA seem to have an important role in food intake regulation, but the mechanisms of inducing GLP-1 and PYY production in intestinal cells are still poorly described and need further investigation. Saturated fatty acids are known to promote inflammation and insulin resistance and are prevalent in the high-fat diet administered to experimental rodents in these studies and in the western diet that has led to epidemic rates of obesity. However, the utilization of unsaturated fatty acid supplementation, which is known as a lipid with anti-inflammatory properties, has not yet been related to gut microbiota modulation. This is an interesting nutritional field, as probiotic and prebiotic strategies can have irrefutable benefits to the host's metabolism.

Ongoing efforts will try to determine the best set of symbiotic bacteria for mammals to harbor in the intestine in a metabolic evaluation, avoid excessive energy uptake from the diet, preserve gut barrier function, and reduce bacteria and the translocation of their inflammatory products; it is hoped that this will culminate in reduced inflammation. This issue is under debate, as articles do not show coherence regarding bacterial prevalence modulation in the gut induced by obesity and its treatment, which will need further studies and standardization. It seems that studies from different facilities bring distinct results. This issue has to be addressed in order to define what bacterial colony is more interesting to have grown in the mammalians gut.

References

[1] C. K. Glass and J. M. Olefsky, "Inflammation and lipid signaling in the etiology of insulin resistance," *Cell Metabolism*, vol. 15, no. 5, pp. 635–645, 2012.

[2] P. G. Kopelman, "Obesity as a medical problem," *Nature*, vol. 404, no. 6778, pp. 635–643, 2000.

[3] M. M. Finucane, G. A. Stevens, M. J. Cowan et al., "National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9·1 million participants," *The Lancet*, vol. 377, no. 9765, pp. 557–567, 2011.

[4] V. A. Ferreira and R. Magalhães, "Obesidade no Brasil: tendências atuais," *Revista Portuguesa De SaúDe Pública*, vol. 24, no. 2, pp. 71–91, 2006.

[5] WHO, "Obesity and overweight," Fact sheet 311, 2013, http://www.who.int/mediacentre/factsheets/fs311/en/.

[6] K. M. Flegal, M. D. Carroll, C. L. Ogden, and L. R. Curtin, "Prevalence and trends in obesity among US adults, 1999–2008," *The Journal of the American Medical Association*, vol. 303, no. 3, pp. 235–241, 2010.

[7] A. B. de Gonzalez, P. Hartge, J. R. Cerhan et al., "Body-mass index and mortality among 1.46 million white adults," *The New England Journal of Medicine*, vol. 363, no. 23, pp. 2211–2219, 2010.

[8] G. S. Hotamisligil, "Inflammation and metabolic disorders," *Nature*, vol. 444, no. 7121, pp. 860–867, 2006.

[9] G. S. Hotamisligil, N. S. Shargill, and B. M. Spiegelman, "Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance," *Science*, vol. 259, no. 5091, pp. 87–91, 1993.

[10] J. Qin, R. Li, J. Raes et al., "A human gut microbial gene catalogue established by metagenomic sequencing," *Nature*, vol. 464, no. 7285, pp. 59–65, 2010.

[11] J. Xu and J. I. Gordon, "Honor thy symbionts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10452–10459, 2003.

[12] V. Bocci, "The neglected organ: bacterial flora has a crucial immunostimulatory role," *Perspectives in Biology and Medicine*, vol. 35, no. 2, pp. 251–260, 1992.

[13] P. D. Cani and N. M. Delzenne, "The role of the gut microbiota in energy metabolism and metabolic disease," *Current Pharmaceutical Design*, vol. 15, no. 13, pp. 1546–1558, 2009.

[14] F. Bäckhed, H. Ding, T. Wang et al., "The gut microbiota as an environmental factor that regulates fat storage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 44, pp. 15718–15723, 2004.

[15] F. Bäckhed, J. K. Manchester, C. F. Semenkovich, and J. I. Gordon, "Mechanisms underlying the resistance to diet-induced obesity in germ-free mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 3, pp. 979–984, 2007.

[16] P. B. Eckburg, E. M. Bik, C. N. Bernstein et al., "Microbiology: diversity of the human intestinal microbial flora," *Science*, vol. 308, no. 5728, pp. 1635–1638, 2005.

[17] A. F. Andersson, M. Lindberg, H. Jakobsson, F. Bäckhed, P. Nyrén, and L. Engstrand, "Comparative analysis of human gut microbiota by barcoded pyrosequencing," *PLoS ONE*, vol. 3, no. 7, Article ID e2836, 2008.

[18] S. R. Gill, M. Pop, R. T. DeBoy et al., "Metagenomic analysis of the human distal gut microbiome," *Science*, vol. 312, no. 5778, pp. 1355–1359, 2006.

[19] R. E. Ley, F. Bäckhed, P. Turnbaugh, C. A. Lozupone, R. D. Knight, and J. I. Gordon, "Obesity alters gut microbial ecology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 31, pp. 11070–11075, 2005.

[20] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest," *Nature*, vol. 444, no. 7122, pp. 1027–1031, 2006.

[21] R. P. Ferraris and R. R. Vinnakota, "Intestinal nutrient transport in genetically obese mice," *The American Journal of Clinical Nutrition*, vol. 62, no. 3, pp. 540–546, 1995.

[22] B. P. Warwick and D. R. Romsos, "Energy balance in adrenalectomized ob/ob mice: effects of dietary starch and glucose," *The American Journal of Physiology*, vol. 255, no. 1, part 2, pp. R141–R148, 1988.

[23] J. R. Mujico, G. C. Baccan, A. Gheorghe, L. E. Díaz, and A. Marcos, "Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice," *The British Journal of Nutrition*, 2013.

[24] J. A. Parnell and R. A. Reimer, "Prebiotic fiber modulation of the gut microbiota improves risk factors for obesity and the metabolic syndrome," *Gut Microbes*, vol. 3, no. 1, pp. 29–34, 2012.

[25] M. Ferrer, A. Ruiz, F. Lanza et al., "Microbiota from the distal guts of lean and obese adolescents exhibit partial functional redundancy besides clear differences in community structure," *Environmental Microbiology*, vol. 15, no. 1, pp. 211–226, 2013.

[26] A. M. Caricilli, P. K. Picardi, L. L. de Abreu et al., "Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice," *PLOS Biology*, vol. 9, no. 12, Article ID e1001212, 2011.

[27] B. M. Carvalho, D. Guadagnini, D. M. Tsukumo et al., "Modulation of gut microbiota by antibiotics improves insulin signalling in high-fat fed mice," *Diabetologia*, vol. 55, no. 10, pp. 2823–2834, 2012.

[28] J. Henao-Mejia, E. Elinav, C. Jin et al., "Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity," *Nature*, vol. 482, no. 7384, pp. 179–185, 2012.

[29] A. Schwiertz, D. Taras, K. Schäfer et al., "Microbiota and SCFA in lean and overweight healthy subjects," *Obesity*, vol. 18, no. 1, pp. 190–195, 2010.

[30] M. C. Collado, E. Isolauri, K. Laitinen, and S. Salminen, "Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women," *The American Journal of Clinical Nutrition*, vol. 88, no. 4, pp. 894–899, 2008.

[31] J. C. Arthur, E. Perez-Chanona, M. Muhlbauer et al., "Intestinal inflammation targets cancer-inducing activity of the microbiota," *Science*, vol. 338, no. 6103, pp. 120–123, 2012.

[32] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 4, no. 7, pp. 499–511, 2004.

[33] R. Medzhitov and T. Horng, "Transcriptional control of the inflammatory response," *Nature Reviews Immunology*, vol. 9, no. 10, pp. 692–703, 2009.

[34] A. G. Oliveira, B. M. Carvalho, N. Tobar et al., "Physical exercise reduces circulating lipopolysaccharide and TLR4 activation and improves insulin signaling in tissues of DIO rats," *Diabetes*, vol. 60, no. 3, pp. 784–796, 2011.

[35] P. D. Cani, R. Bibiloni, C. Knauf et al., "Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice," *Diabetes*, vol. 57, no. 6, pp. 1470–1481, 2008.

[36] S. J. Creely, P. G. McTernan, C. M. Kusminski et al., "Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes," *The American Journal of Physiology*, vol. 292, no. 3, pp. E740–E747, 2007.

[37] P. Brun, I. Castagliuolo, V. Di Leo et al., "Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis," *The American Journal of Physiology*, vol. 292, no. 2, pp. G518–G525, 2007.

[38] R. Burcelin, L. Garidou, and C. Pomie, "Immuno-microbiota cross and talk: the new paradigm of metabolic diseases," *Seminars in Immunology*, vol. 24, no. 1, pp. 67–74, 2012.

[39] J. Amar, C. Chabo, A. Waget et al., "Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment," *EMBO Molecular Medicine*, vol. 3, no. 9, pp. 559–572, 2011.

[40] S. Ghoshal, J. Witta, J. Zhong, W. de Villiers, and E. Eckhardt, "Chylomicrons promote intestinal absorption of lipopolysaccharides," *Journal of Lipid Research*, vol. 50, no. 1, pp. 90–97, 2009.

[41] E. Lien, T. K. Means, H. Heine et al., "Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide," *Journal of Clinical Investigation*, vol. 105, no. 4, pp. 497–504, 2000.

[42] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[43] S. I. Miller, R. K. Ernst, and M. W. Bader, "LPS, TLR4 and infectious disease diversity," *Nature Reviews Microbiology*, vol. 3, no. 1, pp. 36–46, 2005.

[44] V. T. Samuel and G. I. Shulman, "Mechanisms for insulin resistance: common threads and missing links," *Cell*, vol. 148, no. 5, pp. 852–871, 2012.

[45] M. Vijay-Kumar, J. D. Aitken, F. A. Carvalho et al., "Metabolie syndrome and altered gut microbiota in mice lacking toll-like receptor 5," *Science*, vol. 328, no. 5975, pp. 228–231, 2010.

[46] C. Ubeda, L. Lipuma, A. Gobourne et al., "Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice," *Journal of Experimental Medicine*, vol. 209, no. 8, pp. 1445–1456, 2012.

[47] K. L. Calisto, B. M. Carvalho, E. R. Ropelle et al., "Atorvastatin improves survival in septic rats: effect on tissue inflammatory pathway and on insulin signaling," *PLoS ONE*, vol. 5, no. 12, Article ID e14232, 2010.

[48] M. Milanski, G. Degasperi, A. Coope et al., "Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity," *Journal of Neuroscience*, vol. 29, no. 2, pp. 359–370, 2009.

[49] K. A. Kim, W. Gu, I. A. Lee, E. H. Joh, and D. H. Kim, "High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway," *PLoS ONE*, vol. 7, no. 10, Article ID e47713, 2012.

[50] D. M. L. Tsukumo, M. A. Carvalho-Filho, J. B. C. Carvalheira et al., "Loss-of-function mutation in toll-like receptor 4 prevents diet-induced obesity and insulin resistance," *Diabetes*, vol. 56, no. 8, pp. 1986–1998, 2007.

[51] P. D. Cani, J. Amar, M. A. Iglesias et al., "Metabolic endotoxemia initiates obesity and insulin resistance," *Diabetes*, vol. 56, no. 7, pp. 1761–1772, 2007.

[52] H. Shi, M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier, "TLR4 links innate immunity and fatty acid-induced insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 3015–3025, 2006.

[53] A. Schaeffler, P. Gross, R. Buettner et al., "Fatty acid-induced induction of toll-like receptor-4/nuclear factor-κB pathway in adipocytes links nutritional signalling with innate immunity," *Immunology*, vol. 126, no. 2, pp. 233–245, 2009.

[54] C. Erridge and N. J. Samani, "Saturated fatty acids do not directly stimulate toll-like receptor signaling," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 11, pp. 1944–1949, 2009.

[55] A. J. Cayatte, L. Kumbla, and M. T. R. Subbiah, "Marked acceleration of exogenous fatty acid incorporation into cellular triglycerides by fetuin," *The Journal of Biological Chemistry*, vol. 265, no. 10, pp. 5883–5888, 1990.

[56] D. Pal, S. Dasgupta, R. Kundu et al., "Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance," *Nature Medicine*, vol. 18, pp. 1279–1285, 2012.

[57] H. Y. Ou, H. T. Wu, H. C. Hung, Y. C. Yang, J. S. Wu, and C. J. Chang, "Endoplasmic reticulum stress induces the expression of fetuin-A to develop insulin resistance," *Endocrinology*, vol. 153, no. 7, pp. 2974–2984, 2012.

[58] N. A. Ismail, S. Ragab, S. M. El Dayem et al., "Fetuin-A levels in obesity: differences in relation to metabolic syndrome and correlation with clinical and laboratory variables," *Archives of Medical Science*, vol. 8, no. 5, pp. 826–833, 2012.

[59] T. Reinehr and C. L. Roth, "Fetuin-A and its relation to metabolic syndrome and fatty liver disease in obese children before and after weight loss," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 11, pp. 4479–4485, 2008.

[60] S. T. Mathews, G. P. Singh, M. Ranalletta et al., "Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene," *Diabetes*, vol. 51, no. 8, pp. 2450–2458, 2002.

[61] S. T. Mathews, S. Rakhade, X. Zhou, G. C. Parker, D. V. Coscina, and G. Grunberger, "Fetuin-null mice are protected against obesity and insulin resistance associated with aging," *Biochemical and Biophysical Research Communications*, vol. 350, no. 2, pp. 437–443, 2006.

[62] E. Elinav, T. Strowig, A. L. Kau et al., "NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis," *Cell*, vol. 145, no. 5, pp. 745–757, 2011.

[63] T. Strowig, J. Henao-Mejia, E. Elinav, and R. Flavell, "Inflammasomes in health and disease," *Nature*, vol. 481, no. 7381, pp. 278–286, 2012.

[64] K. Schroder, V. Sagulenko, A. Zamoshnikova et al., "Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction," *Immunobiology*, vol. 217, no. 12, pp. 1325–1329, 2012.

[65] Y. Qiao, P. Wang, J. Qi, L. Zhang, and C. Gao, "TLR-induced NF-κB activation regulates NLRP3 expression in murine macrophages," *FEBS Letters*, vol. 586, no. 7, pp. 1022–1026, 2012.

[66] C. E. Samuel, "The eIF-2α protein kinases, regulators of translation in eukaryotes from yeasts to humans," *The Journal of Biological Chemistry*, vol. 268, no. 11, pp. 7603–7606, 1993.

[67] M. A. García, J. Gil, I. Ventoso et al., "Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action," *Microbiology and Molecular Biology Reviews*, vol. 70, no. 4, pp. 1032–1060, 2006.

[68] J. Hirosumi, G. Tuncman, L. Chang et al., "A central, role for JNK in obesity and insulin resistance," *Nature*, vol. 420, no. 6913, pp. 333–336, 2002.

[69] M. C. Arkan, A. L. Hevener, F. R. Greten et al., "IKK-β links inflammation to obesity-induced insulin resistance," *Nature Medicine*, vol. 11, no. 2, pp. 191–198, 2005.

[70] V. Aguirre, E. D. Werner, J. Giraud, Y. H. Lee, S. E. Shoelson, and M. F. White, "Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action," *The Journal of Biological Chemistry*, vol. 277, no. 2, pp. 1531–1537, 2002.

[71] T. Nakamura, M. Furuhashi, P. Li et al., "Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis," *Cell*, vol. 140, no. 3, pp. 338–348, 2010.

[72] M. A. Carvalho-Filho, B. M. Carvalho, A. G. Oliveira et al., "Double-stranded RNA-activated protein kinase is a key modulator of insulin sensitivity in physiological conditions and in obesity in mice," *Endocrinology*, vol. 153, no. 11, pp. 5261–5274, 2012.

[73] L. C. Hsu, J. M. Park, K. Zhang et al., "The protein kinase PKR is required for macrophage apoptosis after activation of toll-like receptor 4," *Nature*, vol. 428, no. 6980, pp. 341–345, 2004.

[74] M. Cabanski, M. Steinmüller, L. M. Marsh, E. Surdziel, W. Seeger, and J. Lohmeyer, "PKR regulates TLR2/TLR4-dependent signaling in murine alveolar macrophages," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 38, no. 1, pp. 26–31, 2008.

[75] G. A. Peters, R. Hartmann, J. Qin, and G. C. Sen, "Modular structure of PACT: distinct domains for binding and activating PKR," *Molecular and Cellular Biology*, vol. 21, no. 6, pp. 1908–1920, 2001.

[76] B. R. Williams, "Signal integration via PKR," *Science's STKE*, vol. 2001, no. 89, p. RE2, 2001.

[77] S. Symeonides and R. A. Balk, "Nitric oxide in the pathogenesis of sepsis," *Infectious Disease Clinics of North America*, vol. 13, no. 2, pp. 449–463, 1999.

[78] H. Sugita, M. Kaneki, E. Tokunaga et al., "Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance," *The American Journal of Physiology*, vol. 282, no. 2, pp. E386–E394, 2002.

[79] J. S. Stamler, D. I. Simon, J. A. Osborne et al., "S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 1, pp. 444–448, 1992.

[80] J. S. Stamler, E. J. Toone, S. A. Lipton, and N. J. Sucher, "(S)NO signals: translocation, regulation, and a consensus motif," *Neuron*, vol. 18, no. 5, pp. 691–696, 1997.

[81] S. Shinozaki, C. S. Choi, N. Shimizu et al., "Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice," *The Journal of Biological Chemistry*, vol. 286, no. 40, pp. 34959–34975, 2011.

[82] M. A. Carvalho-Filho, M. Ueno, S. M. Hirabara et al., "S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: a novel mechanism of insulin resistance," *Diabetes*, vol. 54, no. 4, pp. 959–967, 2005.

[83] H. Ovadia, Y. Haim, O. Nov et al., "Increased adipocyte S-nitrosylation targets anti-lipolytic action of insulin: relevance to adipose tissue dysfunction in obesity," *The Journal of Biological Chemistry*, vol. 286, no. 35, pp. 30433–30443, 2011.

[84] M. A. Carvalho-Filho, M. Ueno, J. B. C. Carvalheira, L. A. Velloso, and M. J. A. Saad, "Targeted disruption of iNOS prevents LPS-induced S-nitrosation of IRβ/IRS-1 and Akt and insulin resistance in muscle of mice," *The American Journal of Physiology*, vol. 291, no. 3, pp. E476–E482, 2006.

[85] E. R. Ropelle, J. R. Pauli, D. E. Cintra et al., "Targeted disruption of inducible nitric oxide synthase protects against aging, S-nitrosation, and insulin resistance in muscle of male mice," *Diabetes*, vol. 62, no. 2, pp. 466–470, 2013.

[86] J. R. Pauli, E. R. Ropelle, D. E. Cintra et al., "Acute physical exercise reverses S-nitrosation of the insulin receptor, insulin receptor substrate 1 and protein kinase B/Akt in diet-induced obese Wistar rats," *Journal of Physiology*, vol. 586, no. 2, pp. 659–671, 2008.

[87] M. A. Carvalho-Filho, E. R. Ropelle, R. J. Pauli et al., "Aspirin attenuates insulin resistance in muscle of diet-induced obese rats by inhibiting inducible nitric oxide synthase production and S-nitrosylation of IRβ/IRS-1 and Akt," *Diabetologia*, vol. 52, no. 11, pp. 2425–2434, 2009.

[88] G. T. Macfarlane and S. Macfarlane, "Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics," *Journal of Clinical Gastroenterology*, vol. 45, pp. S120–S127, 2011.

[89] J. H. Cummings, E. W. Pomare, W. J. Branch, C. P. E. Naylor, and G. T. Macfarlane, "Short chain fatty acids in human large intestine, portal, hepatic and venous blood," *Gut*, vol. 28, no. 10, pp. 1221–1227, 1987.

[90] P. Louis and H. J. Flint, "Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine," *FEMS Microbiology Letters*, vol. 294, no. 1, pp. 1–8, 2009.

[91] P. Louis, K. P. Scott, S. H. Duncan, and H. J. Flint, "Understanding the effects of diet on bacterial metabolism in the large intestine," *Journal of Applied Microbiology*, vol. 102, no. 5, pp. 1197–1208, 2007.

[92] M. S. M. Ardawi and E. A. Newsholme, "Fuel utilization in colonocytes of the rat," *Biochemical Journal*, vol. 231, no. 3, pp. 713–719, 1985.

[93] W. E. Roediger, "Short chain fatty acids as metabolic regulators of ion absorption in the colon," *Acta Veterinaria Scandinavica Supplementum*, vol. 86, pp. 116–125, 1989.

[94] N. M. Delzenne and C. M. Williams, "Prebiotics and lipid metabolism," *Current Opinion in Lipidology*, vol. 13, no. 1, pp. 61–67, 2002.

[95] S. H. Al-Lahham, M. P. Peppelenbosch, H. Roelofsen, R. J. Vonk, and K. Venema, "Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms," *Biochimica et Biophysica Acta*, vol. 1801, no. 11, pp. 1175–1183, 2010.

[96] M. Elia and J. H. Cummings, "Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates," *European Journal of Clinical Nutrition*, vol. 61, supplement 1, pp. S40–S74, 2007.

[97] E. Devillard, F. M. McIntosh, S. H. Duncan, and R. J. Wallace, "Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid," *Journal of Bacteriology*, vol. 189, no. 6, pp. 2566–2570, 2007.

[98] S. Kishino, J. Ogawa, K. Yokozeki, and S. Shimizu, "Metabolic diversity in biohydrogenation of polyunsaturated fatty acids by lactic acid bacteria involving conjugated fatty acid production," *Applied Microbiology and Biotechnology*, vol. 84, no. 1, pp. 87–97, 2009.

[99] J. R. Swann, E. J. Want, F. M. Geier et al., "Systemic gut microbial modulation of bile acid metabolism in host tissue compartments," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 1, pp. 4523–4530, 2011.

[100] A. B. Sahakian, S. R. Jee, and M. Pimentel, "Methane and the gastrointestinal tract," *Digestive Diseases and Sciences*, vol. 55, no. 8, pp. 2135–2143, 2010.

[101] P. Marquet, S. H. Duncan, C. Chassard, A. Bernalier-Donadille, and H. J. Flint, "Lactate has the potential to promote hydrogen sulphide formation in the human colon," *FEMS Microbiology Letters*, vol. 299, no. 2, pp. 128–134, 2009.

[102] S. Tedelind, F. Westberg, M. Kjerrulf, and A. Vidal, "Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 13, no. 20, pp. 2826–2832, 2007.

[103] S. H. Al-Lahham, H. Roelofsen, M. Priebe et al., "Regulation of adipokine production in human adipose tissue by propionic acid," *European Journal of Clinical Investigation*, vol. 40, no. 5, pp. 401–407, 2010.

[104] J. Fukae, Y. Amasaki, Y. Yamashita et al., "Butyrate suppresses tumor necrosis factor α production by regulating specific messenger RNA degradation mediated through a cis-acting AU-rich element," *Arthritis and Rheumatism*, vol. 52, no. 9, pp. 2697–2707, 2005.

[105] M. D. Säemann, G. A. Böhmig, C. H. Osterreicher et al., "Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production," *The FASEB Journal*, vol. 14, no. 15, pp. 2380–2382, 2000.

[106] K. M. Maslowski, A. T. Vieira, A. Ng et al., "Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43," *Nature*, vol. 461, no. 7268, pp. 1282–1286, 2009.

[107] A. Barcelo, J. Claustre, F. Moro, J. A. Chayvialle, J. C. Cuber, and P. Plaisancié, "Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon," *Gut*, vol. 46, no. 2, pp. 218–224, 2000.

[108] E. Gaudier, A. Jarry, H. M. Blottière et al., "Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose," *The American Journal of Physiology*, vol. 287, no. 6, pp. G1168–G1174, 2004.

[109] M. Bordin, F. D'Atri, L. Guillemot, and S. Citi, "Histone deacetylase inhibitors up-regulate the expression of tight junction proteins," *Molecular Cancer Research*, vol. 2, no. 12, pp. 692–701, 2004.

[110] L. Peng, Z. He, W. Chen, I. R. Holzman, and J. Lin, "Effects of butyrate on intestinal barrier function in a caco-2 cell monolayer model of intestinal barrier," *Pediatric Research*, vol. 61, no. 1, pp. 37–41, 2007.

[111] S. Fukuda, H. Toh, K. Hase et al., "Bifidobacteria can protect from enteropathogenic infection through production of acetate," *Nature*, vol. 469, no. 7331, pp. 543–547, 2011.

[112] T. Suzuki, S. Yoshida, and H. Hara, "Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability," *The British Journal of Nutrition*, vol. 100, no. 2, pp. 297–305, 2008.

[113] T. Kondo, M. Kishi, T. Fushimi, S. Ugajin, and T. Kaga, "Vinegar intake reduces body weight, body fat mass, and serum triglyceride levels in obese Japanese subjects," *Bioscience, Biotechnology and Biochemistry*, vol. 73, no. 8, pp. 1837–1843, 2009.

[114] B. B. Kahn, T. Alquier, D. Carling, and D. G. Hardie, "AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism," *Cell Metabolism*, vol. 1, no. 1, pp. 15–25, 2005.

[115] S. Sakakibara, T. Yamauchi, Y. Oshima, Y. Tsukamoto, and T. Kadowaki, "Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-A(y) mice," *Biochemical and Biophysical Research Communications*, vol. 344, no. 2, pp. 597–604, 2006.

[116] Z. Gao, J. Yin, J. Zhang et al., "Butyrate improves insulin sensitivity and increases energy expenditure in mice," *Diabetes*, vol. 58, no. 7, pp. 1509–1517, 2009.

[117] C. Grootaert, T. van de Wiele, I. van Roosbroeck et al., "Bacterial monocultures, propionate, butyrate and H_2O_2 modulate the expression, secretion and structure of the fasting-induced adipose factor in gut epithelial cell lines," *Environmental Microbiology*, vol. 13, no. 7, pp. 1778–1789, 2011.

[118] S. Alex, K. Lange, T. Amolo et al., "Short-chain fatty acids stimulate angiopoietin-like 4 synthesis in human colon adenocarcinoma cells by activating peroxisome proliferator-activated receptor γ," *Molecular and Cellular Biology*, vol. 33, no. 7, pp. 1303–1316, 2013.

[119] Y. H. Hong, Y. Nishimura, D. Hishikawa et al., "Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43," *Endocrinology*, vol. 146, no. 12, pp. 5092–5099, 2005.

[120] C. J. Small and S. R. Bloom, "Gut hormones and the control of appetite," *Trends in Endocrinology and Metabolism*, vol. 15, no. 6, pp. 259–263, 2004.

[121] O. B. Chaudhri, V. Salem, K. G. Murphy, and S. R. Bloom, "Gastrointestinal satiety signals," *Annual Review of Physiology*, vol. 70, pp. 239–255, 2008.

[122] Y. Xiong, N. Miyamoto, K. Shibata et al., "Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 4, pp. 1045–1050, 2004.

[123] K. R. Freeland and T. M. S. Wolever, "Acute effects of intravenous and rectal acetate on glucagon-like peptide-1, peptide YY, ghrelin, adiponectin and tumour necrosis factor-α," *The British Journal of Nutrition*, vol. 103, no. 3, pp. 460–466, 2010.

[124] C. A. Baile, "Metabolites as feedbacks for control of feed intake and receptor sites in goats and sheep," *Physiology and Behavior*, vol. 7, no. 6, pp. 819–826, 1971.

[125] M. H. Anil and J. M. Forbes, "Feeding in sheep during intraportal infusions of short-chain fatty acids and the effect of liver denervation," *Journal of Physiology*, vol. 298, pp. 407–414, 1980.

[126] H. V. Lin, A. Frassetto, E. J. Kowalik Jr. et al., "Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms," *PLoS ONE*, vol. 7, no. 4, Article ID e35240, 2012.

[127] R. M. A. J. Ruijschop, A. E. M. Boelrijk, and M. C. T. Giffel, "Satiety effects of a dairy beverage fermented with propionic acid bacteria," *International Dairy Journal*, vol. 18, no. 9, pp. 945–950, 2008.

[128] J. Zhou, M. Hegsted, K. L. McCutcheon et al., "Peptide YY and proglucagon mRNA expression patterns and regulation in the gut," *Obesity*, vol. 14, no. 4, pp. 683–689, 2006.

[129] B. S. Samuel, A. Shaito, T. Motoike et al., "Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 43, pp. 16767–16772, 2008.

[130] M. Membrez, F. Blancher, M. Jaquet et al., "Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice," *The FASEB Journal*, vol. 22, no. 7, pp. 2416–2426, 2008.

[131] C. J. Chou, M. Membrez, and F. Blancher, "Gut decontamination with norfloxacin and ampicillin enhances insulin sensitivity in mice," *Nestle Nutrition Workshop Series: Pediatric Program*, vol. 62, pp. 127–140, 2008.

[132] I. Cho, S. Yamanishi, L. Cox et al., "Antibiotics in early life alter the murine colonic microbiome and adiposity," *Nature*, vol. 488, no. 7413, pp. 621–626, 2012.

[133] G. Ternak, "Antibiotics may act as growth/obesity promoters in humans as an inadvertent result of antibiotic pollution?" *Medical Hypotheses*, vol. 64, no. 1, pp. 14–16, 2005.

[134] M. Caselli, F. Cassol, G. Calò, J. Holton, G. Zuliani, and A. Gasbarrini, "Actual concept of "probiotics": is it more functional to science or business?" *World Journal of Gastroenterology*, vol. 19, no. 10, pp. 1527–1540, 2013.

[135] L. Macia, A. N. Thorburn, L. C. Binge et al., "Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases," *Immunological Reviews*, vol. 245, no. 1, pp. 164–176, 2012.

[136] N. Takemura, T. Okubo, and K. Sonoyama, "*Lactobacillus plantarum* strain No. 14 reduces adipocyte size in mice fed high-fat diet," *Experimental Biology and Medicine*, vol. 235, no. 7, pp. 849–856, 2010.

[137] Y. Kadooka, M. Sato, K. Imaizumi et al., "Regulation of abdominal adiposity by probiotics (*Lactobacillus gasseri* SBT2055) in adults with obese tendencies in a randomized controlled trial," *European Journal of Clinical Nutrition*, vol. 64, no. 6, pp. 636–643, 2010.

[138] A. S. Andreasen, N. Larsen, T. Pedersen-Skovsgaard et al., "Effects of *Lactobacillus acidophilus* NCFM on insulin sensitivity and the systemic inflammatory response in human subjects," *The British Journal of Nutrition*, vol. 104, no. 12, pp. 1831–1838, 2010.

[139] R. Luoto, M. Kalliomäki, K. Laitinen, and E. Isolauri, "The impact of perinatal probiotic intervention on the development of overweight and obesity: follow-up study from birth to 10 years," *International Journal of Obesity*, vol. 34, no. 10, pp. 1531–1537, 2010.

[140] L. Aronsson, Y. Huang, P. Parini et al., "Decreased fat storage by *Lactobacillus paracasei* is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4)," *PLoS ONE*, vol. 5, no. 9, Article ID e13087, 2010.

[141] J. M. Uronis, J. C. Arthur, T. Keku et al., "Gut microbial diversity is reduced by the probiotic VSL#3 and correlates with decreased TNBS-induced colitis," *Inflammatory Bowel Diseases*, vol. 17, no. 1, pp. 289–297, 2011.

[142] R. Mennigen, K. Nolte, E. Rijcken et al., "Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis," *The American Journal of Physiology*, vol. 296, no. 5, pp. G1140–G1149, 2009.

[143] A. Mencarelli, S. Cipriani, B. Renga et al., "VSL#3 resets insulin signaling and protects against NASH and atherosclerosis in a model of genetic dyslipidemia and intestinal inflammation," *PLoS ONE*, vol. 7, no. 9, Article ID e45425, 2012.

[144] C. Alberda, L. Gramlich, J. Meddings et al., "Effects of probiotic therapy in critically ill patients: a randomized, double-blind, placebo-controlled trial," *The American Journal of Clinical Nutrition*, vol. 85, no. 3, pp. 816–823, 2007.

[145] X. Ma, J. Hua, and Z. Li, "Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells," *Journal of Hepatology*, vol. 49, no. 5, pp. 821–830, 2008.

[146] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[147] S. Resta-Lenert and K. E. Barrett, "Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC)," *Gut*, vol. 52, no. 7, pp. 988–997, 2003.

[148] A. A. Zyrek, C. Cichon, S. Helms, C. Enders, U. Sonnenborn, and M. A. Schmidt, "Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCζ redistribution resulting in tight junction and epithelial barrier repair," *Cellular Microbiology*, vol. 9, no. 3, pp. 804–816, 2007.

[149] M. Pineiro, N. G. Asp, G. Reid et al., "FAO Technical meeting on prebiotics," *Journal of Clinical Gastroenterology*, vol. 42, supplement 3, part 2, pp. S156–S159, 2008.

[150] A. M. Bakker-Zierikzee, E. A. F. van Tol, H. Kroes, M. S. Alles, F. J. Kok, and J. G. Bindels, "Faecal SIgA secretion in infants fed on pre- or probiotic infant formula," *Pediatric Allergy and Immunology*, vol. 17, no. 2, pp. 134–140, 2006.

[151] F. Campeotto, A. Suau, N. Kapel et al., "A fermented formula in pre-term infants: clinical tolerance, gut microbiota, down-regulation of faecal calprotectin and up-regulation of faecal secretory IgA," *The British Journal of Nutrition*, vol. 105, no. 12, pp. 1843–1851, 2011.

[152] P. D. Cani, C. Knauf, M. A. Iglesias, D. J. Drucker, N. M. Delzenne, and R. Burcelin, "Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor," *Diabetes*, vol. 55, no. 5, pp. 1484–1490, 2006.

[153] P. D. Cam, A. M. Neyrinck, N. Maton, and N. M. Delzenne, "Oligofructose promotes satiety in rats fed a high-fat diet: involvement of glucagon-like peptide," *Obesity Research*, vol. 13, no. 6, pp. 1000–1007, 2005.

[154] C. Daubioul, N. Rousseau, R. Demeure et al., "Dietary fructans, but not cellulose, decrease triglyceride accumulation in the liver of obese Zucker fa/fa rats," *Journal of Nutrition*, vol. 132, no. 5, pp. 967–973, 2002.

[155] P. D. Cani, A. M. Neyrinck, F. Fava et al., "Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia," *Diabetologia*, vol. 50, no. 11, pp. 2374–2383, 2007.

[156] S. A. Abrams, I. J. Griffin, K. M. Hawthorne, and K. J. Ellis, "Effect of prebiotic supplementation and calcium intake on body mass index," *Journal of Pediatrics*, vol. 151, no. 3, pp. 293–298, 2007.

[157] S. Genta, W. Cabrera, N. Habib et al., "Yacon syrup: beneficial effects on obesity and insulin resistance in humans," *Clinical Nutrition*, vol. 28, no. 2, pp. 182–187, 2009.

[158] M. Roberfroid, G. R. Gibson, L. Hoyles et al., "Prebiotic effects: metabolic and health benefits," *The British Journal of Nutrition*, vol. 104, supplement 2, pp. S1–S63, 2010.

[159] P. D. Cani, S. Hoste, Y. Guiot, and N. M. Delzenne, "Dietary non-digestible carbohydrates promote L-cell differentiation in the proximal colon of rats," *The British Journal of Nutrition*, vol. 98, no. 1, pp. 32–37, 2007.

[160] N. M. Delzenne, P. D. Cani, C. Daubioul, and A. M. Neyrinck, "Impact of inulin and oligofructose on gastrointestinal peptides," *The British Journal of Nutrition*, vol. 93, pp. S157–S161, 2005.

[161] J. M. Gee and I. T. Johnson, "Dietary lactitol fermentation increases circulating peptide YY and glucagon-like peptide-1 in rats and humans," *Nutrition*, vol. 21, no. 10, pp. 1036–1043, 2005.

[162] A. A. Aziz, L. S. Kenney, B. Goulet, and E. S. Abdel-Aal, "Dietary starch type affects body weight and glycemic control in freely fed but not energy-restricted obese rats," *Journal of Nutrition*, vol. 139, no. 10, pp. 1881–1889, 2009.

[163] M. J. Keenan, J. Zhou, K. L. McCutcheon et al., "Effects of resistant starch, a non-digestible fermentable fiber, on reducing body fat," *Obesity*, vol. 14, no. 9, pp. 1523–1534, 2006.

[164] L. Shen, M. J. Keenan, R. J. Martin et al., "Dietary resistant starch increases hypothalamic POMC expression in rats," *Obesity*, vol. 17, no. 1, pp. 40–45, 2009.

[165] J. Zhou, R. J. Martin, R. T. Tulley et al., "Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents," *The American Journal of Physiology*, vol. 295, no. 5, pp. E1160–E1166, 2008.

[166] J. A. Parnell and R. A. Reimer, "Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults," *The American Journal of Clinical Nutrition*, vol. 89, no. 6, pp. 1751–1759, 2009.

[167] J. Tarini and T. M. S. Wolever, "The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects," *Applied Physiology, Nutrition and Metabolism*, vol. 35, no. 1, pp. 9–16, 2010.

[168] P. D. Cani, S. Possemiers, T. van de Wiele et al., "Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability," *Gut*, vol. 58, no. 8, pp. 1091–1103, 2009.

[169] H. Zhang, J. K. DiBaise, A. Zuccolo et al., "Human gut microbiota in obesity and after gastric bypass," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 7, pp. 2365–2370, 2009.

[170] A. P. Liou, M. Paziuk, J. M. Luevano Jr., S. Machineni, P. J. Turnbaugh, and L. M. Kaplan, "Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity," *Science Translational Medicine*, vol. 5, no. 178, Article ID 178ra141, 2013.

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Review Article

Adipo-Myokines: Two Sides of the Same Coin—Mediators of Inflammation and Mediators of Exercise

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This review summarizes the current literature regarding the most discussed contraction-regulated moykines like IL-6, IL-15, irisin, BDNF, ANGPTL4, FGF21, myonectin and MCP-1. It is suggested that the term myokine is restricted to proteins secreted from skeletal muscle cells, excluding proteins that are secreted by other cell types in skeletal muscle tissue and excluding proteins which are only described on the mRNA level. Interestingly, many of the contraction-regulated myokines described in the literature are additionally known to be secreted by adipocytes. We termed these proteins adipo-myokines. Within this review, we try to elaborate on the question why pro-inflammatory adipokines on the one hand are upregulated in the obese state, and have beneficial effects after exercise on the other hand. Both, adipokines and myokines do have autocrine effects within their corresponding tissues. In addition, they are involved in an endocrine crosstalk with other tissues. Depending on the extent and the kinetics of adipo-myokines in serum, these molecules seem to have a beneficial or an adverse effect on the target tissue.

1. Skeletal Muscle and Adipose Tissue as Endocrine Organs

In line with the acceptance of adipose tissue as an endocrine organ [1–3], path-breaking work during the last decade demonstrated that skeletal muscle is an active endocrine organ releasing myokines, which might in part be responsible for the beneficial effect of exercise [4–6]. These myokines are described to communicate with cells in an autocrine/paracrine manner, locally within the muscles, or in an endocrine fashion to distant tissues.

Obesity in a combination with a lack of exercise is a strong risk factor to develop metabolic diseases and type 2 diabetes. Physical inactivity causes the accumulation of visceral fat and the health consequences of both are related to systemic low-grade inflammation [7, 8]. Adipocytes from obese patients are characterized by altered endocrine function, leading to increased secretion of proinflammatory adipokines, such as TNFα, chemerin, MCP-1, dipeptidyl peptidase 4 (DPP4), and others [9–14]. Thus, the dysregulation of adipokine secretion is related to metabolic diseases. The activation of inflammatory pathways leads to insulin resistance in peripheral tissues such as skeletal muscle and adipose tissue itself, constituting an early defect in the pathogenesis of type 2 diabetes [15]. Different research strategies revealed the complexity of the adipocyte secretome, and to date more than 600 potentially secretory proteins were identified [2].

In addition, it is well accepted that contracting skeletal muscle secretes enhanced levels of myokines which have a beneficial endocrine effect on other organs, presenting novel targets for the treatment of metabolic diseases and type 2 diabetes [16].

2. Identification of Contraction-Regulated Myokines

It is well accepted that physical activity exerts multiple beneficial effects on the prevention of chronic diseases, both due to an improved energy balance and due to effects independent of obesity. It is assumed that contraction-regulated myokines play a pivotal role in the communication between muscle and other tissues such as adipose tissue, liver, and pancreatic cells [16–18].

Table 1: Contraction-regulated myokines. A search of original articles in pubMed was performed for all myokines described to identify contraction regulation of a myokine on the level of enhanced muscle mRNA expression and enhanced serum level. In addition, studies describing basal secretion of the indicated myokine from myotubes (*in vitro* studies) are given. The search terms used were "skeletal muscle," "myokine," "exercise," "secretion," and the indicated myokine. Reference lists of identified articles were also used to search for further papers.

Myokine	Secreted by cells	Enhanced muscle mRNA level after exercise	Enhanced serum level after exercise
ANGPTL4	√ [157]	√ [32]	√ [75]
BDNF	n.d. [19]	√ [19]	√ [63, 64]
FGF21	√ [79]	—	√ [83]#
FSTL1	√ [112, 113]	√ [31]	√ [112]
IL-6	√ [24]	√ [158]	√ [35]
IL-7	√ [159]	√ [159]	—
IL-8	√ [140]	√ [51, 70, 160, 161]	—
IL-15	n.d. [162–164]	√ [48, 52, 55] × [47]	√ [49–51] × [52, 165]
Irisin		√ [55]	√ [55, 57] × [56]
LIF	√ [166]	√ [166, 167]	—
MCP-1	√ [68, 140]	√ [70, 72]	√ [32, 69]
Myonectin	√ [86, 87]	√ [86]	√ [86] [88]#
Myostatin	√ [147]	√ [168–172]#	√ [173]#
PAI-1	√ [31]	√ [31]	
PEDF	√ [31]	√ [31]	
VEGF	√ [24]	√ [174]	√ [51]

√: secretion, enhanced muscle mRNA level, or serum level of myokines have been shown in indicated publications. ×: contraction regulation of myokine has not been shown. #Myokine serum levels are described to be decreased after exercise, n.d.: not detected in supernatants of myotubes.

Research of the last decade revealed that several myokines are regulated by contraction, like angiopoietin-like 4 (ANGPTL4), brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF) 21, follistatin-like 1 (FSTL1), interleukin (IL)-6, IL-7, IL-15, irisin, leukemia inhibitory factor (LIF), myonectin, myostatin, and vascular endothelial growth factor (VEGF) (for references see Table 1). For some of these reported myokines, the description as a myokine is based on mRNA data of skeletal muscle biopsies. For example, Matthews et al. [19] report increased BNDF mRNA level in human contracting skeletal muscle biopsies. Although the authors could prove enhanced serum levels after exercise in humans and increased BDNF protein level after electrical pulse stimulation of C2C12 cells, BDNF basal secretion could not be detected in the media from skeletal muscle cells *in vitro* [19]. Secretion is the critical characteristic of a myokine and it is preferable to restrict the term myokine to those proteins that are released by skeletal muscle cells themselves.

Nevertheless, the term myokine has also been employed to describe a protein that is synthesized by skeletal muscle tissue, rather than by the skeletal muscle cell. The initial characterization of a candidate myokine is frequently the detection of the gene expression in skeletal muscle tissue by mRNA expression or immunodetection of protein lysates. One dilemma in only determining gene expression or protein level in skeletal muscle biopsies is that aside from skeletal muscle fibres, skeletal muscle contains extended layers of connective tissues, capillaries, and nerve cells among others. Thus satellite cells, endothelial cells, fibroblasts, and motor neurons are included in the analysis. Gene expression must be followed by the detection of the encoded protein in skeletal muscle fibers. Additional immunostaining of the skeletal muscle tissue sections shows that the protein production is indeed intramyocellular. When the expression is first identified in skeletal muscle tissue, the validation of a protein as a myokine has to include that secretion from skeletal muscle cells is demonstrated. In practice, this will generally reflect selective release from skeletal muscle cells *in vitro* either by the use of primary human or animal skeletal muscle cells or from clonal cell lines. Equally, proteins that have been identified in skeletal muscle cells needs to be verified for the native tissue. We recommend that the term myokine is used for a protein that is synthesized and secreted by skeletal muscle cells.

The identification of a protein as a contraction-regulated myokine represents an additional critical step in the analysis. Repeated biopsy sampling from one muscle is necessary to investigate muscular adaptation to different forms of exercise. The adaptation is thought to be the result of cumulative effects of transient changes in gene expression in response to single exercise bouts. Nevertheless, it was shown that multiple fine needle biopsies obtained from the same muscle region can per se influence the expression of marker genes

induced by an acute bout of resistance exercise [20]. Thus, repeated biopsies have to be taken carefully in regard to avoiding an inflammatory response in the tissue. In the case that contraction regulation of a protein is first identified in muscle biopsies on the mRNA level, it is essential to determine whether the enhanced mRNA expression is translated to enhanced protein level. An additional elegant approach is to induce contraction of human skeletal muscle cells or clonal cell lines by electrical pulse stimulation [21–24]. The potentially contraction-regulated myokine can be analyzed on the mRNA and protein level. Most importantly, enhanced secretion can be determined in the supernatants by immunodetection.

3. Secretome of Muscle Cells

To gain a broader view, recent efforts have focused on exploring the complete secretome of skeletal muscle by proteomic studies. New technological advances, like array studies and proteomic analysis, made the analysis of the qualitative and quantitative analysis of the secretome of skeletal muscle possible. These approaches extended the list of described myokines rapidly. Chan et al. and Henningsen et al. have investigated altered regulation of secretome components at different time points of muscle differentiation of murine C2C12 cells by a quantitative proteomics approach [25–27], while Yoon et al. have studied the regulation of myokine secretion by rat skeletal muscle cells after insulin stimulation [28] and TNFα treatment [29]. Recently, Hittel et al. have explored the secretome from cultured myotubes derived from extremely obese compared with healthy nonobese women [30]. All these studies found hundreds of secreted proteins from skeletal muscle, some regulated by insulin or TNFα, others during differentiation. A drawback of all these studies is the use of noncontracting cells although contraction is a major characteristic of skeletal muscle activating intracellular signalling pathways, changing the secretory profile, inducing metabolic adaption and the change of its plasticity. To overcome this problem, Norheim et al. combined the proteomic analysis of the secretome of human myotubes with mRNA expression data of muscle biopsies in response to strength training. Using this approach the authors identified 15 novel contraction-regulated myokines [31]. Recently, Catoire et al. described the effect of endurance exercise on gene expression in exercising and nonexercising human muscle by one-legged cycling [32]. Noticeably, acute exercise also caused substantial gene expression changes in nonexercising leg [32]. This effect might be mediated by changes in circulating factors such as free fatty acids, adrenalin, and lactate, but might also support the myokine concept.

Nevertheless, all these studies indicate that skeletal muscle cells are, like adipocytes, major secretory cells. Clustering these skeletal muscle-derived proteins according to their postulated function revealed that these myokines can be sorted to several groups, including myokines contributing to energy metabolism, angiogenesis, blood vessel regulation, and myogenesis [33].

4. IL-6: The Best Characterized Myokine

Some of the first reports in this research field identified IL-6 as a secreted protein from skeletal muscle [34, 35]. The identification that contracting human skeletal muscle releases significant amounts of IL-6 into the circulation during prolonged single-limb exercise was a milestone in this research field and identified skeletal muscle as an endocrine organ [36]. Up to now, IL-6 is the most prominent muscle-derived protein, which was demonstrated to be upregulated in plasma after exercise without muscle damage [34, 37]. The level of circulating IL-6 increases in an exponential fashion in response to exercise [36, 38, 39] and declines in the postexercise period [40]. The magnitude by which plasma levels increase is related to exercise duration, intensity, and the muscle mass involved in the mechanical work [36, 38, 39, 41]. However, during one year of training intervention plasma levels of IL-6 remained unchanged [42]. Plasma IL-6 levels can increase up to 100-fold in response to exercise although less strong effects are more frequent [43]. In addition to human serum and skeletal muscle biopsy data, IL-6 has been shown to be secreted by primary human skeletal muscle cells *in vitro* and its secretion was increased by contraction [24].

5. IL-15: A Contraction-Regulated Myokine?

IL-15 is discussed as a contraction-regulated myokine in the literature which may play a role in muscle-fat crosstalk [44, 45] mediating some of the beneficial effects of physical activity [46]. Until today, five groups analyzed the regulation of IL-15 after different exercise protocols in humans. However, conflicting data are published whether physical activity affects IL-15 expression, protein level, and secretion from skeletal muscle.

In a first report, no change in IL-15 mRNA level was described in human *vastus lateralis* muscle biopsy samples, which were taken immediately after 2 h intensive resistance training [47]. Although, Nielsen et al. observed that IL-15 mRNA content was upregulated twofold in human *vastus lateralis* muscle 24 hours following a single bout of resistance exercise, this increase in mRNA level was not accompanied by an increase in muscular IL-15 protein level or plasma IL-15 [48]. In addition, Riechman et al. demonstrated that immediately after the end of one resistance exercise bout, plasma IL-15 increased slightly (approximately 5%) [49]. Recently, it has been shown that 30 min treadmill running at 70% of maximum heart rate resulted in a significant increase in circulating IL-15 level in untrained healthy young men (about 12%), measured 10 min after exercise [50]. Different from these acute exercise studies, Yeo et al. described that both 8-week moderate- and high-intensity resistance exercises enhanced IL-15 serum levels [51]. In this study, the authors showed that IL-15 blood level was significantly enhanced after 8 weeks of moderate intensity resistance exercise (250%), while the increase of the myokine prototype IL-6 was rather small (115%). High intensity resistance training also enhanced IL-15 blood levels, but to a lower extent (150%).

In addition, training studies were performed in mice and rats. It has been shown that IL-15 mRNA expression in *soleus* and *gastrocnemius* muscle is increased after 8-week treadmill running training in rats, while plasma IL-15 level was not changed [52]. Yang et al. observed about 1.7-fold increase in IL-15 mRNA expression after three weeks of free wheel running in mice but did not analyze protein or serum level.

Nevertheless, while the observed increase in plasma IL-15 levels in humans is rather small (5–12%) after acute exercise and not depending on the mode of exercise, particularly moderate intensity resistance exercise had a significant effect on IL-15 blood levels. However, to the best of our knowledge, secretion of IL-15 from muscle cells has not been described yet, and it has not been shown that the observations on muscle mRNA level are translated to meaningful contributions to IL-15 serum levels.

6. Irisin: A Novel Myokine

Just recently, a novel identified myokine has drawn the attention as a novel preventive and therapeutic target to treat obesity and metabolic diseases like type 2 diabetes. Bostrom et al. observed that overexpression of PGC1α in mice muscle as well as exercise induces the expression of the FNDC5 (fibronectin type III domain containing protein 5) gene, a gene which has scarcely been studied before. FNDC5 is described as a protein containing a signal peptide, fibronectin type III repeats, and hydropathy analysis revealed a hydrophobic region, which is likely to encode a transmembrane domain. Previous studies linked the gene to differentiation of myoblasts and neurones [53, 54], and it has been suggested that FNDC5 is located in the matrix of peroxisomes [53]. Bostrom et al. described that the C-terminal tail of the protein is located in the cytoplasm, whereas the extracellular N-terminal part is supposed to be cleaved and released as novel messenger molecule called irisin [55]. Mice subjected to three weeks of free wheel running showed enhanced muscle mRNA expression and elevated irisin plasma concentrations (65%). In addition, ten weeks of supervised endurance exercise training revealed a twofold increase in circulating irisin levels compared to the nonexercised state in a cohort of older subjects [55]. Both the mice and human study analyzed the long-term effect of exercise on irisin plasma levels. It is not described if enhanced serum levels of FNDC5 after muscle contraction are dependent on enhanced gene expression or dependent on enhanced cleavage of the membrane protein.

However, using gene-chip probe sets Timmons et al. observed no effect on FNDC5 mRNA level neither after 6 weeks of intense endurance cycling in younger subjects nor after supervised resistance training [56]. Timmons et al. demonstrated that FNDC5 induction in muscle occurred only in highly active elderly subjects compared to sedentary controls (1.3fold), which were a minority of analyzed subjects. They failed to confirm FNDC5 gene expression by aerobic exercise in younger subjects. Huh et al. observed minor effects on irisin plasma levels after 1 week of exercise (increase of about 18%) and no effect after prolonged training over 8 weeks [57]. Up to now, there is only one study showing a robust activation of FNDC5 after exercise in humans measured by RT-PCR in muscle biopsies [24], however, limited to a very small number of subjects.

Although Bostrom et al. described the discovery of the novel myokine irsin, the authors showed that the release of irisin exclusively in HEK 293 cells transfected with a vector expressing FNDC5 followed immunodetection of culture media protein. It has not been confirmed that muscle FNDC5 mRNA is translated to protein in primary or clonal skeletal muscle cells, and, most importantly, it has not been shown that irisin is secreted from skeletal muscle cells. Furthermore, to demonstrate the secretion of irisin from HEK293 cells and to analyze murine and human serum samples, the authors used an antibody, which most likely cannot detect the cleaved irisin, since the antibody used is directed against the C-terminal part of the protein (Abcam 149–178, C-terminal) [58]. Taken together, future studies should address FNDC5/irisin precise expression and cleavage mechanism to clarify the controversy of current literature.

7. BDNF: Released from the Muscle or the Brain?

BDNF belongs to the family of neurotrophins (NT), which includes nerve growth factor, BDNF, NT-3, NT-4/5, and NT-6. These proteins are produced as large precursor proteins that are then cleaved to form the mature neurotrophic protein (reviewed in [59]). In the literature BDNF is discussed as a contraction-regulated myokine [6]. During myogenic differentiation, the expression of BDNF is drastically reduced and is hardly detectable in adult rat skeletal myofibers [60]. By reverse transcription PCR, *in situ* hybridization, and immunofluorescence, it was shown that BDNF is not expressed at significant levels within mature myofibers [60]. *In situ* hybridisation analysis revealed that in adult rat muscle the constitutive expression of muscular BDNF is confined to the myofibres. Satellite cells, Schwann cells, endothelial cells, fibroblasts, or axons did not appear to contribute to BDNF production in normal muscle [61]. In complementary cell culture experiments, it has been shown that levels of BDNF correlate with the population of satellite cells [60]. BDNF is required for early phases of myogenic differentiation, which is delayed in the absence of BDNF [62]. Nevertheless, BDNF protein level was determined in lysates of rat L6 cells [60] and murine C2C12 cells [19].

Serum BDNF levels increased after a graded cycling exercise test in humans (30%) [63] and at the point of exhaustion at the end of a ramp test (about 25%) [64]. Matthews et al. reported enhanced BDNF mRNA and protein expression in human skeletal muscle and after bicycle exercise [19]. Cell culture experiments using murine C2C12 cells stimulated by electrical pulse stimulation confirmed that contractile activity enhanced BNDF mRNA and protein level. Although Matthews et al. report increased serum levels after exercise in humans, BDNF basal secretion by C2C12 that underwent contraction has not been proven and overexpression of BDNF in mouse skeletal muscle did not lead to differences in plasma

BDNF [19], leading the authors to the conclusion that BNDF exerts its action locally and is not released into the circulation. Matthews et al. reported an autocrine effect of BNDF since treatment of skeletal muscle cells with recombinant BDNF resulted in enhanced phosphorylation of AMP-activated protein kinase (AMPK) and ACC in rat L6 cells which leads to enhanced fatty acid oxidation [19].

In mice, treadmill exercise induced an increase in BDNF mRNA expression in the hippocampus and cortex (three- to fivefold) [65]. In humans, a BDNF release from the brain was observed at rest and increased two- to threefold during exercise. Both at rest and during exercise, the brain contributed 70–80% of circulating BDNF [65]. These results suggest that the brain is a major, but not the sole contributor to circulating BDNF after exercise.

8. MCP-1

MCP-1 is a chemokine and member of the small inducible cytokine family. It plays a crucial role in the recruitment of monocytes and T lymphocytes into tissues [66, 67]. MCP-1 was detected in supernatants of C2C12 cells [68]. In mice, plasma IL-6 levels were markedly increased 3 h following maximum progressive swimming, while MCP-1 plasma levels were not altered by exercise [69]. However, a single bout of intense resistance exercise increased MCP-1 mRNA expression in muscle biopsy samples obtained from *vastus lateralis* muscle about 35-fold after two hours. In comparison, IL-6 mRNA expression, the myokine prototype, was enhanced about 400-fold [70]. One bout of moderate-intensity cycle exercise increased MCP-1 mRNA levels in *vastus lateralis* muscle biopsy samples after 40 min [71]. One-legged cycling of male subjects induced a significant change in MCP-1 mRNA levels in the exercising leg and enhanced MCP-1 plasma levels after exercise and after 3 hours of recovery [72]. In addition, increased MCP-1 mRNA expression in skeletal muscle was reported in elderly individuals following one bout of resistance exercise [73] and in young men after a repeated eccentric exercise bout [74]. Immunohistochemistry analysis of muscle biopsies colocalized MCP-1 with resident macrophage and satellite cell populations, suggesting that alterations in cytokine signalling between these cell populations may play a role in muscle adaptation to exercise [74].

9. ANGPTL4: Regulated by Free Fatty Acids

ANGPTL4 represents a prominent long chain fatty acid-responsive gene in human myotubes. Kersten et al. reported that plasma ANGPTL4 levels in humans increased significantly in response to long-term fasting, chronic caloric restriction, and endurance training. All these states are characterized by enhanced circulating FFA [75]. Fasting plasma ANGPTL4 levels of healthy, untrained male volunteers increased during endurance exercise at 50% VO_2 max for 2 h and especially during subsequent recovery. Importantly, the increase in plasma ANGPTL4 was abolished when subjects were given oral glucose, which induces insulin release and thereby suppresses plasma FFA levels [75]. While ANGPTL4 is below the detection limit in supernatants of differentiated C2C12 cells, long-term treatment of human myotubes (48 h) with the PPARδ-specific activator GW501516 results in the accumulation of ANGPTL4 in the supernatant [76]. In addition, incubation of human primary myocytes with oleic acid and linoleic acid enhanced ANGPTL4 mRNA expression. Nevertheless, this effect was not only observed in primary human myocytes, but also in FAO hepatoma cells and mouse intestinal MSIE cells [75].

Catoire et al. have shown in a human one-legged exercise study that target genes of PPAR transcription factors including ANGPTL4 were induced equally in exercising and nonexercising muscle [77]. Although PPARδ is known to be activated by high-intensity exercise [78], Catoire et al. have concluded that the increase of plasma free fatty acid levels due to acute exercise activates PPARs and therefore ANGPTL4 [77].

Long-term changes in plasma ANGPTL4 levels are most likely mediated by changes in plasma free fatty acids, which raise ANGPTL4 gene expression in target tissues. Nevertheless, ANGPTL4 is ubiquitously expressed in human tissues and highest expression levels were found in liver, followed by adipose tissue, thyroid, brain, small intestine, and less in skeletal muscle [75]. Thus, skeletal muscle might not be the only tissue which is responsible for enhanced ANGPTL4 plasma levels in states of increased FFA levels like endurance training. Furthermore, ANGPTL4 stimulates adipose tissue lipolysis, leading to elevation of plasma free fatty acid levels. Kersten et al. speculated that both mechanisms operate as a positive feedback loop. Free fatty acids raises plasma ANGPTL4 and ANGPTL4 raises plasma free fatty acids by the stimulation of adipose tissue lipolysis [75].

10. FGF21

FGF21 is a member of the fibroblast growth factor super family, a large family of proteins involved in cell proliferation, growth, and differentiation. The first evidence that FGF21 is an Akt-regulated myokine was published by Izumiya et al. [79]. FGF21 protein expression and secretion are upregulated by insulin and inhibited by PI3-kinase inhibitor in cultured C2C12 myocytes [79]. Skeletal muscle mRNA level and plasma level are induced by hyperinsulinemia studied in young healthy men during a hyperinsulinemic-euglycemic clamp [80]. In line with this observation, circulating FGF21 is elevated in impaired glucose tolerance and type 2 diabetes patients and correlates with muscle and hepatic insulin resistance [81].

Interestingly, an acute bout of treadmill exercise did not change FGF21 serum levels in sedentary young women. However, after two weeks of exercising there was a 1.6-fold increase in serum FGF21 [82]. In contrast, twelve-week exercise program combining aerobic and resistance exercise, five times per week, reduced FGF21 plasma levels in nondiabetic, obese women (from 230.2 ± 135.9 versus 102.6 ± 117.8 pg/mL) [83]. Nevertheless, nothing is known about the acute effect of contraction on the expression, protein level, and secretion of FGF21 from skeletal muscle cells.

11. Myonectin

Myonectin belongs to the C1q/TNF-related protein family (C1QTNF isoform 5) and shows a sequence homology with adiponectin in the shared C1q domain, the signature that defines this protein family [84]. Before myonectin was described as a myokine, the protein was reported to be expressed in the retinal pigment epithelium, and mutations in this gene caused abnormal high molecular weight aggregate formation, which results in late-onset retinal macular degeneration in humans [85].

Myonectin is supposed to be myokine due to the detection of the myonectin transcript in mice skeletal muscle, with significantly lower expression in other tissues, immunoblot detection of myonectin in mouse skeletal muscle lysates [86], and L6 supernatants [87] as well as induced expression during differentiation of mouse C2C12 cells [86].

Currently, one human and one mice exercise studies report divergent results regarding the regulation of myonectin by contraction. Lim et al. reported that a 10-week exercise training program in younger and older groups of healthy women decreased significantly myonectin serum levels, while training increased VO_2 max, mitochondrial DNA density in skeletal muscle, and plasma adiponectin levels significantly [88].

On the other hand, free wheel running for two weeks increased myonectin expression in soleus und plantaris muscle of mice and circulating serum levels, suggesting a potential role of myonectin in exercise-induced physiology [86]. Recombinant myonectin induced the phosphorylation of AMPK, leading to increased cell surface recruitment of GLUT4, enhanced glucose uptake, and stimulated fatty acid oxidation [87]. Thus, enhanced myonectin secretion induced by contraction could activate signaling pathways providing enhanced energy demands during contraction.

Most intriguingly, Seldin et al. reported that recombinant myonectin promotes fatty acid uptake in mouse adipocytes and rat hepatocytes *in vitro* by enhancing CD36, FATP1, and Fabp4 mRNA expressions, which are known to play important roles in fatty acid uptake. In addition, recombinant myonectin had no effect on adipose tissue lipolysis [86]. A question that should be addressed by future studies is why physical activity activates the secretion of a myokine that induces fatty acid uptake by adipose tissue. Enhanced myonectin serum levels after exercise would therefore lead to an endocrine signal that would deplete energy sources in the blood which is needed by the exercising muscle. On the other hand, long-lasting increase in myonectin serum levels could increase fatty acid uptake in adipose tissue and therefore improve fat metabolism and lipid handling.

12. Adipo-Myokines

In a recently published review, Pedersen and Febbraio suggest that skeletal muscle might mediate some of the well-established protective effects of exercise via the secretion of myokines that counteract the harmful effects of proinflammatory adipokines [6].

Table 1 summarizes the most prominent myokines, which are described to be contraction regulated. For more than half of the described myokines, ten out of seventeen, secretion by adipocytes has also been described (Figure 1). We termed these cytokines adipo-myokines. How should a cytokine exert on the one hand inflammatory signalling in the obese state and have beneficial effects after exercise? Is it likely that a bidirectional communication between fat and muscle cells takes place? Just recently, Christiansen et al. reported that acute exercise increases circulating inflammatory markers in overweight and obese compared with lean subjects [89]. Why should inflammatory markers increase after exercise?

13. IL-6: The Prototype Adipo-Myokine

IL-6 seems to be a good example for an adipo-myokine that is released by both tissues and has the potential to act on both tissues. As described before, the level of circulating IL-6 increases after an acute bout of exercise in an exponential fashion [36, 38, 39] and declines in the postexercise period [40].

In addition, the quantitative release from adipose tissue correlates positively with increased body fat content, which results in systemic elevation of IL-6 plasma levels [90]. It is overexpressed in human fat cells from insulin-resistant subjects [91], increased in the plasma of obese patients [92, 93], and associated with type 2 diabetes [90, 94], while it was described to be decreased after bariatric surgery [95]. IL-6 expression was known to be activated by proinflammatory IKKβ/NFκB signalling pathway which is thought to contribute to the development of obesity-induced insulin resistance [96, 97]. In addition, it has been shown to inhibit insulin-signalling pathways in the liver [98, 99] and adipocytes [91]. *In vitro* experiments revealed that IL-6 induced insulin resistance in hepatocytes [99], adipocytes [91], and in skeletal muscle cells after treatment with high doses [100]. Incubation of the rat L6 myotubes with 200 ng/mL recombinant IL-6 induced insulin resistance on the level of diminished Akt phosphorylation after 96 h [101] and in primary human myotubes after 48 h [100].

Since exercise is thought to increase insulin sensitivity, the observation that IL-6 is also increased after exercise seemed quite paradoxical. Interestingly, for skeletal muscle cells, *in vitro* studies showed that a rather brief challenge of minutes to few hours with recombinant IL-6 had a positive autocrine effect on skeletal muscle cells. Recombinant IL-6 enhanced insulin-stimulated Akt phosphorylation in primary human myotubes (20 ng/mL) [102, 103] and rat L6 myotubes (about 200 ng/mL) [101]. In addition, basal and insulin-stimulated glucose uptake and translocation of GLUT4 to the plasma membrane were enhanced after 5–120 min in L6 myotubes (1–100 ng/mL) [104]. Furthermore, IL-6 rapidly and markedly increased AMPK and increased fatty acid oxidation [104, 105]. Interestingly, the regulation of intracellular signalling mechanisms, mediating IL-6 expression, differs from the classical proinflammatory pathway. IL-6 expression in contracting muscle is regulated by c-Jun terminal kinase (JNK)/activator protein-1 [106] and increases

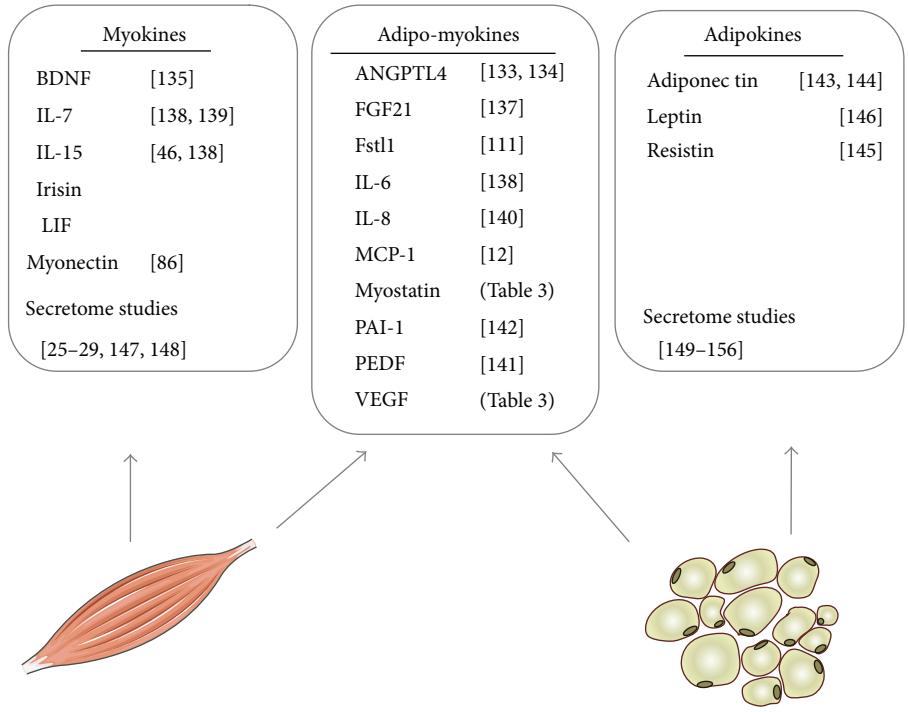

Figure 1: Adipokines, myokines, and adipo-myokines. A search of original articles in pubMed was performed for all myokines described in Table 1 to identify myokines that were also secreted by adipocytes. The search terms used were "adipose tissue," "adipocyte," and the indicated cytokine/protein. Reference lists of identified articles were also used to search for further papers. Indicated references published data that the cytokines/proteins are secreted or not secreted by adipocytes or adipose tissue. No data was published on the secretion of LIF and irisin from adipocytes or adipose tissue.

insulin-stimulated glucose disposal in humans and glucose uptake as well as fatty acid oxidation in rat myotubes *in vitro* [104].

Although one study reported that acute IL-6 administration in resting healthy young men in physiological concentrations did not affect whole-body glucose disposal, net leg-glucose uptake, or endogenous glucose production [107], some evidence is published that IL-6 might have systemic effects. IL-6 knock-out mice develop late onset obesity and impaired glucose tolerance [108]. In healthy humans IL-6 infusion increases glucose disposal without affecting the complete suppression of endogenous glucose production during a hyperinsulinemic-euglycemic clamp [104]. This insulin-sensitizing effect of IL-6, without influencing glucose output from the liver, indicates that the main effect of IL-6 on insulin-stimulated glucose metabolism is likely to occur in peripheral tissues and might just affect skeletal muscle itself or adipose tissue as well. IL-6 was described as a potent modulator of fat metabolism in humans, increasing fat oxidation and fatty acid reesterification without causing hypertriacylglyceridemia [109]. Infusion of rhIL-6 in physiological concentrations into healthy humans increased whole-body fat oxidation [109].

In summary, IL-6 is released by contracting human skeletal muscle and seems to have a beneficial effect on insulin-stimulated glucose disposal and fatty acid oxidation after acute stimulation. These findings support the hypothesis that the myokine IL-6 is important for muscle metabolism during contraction, whereas the chronic elevation of IL-6 released from adipocytes may induce insulin resistance. In addition, Weigert et al. propose a different influence of IL-6 depending on the target tissue. In energy-supplying tissues like the liver and fat the insulin signal is attenuated, whereas in energy-utilizing tissues like the skeletal muscle insulin action is improved [102].

14. Follistatin Like 1: An Adipo-Myokine

FSTL1 is the smallest member of the SPARC protein family and a secreted glycoprotein of 45–55 kDa that, despite limited homology, has been grouped in the follistatin family of proteins. A proteomic approach found FSTL1 in the supernatant of primary human adipocytes [110]. FSTL1 is highly expressed and secreted in 3T3-L1 preadipocytes and dramatically downregulated early in their differentiation to adipocytes [111]. Nevertheless, subcutaneous white adipose tissue, lung and heart are the primary sites of FSTL1 transcript expression, compared to brown adipose tissue and muscle of adult murine tissues [111]. Three proteomics studies performed in murine C2C12 and rat L6 cell lines identified FSTL1 as a myokine which is secreted by skeletal muscle cells [25, 27, 28]. These studies found that FSTL1 is secreted by murine and rat skeletal muscle cells, and its secretion is decreased during insulin stimulation and myogenesis.

In addition, Görgens et al. recently showed that FSTL1 is also secreted by primary human skeletal muscle cells [112]. Data have shown that FSTL1 is secreted into the media by cultured C2C12 skeletal muscle cells, and it can directly act on endothelial cell-signalling pathways that promote function and survival. FSTL1 overexpression in endothelial cells was found to enhance endothelial cell differentiation and migration and diminish endothelial apoptosis [113]. Thus, FSTL1 might be a myokine that mediates some of the well-established protective effects of exercise that counteract the harmful effects of proinflammatory adipokines on the vasculature. In addition, treatment of neonatal rat ventricular cardiomyocytes with recombinant FSTL1 induced a time- and dose-dependent increase in AMPK and ACC phosphorylation [114]. Given that FSTL1 mRNA expression was increased after strength training [31] and after an acute bout of cycling at 70% VO_2 max [112], it might be speculated that exercise activates FSTL1 expression and secretion in skeletal muscle, which might act in an autocrine and/or endocrine manner and activate muscular and/or adipogenic AMPK. However, the biological role of adipocyte-derived and muscle-derived FSTL1 has still to be defined.

15. Leptin: An Adipokine rather than a Myokine

Originally, leptin was described as an adipokine which controls food intake [115]. It is synthesized and released in response to increased energy storage in adipose tissue [115–117]. Leptin was detected on the mRNA level in several tissues including skeletal muscle tissue [118]. Recently, Wolsk et al. published that human skeletal muscle releases leptin *in vivo* [119]. The secretion of leptin from skeletal muscle was measured by insertion of catheters into the femoral artery and vein draining the skeletal muscle. The secretion from adipose tissue was measured by an epigastric vein draining the abdominal subcutaneous adipose tissue. The authors measured a leptin release of $0.8 \pm 0.3\,\text{ng}\,\text{min}^{-1}\,100\,\text{g}\,\text{tissue}^{-1}$ from adipose tissue and $0.5 \pm 0.1\,\text{ng}\,\text{min}^{-1}\,100\,\text{g}\,\text{tissue}^{-1}$ from skeletal muscle. From these results the authors conclude that the contribution of whole-body leptin production could be substantial greater than skeletal muscle compared to fat due to the greater muscle mass in lean subjects. Earlier work also observed leptin release from human skeletal muscle tissue and subcutaneous adipose explants, with more than ten times less secretion from muscle explants compared to fat explants [120]. Here, we measured the leptin release of differentiated primary human adipocytes and myotubes. The myotubes do not secrete leptin or at levels close to the detection limit (Figure 2). Even in concentrated supernatants of myotubes leptin secretion was barely detectable and contraction induced by electrical pulse stimulation had no effect on leptin secretion (Raschke, unpublished observation). Nevertheless, increasing evidence revealed inter- and intramuscular accumulation of nonmyogenic cell types, which might contribute to the observed leptin secretion of skeletal muscle tissue. Preadipocytes of unknown origin are observed in skeletal muscle [121, 122], and macrophage accumulation is slightly enhanced in skeletal muscle in obese type 2 diabetic subjects or in elderly individuals [123]. From the current data, we would conclude that leptin is rather a true adipokine, instead of an adipo-myokine.

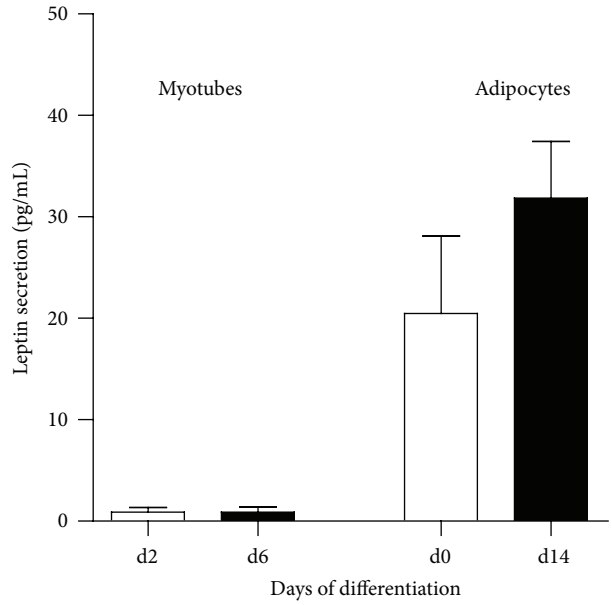

Figure 2: Leptin secretion from primary human myotubes and adipocytes. Primary human skeletal muscle cells and preadipocytes were differentiated *in vitro* for 6 and 14 days, respectively. Supernatants were collected on day 2/6 and day 0/14 of differentiation, respectively. Leptin secretion was measured by ELISA (R&D Systems), $n \geq 8$. Samples were measured according to the manufacturer's instructions.

16. IL-15: A Myokine rather than an Adipokine

Besides BDNF, IL-7, irisin, LIF, and myonectin, IL-15 is a myokine, which is mainly expressed in skeletal muscle and not in adipose tissue. The most prominent effect of exercise on IL-15 serum levels was observed after moderate intensity resistance training [51]. IL-15 mRNA level in muscle biopsies taken from marathon runners increased more compared to other cytokines like IL-6, IL8, and TNFα [47]. Most interestingly, it is higher expressed in skeletal muscle compared to adipocytes [46], while most adipo-myokines are higher expressed in adipocytes compared to myotubes (Table 3). Since IL-15 has been described to have anabolic effects, it may play a role in reducing adipose tissue mass as part of muscle-adipose tissue crosstalk [124]. In 3T3-L1 adipocytes, the administration of IL-15 inhibited lipid accumulation and stimulated secretion of the adipocyte-specific hormone adiponectin [46]. In addition, IL-15 overexpression in mice promotes endurance and oxidative energy metabolism and enhances exercise-related transcription factors in muscle [125] and most interestingly IL-15 treatment improves glucose homeostasis and insulin sensitivity in obese mice [126]. In human subjects, negative correlations between circulating

Table 2: Overview of selected adipo-myokines which are associated with obesity and insulin resistance.

Adipo-Myokine	Associated with obesity	Associated with insulin resistance/T2D	Associated with improved glucose metabolism
IL-6	√ Plasma IL-6 is positively related to fat mass [175], elevated in type 2 diabetics [90, 94]	√ IL-6 promotes insulin resistance [91, 98, 99]	√ Insulin-sensitizing effect on skeletal muscle [102, 104] increases whole body fat oxidation [109]
IL-7	? Increased mRNA level in omental adipose tissue [139] although mice overexpressing IL-7 have reduced adipose tissue mass [177]	n.d.	n.d.
IL-8	√ Higher expression in visceral adipose tissue in type 2 diabetics and insulin resistant subjects [91, 178]	√ IL-8 plasma levels correlate with measures of insulin resistance [97, 179]	n.d.
MCP-1	√ Serum MCP-1 is increased in obesity [129]	√ Promotes insulin resistance [12, 129]	n.d.
PEDF	√ PEDF serum levels increased in obesity [180, 181]	√ PEDF serum levels associated with insulin resistance [180–183], PEDF promotes insulin resistance [141]	n.d.

√: association has been shown in indicated publications; √: contradictory data published; n.d.: not described.

Table 3: Concentrations of various factors in conditioned medium from primary human adipocytes and primary human myotubes. Primary human skeletal muscle cells were differentiated for 5 days and primary human preadipocytes were differentiated for 14 days *in vitro* to mature cells. During the last 24 h cells were incubated with serum-free medium to obtain conditioned medium. Concentrations of secreted factors from cells within this conditioned medium were analyzed by enzyme-linked immunosorbent assay. Data are means ± SEM, $n \geq 3$.

Secreted factor	Concentration in adipocyte-conditioned medium (ng/mL)	Concentration in skeletal muscle-conditioned medium (ng/mL)
Chemerin	2.12 ± 0.3 [141]	0.006 ± 0.001
DPP4	2.19 ± 1.4 [11]	0.69 ± 0.18
IL-6	0.03 ± 0.002 [141]	0.02 ± 0.003
IL-8	0.15 ± 0.04	0.07 ± 0.01
MCP-1	0.35 ± 0.06	0.33 ± 0.08
Myostatin	12.64 ± 4.44	3.44 ± 1.64
PEDF	45.7 ± 0.82 [141]	5.4 ± 0.86
VEGF	0.33 ± 0.09 [141]	0.05 ± 0.03

IL-15 levels and both total and abdominal fat have been demonstrated [127]. Since both IL-15 and physical exercise have positive effects on body composition, IL-15 is discussed as a contraction-regulated myokine in the literature which may play a role in muscle-fat cross-talk [44, 45] mediating some of the beneficial effects of physical activity. Yang et al. published just recently a direct link between treadmill exercise of high-fat diet rats, enhanced expression of IL-15 in muscle, and increased IL-15 receptor alpha expression in adipose tissue [52].

17. Other Adipo-Myokines

MCP-1 is one of these adipo-myokines. Before MCP-1 was characterized as a myokine, it was described to be produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects [128]. It is overexpressed in obese rodents [129, 130] and reaches significantly higher plasma levels in diabetic patients [131]. In addition, MCP-1-induced macrophage infiltration in adipose tissue leads to a chronic state of low-grade inflammation [132], which is linked to insulin resistance. *In vitro* data demonstrate that this factor has the ability to induce insulin resistance in adipocytes and skeletal muscle cells [12].

Nevertheless, while IL-6, IL-7, IL-8, MCP-1, and pigment endothelial derived factor (PEDF) are associated with obesity and insulin resistance, these proteins are contraction-regulated myokines (Table 1), and only for IL-6 a beneficial effect has been described (Table 2). The description of a beneficial effect for these myokines is lacking and is an interesting

open question for future studies (Table 2). Myostatin is a well-known myokine, but our group recently identified myostatin as an adipokine [2].

18. Conclusion

Taken together, one protein can be a myokine as well as an adipokine, indeed two sides of the same coin. In healthy, normal weight subjects skeletal muscle is the largest tissue in the human body, accounting for 40–50% of total human body mass, while body fat accounts for 20–35%. In obese subjects the percentage of total body fat increases to 40–60% resulting in an increased secretion of proinflammatory adipokines, while the percentage of proteins secreted from skeletal muscle during a sedentary lifestyle is decreased. However, for many adipo-myokines the local tissue concentration may be divergent from the serum level, and substantial differences between auto- and endocrine effects of these molecules need to be considered.

As Paracelsus (1493–1541) already coined the famous phrase; "dosis sola facit venenum," "Only the dose makes the poison". This might also be true for adipo-myokines. Findings support the hypothesis that the myokines are essential for muscle metabolism during contraction, whereas the chronic elevation of adipokines released from adipocytes may induce adverse effects, even leading to insulin resistance.

Abbreviations

AMPK:	AMP-activated protein kinase
ANGPTL4:	Angiopoietin like 4
BDNF:	Brain-derived neurotrophic factor
DPP4:	Dipeptidyl peptidase 4
FGF:	Fibroblast growth factor
FNDC5:	Fibronectin type III domain containing protein 5
FSTL1:	Follistatin-like 1
IL:	Interleukin
LIF:	Leukemia inhibitory factor
MCP-1:	Monocyte chemoattractant protein 1
NT:	Neurotrophins
PEDF:	Pigment endothelial derived factor
TNFα:	Tumor necrosis factor α
VEGF:	Vascular endothelial growth factor.

Conflict of Interests

The authors have no conflict of interests in relation to the contents of this paper.

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References

[1] M. Adamczak and A. Wiecek, "The adipose tissue as an endocrine organ," *Seminars in Nephrology*, vol. 33, pp. 2–13, 2013.

[2] S. Lehr, S. Hartwig, and H. Sell, "Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders," *Proteomics. Clinical Applications*, vol. 6, pp. 91–101, 2012.

[3] N. Ouchi, J. L. Parker, J. J. Lugus, and K. Walsh, "Adipokines in inflammation and metabolic disease," *Nature Reviews Immunology*, vol. 11, no. 2, pp. 85–97, 2011.

[4] B. K. Pedersen, "The diseasome of physical inactivity- and the role of myokines in muscle-fat cross talk," *Journal of Physiology*, vol. 587, no. 23, pp. 5559–5568, 2009.

[5] B. K. Pedersen, "Exercise-induced myokines and their role in chronic diseases," *Brain, Behavior, and Immunity*, vol. 25, no. 5, pp. 811–816, 2011.

[6] B. K. Pedersen and M. A. Febbraio, "Muscles, exercise and obesity: skeletal muscle as a secretory organ," *Nature Reviews Endocrinology*, vol. 8, pp. 457–465, 2012.

[7] C. Handschin and B. M. Spiegelman, "The role of exercise and PGC1α in inflammation and chronic disease," *Nature*, vol. 454, no. 7203, pp. 463–469, 2008.

[8] J. S. Yudkin, "Inflammation, obesity, and the metabolic syndrome," *Hormone and Metabolic Research*, vol. 39, no. 10, pp. 707–709, 2007.

[9] P. Arner, "Regional differences in protein production by human adipose tissue," *Biochemical Society Transactions*, vol. 29, no. 2, pp. 72–75, 2001.

[10] P. Trayhurn and J. H. Beattie, "Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ," *Proceedings of the Nutrition Society*, vol. 60, no. 3, pp. 329–339, 2001.

[11] D. Lamers, S. Famulla, N. Wronkowitz et al., "Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome," *Diabetes*, vol. 60, pp. 1917–1925, 2011.

[12] H. Sell, D. Dietze-Schroeder, U. Kaiser, and J. Eckel, "Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle," *Endocrinology*, vol. 147, no. 5, pp. 2458–2467, 2006.

[13] H. Sell, J. Eckel, and D. Dietze-Schroeder, "Pathways leading to muscle insulin resistance—the muscle-fat connection," *Archives of Physiology and Biochemistry*, vol. 112, no. 2, pp. 105–113, 2006.

[14] H. Sell, A. Divoux, C. Poitou et al., "Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 6, pp. 2892–2896, 2010.

[15] R. A. DeFronzo and D. Tripathy, "Skeletal muscle insulin resistance is the primary defect in type 2 diabetes," *Diabetes Care*, vol. 32, supplement 2, pp. S157–S163, 2009.

[16] B. K. Pedersen, "Muscles and their myokines," *Journal of Experimental Biology*, vol. 214, no. 2, pp. 337–346, 2011.

[17] H. Ellingsgaard, I. Hauselmann, B. Schuler et al., "Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells," *Nature Medicine*, vol. 17, pp. 1481–1489, 2011.

[18] B. K. Pedersen and M. A. Febbraio, "Muscle as an endocrine organ: focus on muscle-derived interleukin-6," *Physiological Reviews*, vol. 88, no. 4, pp. 1379–1406, 2008.

[19] V. B. Matthews, M. B. Åström, M. H. S. Chan et al., "Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase," *Diabetologia*, vol. 52, no. 7, pp. 1409–1418, 2009.

[20] B. Friedmann-Bette, F. R. Schwartz, H. Eckhardt, R. Billeter, G. Bonaterra, and R. Kinscherf, "Similar changes of gene expression in human skeletal muscle after resistance exercise and multiple fine needle biopsies," *Journal of Applied Physiology*, vol. 112, pp. 289–295, 2012.

[21] N. Nikolic, S. S. Bakke, E. T. Kase et al., "Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise," *PLoS ONE*, vol. 7, Article ID e33203, 2012.

[22] T. Nedachi, H. Fujita, and M. Kanzaki, "Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle," *American Journal of Physiology*, vol. 295, no. 5, pp. E1191–E1204, 2008.

[23] H. Fujita, T. Nedachi, and M. Kanzaki, "Accelerated de novo sarcomere assembly by electric pulse stimulation in C2C12 myotubes," *Experimental Cell Research*, vol. 313, no. 9, pp. 1853–1865, 2007.

[24] S. Lambernd, A. Taube, A. Schober et al., "Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways," *Diabetologia*, vol. 55, pp. 1128–1113, 2012.

[25] X. C. Y. Chan, J. C. McDermott, and K. W. M. Siu, "Identification of secreted proteins during skeletal muscle development," *Journal of Proteome Research*, vol. 6, no. 2, pp. 698–710, 2007.

[26] C. Y. Chan, J. C. McDermott, and K. W. Siu, "Secretome analysis of skeletal myogenesis using SILAC and shotgun proteomics," *International Journal of Proteomics*, vol. 2011, Article ID 329467, 13 pages, 2011.

[27] J. Henningsen, K. T. G. Rigbolt, B. Blagoev, B. K. Pedersen, and I. Kratchmarova, "Dynamics of the skeletal muscle secretome during myoblast differentiation," *Molecular and Cellular Proteomics*, vol. 9, no. 11, pp. 2482–2496, 2010.

[28] J. H. Yoon, K. Yea, J. Kim et al., "Comparative proteomic analysis of the insulin-induced L6 myotube secretome," *Proteomics*, vol. 9, no. 1, pp. 51–60, 2009.

[29] J. H. Yoon, P. Song, J. H. Jang et al., "Proteomic analysis of tumor necrosis factor-alpha (TNF-alpha)-induced L6 myotube secretome reveals novel TNF-alpha-dependent myokines in diabetic skeletal muscle," *Journal of Proteome Research*, vol. 10, pp. 5315–5325, 2011.

[30] D. S. Hittel, Y. Hathout, and E. P. Hoffman, "Proteomics and systems biology in exercise and sport sciences research," *Exercise and Sport Sciences Reviews*, vol. 35, no. 1, pp. 5–11, 2007.

[31] F. Norheim, T. Raastad, B. Thiede, A. C. Rustan, C. A. Drevon, and F. Haugen, "Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training," *American Journal of Physiology*, vol. 301, pp. E1013–E1021, 2011.

[32] M. Catoire, M. Mensink, M. V. Boekschoten et al., "Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle," *PLoS ONE*, vol. 7, Article ID e51066, 2012.

[33] J. H. Yoon, J. Kim, P. Song, T. G. Lee, P. G. Suh, and S. H. Ryu, "Secretomics for skeletal muscle cells: a discovery of novel regulators?" *Advances in Biological Regulation*, vol. 52, pp. 340–350, 2012.

[34] K. Ostrowski, T. Rohde, S. Asp, P. Schjerling, and B. K. Pedersen, "Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans," *Journal of Physiology*, vol. 515, no. 1, pp. 287–291, 1999.

[35] D. A. Papanicolaou, J. S. Petrides, C. Tsigos et al., "Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines," *American Journal of Physiology*, vol. 271, no. 3, pp. E601–E605, 1996.

[36] A. Steensberg, G. Van Hall, T. Osada, M. Sacchetti, B. Saltin, and B. K. Pedersen, "Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6," *Journal of Physiology*, vol. 529, no. 1, pp. 237–242, 2000.

[37] J. L. Croisier, G. Camus, I. Venneman et al., "Effects of training on exercise-induced muscle damage and interleukin 6 production," *Muscle & Nerve*, vol. 22, pp. 208–212, 1999.

[38] D. C. Nieman, S. L. Nehlsen-Cannarella, O. R. Fagoaga et al., "Influence of mode and carbohydrate on the cytokine response to heavy exertion," *Medicine and Science in Sports and Exercise*, vol. 30, no. 5, pp. 671–678, 1998.

[39] R. L. Starkie, M. J. Arkinstall, I. Koukoulas, J. A. Hawley, and M. A. Febbraio, "Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans," *Journal of Physiology*, vol. 533, no. 2, pp. 585–591, 2001.

[40] A. Steensberg, M. A. Febbraio, T. Osada et al., "Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content," *Journal of Physiology*, vol. 537, no. 2, pp. 633–639, 2001.

[41] M. A. Febbraio and B. K. Pedersen, "Muscle-derived interleukin-6: mechanisms for activation and possible biological roles," *The FASEB Journal*, vol. 16, no. 11, pp. 1335–1347, 2002.

[42] M. H. Rokling-Andersen, J. E. Reseland, M. B. Veierød et al., "Effects of long-term exercise and diet intervention on plasma adipokine concentrations," *American Journal of Clinical Nutrition*, vol. 86, no. 5, pp. 1293–1301, 2007.

[43] C. P. Fischer, "Interleukin-6 in acute exercise and training: what is the biological relevance?" *Exercise immunology review*, vol. 12, pp. 6–33, 2006.

[44] A. R. Nielsen and B. K. Pedersen, "The biological roles of exercise-induced cytokines: IL-6, IL-8, and IL-15," *Applied Physiology, Nutrition and Metabolism*, vol. 32, no. 5, pp. 833–839, 2007.

[45] J. M. Argilés, J. López-Soriano, V. Almendro, S. Busquets, and F. J. López-Soriano, "Cross-talk between skeletal muscle and adipose tissue: a link with obesity?" *Medicinal Research Reviews*, vol. 25, no. 1, pp. 49–65, 2005.

[46] L. S. Quinn, L. Strait-Bodey, B. G. Anderson, J. M. Argilés, and P. J. Havel, "Interleukin-15 stimulates adiponectin secretion by 3T3-L1 adipocytes: evidence for a skeletal muscle-to-fat signaling pathway," *Cell Biology International*, vol. 29, no. 6, pp. 449–457, 2005.

[47] D. C. Nieman, J. M. Davis, V. A. Brown et al., "Influence of carbohydrate ingestion on immune changes after 2 h of

intensive resistance training," *Journal of Applied Physiology*, vol. 96, no. 4, pp. 1292–1298, 2004.

[48] A. R. Nielsen, R. Mounier, P. Plomgaard et al., "Expression of interleukin-15 in human skeletal muscle—effect of exercise and muscle fibre type composition," *Journal of Physiology*, vol. 584, no. 1, pp. 305–312, 2007.

[49] S. E. Riechman, G. Balasekaran, S. M. Roth, and R. E. Ferrell, "Association of interleukin-15 protein and interleukin-15 receptor genetic variation with resistance exercise training responses," *Journal of Applied Physiology*, vol. 97, no. 6, pp. 2214–2219, 2004.

[50] Y. Tamura, K. Watanabe, T. Kantani, J. Hayashi, N. Ishida, and M. Kaneki, "Upregulation of circulating IL-15 by treadmill running in healthy individuals: is IL-15 an endocrine mediator of the beneficial effects of endurance exercise?" *Endocrine Journal*, vol. 58, no. 3, pp. 211–215, 2011.

[51] N. H. Yeo, J. Woo, K. O. Shin, J. Y. Park, and S. Kang, "The effects of different exercise intensity on myokine and angiogenesis factors," *Journal of Sports Medicine and Physical Fitness*, vol. 52, pp. 448–454, 2012.

[52] H. Yang, J. Chang, W. Chen et al., "Treadmill exercise promotes interleukin 15 expression in skeletal muscle and interleukin 15 receptor alpha expression in adipose tissue of high-fat diet rats," *Endocrine*, vol. 43, no. 3, pp. 579–585, 2012.

[53] A. Ferrer-Martínez, P. Ruiz-Lozano, and K. R. Chien, "Mouse PeP: a novel peroxisomal protein linked to myoblast differentiation and development," *Developmental Dynamics*, vol. 224, no. 2, pp. 154–167, 2002.

[54] M. S. Hashemi, K. Ghaedi, A. Salamian et al., "Fndc5 knockdown significantly decreased neural differentiation rate of mouse embryonic stem cells," *Neuroscience*, vol. 231, pp. 296–304, 2013.

[55] P. Bostrom, J. Wu, M. P. Jedrychowski et al., "A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis," *Nature*, vol. 481, pp. 463–468, 2012.

[56] J. A. Timmons, K. Baar, P. K. Davidsen, and P. J. Atherton, "Is irisin a human exercise gene?" *Nature*, vol. 488, pp. 9–10, 2012.

[57] J. Y. Huh, G. Panagiotou, V. Mougios et al., "FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise," *Metabolism*, vol. 61, no. 12, pp. 1725–1738, 2012.

[58] A. Roca-Rivada, C. Castelao, L. L. Senin et al., "FNDC5/Irisin is not only a myokine but also an adipokine," *PLOS ONE*, vol. 8, Article ID e60563, 2013.

[59] G. Chevrel, R. Hohlfeld, and M. Sendtner, "The role of neurotrophins in muscle under physiological and pathological conditions," *Muscle & Nerve*, vol. 33, no. 4, pp. 462–476, 2006.

[60] K. Mousavi and B. J. Jasmin, "BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation," *Journal of Neuroscience*, vol. 26, no. 21, pp. 5739–5749, 2006.

[61] R. S. B. Liem, N. Brouwer, and J. C. V. M. Copray, "Ultrastructural localisation of intramuscular expression of BDNF mRNA by silver-gold intensified non-radioactive in situ hybridisation," *Histochemistry and Cell Biology*, vol. 116, no. 6, pp. 545–551, 2001.

[62] C. Clow and B. J. Jasmin, "Brain-derived neurotrophic factor regulates satellite cell differentiation and skeltal muscle regeneration," *Molecular Biology of the Cell*, vol. 21, no. 13, pp. 2182–2190, 2010.

[63] L. T. Ferris, J. S. Williams, and C. L. Shen, "The effect of acute exercise on serum brain-derived neurotrophic factor levels and cognitive function," *Medicine and Science in Sports and Exercise*, vol. 39, no. 4, pp. 728–734, 2007.

[64] S. Rojas Vega, H. K. Strüder, B. Vera Wahrmann, A. Schmidt, W. Bloch, and W. Hollmann, "Acute BDNF and cortisol response to low intensity exercise and following ramp incremental exercise to exhaustion in humans," *Brain Research*, vol. 1121, no. 1, pp. 59–65, 2006.

[65] P. Rasmussen, P. Brassard, H. Adser et al., "Evidence for a release of brain-derived neurotrophic factor from the brain during exercise," *Experimental Physiology*, vol. 94, no. 10, pp. 1062–1069, 2009.

[66] S. Qin, G. LaRosa, J. J. Campbell et al., "Expression of monocyte chemoattractant protein-1 and interleukin-8 receptors on subsets of T cells: correlation with transendothelial chemotactic potential," *European Journal of Immunology*, vol. 26, no. 3, pp. 640–647, 1996.

[67] M. Baggiolini, B. Dewald, and B. Moser, "Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines," *Advances in Immunology*, vol. 55, pp. 97–179, 1994.

[68] J. Henningsen, B. K. Pedersen, and I. Kratchmarova, "Quantitative analysis of the secretion of the MCP family of chemokines by muscle cells," *Molecular BioSystems*, vol. 7, no. 2, pp. 311–321, 2011.

[69] L. S. Cleto, A. F. Oleto, L. P. Sousa et al., "Plasma cytokine response, lipid peroxidation and NF-κB activation in skeletal muscle following maximumprogressive swimming," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 6, pp. 546–552, 2011.

[70] L. Vella, M. K. Caldow, A. E. Larsen et al., "Resistance exercise increases NF-kappaB activity in human skeletal muscle," *American Journal of Physiology*, vol. 302, pp. R667–R673, 2012.

[71] P. Tantiwong, K. Shanmugasundaram, A. Monroy et al., "NF-κB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions," *American Journal of Physiology*, vol. 299, no. 5, pp. E794–E801, 2010.

[72] M. Catoire, M. Mensink, P. Schrauwen, and S. Kersten, "Effects of acute endurance exercise on gene expression in human skeletal muscle: search for putative myokines," *Diabetologia*, vol. 55, article S598, 2012.

[73] J. L. Mathers, M. M. Farnfield, A. P. Garnham, M. K. Caldow, D. Cameron-Smith, and J. M. Peake, "Early inflammatory and myogenic responses to resistance exercise in the elderly," *Muscle & Nerve*, vol. 46, pp. 407–412, 2012.

[74] M. J. Hubal, T. C. Chen, P. D. Thompson, and P. M. Clarkson, "Inflammatory gene changes associated with the repeated-bout effect," *American Journal of Physiology*, vol. 294, no. 5, pp. R1628–R1637, 2008.

[75] S. Kersten, L. Lichtenstein, E. Steenbergen et al., "Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 6, pp. 969–974, 2009.

[76] H. Staiger, C. Haas, J. Machann et al., "Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-δ and is of metabolic relevance in humans," *Diabetes*, vol. 58, no. 3, pp. 579–589, 2009.

[77] M. Catoire, M. Mensink, M. V. Boekschoten et al., "Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle," *PLoS ONE*, vol. 7, Article ID e51066, 2012.

[78] R. Barres, J. Yan, B. Egan et al., "Acute exercise remodels promoter methylation in human skeletal muscle," *Cell Metabolism*, vol. 15, pp. 405–411, 2012.

[79] Y. Izumiya, H. A. Bina, N. Ouchi, Y. Akasaki, A. Kharitonenkov, and K. Walsh, "FGF21 is an Akt-regulated myokine," *FEBS Letters*, vol. 582, no. 27, pp. 3805–3810, 2008.

[80] P. Hojman, M. Pedersen, A. R. Nielsen et al., "Fibroblast growth factor-21 is induced in human skeletal muscles by hyperinsulinemia," *Diabetes*, vol. 58, no. 12, pp. 2797–2801, 2009.

[81] A. O. Chavez, M. Molina-Carrion, M. A. Abdul-Ghani, F. Folli, R. A. DeFronzo, and D. Tripathy, "Circulating fibroblast growth factor-21 is elevated in impaired glucose tolerance and type 2 diabetes and correlates with muscle and hepatic insulin resistance," *Diabetes Care*, vol. 32, no. 8, pp. 1542–1546, 2009.

[82] D. Cuevas-Ramos, P. Almeda-Valdes, C. E. Meza-Arana et al., "Exercise increases serum fibroblast growth factor 21 (FGF21) levels," *PLOS ONE*, vol. 7, Article ID e38022, 2012.

[83] S. J. Yang, H. C. Hong, H. Y. Choi et al., "Effects of a three-month combined exercise programme on fibroblast growth factor 21 and fetuin-A levels and arterial stiffness in obese women," *Clinical Endocrinology*, vol. 75, pp. 464–469, 2011.

[84] U. Kishore and K. B. M. Reid, "C1q: structure, function, and receptors," *Immunopharmacology*, vol. 49, no. 1-2, pp. 159–170, 2000.

[85] C. Hayward, X. Shu, A. V. Cideciyan et al., "Mutation in a short-chain collagen gene, CTRP5, results in extracellular deposit formation in late-onset retinal degenaration: a genetic model for age-related macular degeneration," *Human Molecular Genetics*, vol. 12, no. 20, pp. 2657–2667, 2003.

[86] M. M. Seldin, J. M. Peterson, M. S. Byerly, Z. Wei, and G. W. Wong, "Myonectin (CTRP15), a novel myokine that links skeletal muscle to systemic lipid homeostasis," *Journal of Biological Chemistry*, vol. 287, pp. 11968–11980, 2012.

[87] S. Y. Park, J. H. Choi, H. S. Ryu et al., "C1q tumor necrosis factor α-related protein isoform 5 is increased in mitochondrial DNA-depleted myocytes and activates AMP-activated protein kinase," *Journal of Biological Chemistry*, vol. 284, no. 41, pp. 27780–27789, 2009.

[88] S. Lim, S. H. Choi, B. K. Koo et al., "Effects of aerobic exercise training on C1q tumor necrosis factor alpha-related protein isoform 5 (myonectin): association with insulin resistance and mitochondrial DNA density in women," *Journal of Clinical Endocrinology & Metabolism*, vol. 97, pp. E88–E93, 2012.

[89] T. Christiansen, J. M. Bruun, S. K. Paulsen et al., "Acute exercise increases circulating inflammatory markers in overweight and obese compared with lean subjects," *European Journal of Applied Physiology*. In press.

[90] B. Vozarova, C. Weyer, K. Hanson, P. A. Tataranni, C. Bogardus, and R. E. Pratley, "Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion," *Obesity Research*, vol. 9, no. 7, pp. 414–417, 2001.

[91] V. Rotter, I. Nagaev, and U. Smith, "Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects," *Journal of Biological Chemistry*, vol. 278, no. 46, pp. 45777–45784, 2003.

[92] S. J. Piva, E. Tatsch, and J. A. De Carvalho, "Assessment of inflammatory and oxidative biomarkers in obesity and their associations with body mass index," *Inflammation*, vol. 36, no. 1, pp. 226–231, 2012.

[93] I. Stelzer, S. Zelzer, R. B. Raggam et al., "Link between leptin and interleukin-6 levels in the initial phase of obesity related inflammation," *Translational Research*, vol. 159, pp. 118–124, 2012.

[94] J. P. Bastard, M. Maachi, J. T. Van Nhieu et al., "Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 5, pp. 2084–2089, 2002.

[95] F. Illan-Gomez, M. Gonzalvez-Ortega, I. Orea-Soler et al., "Obesity and inflammation: change in adiponectin, C-reactive protein, tumour necrosis factor-alpha and interleukin-6 after bariatric surgery," *Obesity Surgery*, vol. 22, pp. 950–955, 2012.

[96] J. M. Bruun, A. S. Lihn, C. Verdich et al., "Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans," *American Journal of Physiology*, vol. 285, no. 3, pp. E527–E533, 2003.

[97] J. M. Bruun, C. Verdich, S. Toubro, A. Astrup, and B. Richelsen, "Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-α. Effect of weight loss in obese men," *European Journal of Endocrinology*, vol. 148, no. 5, pp. 535–542, 2003.

[98] P. J. Klover, T. A. Zimmers, L. G. Koniaris, and R. A. Mooney, "Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice," *Diabetes*, vol. 52, no. 11, pp. 2784–2789, 2003.

[99] J. J. Senn, P. J. Klover, I. A. Nowak, and R. A. Mooney, "Interleukin-6 induces cellular insulin resistance in hepatocytes," *Diabetes*, vol. 51, no. 12, pp. 3391–3399, 2002.

[100] D. Dietze, S. Ramrath, O. Ritzeler, N. Tennagels, H. Hauner, and J. Eckel, "Inhibitor κB kinase is involved in the paracrine crosstalk between human fat and muscle cells," *International Journal of Obesity*, vol. 28, no. 8, pp. 985–992, 2004.

[101] B. Seyoum, A. Fite, and A. B. Abou-Samra, "Effects of 3T3 adipocytes on interleukin-6 expression and insulin signaling in L6 skeletal muscle cells," *Biochemical and Biophysical Research Communications*, vol. 410, no. 1, pp. 13–18, 2011.

[102] C. Weigert, A. M. Hennige, K. Brodbeck, H. U. Häring, and E. D. Schleicher, "Interleukin-6 acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser473 of Akt," *American Journal of Physiology*, vol. 289, no. 2, pp. E251–E257, 2005.

[103] C. Weigert, A. M. Hennige, R. Lehmann et al., "Direct cross-talk of interleukin-6 and insulin signal transduction via insulin receptor substrate-1 in skeletal muscle cells," *Journal of Biological Chemistry*, vol. 281, no. 11, pp. 7060–7067, 2006.

[104] A. L. Carey, G. R. Steinberg, S. L. Macaulay et al., "Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase," *Diabetes*, vol. 55, no. 10, pp. 2688–2697, 2006.

[105] E. Wolsk, H. Mygind, T. S. Grøndahl, B. K. Pedersen, and G. Van Hall, "IL-6 selectively stimulates fat metabolism in human skeletal muscle," *American Journal of Physiology*, vol. 299, no. 5, pp. E832–E840, 2010.

[106] M. Whitham, M. H. Chan, M. Pal et al., "Contraction-induced interleukin-6 gene transcription in skeletal muscle is regulated by c-Jun terminal kinase/activator protein-1," *Journal of Biological Chemistry*, vol. 287, pp. 10771–10779, 2012.

[107] A. Steensberg, C. P. Fischer, M. Sacchetti et al., "Acute interleukin-6 administration does not impair muscle glucose uptake or whole-body glucose disposal in healthy humans," *Journal of Physiology*, vol. 548, no. 2, pp. 631–638, 2003.

[108] V. Wallenius, K. Wallenius, B. Ahrén et al., "Interleukin-6-deficient mice develop mature-onset obesity," *Nature Medicine*, vol. 8, no. 1, pp. 75–79, 2002.

[109] G. Van Hall, A. Steensberg, M. Sacchetti et al., "Interleukin-6 stimulates lipolysis and fat oxidation in humans," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 7, pp. 3005–3010, 2003.

[110] S. Lehr, S. Hartwig, D. Lamers et al., "Identification and validation of novel adipokines released from primary human adipocytes," *Molecular & Cellular Proteomics*, vol. 11, p. M111, 2012.

[111] Y. Wu, S. Zhou, and C. M. Smas, "Downregulated expression of the secreted glycoprotein follistatin-like 1 (Fstl1) is a robust hallmark of preadipocyte to adipocyte conversion," *Mechanisms of Development*, vol. 127, no. 3-4, pp. 183–202, 2010.

[112] S. W. Gorgens, S. Raschke, K. B. Holven, J. Jensen, K. Eckardt, and J. Eckel, "Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells," *Archives of Physiology and Biochemistry*, vol. 119, no. 2, pp. 75–80, 2013.

[113] N. Ouchi, Y. Oshima, K. Ohashi et al., "Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism," *Journal of Biological Chemistry*, vol. 283, no. 47, pp. 32802–32811, 2008.

[114] M. Shimano, N. Ouchi, K. Nakamura et al., "Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy following pressure overload," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, pp. E899–E906, 2011.

[115] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, and J. M. Friedman, "Positional cloning of the mouse obese gene and its human homologue," *Nature*, vol. 372, no. 6505, pp. 425–432, 1994.

[116] M. A. Pelleymounter, M. J. Cullen, M. B. Baker et al., "Effects of the obese gene product on body weight regulation in ob/ob mice," *Science*, vol. 269, no. 5223, pp. 540–543, 1995.

[117] L. A. Campfield, F. J. Smith, Y. Guisez, R. Devos, and P. Burn, "Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks," *Science*, vol. 269, no. 5223, pp. 546–549, 1995.

[118] J. Wang, R. Liu, M. Hawkins, N. Barzilial, and L. Rossetti, "A nutrient-sensing pathway regulates leptin gene expression in muscle and fat," *Nature*, vol. 393, no. 6686, pp. 684–688, 1998.

[119] E. Wolsk, H. Mygind, T. S. Grondahl, B. K. Pedersen, and H. G. van, "Human skeletal muscle releases leptin in vivo," *Cytokine*, vol. 60, pp. 667–673, 2012.

[120] M. Lappas, K. Yee, M. Permezel, and G. E. Rice, "Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies," *Journal of Endocrinology*, vol. 186, no. 3, pp. 457–465, 2005.

[121] J. D. Starkey, M. Yamamoto, S. Yamamoto, and D. J. Goldhamer, "Skeletal muscle satellite cells are committed to myogenesis and do not spontaneously adopt nonmyogenic fates," *Journal of Histochemistry and Cytochemistry*, vol. 59, no. 1, pp. 33–46, 2011.

[122] A. P. Russell, M. Crisan, B. Leger et al., "Brown adipocyte progenitor population is modified in obese and diabetic skeletal muscle," *International Journal of Obesity*, vol. 36, pp. 155–158, 2012.

[123] C. S. Tam, L. M. Sparks, D. L. Johannsen, J. D. Covington, T. S. Church, and E. Ravussin, "Low macrophage accumulation in skeletal muscle of obese type 2 diabetics and elderly subjects," *Obesity*, vol. 20, pp. 1530–1533, 2012.

[124] N. Carbó, J. López-Soriano, P. Costelli et al., "Interleukin-15 mediates reciprocal regulation of adipose and muscle mass: a potential role in body weight control," *Biochimica et Biophysica Acta*, vol. 1526, no. 1, pp. 17–24, 2001.

[125] L. S. Quinn, B. G. Anderson, J. D. Conner, and T. Wolden-Hanson, "IL-15 overexpression promotes endurance, oxidative energy metabolism, and muscle PPARdelta, SIRT1, PGC-1alpha, and PGC-1beta expression in male mice," *Endocrinology*, vol. 154, no. 1, pp. 232–245, 2013.

[126] N. G. . Barra, M. V. Chew, A. C. Holloway, and A. A. Ashkar, "Interleukin-15 treatment improves glucose homeostasis and insulin sensitivity in obese mice," *Diabetes, Obesity and Metabolism*, vol. 14, pp. 190–193, 2012.

[127] A. R. Nielsen, P. Hojman, C. Erikstrup et al., "Association between interleukin-15 and obesity: interleukin-15 as a potential regulator of fat mass," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 11, pp. 4486–4493, 2008.

[128] T. Christiansen, B. Richelsen, and J. M. Bruun, "Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects," *International Journal of Obesity*, vol. 29, no. 1, pp. 146–150, 2005.

[129] P. Sartipy and D. J. Loskutoff, "Monocyte chemoattractant protein 1 in obesity and insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7265–7270, 2003.

[130] K. Takahashi, S. Mizuarai, H. Araki et al., "Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice," *Journal of Biological Chemistry*, vol. 278, no. 47, pp. 46654–46660, 2003.

[131] S. Nomura, A. Shouzu, S. Omoto, M. Nishikawa, and S. Fukuhara, "Significance of chemokines and activated platelets in patients with diabetes," *Clinical and Experimental Immunology*, vol. 121, no. 3, pp. 437–443, 2000.

[132] J. M. Bruun, A. S. Lihn, S. B. Pedersen, and B. Richelsen, "Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 4, pp. 2282–2289, 2005.

[133] N. E. Gray, L. N. Lam, K. Yang, A. Y. Zhou, S. Koliwad, and J. C. Wang, "Angiopoietin-like 4 (Angptl4) protein is a physiological mediator of intracellular lipolysis in murine adipocytes," *Journal of Biological Chemistry*, vol. 287, pp. 8444–8456, 2012.

[134] T. Yamada, N. Ozaki, Y. Kato, Y. Miura, and Y. Oiso, "Insulin downregulates angiopoietin-like protein 4 mRNA in 3T3-L1 adipocytes," *Biochemical and Biophysical Research Communications*, vol. 347, no. 4, pp. 1138–1144, 2006.

[135] E. Golden, A. Emiliano, S. Maudsley et al., "Circulating brain-derived neurotrophic factor and indices of metabolic and cardiovascular health: data from the baltimore longitudinal study of aging," *PLoS ONE*, vol. 5, no. 4, Article ID e10099, 2010.

[136] F. Sornelli, M. Fiore, G. N. Chaldakov, and L. Aloe, "Adipose tissue-derived nerve growth factor and brain-derived neurotrophic factor: results from experimental stress and diabetes," *General Physiology and Biophysics*, vol. 28, pp. 179–183, 2009.

[137] F. M. Fisher, S. Kleiner, N. Douris et al., "FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive

thermogenesis," *Genes & Development*, vol. 26, pp. 271–281, 2012.

[138] K. Meijer, M. de Vries, S. Al-Lahham et al., "Human primary adipocytes exhibit immune cell function: adipocytes prime inflammation independent of macrophages," *PLoS ONE*, vol. 6, no. 3, Article ID e17154, 2011.

[139] E. Maury, K. Ehala-Aleksejev, Y. Guiot, R. Detry, A. Vandenhooft, and S. M. Brichard, "Adipokines oversecreted by omental adipose tissue in human obesity," *American Journal of Physiology*, vol. 293, no. 3, pp. E656–E665, 2007.

[140] H. Sell, K. Eckardt, A. Taube et al., "Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility," *American Journal of Physiology*, vol. 294, no. 6, pp. E1070–E1077, 2008.

[141] S. Famulla, D. Lamers, S. Hartwig et al., "Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells," *International Journal of Obesity*, vol. 35, no. 6, pp. 762–772, 2011.

[142] S. M. Lobo, B. M. Quinto, L. Oyama et al., "TNF-alpha modulates statin effects on secretion and expression of MCP-1, PAI-1 and adiponectin in 3T3-L1 differentiated adipocytes," *Cytokine*, vol. 60, pp. 150–156, 2012.

[143] E. Hu, P. Liang, and B. M. Spiegelman, "AdipoQ is a novel adipose-specific gene dysregulated in obesity," *Journal of Biological Chemistry*, vol. 271, no. 18, pp. 10697–10703, 1996.

[144] P. E. Scherer, S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish, "A novel serum protein similar to C1q, produced exclusively in adipocytes," *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 26746–26749, 1995.

[145] K. H. Kim, K. Lee, Y. S. Moon, and H. S. Sul, "A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation," *Journal of Biological Chemistry*, vol. 276, no. 14, pp. 11252–11256, 2001.

[146] L. J. Hardie, N. Guilhot, and P. Trayhurn, "Regulation of leptin production in cultured mature white adipocytes," *Hormone and Metabolic Research*, vol. 28, no. 12, pp. 685–689, 1996.

[147] D. S. Hittel, J. R. Berggren, J. Shearer, K. Boyle, and J. A. Houmard, "Increased secretion and expression of myostatin in skeletal muscle from extremely obese women," *Diabetes*, vol. 58, no. 1, pp. 30–38, 2009.

[148] M. C. Le Bihan, A. Bigot, and S. S. Jensen, "In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts," *Journal of Proteomics*, vol. 77, pp. 344–356, 2012.

[149] P. Wang, E. Mariman, J. Keijer et al., "Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines," *Cellular and Molecular Life Sciences*, vol. 61, no. 18, pp. 2405–2417, 2004.

[150] X. Chen, S. W. Cushman, L. K. Pannell, and S. Hess, "Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach," *Journal of Proteome Research*, vol. 4, no. 2, pp. 570–577, 2005.

[151] G. Alvarez-Llamas, E. Szalowska, M. P. de Vries et al., "Characterization of the human visceral adipose tissue secretome," *Molecular and Cellular Proteomics*, vol. 6, no. 4, pp. 589–600, 2007.

[152] S. Zvonic, M. Lefevre, G. Kilroy et al., "Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis," *Molecular and Cellular Proteomics*, vol. 6, no. 1, pp. 18–28, 2007.

[153] H. Molina, Y. Yang, T. Ruch et al., "Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy," *Journal of Proteome Research*, vol. 8, no. 1, pp. 48–58, 2009.

[154] J. Kim, Y. S. Choi, S. Lim et al., "Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis," *Proteomics*, vol. 10, no. 3, pp. 394–405, 2010.

[155] A. Rosenow, T. N. Arrey, F. G. Bouwman et al., "Identification of novel human adipocyte secreted proteins by using SGBS cells," *Journal of Proteome Research*, vol. 9, no. 10, pp. 5389–5401, 2010.

[156] J. Zhong, S. A. Krawczyk, R. Chaerkady et al., "Temporal profiling of the secretome during adipogenesis in humans," *Journal of Proteome Research*, vol. 9, no. 10, pp. 5228–5238, 2010.

[157] H. Staiger, C. Haas, J. Machann et al., "Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (PPAR)-δ and is of metabolic relevance in humans," *Diabetes*, vol. 58, no. 3, pp. 579–589, 2009.

[158] C. Keller, A. Steensberg, H. Pilegaard et al., "Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content," *The FASEB Journal*, vol. 15, no. 14, pp. 2748–2750, 2001.

[159] F. Haugen, F. Norheim, H. Lian et al., "IL-7 is expressed and secreted by human skeletal muscle cells," *American Journal of Physiology*, vol. 298, no. 4, pp. C807–C816, 2010.

[160] D. C. Nieman, J. M. Davis, D. A. Henson et al., "Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run," *Journal of Applied Physiology*, vol. 94, no. 5, pp. 1917–1925, 2003.

[161] K. Ostrowski, T. Rohde, S. Asp, P. Schjerling, and B. K. Pedersen, "Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans," *Journal of Physiology*, vol. 515, no. 1, pp. 287–291, 1999.

[162] T. Sugiura, M. Harigai, Y. Kawaguchi et al., "Increased IL-15 production of muscle cells in polymyositis and dermatomyositis," *International Immunology*, vol. 14, no. 8, pp. 917–924, 2002.

[163] P. L. Lollini, G. Palmieri, G. C. De et al., "Expression of interleukin 15 (IL-15) in human rhabdomyosarcoma, osteosarcoma and Ewing's sarcoma," *International Journal of Cancer*, vol. 71, pp. 732–736, 1997.

[164] T. Stegall and K. A. Krolick, "Myocytes respond to both interleukin-4 and interferon-γ: cytokine responsiveness with the potential to influence the severity and course of experimental myasthenia gravis," *Clinical Immunology*, vol. 94, no. 2, pp. 133–139, 2000.

[165] A. R. Nielsen, R. Mounier, P. Plomgaard et al., "Expression of interleukin-15 in human skeletal muscle—effect of exercise and muscle fibre type composition," *Journal of Physiology*, vol. 584, no. 1, pp. 305–312, 2007.

[166] C. Broholm, M. J. Laye, C. Brandt et al., "LIF is a contraction-induced myokine stimulating human myocyte proliferation," *Journal of Applied Physiology*, vol. 111, no. 1, pp. 251–259, 2011.

[167] C. Broholm, O. H. Mortensen, S. Nielsen et al., "Exercise induces expression of leukaemia inhibitory factor in human skeletal muscle," *Journal of Physiology*, vol. 586, no. 8, pp. 2195–2201, 2008.

[168] J. L. Ruas, J. P. White, R. R. Rao et al., "A PGC-1alpha isoform induced by resistance training regulates skeletal muscle hypertrophy," *Cell*, vol. 151, pp. 1319–1331, 2012.

[169] G. C. Laurentino, C. Ugrinowitsch, H. Roschel et al., "Strength training with blood flow restriction diminishes myostatin gene expression," *Medicine & Science in Sports & Exercise*, vol. 44, pp. 406–412, 2012.

[170] E. Louis, U. Raue, Y. Yang, B. Jemiolo, and S. Trappe, "Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle," *Journal of Applied Physiology*, vol. 103, no. 5, pp. 1744–1751, 2007.

[171] S. M. Roth, G. F. Martel, R. E. Ferrell, E. J. Metter, B. F. Hurley, and M. A. Rogers, "Myostatin gene expression is reduced in humans with heavy-resistance strength training: a brief communication," *Experimental Biology and Medicine*, vol. 228, no. 6, pp. 706–709, 2003.

[172] H. Mascher, J. Tannerstedt, T. Brink-Elfegoun, B. Ekblom, T. Gustafsson, and E. Blomstrand, "Repeated resistance exercise training induces different changes in mRNA expression of MAFbx and MuRF-1 in human skeletal muscle," *American Journal of Physiology*, vol. 294, no. 1, pp. E43–E51, 2008.

[173] A. Saremi, R. Gharakhanloo, S. Sharghi, M. R. Gharaati, B. Larijani, and K. Omidfar, "Effects of oral creatine and resistance training on serum myostatin and GASP-1," *Molecular and Cellular Endocrinology*, vol. 317, no. 1-2, pp. 25–30, 2010.

[174] B. Hoier, N. Nordsborg, and S. Andersen, "Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training," *Journal of Physiology*, vol. 590, pp. 595–606, 2012.

[175] A. L. Carey, C. R. Bruce, M. Sacchetti et al., "Interleukin-6 and tumor necrosis factor-α are not increased in patients with Type 2 diabetes: evidence that plasma interleukin-6 is related to fat mass and not insulin responsiveness," *Diabetologia*, vol. 47, no. 6, pp. 1029–1037, 2004.

[176] A. L. Carey, G. R. Steinberg, S. L. Macaulay et al., "Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase," *Diabetes*, vol. 55, no. 10, pp. 2688–2697, 2006.

[177] S. Lucas, S. Taront, C. Magnan et al., "Interleukin-7 regulates adipose tissue mass and insulin sensitivity in high-fat diet-fed mice through lymphocyte-dependent and independent mechanisms," *PLOS ONE*, vol. 7, Article ID e40351, 2012.

[178] K. Samaras, N. K. Botelho, D. J. Chisholm, and R. V. Lord, "Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes," *Obesity*, vol. 18, no. 5, pp. 884–889, 2010.

[179] C. S. Kim, H. S. Park, T. Kawada et al., "Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters," *International Journal of Obesity*, vol. 30, no. 9, pp. 1347–1355, 2006.

[180] A. Jenkins, S. X. Zhang, A. Gosmanova et al., "Increased serum pigment epithelium derived factor levels in Type 2 diabetes patients," *Diabetes Research and Clinical Practice*, vol. 82, no. 1, pp. e5–e7, 2008.

[181] M. Sabater, J. M. Moreno-Navarrete, F. J. Ortega et al., "Circulating pigment epithelium-derived factor levels are associated with insulin resistance and decrease after weight loss," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 10, pp. 4720–4728, 2010.

[182] S. I. Yamagishi, H. Adachi, A. Abe et al., "Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 6, pp. 2447–2450, 2006.

[183] A. K. Gattu, A. L. Birkenfeld, F. Jornayvaz et al., "Insulin resistance is associated with elevated serum pigment epithelium-derived factor (PEDF) levels in morbidly obese patients," *Acta Diabetologica*, vol. 49, no. 1, pp. 161–169, 2012.

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Research Article

Carrageenan-Induced Colonic Inflammation Is Reduced in Bcl10 Null Mice and Increased in IL-10-Deficient Mice

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The common food additive carrageenan is a known activator of inflammation in mammalian tissues and stimulates both the canonical and noncanonical pathways of NF-κB activation. Exposure to low concentrations of carrageenan (10 μg/mL in the water supply) has produced glucose intolerance, insulin resistance, and impaired insulin signaling in C57BL/6 mice. B-cell leukemia/lymphoma 10 (Bcl10) is a mediator of inflammatory signals from Toll-like receptor (TLR) 4 in myeloid and epithelial cells. Since the TLR4 signaling pathway is activated in diabetes and by carrageenan, we addressed systemic and intestinal inflammatory responses following carrageenan exposure in Bcl10 wild type, heterozygous, and null mice. Fecal calprotectin and circulating keratinocyte chemokine (KC), nuclear RelA and RelB, phospho(Thr559)-NF-κB-inducing kinase (NIK), and phospho(Ser36)-IκBα in the colonic epithelial cells were significantly less ($P < 0.001$) in the carrageenan-treated Bcl10 null mice than in controls. IL-10-deficient mice exposed to carrageenan in a germ-free environment showed an increase in activation of the canonical pathway of NF-κB (RelA) activation, but without increase in RelB or phospho-Bcl10, and exogenous IL-10 inhibited only the canonical pathway of NF-κB activation in cultured colonic cells. These findings demonstrate a Bcl10 requirement for maximum development of carrageenan-induced inflammation and lack of complete suppression by IL-10 of carrageenan-induced inflammation.

1. Introduction

Carrageenans are sulfated polygalactans and are obtained from several species of red seaweed (*Rhodophyceae*). Chemically, they are composed of repeating disaccharide units and consist of sulfated or unsulfated D-galactose residues that are linked in alternating β-1,4 and α-1,3 bonds. They resemble the endogenous, human, galactose-containing sulfated glycosaminoglycans, chondroitin sulfate, and keratan sulfate, but differ by the presence of the unusual α-1,3 linkages [1, 2]. Carrageenans predictably induce an inflammatory response and can activate immune responses that are mediated by B-cell leukemia/lymphoma (Bcl) 10 and Toll-like receptor (TLR) 4 [3, 4]. Due to their marked chemical reactivity, carrageenans are widely used in processed foods to improve the texture of food products, although their harmful effects are well recognized [5, 6].

Animal models have demonstrated that ingestion of carrageenan induces colonic inflammation, with development of inflammatory infiltrates, ulcerations, and clinical evidence of colitis [5, 6]. In studies of the effects of carrageenan on human colonic epithelial cells in cell culture, carrageenan stimulated NF-κB activation and increased secretion of IL-8 by TLR4-Bcl10-dependent and Bcl10-independent pathways [3, 4, 7, 8]. Specific phosphorylations of Bcl10 were required for the NF-κB responses [9], and prolongation of the effects of

carrageenan in colonic epithelial cells on both noncanonical and canonical inflammatory pathways occurred due to the presence of an NF-κB binding site in the Bcl10 promoter [10].

The carrageenan-induced activation of the innate immune response in the colonic epithelial cells was attributable to its distinctive chemical structure, and, in particular, its unusual α-D-Gal $(1 \rightarrow 3)$ D-Gal link [11]. Carrageenan exposure was shown to produce NF-κB activation by three distinct mechanisms, including (1) TLR4-Bcl10-IKKγ-IKKβ-inhibitory factor (IκB)α-NF-κB (RelA) pathway; (2) TLR4-Bcl10-NF-κB-inducing kinase (NIK)-IKKα-NF-κB (RelB) pathway; and (3) a reactive-oxygen-species-(ROS-) mediated pathway that involves Hsp27-IKKβ-IκBα-NF-κB (RelA) that does not require Bcl10 [3, 4, 7, 8]. These pathways indicate that Bcl10 is a key signaling molecule in the carrageenan-initiated inflammation *in vitro* in colonic epithelial cells. Recently, the impact of carrageenan on glucose tolerance, insulin resistance, and impaired insulin signaling in a mouse model was reported [12], consistent with the evidence that TLR4-mediated inflammation is involved in diabetes [13–15]. Inhibition of inflammation by Bcl10 silencing or TLR4 blocking antibody reduced the carrageenan-induced impairment of insulin signaling [12].

Many recent investigations involve carrageenan, including almost 500 published reports in 2012. Most of these publications address the effects of anti-inflammatory treatments to alleviate carrageenan-induced inflammation, including effects of lactobacilli [16], but without specific attention to the mechanistic pathways by which carrageenan causes inflammation. Other reports consider a variety of topics, including the chemical characteristics of carrageenan as a gel in food products [17], the use of carrageenan hydrogels as therapeutic delivery vehicles [18], carrageenan as a therapy, including for the common cold [19], and thrombotic effects of carrageenan [20].

This is the first report to address the requirement for Bcl10 as a mediator of carrageenan-induced inflammation in the *in vivo* animal model and extends previous findings about the mechanisms by which carrageenan produces inflammation. Prior work has shown the role of Bcl10 in normal development and function of lymphocyte populations [21]. Overexpression of Bcl10 by the translocation t(1; 14)(p22; q32) was associated with development of the mucosa-associated lymphoid tissue (MALT) lymphomas and with the constitutive overexpression of NF-κB [22] by both non-canonical and canonical pathways of NF-κB activation [23]. Bcl10 has been reported to mediate inflammatory cascades in colonic epithelial cells, mouse embryonic fibroblasts, hepatocytes, and human embryonic kidney 293T cells, as well as in cells of myeloid origin [3, 24–26]. Other recent work has identified Bcl10 as a link between fat (palmitate) intake and hepatic NF-κB activation and insulin resistance [27], and Bcl10 was reported to coordinate the NF-κB-mediated response associated with endosomal trafficking and F-actin remodeling in human macrophages [28]. Recognition of the participation of the CARMA-Bcl10-Malt1 (CBM) signalosome in vital processes in cells continues to emerge, including distinct roles of CARMA1 in immune cells and CARMA3 in epithelial/endothelial cells. The CBM complex appears to be a signaling platform, enabling the integration of effects from receptors with downstream cascades, including effects from G-protein coupled receptors [29]. The CBM complex was shown to promote angiotensin II-dependent vascular inflammation and atherogenesis, and these effects were reported to be reduced in Bcl10-deficient mice [30]. The protein A20, also known as tumor necrosis factor alpha-induced protein 3 (TNFAIP3), acts as a negative regulator of the Bcl10-CARMA interactions in both the lymphoid and nonlymphoid cells due to its effects as a ubiquitin ligase and deubiquitinase [31].

In this report, we evaluate the effect of carrageenan in Bcl10 null mice and the pathways of carrageenan-induced inflammation mediated by activation of canonical and non-canonical cascades. Also, since the microbiome and IL-10 are both widely recognized to impact intestinal inflammation, the effects of the germ-free environment and of exogenous IL-10 on carrageenan-induced inflammation are evaluated in order to provide additional insights into the mechanisms and mediators by which carrageenan provokes inflammation.

2. Materials and Methods

2.1. Animal Care and Carrageenan Exposure. Eight-week-old male C57BL/6J mice were purchased (Jackson Laboratories, Bar Harbor, ME, USA) and housed in the Veterinary Medicine Unit (VMU) at the Jesse Brown VA Medical Center (JBVAMC, Chicago, IL, USA). Principles of laboratory animal care were followed, and all procedures were approved by the Animal Care Committee. Adult mice were fed a standard diet and maintained in individual cages with routine light-dark cycles. After acclimatizing to the environment, the water supply was changed to ddH_2O with undegraded carrageenan (λ-κ carrageenan 10 mg/L; Sigma Chemical Co., St Louis, MO, USA; $n = 6$) or without carrageenan ($n = 6$) for ~12 weeks. Weight and water consumption were measured regularly. Bcl10 null ($n = 8$), heterozygote ($n = 3$), and wild type ($n = 6$) mice were bred and genotyped at St. Jude Children's Research Hospital by study investigators (Stephen Morris and Liquan Xue) [21–23]. Adult mice were shipped to the JBVAMC VMU. Following quarantine, adult mice were treated with carrageenan in their water supply. Stool was collected, and mice were euthanized after receiving carrageenan for ~14 weeks. Total carrageenan intake averaged ~11.5 mg/30 g mouse. Blood was collected by cardiac puncture at the time of euthanasia, and organs were immediately harvested and frozen. Carrageenan (10 mg/L × 19 days + 100 mg/L × 9 days) was also given in the water supply to adult C57BL/6 mice ($n = 5$) and IL-10-deficient mice ($n = 4$) in the germ-free facility at the University of Chicago under an approved protocol and direction of study investigators (Eugene Chang and Suzanne Devkota) [32]. Control littermates were not given carrageenan. Mice were euthanized by carbon dioxide and exsanguination by cardiac puncture, blood was collected, and stool and organs were harvested and frozen pending further studies. Germ-free status was assessed by detection of 16S rRNA by PCR from stool and cecal contents.

2.2. NCM460 Cells in Tissue Culture. NCM460 cells (INCELL, San Antonio, TX, USA) were grown in M3:10A media under the established conditions as previously described [3, 4, 33]. Bcl10 siRNA was used as previously described [3, 4]. Cells were exposed to λ-CGN (1 mg/L × 24 h) in the presence or absence of bioactive, exogenous IL-10 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA 10 or 20 μg/L × 24 h). At the end of the treatment, spent media were collected from control and treated wells and stored at −80°C. Total cell protein was measured by the BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as the standard.

2.3. Measurement of Bcl10 and Phospho-Bcl10. Bcl10 protein in the colonic epithelium of control and carrageenan-fed mice was determined by a solid-phase ELISA reported previously [34]. To determine phospho-Bcl10 in mouse colonic epithelium and in control and CGN-treated NCM460 cells, Bcl10 in the samples was captured in a 96-well ELISA plate that was precoated with Bcl10 antibody, sandwiched by a phospho(Ser138)-Bcl10 second antibody, and then detected by a specific anti-rabbit IgG and hydrogen peroxide-TMB chromogenic substrate [8, 9]. Intensity of the developed color was proportional to the quantity of phospho-Bcl10 in the sample, and optical density values were determined (FLUOstar, BMG, Cary, NC, USA) and normalized using the total cell protein determined by a protein assay kit (Pierce). Results were expressed as percent of total Bcl10 in the untreated control.

2.4. ELISA for Phospho-IκBα in Colon of Carrageenan-Exposed Mice and in NCM460 Cells. Commercial sandwich ELISA for phospho(Ser32)-IκBα (Cell Signaling Technology, Inc., Danvers, MA, USA) was used to determine the phosphorylation of IκBα that was produced following carrageenan exposure in the mouse colonic epithelial tissue and in NCM460 cells [3, 4]. Briefly, the IκBα in the cell extracts was captured in a 96-well ELISA plate that was precoated with mouse monoclonal antibody against IκBα. Phospho-IκBα was determined by a specific phospho(Ser32)-IκBα antibody and detected by horseradish peroxidase-(HRP-) conjugated secondary antibody and hydrogen peroxide-tetramethylbenzidine (TMB) chromogenic substrate. Phospho-IκBα was expressed as percent of total IκBα in the untreated control.

2.5. Oligonucleotide-Based ELISA for Measurement of Nuclear RelA and RelB. Nuclear extracts were prepared from either the colonic epithelial cells of control and carrageenan-fed mice or carrageenan-treated and control NCM460 cells by a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). Activated NF-κBs, including RelA and RelB, were determined by oligonucleotide-based ELISA (Active Motif), as previously described [8]. Treated and control samples were incubated in 96-well microtiter plates that were coated with the NF-κB consensus nucleotide sequence (5′-GGGACTTTCC-3′). NF-κB from the samples attached to the wells and was captured by specific antibody to either RelA or RelB. The extent of binding of the antibody was detected by anti-rabbit-HRP-conjugated IgG, and color was developed with hydrogen peroxide-TMB chromogenic substrate. Intensity of the developed color was proportional to the quantity of either RelA or RelB in the sample. Specificity of the NF-κB binding to the nucleotide sequence was determined by the premixing of either free consensus nucleotide or mutated consensus nucleotide to the nuclear extract sample before adding the sample to the well. The optical density values were normalized using the total cell protein determined by a protein assay kit (Pierce), and results were expressed as percent of the unexposed control.

2.6. ELISA for Measurement of KC and Other Cytokines in Mouse Serum. Serum keratinocyte chemokine (KC), the mouse homolog of IL-8, was detected in control and carrageenan-exposed Bcl10 wild type, heterozygotic, and null mice, and untreated control C57BL/6J mice (total $n = 12$) by DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), as previously described [4]. In addition to the KC ELISA, a custom cytokine ELISA array that included TNF-α, IL-6, IFNγ, IL-1β, IL-10, IL-12, MCP- (monocyte chemotactic protein-) 1, IL-12, and IL-23 was prepared (Signosis, Sunnyvale, CA, USA) and serum of carrageenan-treated Bcl10 wild type, heterozygotic, null mice, and untreated control C57BL/6J mice (total $n = 12$) was tested. Hydrogen peroxide-TMB chromogenic substrate was used to develop the color; color development was stopped with a stop solution, and the concentrations of the cytokines were directly proportional to the intensity of color measured spectrophotometrically at 450 nm in an ELISA plate reader (FLUOstar; BMG). KC concentrations were extrapolated from a standard curve. Other chemokines and cytokines were expressed as percent of control, based on determinations from mice unexposed to carrageenan. Technical duplicates were performed for all measurements, and the mean of the two readings was used in subsequent comparisons.

2.7. Measurement of Fecal Calprotectin. Fecal calprotectin was measured in stools of control and carrageenan-treated mice by ELISA (Alpco Diagnostics, Salem, NH, USA) [35]. Feces were collected from control and treated animals at the time of euthanasia, and recommended procedures were followed. Stool samples were extracted in the supplied extraction buffer, centrifuged for 5 minutes at 13,000 g, then used in the sandwich ELISA. TMB was used for the development of color, which was read at an OD of 450 nm in a plate reader (FLUOstar) and compared to the standard curve.

2.8. Western Blots for Phospho(Thr559)-NIK and Phospho(Thr184)-Tak1. Tissue homogenates were prepared from colonic epithelial tissue of control and carrageenan-exposed mice in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Pittsburgh, PA, USA). Western blots were performed on 10% SDS gels with commercial antibodies to phospho(Thr559)-NIK, phospho(Thr184)-TAK, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ, USA). ImageJ software (NIH, Bethesda, MD, USA) was used for densitometry. The density of the protein of interest was normalized with the density of β-actin or tubulin from the same specimen, and density of treated and control samples was compared.

2.9. Histopathology of Colon from Bcl10 Wild Type and Null Mice Following Carrageenan. Intestine and other organs from control and carrageenan-fed mice were sampled, processed in 10% neutral-buffered formalin and paraffin blocks were made by standard procedures. The paraffin blocks were sectioned, and tissues were stained with hematoxylin and eosin by the Veterinary Diagnostic Laboratory of the University of Illinois (Urbana, IL, USA). Slides of duodenum, jejunum, ileum, cecum, colon, and rectum from three Bcl10 wild type, three Bcl10 heterozygotic, and three Bcl10 null mice exposed to carrageenan were analyzed by a veterinary pathologist (Susan Ball-Kell Global Path Imaging, Germantown Hills, IL, USA), who examined blindly the tissue sections from the gastrointestinal sites and recorded on a standardized scale ratings of inflammation, corresponding to the Ameho criteria with six gradations [36]. These grades were: occasional = 0–3 inflammatory cells/hpf, grade 0 of normal; normal to trace inflammatory infiltrate = 4–10 inflammatory cells/hpf, grade "1" or small; mild inflammatory infiltrate = 11–20 inflammatory cells/hpf, grade "2" or small-medium; mild-moderate inflammatory infiltrate = 21–30 inflammatory cells/hpf, grade "3" or medium; moderate inflammatory infiltrate = 31–40 inflammatory cells/hpf, grade "4" or medium-large; moderate to severe inflammatory infiltrate = 41–50 cells/hpf, grade "5" or large; severe inflammatory infiltrate =>50 inflammatory cells/hpf, grade "6" or marked. Photomicrographs were taken with 20x objective.

2.10. Statistical Analysis. Data were analyzed using InStat3 software (GraphPad, La Jolla, CA, USA). Mean values ± standard deviation (S.D.) were calculated, and differences between carrageenan-treated and control results were compared by one-way ANOVA with Tukey-Kramer post-test for multiple comparisons or by unpaired t-tests, two-tailed. Unless stated otherwise, the one-way ANOVA test was used for the reported comparisons. All measurements are the result of independent experiments with at least three biological samples and two technical replicates, with the exception of the control IL-10-deficient mice in which the n was 2. In the figures, * is for $P \leq 0.05$, ** is for $P \leq 0.01$, and *** is for $P \leq 0.001$.

3. Results

3.1. Histopathology of Colonic Tissue Following Carrageenan Exposure. Following exposure to ~18 mg of carrageenan in the water supply over 14 weeks, Bcl10 wild type, heterozygotic, and null mice and unexposed C57BL/6J mice were euthanized, and the intestine and other organs were excised. No gross differences in the length of the intestine or in macroscopic lesions were evident. The intestine was immersed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Disruption of the mucosal surface with hemorrhage and inflammatory infiltrate was seen in the cecal tissue of one Bcl10 wild type mouse (Figure 1(a)), but not in any of the other mice (Figure 1(b)). No macroscopic abnormalities were observed, and the intestine did not appear shortened.

The intestines were evaluated for microscopic changes using a six-point scale based on the extent of leukocyte infiltration, as described in Section 2. Throughout the small and large intestines, the wild-type mice had more leukocyte infiltration, including granulocytes, plasma cells, and lymphocytes, than the Bcl10 null mice, consistent with increased inflammation when Bcl10 was present (Figure 1(c)). The differences in the scores among the Bcl10 WT, heterozygous, and null mice were not statistically significant. Leukocyte infiltration was greater in the small intestine than in the colon and rectum for all of the mice ($P < 0.01$). The Bcl10 WT mice had more cecal infiltration than the null or heterozygous mice. No edema was seen in the null mice, in contrast to edema of the lamina propria and submucosa in the cecum and rectum of the heterozygous and WT mice. The initial and final weights of the mice indicated no significant differences among the groups (Figure 1(d)).

3.2. KC and Fecal Calprotectin Increased Less in Bcl10 Null Mice Following Carrageenan. The serum keratinocyte chemokine (KC), the mouse homolog of IL-8, increased to ~2.0 times the no carrageenan baseline value in the wild type and heterozygous Bcl10 mice ($P < 0.001$). The increase in serum KC following carrageenan was less (to ~1.5 times the baseline) in the Bcl10 null mice than in the Bcl10 wild type or heterozygous mice ($P < 0.001$) (Figure 2(a)). Similarly, the increase in fecal calprotectin following carrageenan was less in the Bcl10 null mice than in the Bcl10 wild type or heterozygous mice ($P < 0.001$) (Figure 2(b)). These findings are consistent with reduced intestinal and systemic inflammation in the absence of Bcl10.

3.3. Increased Serum IL-6 and MCP-1 Following Carrageenan Exposure in Bcl10 Mice. Serum was collected from the Bcl10 WT, heterozygous, and null mice after exposure to carrageenan for 14 weeks. Cytokine ELISA was performed and indicated significant and similar increases following carrageenan in MCP-1 and IL-6 in the Bcl10 WT, heterozygous, and control mice, compared to controls that were not exposed to carrageenan ($P < 0.001$) (Figure 3). Serum TNFα, IFNγ, IL-1β, IL-10, IL-12, and IL-23 were unchanged following carrageenan.

3.4. Activation of NF-κB RelA Reduced and of RelB Inhibited in Bcl10 Null Mice Following Carrageenan. Measurements of activated nuclear RelA and RelB in the colonic epithelium of carrageenan-exposed mice and controls revealed that activation of RelA was significantly increased in wild type, heterozygous, and Bcl10 null mice after carrageenan exposure, but the increase was significantly less in the Bcl10 null

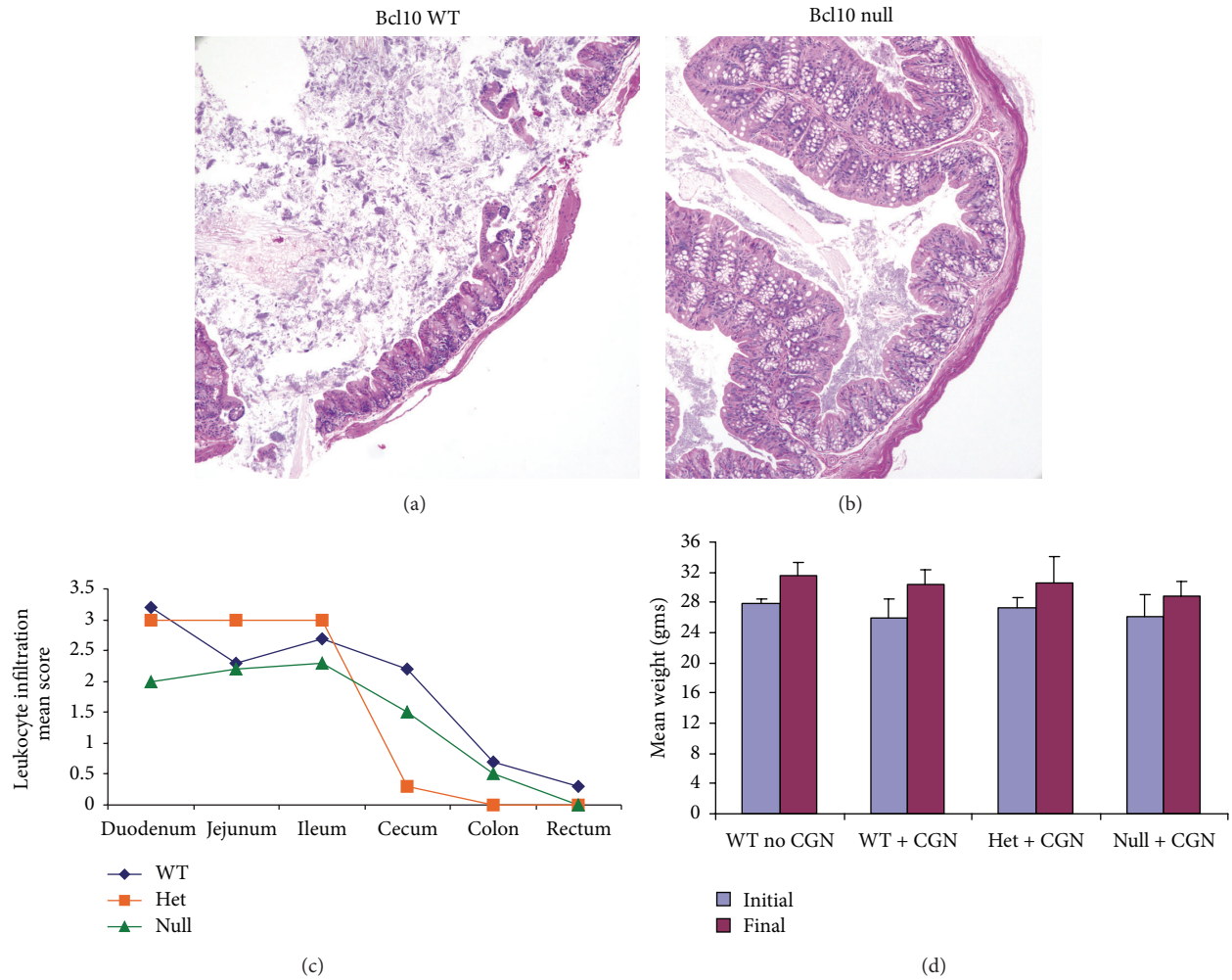

Figure 1: Histopathology of intestinal tissue in Bcl10 wild type and null mice following carrageenan exposure. (a), (b) Bcl10 WT, heterozygous, and null mice were sacrificed after ingestion of carrageenan (10 μg/mL) added in their water for 30 days. Histopathology of H&E sections of cecum demonstrated disruption of the mucosa in a WT mouse, with no similar findings in the Bcl10 null mice, consistent with reduced inflammation in the absence of Bcl10 [WT: wild type]. (c) Throughout the mouse intestine, the extent of inflammatory infiltrate, including granulocytes, lymphocytes, and plasma cells, was greater in the Bcl10 WT mice, than in the Bcl10 null mice. The histopathology was scored for 3 WT, 3 heterozygous, and 3 null mice, and the mean scores for leukocyte infiltration for each site are compared. The scores for the WT mice are higher at each site than for the null mice, although the differences are not statistically significant. The extent of inflammatory infiltrate was significantly greater in the small intestine than in the colon and rectum for each of the groups ($P < 0.01$). Cecal inflammation, including leukocyte infiltration and edema, was greater in the WT mice than in the heterozygous or null mice. (d) The mouse weights at the onset of the carrageenan exposure and at termination are presented and indicate no significant differences in weight and slight weight gain in all groups. (n for no CGN control=3; n for WT with CGN = 6; n for het with CGN = 3; n for null with CGN = 8) (WT: wild type; het: heterozygous; CGN: carrageenan).

mice ($P < 0.001$) (Figure 4(a)). Activated RelB was increased ~75% in wild type and heterozygous mice, but this increase was completely inhibited in the Bcl10 null mice ($P < 0.001$) (Figure 4(b)).

3.5. Phosphorylations of Bcl10 and of IκBα. Phosphorylations of Bcl10 and IκBα were measured by ELISAs in colonic epithelial tissue, following exposure to carrageenan in the Bcl10 transgenic mice and in control, untreated C57BL/6 mice. Four fold increases in phospho(Ser138)-Bcl10 were measured in the carrageenan-exposed Bcl10 wild type and heterozygous mice ($P < 0.001$), but not in the Bcl10 null mice, as anticipated (Figure 5(a)). Phospho(Ser32)-IκBα level was measured by ELISA in the colonic epithelial tissue of the carrageenan-treated Bcl10 wild type, heterozygous, and homozygous null mice and control, untreated C57BL/6 mice. Following carrageenan, phospho-IκBα increased to almost twice the level of the untreated control value in the wild type and heterozygous mice, but in the Bcl10 null mice the increase was nearly 50% less ($P < 0.001$) (Figure 5(b)).

3.6. Phosphorylations of NIK and of Tak1. To compare the activation of the canonical and non-canonical pathways in the Bcl10 transgenic mice, phospho-NIK, which is required

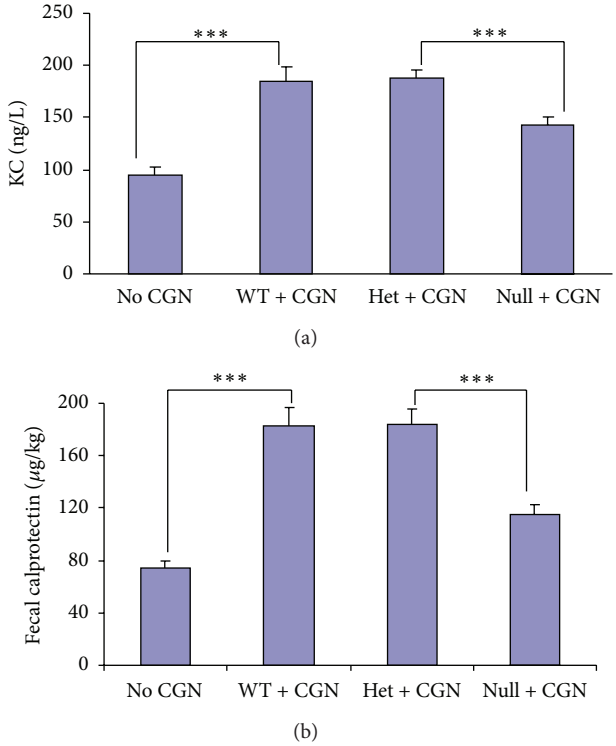

Figure 2: Carrageenan-induced increases in KC and calprotectin are less in Bcl10 null mice. (a) KC, the mouse homolog of IL-8, increased less in the Bcl10 null mice ($n = 8$) than in the wild type ($n = 6$) or heterozygous ($n = 3$) mice following carrageenan to ~77% of the higher value (142.2 ± 9.0 ng/L versus 188.5 ± 6.4 ng/L; $P < 0.001$). (b) Fecal calprotectin, an indicator of colonic inflammation, also increased less following carrageenan in the Bcl10 null mice than in the wild type or heterozygous mice, to 115.1 ± 8.0 μg/kg versus 182.7 ± 14.3 μg/kg in the wild type mice ($P < 0.001$). CGN: carrageenan; KC: keratinocyte chemokine; WT: wild type; het: heterozygous.

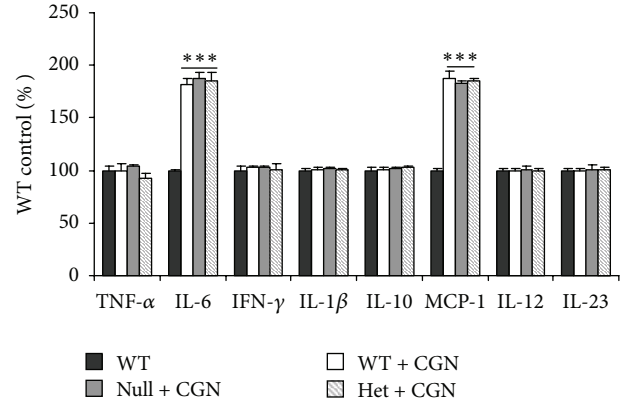

Figure 3: Serum IL-6 and MCP-1 increase post-carrageenan exposure, and increases are similar in Bcl10 WT, heterozygous, and null mice. Carrageenan exposure produced significant increases in serum levels of IL-6 and MCP-1 in the WT, heterozygous, and null mice ($P < 0.001$), compared to the unexposed controls. WT, heterozygotes, and null mice serum levels of MCP-1 and IL-6 both increased ~85%. Serum levels of TNF-α, IFNγ, IL-1β, IL-10, IL-12, and IL-23 did not change.

for RelB activation by the non-canonical pathway, and phospho-Tak, which is involved in the canonical pathway, were determined. Western blots of colonic epithelial tissue from untreated C57/BL6 mice and carrageenan-treated Bcl10 wild type and null mice indicated no increase in phospho(Thr559)-NIK in the Bcl10 null mice, in contrast to the wild type mice (Figure 6(a)). Densitometry confirms the visual impression of the greater increase in phospho-NIK in the Bcl10 wild type than in the Bcl10 null mice ($P = 0.01$) (Figure 6(b)). The carrageenan-induced increase in phospho(Thr184)-Tak in the mouse colonic epithelium was significantly greater in the Bcl10 wild type mice than in the Bcl10 null mice (Figure 6(c)), compared to the untreated control ($P < 0.05$ by densitometry) (Figure 6(d)). Absence of phospho(Thr559)-NIK in the Bcl10 null mice is consistent with the requirement for Bcl10 in the non-canonical RelB pathway of NF-κB activation by carrageenan. In the Bcl10 null mice, the increase in phospho(Thr184)-Tak was less than in the Bcl10 wild type mice, consistent with the presence of both Bcl10-dependent and Bcl10-independent pathways by which carrageenan increases phospho(Thr184) Tak1.

3.7. Effects of Carrageenan in the Germ-Free Environment and in IL-10 Null Mice. C57BL/6 mice were exposed to carrageenan in the germ-free environment. The germ-free environment did not affect the responses of KC (Figure 7(a)), fecal calprotectin (Figure 7(b)), Bcl10 (Figure 7(c)), RelA (Figure 7(d)), RelB (Figure 7(e)), or phospho-Bcl10 (Figure 7(f)). These findings enabled the comparison between the C57BL/6 mice and the IL-10 mice which require the germ-free environment due to their compromised immunity.

In the IL-10 null mice in the germ-free environment, the baseline values without carrageenan of KC ($P < 0.05$) (Figure 7(a)), fecal calprotectin ($P < 0.05$) (Figure 7(b)), Bcl10 ($P < 0.001$) (Figure 7(c)), and RelA ($P < 0.05$) (Figure 7(d)) were all significantly greater than in the C57BL/6 mice. Carrageenan exposure produced larger increases in these measures in the IL-10 null mice than in the C57BL/6 mice ($P < 0.001$). In contrast, the baseline values and carrageenan-stimulated increases in RelB (Figure 7(e)) and phospho-Bcl10 (Figure 7(f)) were similar in the IL-10-deficient mice, in the C57BL/6 mice, and in the germ-free environment, indicating that IL-10 deficiency did not affect activation of the non-canonical NF-κB pathway.

3.8. Inhibition of the Carrageenan-Activated Canonical, but Not the Non-Canonical NF-κB Pathway by Exogenous IL-10. When human colonic NCM460 cells were exposed to carrageenan and exogenous IL-10, the RelA (Figure 8(a)) and phospho-IκBα (Figure 8(b)) responses to carrageenan declined, but the RelB (Figure 8(c)) and phospho-Bcl10 (Figure 8(d)) responses were unaffected. These results are consistent with the *in vivo* findings above and indicate that IL-10 acts only on the canonical pathway of NF-κB activation that involves phospho-IκBα and RelA. Phospho-Bcl10 and RelB, which participate in the non-canonical pathway, are not

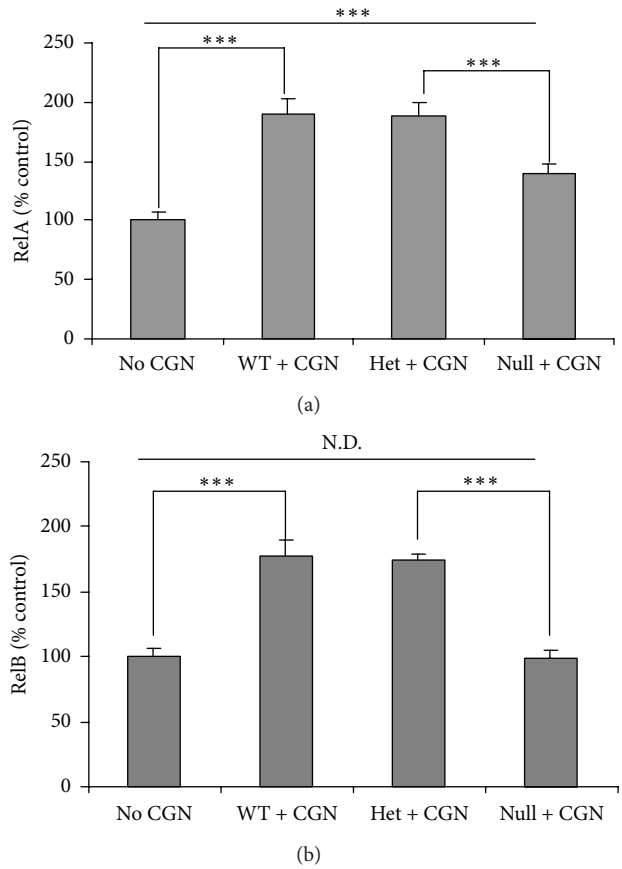

Figure 4: Carrageenan-induced increase in RelA is reduced and is absent in RelB in Bcl10 null mice. (a) Nuclear RelA, measured by oligonucleotide assay, increased by ~89% in the wild type and heterozygous mice following carrageenan but only by about 40% in the Bcl10 null mice ($P < 0.001$). (b) In contrast, the increase in RelB was completely inhibited in the Bcl10 null mice but increased by ~75% in the heterozygous and wild type mice following carrageenan ($P < 0.001$). CGN: carrageenan; WT: wild-type; Het: heterozygous; N.D.: no difference.

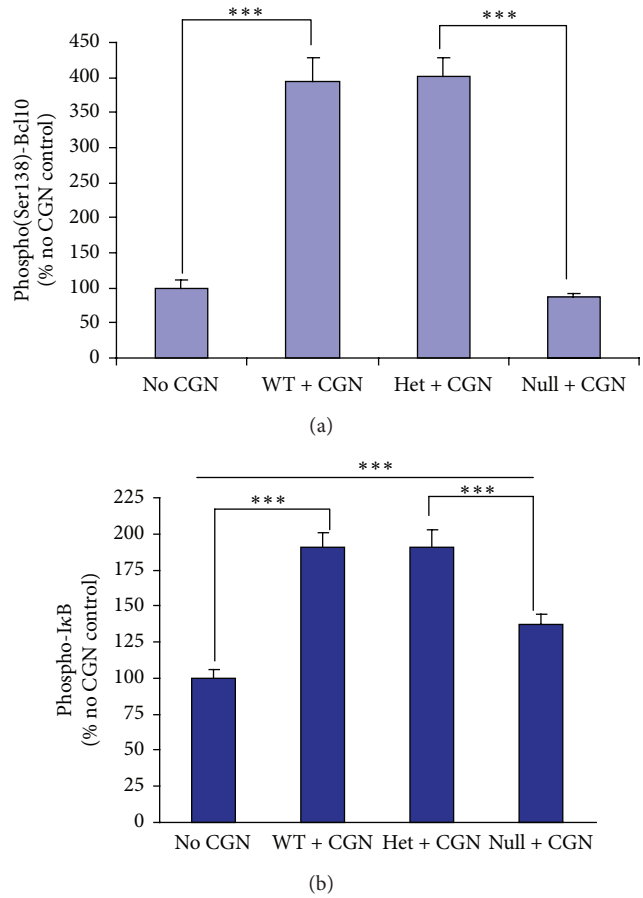

Figure 5: Phosphorylation of Bcl10 is absent and of IκBα is reduced in Bcl10 null mice following carrageenan. (a) Phospho(Ser138)-Bcl10 was increased to ~400% of the no carrageenan baseline when the Bcl10 wild type and heterozygous mice were exposed to carrageenan ($P < 0.001$) but not in the Bcl10 null mice. (b) The increase in phospho(Ser32)-IκBα is less in the Bcl10 null mice, compared to the wild type and heterozygous mice (increase of ~37% versus ~91%), but the increase from the baseline no carrageenan value is also significant ($P < 0.001$). CGN: carrageenan; WT: wild type; Het: heterozygous.

inhibited, since the anti-inflammatory cytokine IL-10 inhibits the activation of the phospho-IκBα-mediated pathway.

4. Discussion

BCL10 has emerged as a key molecule in the activation of the inflammatory cascade in epithelial cells as well as in immune cells [3, 24–26]. Recent investigations in colonic epithelial cells demonstrated the requirement for Bcl10 Ser138 phosphorylation for the activation of the non-canonical pathway of NF-κB activation [9]. The identification of an NF-κB-Bcl10 loop by which an NF-κB binding site in the Bcl10 promoter can lead to transcriptional effects on Bcl10 expression presents a mechanism by which carrageenan, and other Bcl10 stimulators, can lead to prolonged stimulation of inflammatory responses [10].

The studies in this report demonstrate a profound impact of Bcl10-mediated inflammation on the mammalian intestine. In Bcl10 null mice, histopathology following exposure to carrageenan showed less intense inflammatory response than in the wild type mice. Decline in activation of both canonical and non-canonical NF-κB activation occurred when Bcl10 was absent, as indicated by reductions in RelA and RelB. In contrast, studies in IL-10-deficient mice treated with carrageenan demonstrated effects on the canonical, but not the non-canonical pathway of NF-κB activation. Complementary studies in NCM460 cells with exogenous IL-10 following exposure to carrageenan showed effects of IL-10 on RelA, but not on RelB activation. IL-10 gene-knockout mice are known to develop a colitis that resembles IBD [37], and IL-10 gene and IL-10 receptor mutations have been associated with clinical IBD [38]. However, IL-10 therapy has not become as useful clinically as anticipated [39]. By the demonstration of Bcl10-mediated non-canonical inflammation in the IL-10 deficient mouse model, the study findings suggest that specific therapeutic targeting of the non-canonical pathway

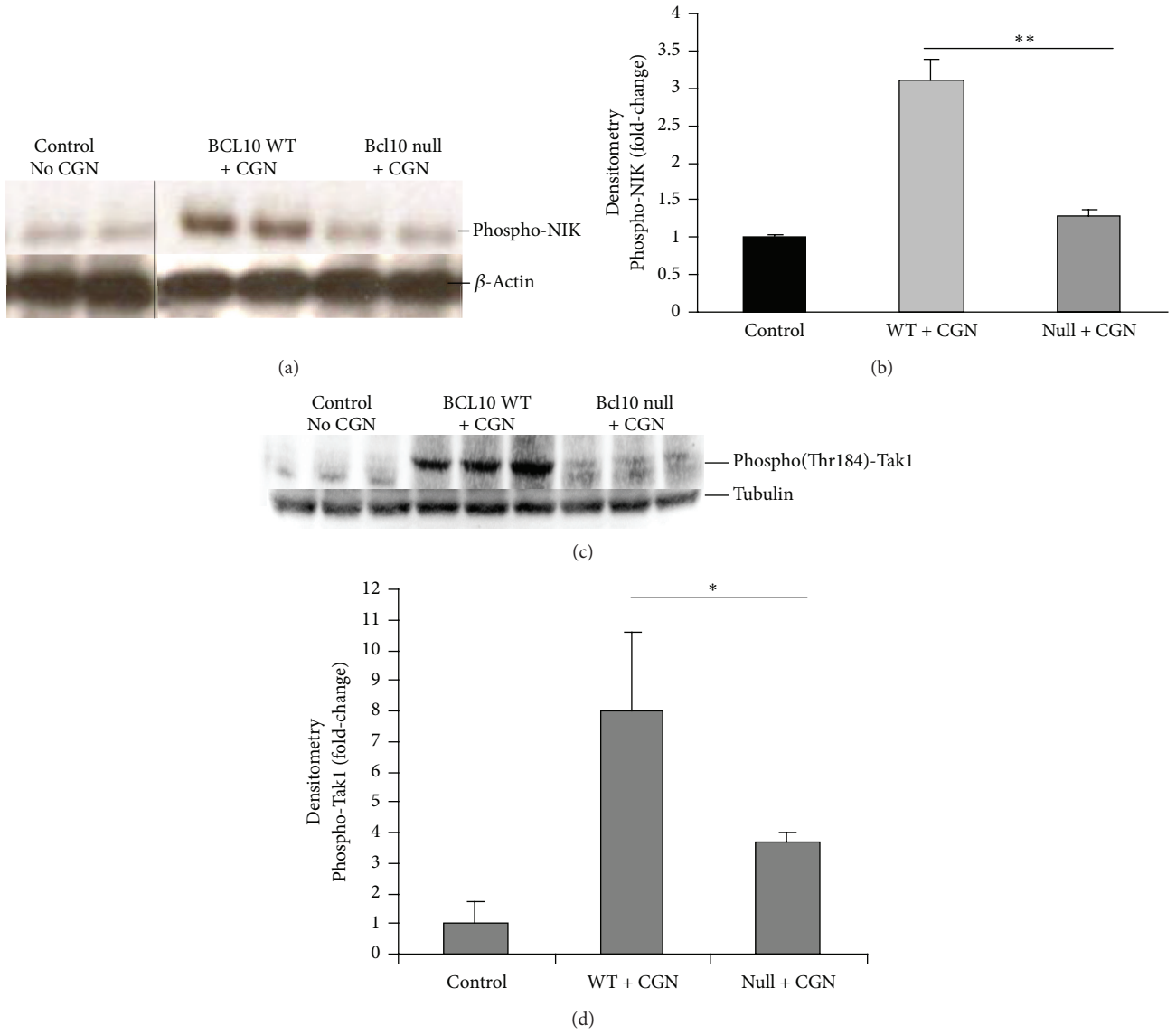

Figure 6: Different responses of phospho(Thr559)-NIK and of phospho(Thr184)-Tak1 following carrageenan in Bcl10 null mice. (a) The immunoblot shows that phospho-NIK does not increase following exposure to carrageenan in the Bcl10 null mice, in contrast to the finding in the wild type mice. (b) Densitometry confirms the increase in phospho-NIK in the Bcl10 WT mice treated with carrageenan compared to the Bcl10 null mice ($P = 0.01$, unpaired t-test, two-tailed). (c) In contrast, phospho-Tak1(Thr184) was somewhat increased in the Bcl10 null mice's intestinal tissue, although less than in the wild type mouse tissue. (d) Densitometry confirms the visual impression that the phospho-Tak1 is significantly reduced in the Bcl10 null versus Bcl10 WT mouse following CGN exposure ($P < 0.05$, unpaired t-test, two-tailed). CGN: carrageenan; WT: wild type; NIK: NF-κB inducing kinase; Tak: TGF-β activated kinase.

of NF-κB activation, as well as targeting of the canonical pathway by IL-10, may lead to improved clinical outcomes. Recent findings that oral *Lactobacillus* significantly reduced carrageenan-induced paw edema and downregulated TNF-α and upregulated IL-10 [16] are suggestive that combined interventions, which impact upon both canonical and non-canonical NF-κB signaling pathways, may lead to improved responses.

The study findings include data that indicate that carageenan-induced inflammation was not suppressed in the germ-free environment. This result suggests that the fecal microflora, as constituted in the C57BL/6 mice under standard conditions, either do not modify carrageenan or do not modify carrageenan in any way that enhances inflammation. The response to *Lactobacillus* noted above suggests that the specific microflora can affect the response to carrageenan, but the effect appears to be indirect, through changes in cytokines or circulating immune cells.

Carrageenan has been widely used in the laboratory for decades to stimulate inflammation in animal models and in cell-based experiments [5, 6]. The animal models have included injection of carrageenan directly into rat hind paw,

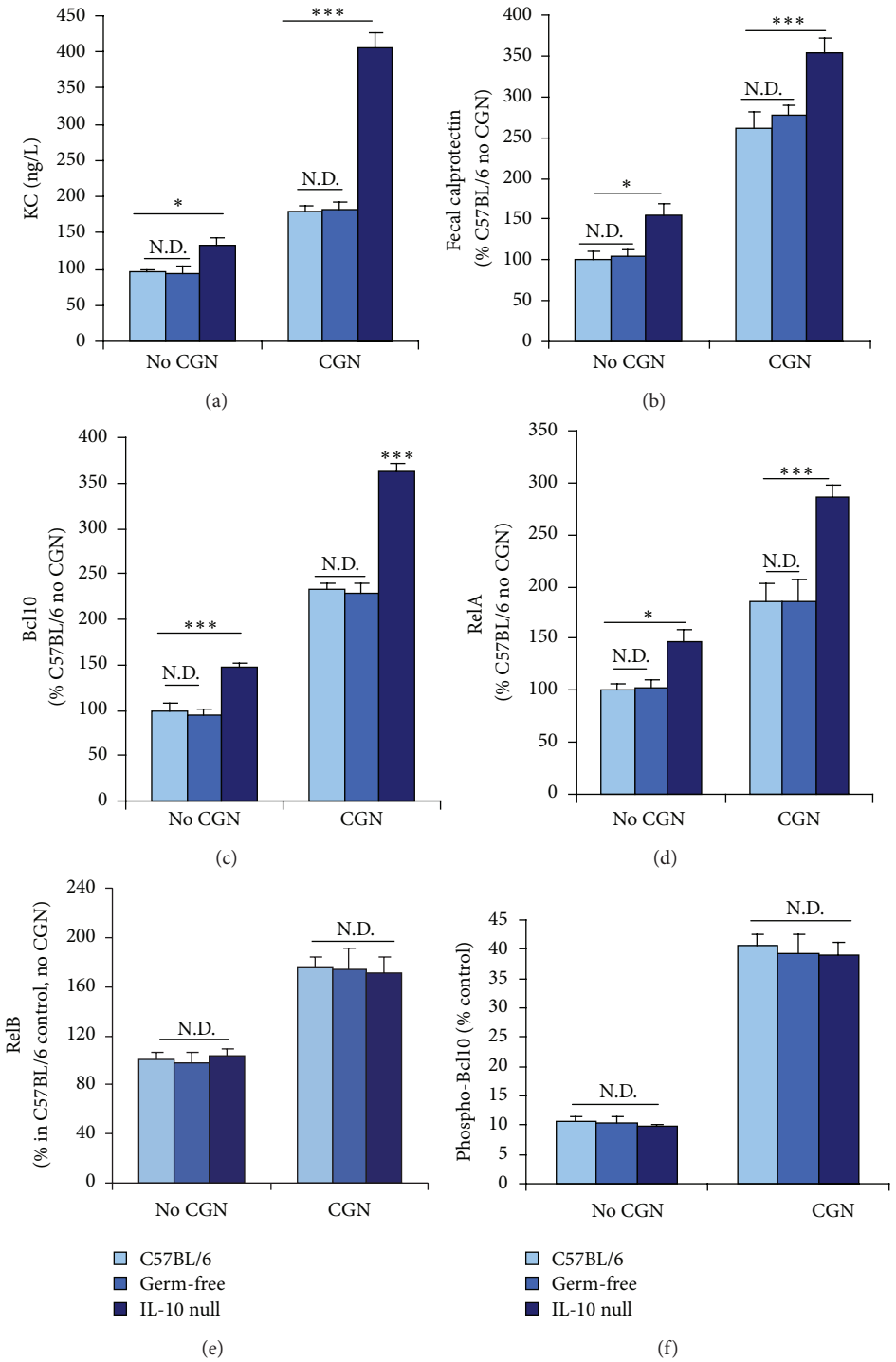

Figure 7: Effects of carrageenan exposure in IL-10-deficient mice compared to C57BL/6 control in germ-free environment and in standard housing. (a) KC was not different in the C57BL/6 germ-free mice versus mice with standard housing at baseline or following carrageenan exposure. In the IL-10-deficient mice, KC was greater at baseline ($P < 0.05$) and following carrageenan ($P < 0.001$) than in the control mice, and the increase in KC was greater in the IL-10-deficient mice than in the controls (~271 ng/L versus ~87 ng/L). (b) Fecal calprotectin was not different in the germ-free mice versus the standard housing control mice with or without carrageenan exposure. Fecal calprotectin was greater in the IL-10-deficient mice at baseline ($P < 0.05$) and following CGN ($P < 0.001$). (c) Bcl10 was similar in the C57BL/6 mice in the germ-free environment as in standard housing. Baseline Bcl10 was significantly greater in the IL-10 null mice than in the controls ($P < 0.001$), and increased more in the IL-10-deficient mice (~229% versus ~144%) following carrageenan ($P < 0.001$) than in the control mice. (d) The germ-free environment did not affect the RelA results in the C57BL/6 mice. Baseline RelA was significantly greater in the IL-10 null mice than in the control C57Bl/6 mice ($P < 0.05$) and increased more following carrageenan in the IL-10-deficient mice than in the controls ($P < 0.001$). (e) Carrageenan-induced increase in RelB was unaffected by IL-10 deficiency. (f) Carrageenan-induced increase in phospho(Ser138)-Bcl10 was unaffected by IL-10 deficiency or the germ-free environment. CGN: carrageenan; N.D.: no difference.

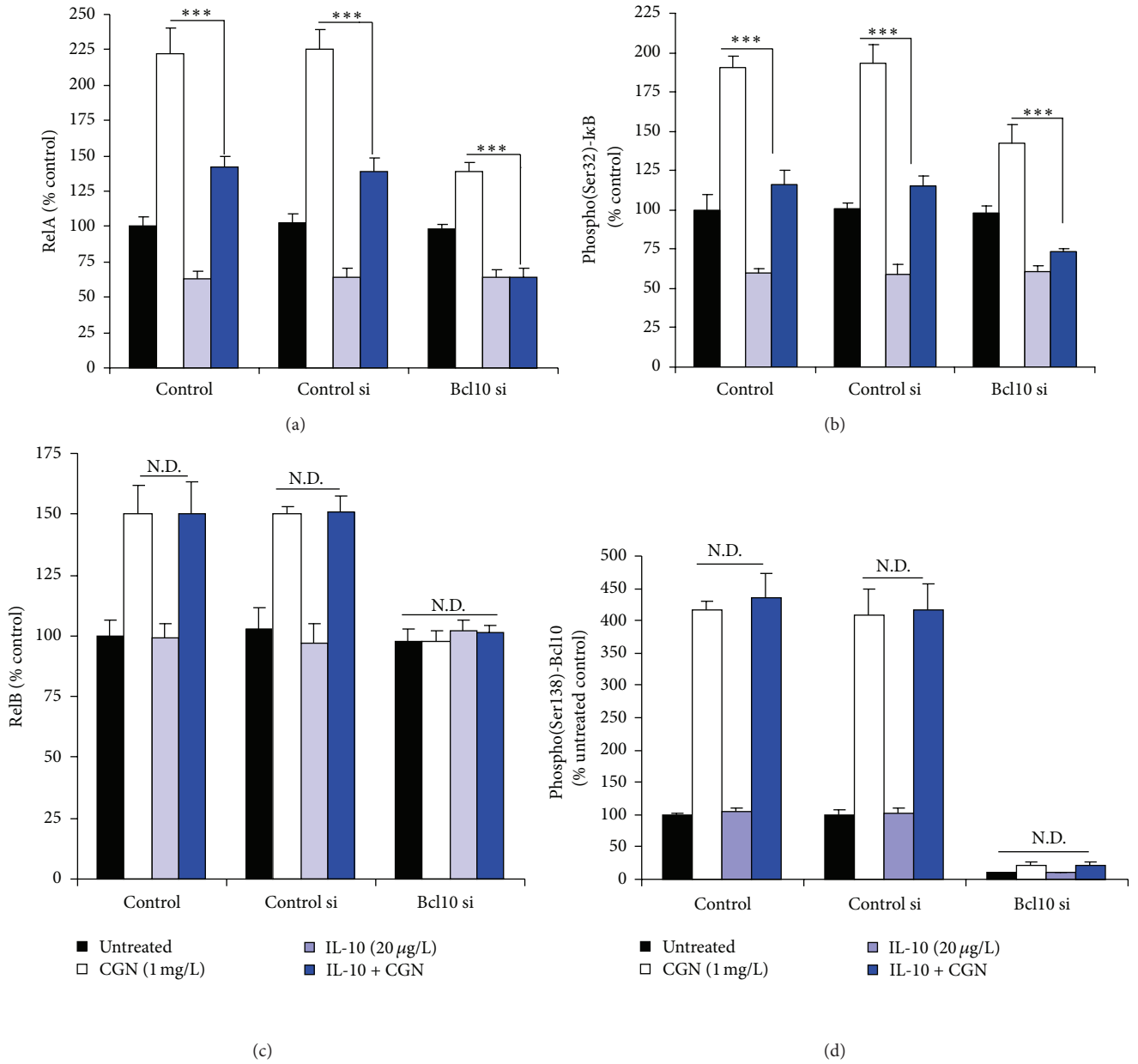

Figure 8: In NCM460 cells, exogenous IL-10 inhibits the canonical but not the non-canonical NF-κB pathway that is activated by carrageenan and mediated by Bcl10. (a) Increase in RelA following exposure to carrageenan was partially inhibited by exogenous IL-10 in the NCM460 cells. When Bcl10 was silenced by siRNA, the increase in RelA was reduced and completely inhibited by IL-10 (20 μg/L × 24 h). (b) The increase in phospho(Ser32)-IκBα was partially inhibited by exogenous IL-10 in the control and control siRNA cells. The increase was less when Bcl10 was silenced and was almost completely inhibited by exogenous IL-10. (c) In contrast to the above findings, the increase in RelB was unaffected by exogenous IL-10. (d) Phospho(Ser138)-Bcl10 increased to over four times the baseline in the control and control siRNA cells, and the increases were not inhibited by exogenous IL-10. ND: no difference; IL-10: interleukin-10; si: small interfering siRNA; CGN: carrageenan.

pleura, peritoneum, bursa, subcutaneous air bleb, or large joints, and oral ingestion to induce colonic inflammation. The signaling mechanisms by which carrageenan induces inflammation have been investigated [3, 4, 7, 8], and the mediators by which carrageenan exposure can lead to systemic effects have been reported [3, 4, 7, 8, 40–42]. The tissue and systemic effects of carrageenan are also mediated by effects on immune cells, including neutrophils, macrophages, and lymphocytes. Mediation of carrageenan-induced inflammation by an inflammatory cell infiltrate was recognized in early experiments in which macrophages in the lamina propria, spleen, and liver were observed to contain fibrillar inclusions and/or have metachromatic staining following oral exposure to carrageenan [43, 44]. Extracolonic manifestations of carrageenan exposure leading to inflammation and impaired insulin signaling in the mouse liver were associated

with glucose intolerance and insulin resistance in recent experiments [12].

In B and T cells, and in epithelial cells, Bcl10 is involved in NF-κB signal transduction involving TLR4 and TAK1 [3, 4, 24–26, 45–47]. The assembly of the lipid raft-associated CARMA-Bcl10-MALT1 (CBM) signalosome involves CARMA1 in the immune cells and CARMA3 in nonimmune cells [23, 48, 49]. The CBM complex promotes the activation and ubiquitination of IKKγ (NEMO), the regulatory component of the IKK signalosome, leading to RelA nuclear translocation. Additional experiments that better characterize the components of the inflammatory infiltrate in the intestine by FACS analysis and that study chimeric mice in which the effects of Bcl10 deficiency in the colonic epithelial cells versus the bone marrow can be differentiated will clarify specific subsets that mediate the response to carrageenan. Immune effects of carrageenan have been recognized for decades [43], and increased attention to the role of Bcl10 provides an opportunity to further differentiate the specific signaling mediators and mechanisms involved in innate and adaptive immunity.

In the experiments in this report, the Bcl10 transgenic mice, weighing about 30 grams, received carrageenan in their water supply at a concentration of 10 μg/mL and drank about 5 mL/day, for a dose of 50 μg/30 g/day (=~1.7 μg/g/day = ~1.7 mg/kg/day). The reported average daily intake of carrageenan in the typical Western diet is reported to be 250 mg/day [50], corresponding to 250 mg/60 kg/day (=~4.2 mg/kg/day), considerably more than the amount ingested by the mice. Greater understanding of the mediators by which carrageenan exerts inflammatory effects *in vivo* and consideration of interventions that might inhibit carrageenan-induced inflammation are relevant to human diseases, such as diabetes and atherosclerosis in which inflammation is involved, and may yield new insights into the role of inflammation on initiation and progression of human diseases.

Conflict of Interests

The authors have no conflict of interests to report.

Acknowledgments

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References

[1] B. A. Macher and U. Galili, "The Galα1,3Galβ1,4GlcNAc-R (α-Gal) epitope: a carbohydrate of unique evolution and clinical relevance," *Biochimica et Biophysica Acta*, vol. 1780, no. 2, pp. 75–88, 2008.

[2] W. R. Blakemore and A. R. Harpell, "Carrageenan," in *Food Stabilisers, Thickeners and Gelling Agents*, Ullman's Encyclopedia of Industrial Chemistry, chapter 5, pp. 73–94, John Wiley & Sons, New York, NY, USA, 2009.

[3] S. Bhattacharyya, A. Borthakur, P. K. Dudeja, and J. K. Tobacman, "Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells," *The American Journal of Physiology*, vol. 292, no. 3, pp. G829–G838, 2007.

[4] S. Bhattacharyya, R. Gill, L. C. Mei et al., "Toll-like receptor 4 mediates induction of the Bcl10-NFκB-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells," *The Journal of Biological Chemistry*, vol. 283, no. 16, pp. 10550–10558, 2008.

[5] J. K. Tobacman, "Review of harmful gastrointestinal effects of carrageenan in animal experiments," *Environmental Health Perspectives*, vol. 109, no. 10, pp. 983–994, 2001.

[6] IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, "Carrageenan," *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, vol. 31, pp. 79–94, 1983.

[7] S. Bhattacharyya, P. K. Dudeja, and J. K. Tobacman, "Carrageenan-induced NFκB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10," *Biochimica et Biophysica Acta*, vol. 1780, no. 7-8, pp. 973–982, 2008.

[8] S. Bhattacharyya, A. Borthakur, S. Tyagi et al., "B-cell CLL/lymphoma 10 (BCL10) is required for NF-κB production by both canonical and noncanonical pathways and for NF-κB-inducing kinase (NIK) phosphorylation," *The Journal of Biological Chemistry*, vol. 285, no. 1, pp. 522–530, 2010.

[9] S. Bhattacharyya, A. Borthakur, A. N. Anbazhagan, S. Katyal, P. K. Dudeja, and J. K. Tobacman, "Specific effects of BCL10 serine mutations on phosphorylations in canonical and noncanonical pathways of NF-κB activation following carrageenan," *The American Journal of Physiology*, vol. 301, no. 3, pp. G475–G486, 2011.

[10] A. Borthakur, S. Bhattacharyya, A. N. Anbazhagan, A. Kumar, P. K. Dudeja, and J. K. Tobacman, "Prolongation of carrageenan-induced inflammation in human colonic epithelial cells by activation of an NFκB-BCL10 loop," *Biochimica et Biophysica Acta*, vol. 1822, no. 8, pp. 1300–1307, 2012.

[11] S. Bhattacharyya, H. Liu, Z. Zhang et al., "Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds," *Journal of Nutritional Biochemistry*, vol. 21, no. 10, pp. 906–913, 2010.

[12] S. Bhattacharyya, I. O-Sullivan, S. Katyal, T. Unterman, and J. K. Tobacman, "Exposure to common food additive carrageenan leads to glucose intolerance, insulin resistance, and inhibition of insulin signaling in HepG2 cells and C57BL/6J mice," *Diabetologia*, vol. 55, no. 1, pp. 194–208, 2012.

[13] H. Shi, M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier, "TLR4 links innate immunity and fatty acid-induced insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 3015–3025, 2006.

[14] M. J. Song, K. H. Kim, J. M. Yoon, and J. B. Kim, "Activation of toll-like receptor 4 is associated with insulin resistance in adipocytes," *Biochemical and Biophysical Research Communications*, vol. 346, no. 3, pp. 739–745, 2006.

[15] M. K. Mohammad, M. Morran, B. Slotterbeck et al., "Dysregulated toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse," *International Immunology*, vol. 18, no. 7, pp. 1101–1113, 2006.

[16] S. Amdekar, P. Roy, V. Singh, A. Kumar, R. Singh, and P. Sharma, "Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats," *International Journal of Inflammation*, vol. 2012, Article ID 752015, 6 pages, 2012.

[17] F. Ma, C. Chen, L. Zheng, C. Zhou, K. Cai, and Z. Han, "Effect of high pressure processing on the gel properties of salt-soluble meat protein contain $CaCl_2$ and κ-carrageenan," *Meat Science*, vol. 95, no. 1, pp. 22–26, 2013.

[18] E. Popa, R. Reis, and M. Gomes, "Chondroigenic phenotype of different cells encapsulated in κ-carrageenan hydrogels for cartilage regeneration strategies," *Biotechnology and Applied Biochemistry*, vol. 59, no. 2, pp. 132–141, 2012.

[19] R. Eccles, C. Meier, M. Jawad, R. Weinmüllner, A. Grassauer, and E. Prieschl-Grassauer, "Efficacy and safety of an antiviral Iota-Carrageenan nasal spray: a randomized, double-blind, placebo-controlled exploratory study in volunteers with early symptoms of the common cold," *Respiratory Research*, vol. 11, article 108, 2010.

[20] J. R. Simkhada, S. S. Cho, P. Mander, Y. H. Choi, and J. C. Yoo, "Purification, biochemical properties and antithrombotic effect of a novel Streptomyces enzyme on carrageenan-induced mice tail thrombosis model," *Thrombosis Research*, vol. 129, no. 2, pp. 176–182, 2012.

[21] L. Xue, S. W. Morris, C. Orihuela et al., "Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells," *Nature Immunology*, vol. 4, no. 9, pp. 857–865, 2003.

[22] Q. Zhang, R. Siebert, M. Yan et al., "Inactivating mutations and overexpression of BCL 10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32)," *Nature Genetics*, vol. 22, no. 1, pp. 63–68, 1999.

[23] Z. Li, H. Wang, L. Xue et al., "Eμ-BCL10 mice exhibit constitutive activation of both canonical and noncanonical NF-κB pathways generating marginal zone (MZ) B-cell expansion as a precursor to splenic MZ lymphoma," *Blood*, vol. 114, no. 19, pp. 4158–4168, 2009.

[24] D. Wang, Y. You, P. C. Lin et al., "Bcl10 plays a critical role in NF-κB activation induced by G protein-couple receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 1, pp. 145–150, 2007.

[25] S. Klemm, S. Zimmermann, C. Peschel, T. W. Mak, and J. Ruland, "Bcl10 and Malt1 control lysophosphatidic acid-induced NF-κB activation and cytokine production," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 1, pp. 134–138, 2007.

[26] L. M. McAllister-Lucas, J. Ruland, K. Siu et al., "CARMA3/Bcl10/MALT1-dependent NF-κB activation mediates angiotensin II-responsive inflammatory signaling in nonimmune cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 1, pp. 139–144, 2007.

[27] M. Van Beek, K. I. Oravecz-Wilson, P. C. Delekta et al., "Bcl10 links saturated fat overnutrition with hepatocellular NF-κB activation and insulin resistance," *Cell Reports*, vol. 1, no. 5, pp. 444–452, 2012.

[28] S. Marion, J. Mazzolini, F. Herit et al., "The NF-κB signaling protein Bcl10 regulates actin dynamics by controlling AP1 and OCRL-bearing vesicles," *Developmental Cell*, vol. 23, no. 5, pp. 954–967, 2012.

[29] S. Rosebeck, A. O. Rehman, P. C. Lucas, and L. M. McAllister-Lucas, "From MALT lymphoma to the CBM signalosome: three decades of discovery," *Cell Cycle*, vol. 10, no. 15, pp. 2485–2496, 2011.

[30] L. M. McAllister-Lucas, X. Jin, S. Gu et al., "The CARMA3-Bcl10-MALT1 signalosome promotes angiotensin II-dependent vascular inflammation and atherogenesis," *The Journal of Biological Chemistry*, vol. 285, no. 34, pp. 25880–25884, 2010.

[31] R. Stilo, E. Varricchio, D. Liguoro, A. Leonardi, and P. Vito, "A20 is a negative regulator of BCL10- and CARMA3-mediated activation of NF-κB," *Journal of Cell Science*, vol. 121, no. 8, pp. 1165–1171, 2008.

[32] S. Devkota, Y. Wang, M. W. Musch et al., "Dietary fat induced taurocholic acid promotes pathobiont expansion and colitis in IL10-/- mice," *Nature*, vol. 487, no. 7405, pp. 104–108, 2012.

[33] M. P. Moyer, L. A. Manzano, R. L. Merriman, J. S. Stauffer, and L. R. Tanzer, "NCM460, a normal human colon mucosal epithelial cell line," *In vitro Cellular & Developmental Biology, Animal*, vol. 32, no. 6, pp. 315–317, 1996.

[34] S. Bhattacharyya, N. Pant, P. K. Dudeja, and J. K. Tobacman, "Development, evaluation, and application of a highly sensitive microtiter plate ELISA for human Bcl10 protein," *Journal of Immunoassay and Immunochemistry*, vol. 28, no. 3, pp. 173–188, 2007.

[35] F. Costa, M. G. Mumolo, M. Bellini et al., "Role of faecal calprotectin as non-invasive marker of intestinal inflammation," *Digestive and Liver Disease*, vol. 35, no. 9, pp. 642–647, 2003.

[36] C. K. Ameho, A. A. Adjei, E. K. Harrison et al., "Prophylactic effect of dietary glutamine supplementation on interleukin 8 and tumour necrosis factor α production in trinitrobenzene sulphonic acid induced colitis," *Gut*, vol. 41, no. 4, pp. 487–493, 1997.

[37] R. Kuhn, J. Lohler, D. Rennick, K. Rajewsky, and W. Müller, "Interleukin-10-deficient mice develop chronic enterocolitis," *Cell*, vol. 75, no. 2, pp. 263–274, 1993.

[38] E. O. Glocker, D. Kotlarz, C. Klein, N. Shah, and B. Grimbacher, "IL-10 and IL-10 receptor defects in humans," *Annals of the New York Academy of Sciences*, vol. 1246, pp. 102–107, 2011.

[39] M. C. Li and S. H. He, "IL-10 and its related cytokines for treatment of inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 10, no. 5, pp. 620–625, 2004.

[40] S. Cuzzocrea, E. Mazzon, L. Dugo et al., "Absence of endogenous interleukin-10 enhances the evolution of acute lung injury," *European Cytokine Network*, vol. 13, no. 3, pp. 285–297, 2002.

[41] F. D'Acquisto, A. Ianaro, A. Ialenti, T. Iuvone, V. Colantuoni, and R. Carnuccio, "Activation of nuclear transcription factor κB in rat carrageenin-induced pleurisy," *European Journal of Pharmacology*, vol. 369, no. 2, pp. 233–236, 1999.

[42] L. Ellis, V. Gilston, C. C. Y. Soo, C. J. Morris, B. L. Kidd, and P. G. Winyard, "Activation of the transcription factor NF-κB in the rat air pouch model of inflammation," *Annals of the Rheumatic Diseases*, vol. 59, no. 4, pp. 303–307, 2000.

[43] A. W. Thomson and E. F. Fowler, "Carrageenan: a review of its effects on the immune system," *Agents and Actions*, vol. 11, no. 3, pp. 265–273, 1981.

[44] R. Mankes and R. Abraham, "Lysosomal dysfunction in colonic submucosal macrophages of rhesus monkeys caused by degraded iota carrageenan," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 150, no. 1, pp. 166–170, 1975.

[45] H. Zhou, I. Wertz, K. O'Rourke et al., "Bcl10 activates the NF-κB pathway through ubiquitination of NEMO," *Nature*, vol. 427, no. 6970, pp. 167–171, 2004.

[46] W. Dong, Y. Liu, J. Peng et al., "The IRAK-1-BCL10-MALT1-TRAF6-TAK1 cascade mediates signaling to NF-κB from toll-like receptor 4," *The Journal of Biological Chemistry*, vol. 281, no. 36, pp. 26029–26040, 2006.

[47] G. Takaesu, R. M. Surabhi, K. J. Park, J. Ninomiya-Tsuji, K. Matsumoto, and R. B. Gaynor, "TAK1 is critical for IκB kinase-mediated activation of the NF-κB pathway," *Journal of Molecular Biology*, vol. 326, no. 1, pp. 105–115, 2003.

[48] O. Gaide, F. Martinon, O. Micheau, D. Bonnet, M. Thome, and J. Tschopp, "Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-κB activation," *FEBS Letters*, vol. 496, no. 2-3, pp. 121–127, 2001.

[49] A. Borthakur, S. Bhattacharyya, W. A. Alrefai, J. K. Tobacman, K. Ramaswamy, and P. K. Dudeja, "Platelet-activating factor-induced NF-κB activation and IL-8 production in intestinal epithelial cells are Bcl10-dependent," *Inflammatory Bowel Diseases*, vol. 16, no. 4, pp. 593–603, 2010.

[50] Encyclopædia Britannica, "algae," Encyclopædia Britannica, Encyclopædia Britannica Online, 2010, http://search.eb.com/eb.article-31714.

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Review Article

Pathogenesis of the Metabolic Syndrome: Insights from Monogenic Disorders

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Identifying rare human metabolic disorders that result from a single-gene defect has not only enabled improved diagnostic and clinical management of such patients, but also has resulted in key biological insights into the pathophysiology of the increasingly prevalent metabolic syndrome. Insulin resistance and type 2 diabetes are linked to obesity and driven by excess caloric intake and reduced physical activity. However, key events in the causation of the metabolic syndrome are difficult to disentangle from compensatory effects and epiphenomena. This review provides an overview of three types of human monogenic disorders that result in (1) severe, non-syndromic obesity, (2) pancreatic beta cell forms of early-onset diabetes, and (3) severe insulin resistance. In these patients with single-gene defects causing their exaggerated metabolic disorder, the primary defect is known. The lessons they provide for current understanding of the molecular pathogenesis of the common metabolic syndrome are highlighted.

1. Introduction

Insulin resistance is implicated in the pathophysiology of the twin epidemics of type 2 diabetes and obesity. Both conditions are associated with a high burden of premature morbidity and mortality globally; however, the dependence of one on the other is not complete. Despite intensive efforts, the molecular mechanisms underlying the relationship between obesity, insulin resistance, and type 2 diabetes in the metabolic syndrome are incompletely understood.

The accelerating discovery of single-gene defects resulting in rare types of diabetes, obesity, or severe insulin resistance over the past 20 years provides the opportunity to unravel the role of several key mediators in these separate groups of disorders through firmer cause-effect relationships. We summarise the key monogenic disorders that result in non-syndromic obesity, pancreatic beta-cell diabetes, or severe insulin resistance and discuss how the insights they provide may be applied to the understanding of more prevalent metabolic disease (see summary Table 1).

2. Monogenic Nonsyndromic Obesity

Lesson 1: Proof That Humans Can Become Obese as a Result of Single-Gene Defects Controlling Key Central Components of Appetite. At a very fundamental level, obesity is the result of excess energy intake over energy expenditure over a prolonged period of time. The rapid increase in worldwide rates of overweight and obesity over the last 30–40 years suggests a predominant change in environmental, diet, and lifestyle factors rather than any change in genetics as the main cause of the obesity epidemic. However, it is clear that there are important genetic contributions to the susceptibility to becoming obese and to the associated comorbidities. Over the last 2 decades, there has been an increased understanding of mechanisms controlling energy balance and appetite regulation in particular. Much of this has come about through the discovery of genes responsible for appetite regulation (see Figure 1). These genes have been identified by characterization of genetic variants of candidate genes in severely obese rodent models and subsequently humans.

Table 1: Summary of lessons learned from monogenic disorders resulting in nonsyndromic obesity, pancreatic beta cell diabetes, and severe insulin resistance.

	Lessons	Human examples	References
1	Proof that humans can become obese as a result of single-gene defects controlling key central components of appetite	Several etiologies of severe human obesity result from single genes involved in appetite pathways for example, *LEP*, *LEPR*, *POMC*, *MC4R*, *BDNF*, *TrkB*	[1–6]
2	Genetically mediated differences in satiety are likely to underly the difference in body weight seen in the current obesogenic environment	Several common single-nucleotide polymorphisms involving similar appetite components for example, *MC4R* and *BDNF* have been identified at greater frequency in those with common obesity	[7, 8]
3	Proof of key components of pancreatic beta cell function and responsiveness of certain genetic etiologies to oral glucose lowering drugs acting distal to the monogenic defect	Those with mutations in *KCNJ11, ABCC8, HNF1A, HNF4A* are able to be treated with sulphonylurea tablets rather than insulin, given that their molecular defects are upstream of the *SUR1* receptor where sulphonylureas act to promote insulin secretion	[9]
4	Glucose toxicity is not seen in those with lifelong, mild hyperglycaemia resulting from a heterozygous glucokinase mutation	Those with heterozygous *GCK* mutations have stable, mild hyperglycaemia with no deterioration in beta cell function with age	[10]
5	Exposure to mild hyperglycaemia in utero does not program non-mutation carrying offspring to have reduced beta cell function	Non-mutation carrying offspring born to mothers with *GCK* who have experienced mild hyperglycaemia in utero do not have reduced beta cell function compared to those born to fathers with *GCK*	[11]
6	Pancreatic beta cell defects in type 2 diabetes are likely to be multifocal including sites distal to the SUR1 receptor where sulphonylureas act to promote insulin secretion	The progressive failure of sulphonylurea therapy in those with type 2 diabetes compared to durable response seen in monogenic causes upstream of *SUR1* receptor	[12]
7	Insulin receptor signaling on pancreatic islets is not required for beta cell compensatory response to severe insulin resistance	Those with a global defect in their insulin receptor due to *INSR* mutations have dramatically high levels of circulating insulin	[13]
8	Acanthosis nigricans and ovarian hyperandrogenism are likely to be mediated by hyperinsulinemia acting through non-insulin receptor pathways	Those with a global defect in their insulin receptor due to *INSR* mutations have marked acanthosis nigricans and such women have ovarian hyperandrogenism	[14]
9	Development of fatty liver and dyslipidemia are dependent on adequate insulin-receptor signalling	Those with a global defect in their insulin receptor due to *INSR* mutations do not develop fatty liver or dyslipidemia, despite markedly elevated levels of circulating insulin	[15]
10	Selective postreceptor (partial) hepatic insulin resistance occurs in common metabolic dyslipidemia rather than total postreceptor insulin resistance	Fatty liver and dyslipidemia frequently coexist with common metabolic syndrome insulin resistance	[15]
11	Not all fat is bad	Those with inherited defects in fat metabolism resulting in partial or complete loss of body fat have exaggerated dyslipidemia, fatty liver, and insulin resistance	[16]

2.1. Leptin and Leptin Receptor Mutations. Leptin was one of the earliest hormones involved in energy balance to be identified [17] and found to be the responsible factor deficient in the severely obese ObOb mouse model of obesity. Leptin is released from adipocytes in proportion to adipose tissue mass and circulating levels are greater in women than men [18]. Leptin has many biological roles including effects on reproduction, bone mineral density, and the immune system; however, one of the main functions is in appetite regulation by signaling of adipose stores by binding to leptin receptors in the arcuate nucleus of the hypothalamus [19].

The downstream effect of leptin receptor activation is regulation of neuropeptides such as those from the proopiomelanocortin (POMC) and agouti-related peptide (AGRP) neurons. Leptin downregulates orexigenic (appetite promoting) neuropeptides such as neuropeptide Y (NPY) and agouti-related peptide (AGRP) but also upregulates anorexigenic neuropeptides such as α melanocyte stimulating hormone (αMSH) and cocaine and amphetamine-regulated transcripts (CART) [20]. It might therefore be predicted that circulating leptin concentrations would be reduced in those with obesity, therefore resulting in inadequate appetite regulation. However, leptin levels are increased in common obesity in proportion to excess fat mass [21], raising the possibility of so-called "leptin resistance".

Leptin also has an important reproductive function. The development of adequate adipose stores to allow puberty to begin is signalled by leptin to the hypothalamus. Thus leptin has a permissive effect on puberty [22]. Furthermore, if adipose stores reduce and leptin levels fall, gonadotrophin

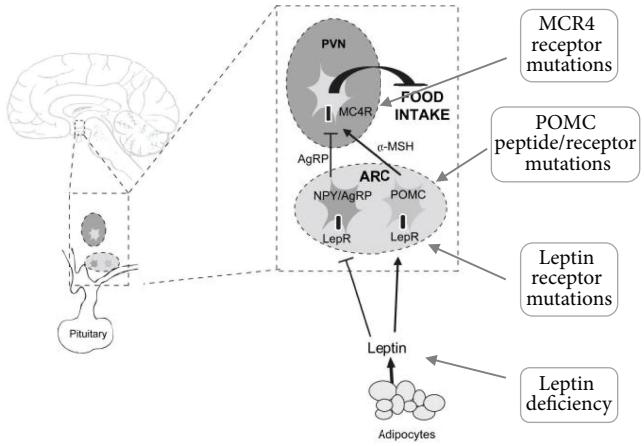

Figure 1: Diagram showing the sites of known monogenic causes of obesity which affect the central regulators of appetite. Leptin is one of the major adiposity signals which circulates to the brain in the region of the arcuate nucleus (ARC) within the hypothalamus and binds to its receptors located in two groups of ARC neurons. Leptin action promotes the synthesis of proopiomelanocortin (POMC) which is cleaved to α-melanocyte stimulating hormone (αMSH), a neurotransmitter which acts at melanocortin 4 receptors (MC4R) on neurons in other hypothalamic areas to reduce food intake. Leptin acts to inhibit the synthesis and secretion of Agouti-related peptide (AgRP) from the second group of ARC neurons, which is an antagonist at MC4R.

levels and pulsatility are affected such that ovulation is inhibited [22].

Evidence for the important role of leptin in energy regulation in humans comes from the observations of O'Rahilly et al., who identified children with severe early onset of obesity who had undetectable levels of leptin [1]. They were found to be homozygous for a frameshift mutation in the *leptin* gene which resulted in a truncated protein which was not secreted. They were morbidly obese with excess fat mass, hyperphagia, but no changes in resting metabolic rate or total energy expenditure adjusted for body composition. These children were the human equivalent of the obese (ob/ob) mouse, resulting from a recessive homozygous mutation in the *leptin* gene and confirmed a critical role for leptin in human appetite regulation. This was reinforced by the observation that replacement of recombinant human leptin in these children resulted in a rapid reversal of the hyperphagia, promoted weight loss, and normalization of body composition [23]. Further support for the importance of leptin comes from identification of a leptin receptor mutation and characterization of humans with homozygous loss of leptin receptor function [2]. These individuals have very high circulating leptin concentrations but a very similar phenotype to those with leptin deficiency. However, as would be predicted, they do not respond to treatment with additional leptin.

2.2. Proopiomelanocortin Gene Mutations. Further evidence for the central role of appetite regulation comes from other monogenic defects of the *POMC* gene, which codes for a number of important proteins including adrenocorticotrophin hormone (ACTH) and melanocortin peptides (α and β melanocyte stimulating hormone (MSH)). Individuals have been identified with homozygous mutations of the *POMC* gene resulting in complete loss of function of all POMC-derived peptides [24]. They present early in life with hypocortisolemia secondary to ACTH deficiency. Obesity with hyperphagia develops even with cortisol deficiency but becomes accelerated after cortisol replacement.

Further insights are provided by mutations of specific POMC-derived peptides. Point mutations that disrupt the α-MSH or β-MSH peptides have been linked with early onset of human obesity depending on the location of the mutation [3, 25, 26].

2.3. Melanocortin Receptor Mutations. The importance of the melanocortin peptides is confirmed by the demonstration of severe obesity associated with mutations of the melanocortin receptor (*MC4R*). The melanocortin receptor MC4R is expressed on neurons in the paraventricular nucleus of the hypothalamus. Activation of MC4R results in release of anorexigenic peptides such as brain-derived neurotrophic factor (BDNF) [4]. Mutations of *MC4R* are found in up to 6% of severe childhood obesity [27, 28]. Whilst obesity appears to be dominantly inherited, the penetrance of obesity with *MC4R* mutations is variable with mutations resulting in complete loss of function having a more severe phenotype. This is demonstrated by the variation in the degree of hyperphagia with ad libitum energy intake. Those with partial *MC4R* deficiency have less excess intake compared with those with complete deficiency [27]. This relationship between degree of function of the mutant receptors and energy intake highlights the importance of the *MC4R* in energy balance.

However, the complexity of the system and the interrelationship of the signals are also demonstrated by the variability in the ad libitum energy intake seen when comparing those with leptin deficiency, inactive and partially active *MC4R* mutations. Those with leptin deficiency have the greatest energy intake, those with inactive MC4R mutations are less hyperphagic, and those with partially active mutations have similar intake to those with treated leptin deficiency [27].

The discovery of peptides and receptors critical to the pathways of energy balance opens the opportunity for therapeutic targets for common obesity. This is demonstrated by the effectiveness of the replacement of leptin in those with leptin deficiency [23]. However, in common obesity, where circulating leptin levels are already high, supplemental leptin therapy has little additional effect on appetite or fat mass [29]. To date no therapeutic agents acting on the other peptides or receptors described have been successfully developed.

2.4. Brain-Derived Neurotrophic Factor and Receptor Mutations. Other players in the central control of appetite have also been identified through rare genetic mutations resulting in reduced production of peptides such as brain-derived neurotrophic factor (BDNF) [5] or loss of function of receptors such as the BDNF receptor tropomyosin-related kinase B (*TrkB*) [6]. Both mutations result in hyperphagia

and obesity. These mutations also highlight the pleiotropic function of these peptides and the activation of their receptors. In addition to obesity, those with *BDNF* mutations also have impaired cognitive function, delayed developmental milestones, and hyperactivity. Those with loss of function of the TrkB receptor also have memory deficits.

Lesson 2: Genetically Mediated Differences in Satiety Are Likely to Underly the Difference in Body Weight Seen in the Current Obesogenic Environment. Defects in single genes causing severe non-syndromic human obesity result mainly through impacts on appetite. The recent rapid rise in obesity epidemic is clearly linked with the technological and social advances which have reduced the need for strenuous physical activity at work and at home, with abundant cheap, high-density food propagated with aggressive advertising. Nonetheless, the persistence of lean people in the current obesogenic environment suggests that either these people are able to withstand the toxic environment through conscious choices about diet and exercise and/or that they may have a genetic predisposition to stronger satiety signals, which enable them to achieve their leanness largely through unconscious mechanisms [30]. Already several common genetic variants predisposing to common obesity have been identified and appear to affect the same processes as monogenic causes of obesity (albeit of small effect size), supporting the latter explanation [7, 8].

3. Monogenic Pancreatic Beta Cell Diabetes

Lesson 3: Proof of Key Components of Pancreatic Beta Cell Function and Responsiveness of Certain Genetic Etiologies to Oral Glucose Lowering Drugs Acting Distal to the Monogenic Defect. The beta-cell monogenic diabetes are characterised by genetic mutations that result in early onset diabetes in absence of obesity or insulin resistance [31–33]. Most of the earlier mutations were identified via the candidate gene approach, that is, the selection of genes known to play a significant role in beta cell function and the subsequent demonstration of critical beta cell dysfunction when a mutation is present both under laboratory conditions and in humans who harbour mutations. More recently, novel and unexpected mutations that result in beta cell dysfunction have been demonstrated and have shed further light on normal beta cell physiology [34].

The beta cell is the major component of the pancreatic islets of Langerhans, which collectively account for 1-2% of the total pancreatic mass. The normal human beta cell has 3 functions that are critical to achieve its contribution to maintaining normoglycaemia. Firstly, the cell must be able to "sense" ambient glucose levels thus allowing any insulin output to be appropriate to requirements; secondly, the beta cell must be able to manufacture and store insulin; and thirdly, the cell must be able to rapidly secrete this insulin when required. Dysfunction of any of these roles will result in impairments of glucose homeostasis (see Figure 2). Typically, the mutations that result in manifestation of diabetes in early life, that is, neonatal diabetes mellitus (NDM), may be interpreted as more intrinsic to this process than those that

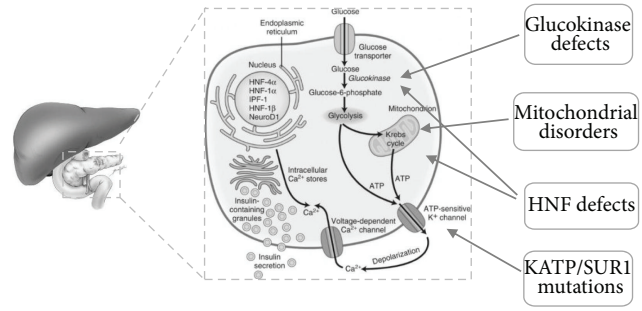

Figure 2: Location of common monogenic pancreatic beta cell defects leading to early onset of diabetes in absence of obesity-related insulin resistance. Hepatocyte nuclear factor (HNF), potassium adenosine triphosphate (KATP) channel, sulphonylurea receptor (SUR).

are manifested later in life; however, the genotype-phenotype relationship for most of the pancreatic beta cell mutations are still unclear.

3.1. Neonatal Diabetes Mellitus (NDM). NDM refers to diabetes diagnosed in the first 6 months of life, which can be transient (approximately 70% of cases) or permanent, and is most commonly the result of a monogenic mutation [9, 35–37]. The incidence of permanent NDM (PNDM) has recently been calculated at 1 in 260,000 live births from a European registry, suggesting that the overall incidence of neonatal diabetes is considerably higher than previously recognized [37, 38]. Transient neonatal diabetes (TNDM) presents in the first weeks of life, remits spontaneously within 1–18 months, and may relapse to permanent diabetes during adolescence or early adulthood [9, 39, 40]. Genetic defects within the imprinted chromosomal 6q24 region are identified in the majority of cases of TNDM, with segmental paternal uniparental disomy, paternally inherited duplication of chromosome 6q24, or loss of methylation in the maternal copy of 6q24 seen. Overexpression of two paternally expressed genes: hydatidiform mole associated and imprinted (*HYMA1,* involved in mRNA encoding) and pleomorphic adenoma gene like 1 (*PLAGL1,* regulating cellular apoptosis) can be identified in 80% of these patients [41].

Activating mutations of the K_{ATP} channel genes (either potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11),* or ATP-binding cassette transporter subfamily C member 8 (*ABCC8*)) or insulin gene (*INS*) are the most common causes of permanent neonatal diabetes mellitus (PNDM), whilst *KCNJ11* and *ABCC8* mutations have been shown to account for a minority of cases of TNDM [35, 40, 42, 43]. The K_{ATP} channels link the ATP produced by cellular glucose metabolism to potassium flux and thus membrane excitability [44]. *KCNJ11* and *ABCC8* mutations cause an increase in the opening of the K_{ATP} channel resulting in hyperpolarisation, preventing beta cell membrane depolarization and insulin release [35, 42, 45]. Oral sulphonylurea (SU) drugs (such as glipizide, gliclazide, and glibenclamide) have long been used to treat type 2 diabetes and act through specific binding to the sulphonylurea receptor (SUR1) located

on the K_{ATP} channel. The resultant closure of these channels stimulates insulin secretion, thus bypassing the effect of the *KCNJ11* and *ABCC8* mutations. Further validation of the function of the K_{ATP} channel is provided by the excellent response of many patients harbouring *KCNJ11/ABCC8* mutations to SU therapy; indeed, it is now standard practice for all patients with diabetes diagnosed before 6 months of age to undergo diagnostic testing for mutations in these two genes, with attempts to transfer those with confirmed mutations from insulin therapy to oral SUs [9, 46]. Furthermore, in those patients with K_{ATP} channel dysfunction evident in extrapancreatic structures (brain and muscle, as seen in cases of DEND (Developmental delay, Epilepsy, and Neonatal Diabetes syndrome)) the use of a nonbeta cell selective SU (glibenclamide) is also effective in treating the neuromuscular phenotype [46–48]. Cases of NDM caused by *INS* gene mutations do not respond to SU medications and currently require lifelong insulin therapy.

The reason why certain mutations within the K_{ATP} channel give rise to TNDM while others result in PNDM is unknown, but changes in pancreatic beta cell turnover or compensation at the level of the beta cell or whole body that is able to overcome the effects of these genes may be possible explanations [49].

Several more rare genetic etiologies of NDM have been identified, most of which are autosomal recessively inherited and are associated with extrapancreatic manifestations. These include pancreas-specific transcription factor 1A (*PTF1A,* pancreatic and cerebellar agenesis) [50], insulin promoter factor 1 (*IPF1,* pancreatic agenesis) [51], GLIS family zinc finger 3 (*GLIS3*, congenital hypothyroidism, glaucoma, kidney cysts and hepatic fibrosis) [52], regulatory factor X6 (*RFX6,* pancreatic hypoplasia, intestinal atresia and gall bladder hypoplasia) [53], GATA-binding factor 6 (*GATA6*, congenital heart defects) [54], neurogenin 3 (*NEUROG3,* congenital malabsorptive diarrhea) [55], neurogenic differentiation 1 (*NEUROD1,* sensorineural deafness and visual impairment) [56], forkhead box P3 (*FOXP3,* enteropathy and dermatitis) [57], eukaryotic translation initiation factor 2-alpha kinase 3 (*EIF2AK3*, exocrine pancreatic dysfunction, spondyloepiphyseal dysplasia, developmental delay, acute liver failure, osteopenia and hypothyroidism) [58], solute carrier family 2 (*SLC2A2,* aminoaciduria, proteinuria, short stature and rickets) [59], and solute carrier family 19 member 2 (*SLC19A2,* thiamine responsive megaloblastic anemia) [60].

The majority of infants with neonatal diabetes of diverse genetic etiologies are born with reduced birth weight, resulting from lower fetal insulin production, as fetal insulin is a major growth factor in utero [61].

3.2. Glucokinase (GCK) Mutations. Glucokinase (GCK) is a hexokinase enzyme that is found in the beta cell, liver, brain, and multiple other tissues in most mammalian species. GCK has many roles within expressing tissues but functions primarily as an ambient glucose sensor for the pancreatic beta cell [62]. The enzyme facilitates the conversion of glucose to glucose-6-phosphate at glucose levels of above 4.0 mmol/L, with adenosine triphosphate (ATP) produced as a secondary product. The increase in intracellular ATP initiates a cascade of membrane depolarisation that results in the extracellular release of preformed insulin containing granules [63]. Homozygous or compound heterozygous inactivating mutations in the *GCK* gene result in a severe diabetes phenotype, presenting at birth as permanent neonatal diabetes mellitus [64, 65]. Heterozygous, inactivating *GCK* mutations result in a higher concentration of glucose being required to stimulate use of the substrate and therefore insulin secretion. However, once this threshold is reached, insulin secretion therafter is relatively normal. Humans who harbour such heterozygous inactivating *GCK* mutations therefore demonstrate mild fasting hyperglycaemia but normal post meal time glucose levels [9]. Those who harbour heterozygous activating *GCK* mutations have the opposite clinical syndrome of hyperinsulinemic hypoglycaemia [66].

The population prevalence of heterozygous inactivating *GCK* mutations is approximately 0.1%, accounting for 20–50% of all cases of monogenic diabetes [67]. Mild fasting hyperglycaemia (5.5–8.0 mmol/L) is present from birth, whilst insulin response, and therefore postprandial glucose levels, are normal such that most patients demonstrate only small glucose excursions after meals (<3.0 mmol/L following a 75 g oral glucose tolerance test) [68]. Furthermore, there is no deterioration in the fasting hyperglycaemia with age, the HbA1c is normal or mildly elevated, and microvascular and macrovascular complications associated with other diabetes subtypes are not seen in persons harbouring *GCK* mutations [9, 69]. For this reason, ownership of a *GCK* mutation resulting in mild hyperglycaemia does not require any glucose lowering therapy.

Lesson 4: Glucose Toxicity Is Not Seen in Those with Lifelong Mild Hyperglycaemia Resulting from Heterozygous GCK Mutations. The glucose toxicity theory proposes that continual exposure to modest increases in blood glucose over a long period of time could has adverse effects on beta cell glucose sensitivity and function [70, 71]. No difference in the deterioration in glucose sensitivity with age has been found among GCK mutation carriers with lifelong mild hyperglycaemia compared with normoglycaemic controls [10]. This is in keeping with the stable glycaemia seen on prolonged followup of patients with heterozygous mutation and the modest decline with age seen in cross-sectional studies of patients with GCK mutations [68, 72]. Continuous exposure to the level of hyperglycaemia experienced by *GCK* mutation carriers has no significant progressive glucose toxic effect on the beta cell.

Up to 17.8% of pregnant women are diagnosed with gestational diabetes on the basis of recent guidelines, and up to 10% of these women will have a *GCK* mutation [73, 74]. However, the importance of suspecting and diagnosing a *GCK* mutation is high in pregnancy; treatment for "hyperglycaemia" in a pregnant woman with a *GCK* mutation may result in reduced birthweight if the foetus has inherited the mutation [75]. Conversely, if the foetus does not inherit the mutation, foetal hyperinsulinemia with resultant increased foetal growth, as a consequence of ambient hyperglycaemia in utero may occur, unless maternal blood glucose is reduced by

insulin therapy. As the likelihood of inheriting the mutation is 50%, and prenatal genetic diagnosis is currently too invasive to perform solely for clarifying *GCK* mutation status, current advice is to perform serial ultrasonography to monitor foetal growth with consideration of treatment only in those with accelerated growth (i.e., those carrying babies presumed not to have inherited the GCK mutation) [74, 76].

Lesson 5: Exposure to Mild Hyperglycaemia in Utero Does Not Program Offspring to Have Reduced Beta Cell Function. There is animal and human evidence for maternal hyperglycaemia exposure in utero programming offspring to have beta cell dysfunction [77], however, these studies are confounded by polygenic predisposition to type 2 diabetes in such offspring. Non-mutation carrying offspring of mothers carrying the heterozygous inactivating GCK mutation was a good human model for studying the impact of exposure to hyperglycaemia in utero on offspring beta cell function. Such offspring had marked increase in birthweight as a result of being exposed to increased glycemia in utero but, despite this, had no evidence of altered beta cell function or glucose intolerance as adults, suggesting a lack of detrimental impact of hyperglycaemia exposure in utero in those without genetic predisposition to type 2 diabetes [11].

The significance of GCK in sensing ambient glucose has led to the hypothesis that pharmaceutical manipulation of this physiological mechanism in the form of glucokinase activators could present an effective method of treating hyperglycaemia as a result of type 2 diabetes [63]. No additional response of incretin hormone stimulation is likely from such GCK activators, as GCK is not the gut glucose sensor by which incretin cells release incretin hormones [10] as was previously hypothesized [78].

3.3. Hepatocyte Nuclear Factor (HNF) Mutations. The hepatocyte nuclear factor (HNF) family of proteins are ubiquitous and function primarily as transcription factors. Whilst predominantly expressed within the liver, their key role in regulating both pancreatic beta cell growth and function is demonstrated by mutations leading to progressive beta cell failure and childhood or early-adult onset of diabetes [79, 80].

Mutations in *HNF1A* are the most commonly encountered form of monogenic diabetes with a minimum prevalence of 50–60 cases per million population (accounting for 52% of all cases of monogenic diabetes) or 1-2% of all patients with diabetes [81]. Patients present in the second or third decade, although marked variability in the clinical phenotype (age of onset of diabetes, presenting features) are seen and relate in part to the type of mutation inherited [82]. Penetrance increases with age such that approximately two thirds of *HNF1A* mutation carriers will have diabetes by the age of 25, and >95% by the age of 40 [9]. Initially, insulin secretion at lower glucose levels is preserved, producing a characteristic glycaemic pattern with a normal fasting glucose and large excursion (>4.5 mmol/L) following a 75 g oral glucose challenge [83]. Progressive beta cell failure over time, however, leads to generalised hyperglycaemia, and unlike *GCK*-related monogenic diabetes, the risk of micro- and macrovascular complications appears to be comparable to type 1 and type 2 diabetes and is directly related to glycaemic control [84]. Patients with *HNF1A* mutations have an increased all-cause and cardiovascular-specific mortality rate when compared with nonaffected relatives (hazard ratios 1.9 and 2.3 resp.) [85]. The risk of coronary heart disease appears to be greater than that seen in type 1 diabetes which appears paradoxical when one considers the elevated high-density lipoprotein levels observed in those with *HNF1A* mutations (traditionally considered cardioprotective) [86]. Patients with *HNF1A* mutations frequently demonstrate high glucose lowering sensitivity to SU therapy [87–89]. As noted previously, SUs stimulate insulin secretion by binding directly to the beta cell membrane K_{ATP} receptor, thereby bypassing the metabolic pathways is rendered dysfunctional by an *HNF* mutation [87]. Consequently, patients misdiagnosed as type 1 diabetes and treated with insulin can be switched to oral SU therapy once the genetic diagnosis of *HNF1A* diabetes is made.

HNF1A directly regulates renal tubular expression of the sodium-glucose cotransporter (SGLT); HNF1A mutations consequently result in a low renal threshold for glucose (i.e, glycosuria despite normal or only slightly elevated blood glucose levels) as a result of impaired tubular glucose reabsorption [90]. This clinical feature often predates the onset of overt diabetes and can therefore be useful as a screening tool [83]. Additionally, HNF1A-binding sites are located at promoter sites in the gene coding for C-reactive peptide (CRP). Consequently, markedly lower high sensitivity CRP (hsCRP) levels are seen in monogenic diabetes as a result of an HNF1A mutation than in other forms of diabetes [91]. An hsCRP value of <0.4 mg/L has a sensitivity of 71% and specificity of 77% for diagnosing HNF1A (as opposed to type 2 diabetes) once type 1 diabetes and glucokinase mutations have been excluded. Furthermore, a urinary c-peptide to creatinine ratio appears useful in distinguishing HNF*1A/4A* monogenic diabetes from type 1 diabetes when diabetes has been present for many years given that C-peptide progressively declines in type 1 diabetes but is retained in HNF*1A/4A* monogenic diabetes [92].

Heterozygous inheritance of an *HNF4A* mutation results in a similar phenotype to the patients with *HNF1A* mutations and accounts for approximately 5–10% of all cases of monogenic diabetes. As with HNF1A mutation carriers, SU therapy remains an effective therapeutic strategy for these patients, but the elevated high density lipoprotein (HDL) levels and glycosuria are not seen in those with HNF4A mutations [93]. Somewhat paradoxically, hyperinsulinaemic hypoglycaemia has been documented in neonates with heterozygous HNF4A mutations, with resultant macrosomia (average increased birth weight of approximately 800 g). Consideration of genetic testing for HNF4A should therefore be considered in patients with clinical features suggestive of HNF1A monogenic diabetes but negative genotyping and those with a positive family history of macrosomia or neonatal hypoglycaemia.

In contrast, mutations in *HNF1B*, which account for approximately 5% of cases of monogenic diabetes, lead to clinical syndromes more reflective of the widespread expression of this transcription factor [94]. Most carriers

of this mutation appear to develop renal disease (cysts and dysplasia, glomerulocystic kidney disease, familial juvenile hyperuricemic nephropathy, and single kidney) which is the predominant clinical feature of this genotype. Indeed, only approximately 50% also develop diabetes. Unlike, HNF1A and HNF4A monogenic diabetes, pancreatic atrophy is common in those with HNF1B mutations, therefore carriers do not display the same sensitivity to SU therapy and often require insulin therapy. Other associated features include genital tract abnormalities, gout, biliary tree abnormalities and abnormal liver function tests.

3.4. Mitochondrial Disorders Associated with Diabetes. Mitochondria are energy-generating organelles identifiable in most human cells [95]. Each mitochondrion, many thousands of which may be found within a single cell, contains abundant copies of mitochondrial DNA (mtDNA) which encode for components of mitochondrial oxidative phosphorylation and respiration. Maternal oocytes contain multiple mitochondria, whereas spermatozoa contain only a few hundred, most of which are destroyed during fertilisation. Thus, mitochondrial DNA, and any mutations contained within, can only be inherited maternally.

Mitochondrial DNA mutations can result in a wide range of multiorgan dysfunction with a number of recognised syndromes. Maternally inherited diabetes and deafness (MIDD) is the most commonly encountered mitochondrial diabetes syndrome and accounts for up to 1% of diabetes [96]. The syndrome is defined by the presence of diabetes and deafness with an inheritance pattern consistent with a mitochondrial disorder and is most commonly the result of a point mutation (*m.3243A>G*) [97]. These defects impair cellular energy generation, and therefore organs that are more metabolically active more frequently display clinical dysfunction. The endocrine pancreas is one such organ, thus diabetes is seen commonly in those with mitochondrial disorders generally. Beta cell failure with deficient insulin production is the characteristic feature, whilst insulin resistance is only seen in the presence of classical risk factors [98]. Most of the patients with MIDD display an insidious onset of glycaemic dysfunction, but occasionally more abrupt loss of beta cell function is seen. Indeed, one multicentre study demonstrated ketoacidosis as the presenting feature in 8% of carriers [99]. There is a wide age range of presentation of diabetes in carriers ranging from childhood to adulthood [96], and the penetrance for glycaemic dysfunction approaches 100% by the age of 70 years [100].

Deafness in MIDD is sensorineural in nature and results from defects in the cochlear and affects the majority of those with mitochondrial (*m.3243A>G*) diabetes [101]. Other systems that are frequently defective in MIDD include cerebrovascular (strokes, cerebral/cerebellar atrophy), opthalmological (macular dystrophy), cardiac (congestive cardiac failure), nephrological (focal glomerulosclerosis), muscular (myopathies), and endocrine systems (short stature secondary to hypothalamic dysfunction) [96]. Cellular heteroplasmy (i.e., a mixture of both functional and dysfunctional with respect to mitochondrial DNA) is likely to explain the significant variation in clinical phenotype in those who harbour these mutations [102].

Lesson 6: Pancreatic Beta Cell Defects in Common Type 2 Diabetes Is Unknown but Is Likely to Be Multiple. Monogenic pancreatic beta cell diabetes arising from key defects within the insulin secretory pathway results in diabetes without the confounding effects of obesity. Despite the contribution of obesity in type 2 diabetes, insufficient pancreatic beta cell function is the most likely cause. However, the site of the beta cell defect in type 2 diabetes is not known. Most common genetic variants identified as associated with type 2 diabetes are within genes important for beta cell function, beta cell development, or the regulation of beta cell mass [103] and overlap with some of the known causes of monogenic diabetes. Certain monogenic subtypes of diabetes have resulted in dramatic success of transferring from insulin therapy to oral sulfonylurea therapy, based on the location of the genetic defect upstream from the target of the sulfonylurea drug. If impaired beta cell regulation of K_{ATP} channel activity or glucokinase activity or HNF activity was the only problem in type 2 diabetes, then its phenotype would resemble that of patients with neonatal diabetes or *GCK* or HNF mutations, which it does not. Impaired mitochondrial metabolism has been postulated to contribute to type 2 diabetes, and an age dependent decline in mitochondrial function might explain why type 2 diabetes develops later in life. Collectively, the characteristics of the various monogenic defects and the temporary response to SU therapy observed in type 2 diabetes suggest that multiple processes downstream of mitochondrial metabolism and the K_{ATP} channel within the pancreatic beta cell, that regulate the amplification response to glucose or insulin exocytosis itself are most likely involved in the progression of type 2 diabetes [12]. The concurrent obesity-related insulin resistance is likely to place an increased functional demand on the beta cell and accelerate beta cell failure as is seen in cases of severe insulin resistance.

4. Monogenic Severe Insulin Resistance

Two groups of monogenic defects have been detected in patients with severe insulin resistance manifested in early life, (a) those that affect insulin signaling and (b) those that affect adipocyte development and/or function.

4.1. Insulin Signalling Disruption

4.1.1. Insulin Receptor Mutations. The most common group of patients with monogenic insulin resistance have a mutation in the insulin receptor (*INSR*), leading to a global reduction of insulin receptor function in target cells. Mutations in the *INSR* gene result in a clinical spectrum of disease severity ranging from mild to severe [13]. Less severe disease is usually caused by an autosomal dominant gene mutation in the *INSR* gene, in which patients present in the peri-pubertal stage or beyond with acanthosis nigricans, dysglycemia (either fasting hypoglycemia and postprandial hyperglycaemia or frank diabetes) in the presence of severe hyperinsulinemia, oligomenorrhea, and hyperandrogenism in women. In men,

the presentation is less obvious with only acanthosis nigricans and sometimes fasting hypoglycaemia. Men often remain undiagnosed even after the development of diabetes requiring high doses of insulin.

The most severe disorders, caused by rare, autosomal recessive mutations in the insulin receptor gene, result in almost complete lack of residual insulin receptor function. Such extreme insulin resistance is seen in patients with Donohue syndrome (or leprechaunism [104, 105]) and Rabson-Mendenhall syndrome [14]. Clinical manifestations include intrauterine and postnatal growth restriction, fasting hypoglycemia, postprandial hyperglycaemia, massive hyperinsulinemia, impaired muscle, and adipose tissue development. Death from intercurrent infection usually occurs in Donohue syndrome; however, Rabson-Mendenhall syndrome differs by the additional presence of dysplastic dentition, coarse facial features, severe diabetic ketoacidosis, pineal hyperplasia, and survival beyond infancy [106].

Patients with *INSR* mutation differ biochemically from patients with more common insulin resistance associated with the metabolic syndrome by having high levels of adiponectin, sex-hormone-binding globulin (SHBG), and insulin-like growth factor-binding protein 1 (IGFBP1) [107]. Adiponectin, SHBG- and IGFBP1 are all commonly reduced in people with obesity-related insulin resistance and various lipodystrophies, so these markers are helpful diagnostically in distinguishing those with insulin receptor dysfunction from those with other causes of severe insulin resistance.

Lesson 7: Insulin Receptor Signalling on Pancreatic Islets Is Not Required for Beta Cell Compensatory Response to Severe Insulin Resistance. The severe hyperinsulinemia seen even in those with almost complete loss of insulin receptor function suggests that the insulin receptor on pancreatic islets is not necessary for beta cell functioning, as has been suggested by some studies in mice [108]. However, the pancreatic beta cells eventually fail to compensate for severe insulin resistance and diabetes that follows over a variable period of time from a few days to beyond 40 years of age [13].

Lesson 8: Acanthosis Nigricans and Ovarian Hyperandrogenism Are Likely to Be Mediated by Hyperinsulinemia Acting through Non-Insulin Receptor Pathways. Acanthosis nigricans (a velvety hyperpigmentation of the skin) and ovarian hyperandrogenism (manifesting as hirsutism, oligomenorrhea, and polycystic ovaries in women) are seen both in patients with severe insulin receptor mutations who have reduced activity of the insulin receptor as well as in those with common obesity-related insulin resistance. This suggests that these clinical features are likely to be mediated by insulin signalling through a noninsulin receptor pathway such as by stimulating the insulin-like growth factor-1 (IGF-1) receptor [109]. Insulin is known to be capable of binding IGF receptors (although at lower affinity than to the insulin receptor) so when present in high concentrations it may bind and activate these receptors which have been found in skin and ovaries [14, 110] (see Figure 3(a)).

Lesson 9: Development of Fatty Liver and Dyslipidemia Is Dependent on Adequate Insulin-Receptor Signalling. Despite severe insulin resistance and compensatory hyperinsulinemia, patients with insulin receptor mutations appear to be protected from fatty liver and atherogenic lipid pattern [15]. This finding suggests that insulin action through the downstream insulin receptor pathway is required to produce these elements of insulin resistance, commonly observed in patients with highly prevalent obesity-related insulin resistance and hyperinsulinemia.

4.1.2. AKT2 Mutations. Surprisingly few mutations have been identified in genes encoding the multiple downstream components of the insulin signaling pathway. A mutation in v-akt murine thymoma viral oncogene homolog 2 (*AKT2)* has been identified accounting for the clinical features of acanthosis nigricans, ovarian hyperandrogenism, diabetes mellitus, and in contrast to those with insulin receptor mutations, both metabolic dyslipidemia and fatty liver in the proband [111]. In addition, the presence of partial lipodystrophy seen in *AKT2* highlights the importance of *AKT2* in adipocyte differentiation as well as its role in the direct effects of insulin signaling.

The presence of metabolic dyslipidemia and fatty liver in those with *AKT2* mutations but not in those with an insulin receptor mutation suggests that the pathway required for hyperinsulinemia to drive liver fat accumulation and atherogenic very low-density lipoprotein (VLDL) secretion depends on the insulin receptor but not on AKT2. This supports the concept of selective postreceptor insulin resistance, also relevant to the highly prevalent form of insulin resistance [15] (see Figure 3(a)).

Lesson 10: Selective Postreceptor (Partial) Hepatic Insulin Resistance Occurs in Common Metabolic Dyslipidemia rather than Total Postreceptor Insulin Resistance. Although hyperinsulinemia in the context of normal blood glucose is often thought to be the hallmark of insulin resistance, the possibility of partial insulin resistance was not clear until studies of monogenic cases of insulin resistance were examined for the effect of hyperinsulinemia on actions such as suppressing hepatic lipogenesis [15]. It has been shown that the lipid metabolism in patients with primary lipodystrophy, AKT2 mutations, or severe insulin resistance of unknown etiology have exaggerated forms of metabolic dyslipidemia and fatty liver in contrast to those with INSR mutations who did not [15]. Those who had INSR mutations were found to have normal hepatic de novo lipogenesis in association with normal lipid profiles, while those with AKT2 mutations or lipodystrophy had elevated de novo lipogenesis and liver fat suggesting that reduced liver fat synthesis plays a key role in the protection from dyslipidemia in patients with insulin receptor mutations. It also supports that the signaling pathway responsible for insulin activation of hepatic de novo lipogenesis diverges from the insulin activation of glucose disposal pathway proximal to AKT2 activation, which is consistent with observations in mice [112] (see Figure 3(a)).

4.2. Monogenic Lipodystrophies. Lipodystrophy refers to heterogeneous disorders characterized by loss of body fat either from discrete or generalized areas, ranging from severe to

mild. The molecular genetic cause of many types of inherited lipodystrophies has recently been discovered.

4.2.1. Congenital Generalized Lipodystrophy. Congenital generalized lipodystrophy (CGL) patients are recognized very early in life due to their near-total lack of body fat and strikingly muscular appearance [113]. Despite lack of body fat, diabetes, acanthosis nigricans, severe dyslipidemia (hypertriglyceridemia, and low high-density lipoprotein cholesterol), and fatty liver develop commonly in the teenage years or later. Most women have polycystic ovaries with impaired ovulation and fertility [114, 115].

Autosomal recessive (either homozygous or compound heterozygous) mutations in several genes have been implicated in the pathogenesis of CGL. Mutations in two genes: 1-acylglycerol-3-phosphate-O-acyltransferase 2 (*AGPAT2)* and Berardinelli-Seip congenital lipodystrophy 2 (*BSCL2)* account for the majority of cases and were identified through linkage analysis with positional cloning in affected families [114, 115]. *AGPAT2* is highly expressed in the adipose tissue and is one of the critical enzymes involved in the biosynthesis of triglyceride and phospholipids from glycerol-3-phosphate [116]. *BSCL2* is highly expressed in the brain and adipose tissue and its protein seipin is thought to be involved in lipid droplet formation and adipocyte differentiation [117, 118]. Patients with both *AGPAT2* and *BSCL2* mutation have similar metabolic abnormalities; however, there are certain distinguishing phenotypic differences. Higher prevalence of mental retardation and cardiomyopathy is seen patients with *BSCL2* mutations, and the presence of lytic lesions in the appendicular skeleton in patients with *AGPAT2* likely reflects the altered tissue expression and function of the two gene products [119].

Autosomal recessive mutations in polymerase 1 and transcript release factor (*PTRF)* [120, 121] and caveolin 1 (*CAV1)* [122] have also been identified in much rarer CGL cases. *PTRF* is involved in the biogenesis of caveolae which are invaginations of the plasma membrane found in many cell types but particularly abundant in adipocytes, muscle, and endothelia. *PTRF* regulates expression of caveolins 1 and 3, which are proteins found within caveolae, that are required for caveolae formation within many tissues [120]. *CAV1* is an integral component of caveolae, which binds fatty acids and translocates them to lipid droplets [123].

Patients with *BSCL2* mutations have the most severe loss of body fat including loss of fat from mechanical sites such as retroorbital, palm, sole, and periarticular regions, in addition to the metabolically active adipose tissue in the subcutaneous, intra-abdominal, intrathoracic, and other regions. However, those with *AGPAT2, CAV1,* and *PTRF* mutations do not have loss of mechanical fat. Both *PTRF* and *CAV1* mutation carriers have preserved bone marrow fat, which is absent in those with *AGPAT2* and *BSCL2* mutations [124].

Lesson 11: Not All Fat Is Bad. Given that obesity is associated with insulin resistance both longitudinally within individuals and cross-sectionally between individuals, this suggests that adipose tissue itself causes insulin resistance. However, the exaggerated appearance of insulin resistance features in rare patients with congenital lack of adipose tissue suggests that a normal amount of adipose tissue is critical for metabolic health. The different adipocyte-related cellular functions of the various congenital generalized lipodystrophy genes suggest that the common severe insulin resistance phenotype (diabetes, dyslipidemia, fatty liver) seen in these disorders is a result of the loss of adipose tissue rather than their specific gene functions in other tissues [16].

4.2.2. Familial Partial Lipodystrophy. The first gene identified as causative of familial partial lipodystrophy (FPL) of the Dunnigan variety was lamin A/C (*LMNA) gene* [125], which remains the most common cause of FPL. *LMNA* encodes the protein lamin A/C which forms part of the nuclear lamina, and mutations have been linked to many other disorders such as Charcot-Marie-Tooth neuropathy, progeria syndromes, restrictive dermopathy, limb-girdle muscular dystrophy, and mandibulo-acral dysplasia [126]. The second gene to be identified in FPL was in peroxisome proliferator-activated receptor gamma (*PPARG)*, which is a nuclear hormone receptor most highly expressed in adipose tissue [127]. It is important in adipocyte differentiation and the target of thiazolidinediones class of diabetes medications. Since then, further genes have been identified including *AKT2* [111], cell-death inducing DFFA-like effector c (*CIDEC)* [128], and perilipin 1 (*PLIN1)* [129]. Both *CIDEC* and *PLIN1* are important in lipid droplet dynamics and regulate triglyceride mobilization.

In all these FPLs, the failure of fat development is often not manifested until puberty and women are much more severely affected than men. This may relate to the higher adiposity seen in females than males, particularly with the onset of puberty. The phenotypic heterogeneity between the various genetic types of FPL is not clear; however, the partial loss of subcutaneous fat from the extremities is seen in all types. In the LMNA variety, the trunk is also affected but face and neck are spared, while there is preserved abdominal fat in the remaining types [124]. Despite this heterogeneity in truncal sparing of fat and overall less loss of body fat compared to CGL, the metabolic derangements in terms of diabetes, fatty liver, and dyslipidemia are similar among the different forms of FPL and comparable to that observed in CGL, yet more severe than that observed with common obesity-related metabolic syndromes.

The lipodystrophies suggest that there is a critical amount of adipose tissue required for buffering episodic excess caloric intake by serving as a sump for free fatty acids and glucose. Loss of this capacity produces increased uptake in other metabolically less suited tissues such as liver, muscle, and pancreatic beta cells where harmful effects include fatty liver, dyslipidemia, and loss of insulin sensitivity and insulin secretion. An exacerbating factor may include the loss of adipocyte-tissue-derived leptin which signals adequacy of adipose tissue to the brain. Loss of leptin in CGL (and to a less extent FPL) suggests that whole body energy stores are low and leads to compensatory hyperphagia. The resulting increase in ingested calories worsens systemic metabolic stress. Proof of this has been illustrated by the dramatic

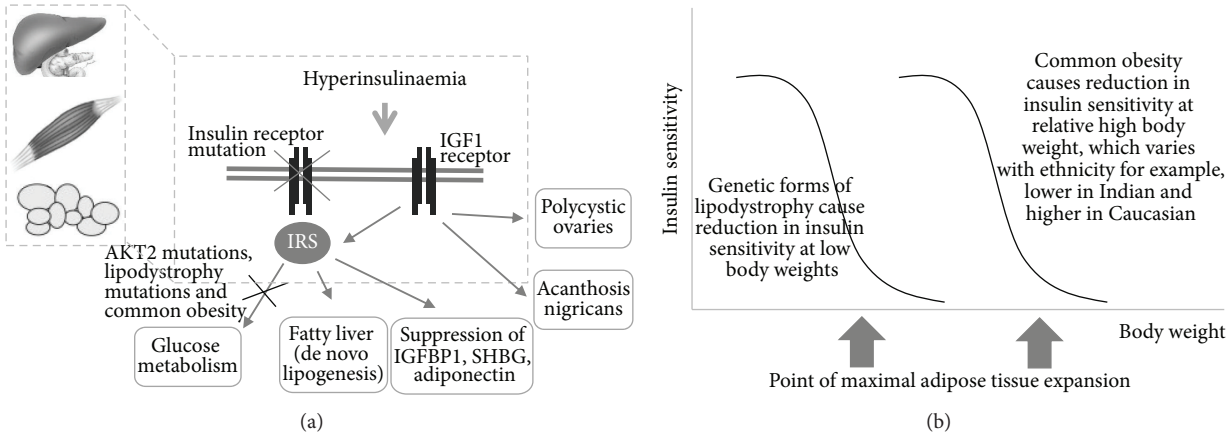

Figure 3: (a) Model of partial insulin resistance within different tissues highlighted by those carrying the insulin receptor mutation who have very high levels of circulating insulin which is able to bind to the insulin-like growth factor-1 (IGF1) receptor and stimulate the development of polycystic ovaries and acanthosis nigricans. In contrast to those with AKT2 (v-akt murine thymoma viral oncogene homolog (2) mutations and those with common obesity-related insulin resistance, those with insulin receptor mutations do not have fatty liver or suppression of insulin-like growth factor binding protein 1 (IGFBP1) or sex hormone binding globulin (SHBG) or adiponectin, which most likely requires active signaling through the nonglucose metabolism arms of the insulin receptor substrate (IRS) downstream pathway. (b) Tissue expandability theory model represents the individual set point up to which adipose tissue can be expanded without metabolic morbidity, which is likely to depend on genetic factors. The two curves represent the relationship of increasing body weight to reducing insulin sensitivity; however, the curve on the left represents the extremely limited adipose expandability in those who have a genetic mutation causing lipodystrophy, while those who have common obesity-associated reduction in insulin sensitivity have a more rightward shifted curve.

efficacy of leptin replacement in patients with most lipodystrophies [130, 131].

The rare lipodystrophy syndromes support the adipose expandability hypothesis [132, 133]. This proposes that individuals have a fixed capacity for adipose tissue expansion to cope with excess energy consumption (very low in those with lipodystrophy but very high in those with morbid obesity without associated features of metabolic syndrome). With chronic consumption of excess calories, this threshold is exceeded (quickly in those with lipodystrophy and more slowly in most people with common metabolic syndrome), and fat is forced to accumulate in other organs such as the liver, skeletal muscle and pancreatic beta cells where they result in fatty liver, dyslipidemia, insulin resistance, and diabetes [13] (see Figure 3(b)).

5. Conclusions

The observation that loss of function of certain genes in humans leads to either severe obesity, early diabetes, or severe insulin resistance (with or without lipodystrophy) is very powerful in illustrating how defects in specific encoded proteins located predominantly in the brain, pancreatic beta cell, muscle, and or fat give rise to these distinct components of the metabolic syndrome. They challenge the view that environmentally driven obesity leads to insulin resistance which leads to type 2 diabetes, as supported by many cross-sectional and longitudinal epidemiological studies. The monogenic disorders provide particular insights that (1) there are clear biological driven origins of obesity affecting appetite, which predisposes to insulin resistance and type 2 diabetes, (2) specific defects in pancreatic beta cell function predispose to diabetes in absence of either obesity or insulin resistance, and (3) specific defects in insulin signaling or fat storage capacity can lead to severe insulin resistance in absence of obesity, which leads to diabetes through pancreatic exhaustion.

All single-gene defects discovered to be responsible for human obesity have been identified to impact on appetite [134]. Common genetic variants predisposing to obesity appear to affect the same processes [135]. This suggests that although the recent obesogenic environment has resulted in an overall tendency to gain weight in all, interindividual differences in susceptibility to this environment may have a biological explanation (likely through differences in satiety), rather than necessarily a moral explanation through conscious individual efforts to diet and exercise [30].

Various monogenic pancreatic beta cell diabetes arising from key defects within the insulin secretory pathway results in diabetes, manifesting from early infancy to early adulthood. Certain monogenic subtypes of diabetes have resulted in dramatic success of transferring from insulin therapy to oral sulfonylurea therapy, based on the location of the genetic defect upstream from the target of the sulfonylurea drug. The confounding effects of obesity are less important in these disorders, so it is possible to translate some of these findings to the etiology of type 2 diabetes. Common genetic variants predisposing to type 2 diabetes have been identified in many beta cell targets overlapping with known causes of monogenic diabetes [103]. Current evidence favours that insufficient glucose-stimulated insulin secretion is primarily responsible for type 2 diabetes; however, the site of the beta cell defect in type 2 diabetes is not known. Collectively, the monogenic diabetes phenotypes and the temporary response of type

2 diabetes to SU therapy suggest that multiple processes downstream of mitochondrial metabolism and the K_{ATP} channel within the pancreatic beta cell, that regulate the amplification response to glucose or insulin exocytosis itself, is involved in type 2 diabetes [12]. The associated obesity-related insulin resistance in type 2 diabetes is likely to place an increased functional demand on the beta cell and accelerate beta cell failure, as is seen in cases of severe insulin resistance.

In contrast to insulin resistance commonly observed in relation to obesity, patients with monogenic defects resulting in severe insulin resistance occur in absence of obesity or in the presence of generalized or regional lack of adipose tissue. Such lipodystrophic patients from diverse genetic etiologies in fat metabolism have a defect of triglyceride storage in adipose tissue, which results in lipid accumulation elsewhere in the body. This leads to severe insulin resistance associated with the usual accompanying characteristics of fatty liver and atherogenic dyslipidemia. At a more direct level, those who have a defect in their insulin receptor have very high levels of insulin resistance and frequently develop diabetes from compensatory beta cell exhaustion, yet are protected from fatty liver and atherogenic dyslipidemia. Patients with insulin receptor defects also differ biochemically from other types of insulin resistance by displaying elevated adiponectin, IGFBP1, and SHBG, which are all commonly suppressed in common insulin resistance or lipodystrophy-associated severe insulin resistance. This highlights the mechanisms behind partial insulin resistance or postreceptor selective defect in signaling through one arm of the insulin signaling pathway evident in common obesity-related insulin resistance.

Several monogenic disorders manifesting the key components of the metabolic syndrome have enabled us to unravel the pathogenic link between the defined molecular defect (or groups of defects) and obesity, early diabetes, or severe insulin resistance, each without the confounding effects of all the other components. Moving forward, we should continue to discover additional monogenic causes for the remaining portion of patients with severe disorders who currently do not have any defined etiology, which will not only help improve their management but may uncover future novel and therapeutic targets for the increasingly common metabolic syndrome.

References

[1] C. T. Montague, I. S. Farooqi, J. P. Whitehead et al., "Congenital leptin deficiency is associated with severe early-onset obesity in humans," *Nature*, vol. 387, no. 6636, pp. 903–908, 1997.

[2] K. Clément, C. Vaisse, N. Lahlou et al., "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction," *Nature*, vol. 392, no. 6674, pp. 398–401, 1998.

[3] H. Krude and A. Grüters, "Implications of proopiomelanocortin (POMC) mutations in humans: the POMC deficiency syndrome," *Trends in Endocrinology and Metabolism*, vol. 11, no. 1, pp. 15–22, 2000.

[4] B. Xu, E. H. Goulding, K. Zang et al., "Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor," *Nature Neuroscience*, vol. 6, no. 7, pp. 736–742, 2003.

[5] J. Gray, G. S. H. Yeo, J. J. Cox et al., "Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene," *Diabetes*, vol. 55, no. 12, pp. 3366–3371, 2006.

[6] G. S. H. Yeo, C. C. C. Hung, J. Rochford et al., "A de novo mutation affecting human TrkB associated with severe obesity and developmental delay," *Nature Neuroscience*, vol. 7, no. 11, pp. 1187–1189, 2004.

[7] E. K. Speliotes, C. J. Willer, S. I. Berndt et al., "Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index," *Nature Genetics*, vol. 42, no. 11, pp. 937–948, 2010.

[8] R. J. Loos, C. M. Lindgren, S. Li et al., "Common variants near MC4R are associated with fat mass, weight and risk of obesity," *Nature Genetics*, vol. 40, no. 6, pp. 768–775, 2008.

[9] R. Murphy, S. Ellard, and A. T. Hattersley, "Clinical implications of a molecular genetic classification of monogenic β-cell diabetes," *Nature Clinical Practice Endocrinology and Metabolism*, vol. 4, no. 4, pp. 200–213, 2008.

[10] R. Murphy, A. Tura, P. M. Clark, J. J. Holst, A. Mari, and A. T. Hattersley, "Glucokinase, the pancreatic glucose sensor, is not the gut glucose sensor," *Diabetologia*, vol. 52, no. 1, pp. 154–159, 2009.

[11] R. Singh, E. R. Pearson, P. M. Clark, and A. T. Hattersley, "The long-term impact on offspring of exposure to hyperglycaemia in utero due to maternal glucokinase gene mutations," *Diabetologia*, vol. 50, no. 3, pp. 620–624, 2007.

[12] F. M. Ashcroft and P. Rorsman, "Diabetes mellitus and the beta cell: the last ten years," *Cell*, vol. 148, no. 6, pp. 1160–1171, 2012.

[13] R. K. Semple, D. B. Savage, E. K. Cochran, P. Gorden, and S. O'Rahilly, "Genetic syndromes of severe insulin resistance," *Endocrine Reviews*, vol. 32, no. 4, pp. 498–514, 2011.

[14] D. Accili, F. Barbetti, A. Cama et al., "Mutations in the insulin receptor gene in patients with genetic syndromes of insulin resistance and acanthosis nigricans," *Journal of Investigative Dermatology*, vol. 98, no. 6, pp. 77S–81S, 1992.

[15] R. K. Semple, A. Sleigh, P. R. Murgatroyd et al., "Postreceptor insulin resistance contributes to human dyslipidemia and hepatic steatosis," *Journal of Clinical Investigation*, vol. 119, no. 2, pp. 315–322, 2009.

[16] V. E. Parker, D. B. Savage, S. O'Rahilly, and R. K. Semple, "Mechanistic insights into insulin resistance in the genetic era," *Diabetic Medicine*, vol. 28, no. 12, pp. 1476–1486, 2011.

[17] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, and J. M. Friedman, "Positional cloning of the mouse obese gene and its human homologue," *Nature*, vol. 372, no. 6505, pp. 425–432, 1994.

[18] L. J. Martin, M. C. Mahaney, L. Almasy et al., "Leptin's sexual dimorphism results from genotype by sex interactions mediated by testosterone," *Obesity Research*, vol. 10, no. 1, pp. 14–21, 2002.

[19] M. Baratta, "Leptin—from a signal of adiposity to a hormonal mediator in peripheral tissues," *Medical Science Monitor*, vol. 8, no. 12, pp. RA282–RA292, 2002.

[20] E. Jéquier, "Leptin signaling, adiposity, and energy balance," *Annals of the New York Academy of Sciences*, vol. 967, pp. 379–388, 2002.

[21] B. M. Spiegelman and J. S. Flier, "Adipogenesis and obesity: rounding out the big picture," *Cell*, vol. 87, no. 3, pp. 377–389, 1996.

[22] G. Martos-Moreno, J. A. Chowen, and J. Argente, "Metabolic signals in human puberty: effects of over and undernutrition," *Molecular and Cellular Endocrinology*, vol. 324, no. 1-2, pp. 70–81, 2010.

[23] I. S. Farooqi, S. A. Jebb, G. Langmack et al., "Effects of recombinant leptin therapy in a child with congenital leptin deficiency," *New England Journal of Medicine*, vol. 341, no. 12, pp. 879–884, 1999.

[24] H. Krude, H. Biebermann, W. Luck, R. Horn, G. Brabant, and A. Grüters, "Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans," *Nature Genetics*, vol. 19, no. 2, pp. 155–157, 1998.

[25] B. Dubern, C. Lubrano-Berthelier, M. Mencarelli et al., "Mutational analysis of the pro-opiomelanocortin gene in French obese children led to the identification of a novel deleterious heterozygous mutation located in the α-melanocyte stimulating hormone domain," *Pediatric Research*, vol. 63, no. 2, pp. 211–216, 2008.

[26] B. G. Challis, L. E. Pritchard, J. W. M. Creemers et al., "A missense mutation disrupting a dibasic prohormone processing site in pro-opiomelanocortin (POMC) increases susceptibility to early-onset obesity through a novel molecular mechanism," *Human Molecular Genetics*, vol. 11, no. 17, pp. 1997–2004, 2002.

[27] I. S. Farooqi, J. M. Keogh, G. S. H. Yeo, E. J. Lank, T. Cheetham, and S. O'Rahilly, "Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene," *New England Journal of Medicine*, vol. 348, no. 12, pp. 1085–1095, 2003.

[28] L. H. Larsen, S. M. Echwald, T. I. A. Sørensen, T. Andersen, B. S. Wulff, and O. Pedersen, "Prevalence of mutations and functional analyses of melanocortin 4 receptor variants identified among 750 men with juvenile-onset obesity," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 1, pp. 219–224, 2005.

[29] S. B. Heymsfield, A. S. Greenberg, K. Fujioka et al., "Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial," *Journal of the American Medical Association*, vol. 282, no. 16, pp. 1568–1575, 1999.

[30] S. O'Rahilly and I. S. Farooqi, "Human obesity: a heritable neurobehavioral disorder that is highly sensitive to environmental conditions," *Diabetes*, vol. 57, no. 11, pp. 2905–2910, 2008.

[31] A. T. Hattersley, "Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity," *Diabetic Medicine*, vol. 15, no. 1, pp. 15–24, 1998.

[32] A. Hattersley, J. Bruining, J. Shield, P. Njolstad, and K. C. Donaghue, "The diagnosis and management of monogenic diabetes in children and adolescents," *Pediatric Diabetes*, vol. 10, supplement 12, pp. 33–42, 2009.

[33] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 35, supplement 1, pp. S64–S71, 2012.

[34] M. I. McCarthy and A. T. Hattersley, "Learning from molecular genetics: novel insights arising from the definition of genes for monogenic and type 2 diabetes," *Diabetes*, vol. 57, no. 11, pp. 2889–2898, 2008.

[35] A. L. Gloyn, D. Phil, E. R. Pearson et al., "Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit Kir6. 2 and permanent neonatal diabetes," *The New England Journal of Medicine*, vol. 350, no. 18, pp. 1838–1849, 2004.

[36] I. K. Temple, R. J. Gardner, D. J. G. Mackay, J. C. K. Barber, D. O. Robinson, and J. P. H. Shield, "Transient neonatal diabetes: widening the understanding of the etiopathogenesis of diabetes," *Diabetes*, vol. 49, no. 8, pp. 1359–1366, 2000.

[37] I. K. Temple and J. P. H. Shield, "Transient neonatal diabetes, a disorder of imprinting," *Journal of Medical Genetics*, vol. 39, no. 12, pp. 872–875, 2002.

[38] A. S. Slingerland, B. M. Shields, S. E. Flanagan et al., "Referral rates for diagnostic testing support an incidence of permanent neonatal diabetes in three European countries of at least 1 in 260,000 live births," *Diabetologia*, vol. 52, no. 8, pp. 1683–1685, 2009.

[39] S. E. Flanagan, A. M. Patch, D. J. G. Mackay et al., "Mutations in ATP-sensitive K+ channel genes cause transient neonatal diabetes and permanent diabetes in childhood or adulthood," *Diabetes*, vol. 56, no. 7, pp. 1930–1937, 2007.

[40] L. E. Docherty, S. Kabwama, A. Lehmann et al., "Clinical presentation of 6q24 transient neonatal diabetes mellitus (6q24 TNDM) and genotype-phenotype correlation in an international cohort of patients," *Diabetologia*, vol. 56, no. 4, pp. 758–762, 2013.

[41] M. Kamiya, H. Judson, Y. Okazaki et al., "The cell cycle control gene ZAC/PLAGL1 is imprinted—a strong candidate gene for transient neonatal diabetes," *Human Molecular Genetics*, vol. 9, no. 3, pp. 453–460, 2000.

[42] A. P. Babenko, M. Polak, H. Cavé et al., "Activating mutations in the ABCC8 gene in neonatal diabetes mellitus," *New England Journal of Medicine*, vol. 355, no. 5, pp. 456–466, 2006.

[43] E. L. Edghill, S. E. Flanagan, A.-M. Patch et al., "Insulin mutation screening in 1, 044 patients with : mutations in the INS gene are a common cause of neonatal diabetes but a rare cause of diabetes diagnosed in childhood or adulthood," *Diabetes*, vol. 57, no. 4, pp. 1034–1042, 2008.

[44] F. M. Ashcroft, "ATP-sensitive potassium channelopathies: focus on insulin secretion," *Journal of Clinical Investigation*, vol. 115, no. 8, pp. 2047–2058, 2005.

[45] P. Proks, J. F. Antcliff, J. Lippiat, A. L. Gloyn, A. T. Hattersley, and F. M. Ashcroft, "Molecular basis of Kir6.2 mutations associated with neonatal diabetes or neonatal diabetes plus neurological features," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 50, pp. 17539–17544, 2004.

[46] E. R. Pearson, I. Flechtner, P. R. Njølstad et al., "Switching from insulin to oral sulfonylureas in patients with diabetes due to Kir6.2 mutations," *New England Journal of Medicine*, vol. 355, no. 5, pp. 467–477, 2006.

[47] A. S. Slingerland, W. Hurkx, K. Noordam et al., "Sulphonylurea therapy improves cognition in a patient with the V59M KCNJ11 mutation," *Diabetic Medicine*, vol. 25, no. 3, pp. 277–281, 2008.

[48] A. S. Slingerland, R. Nuboer, M. Hadders-Algra, A. T. Hattersley, and G. J. Bruining, "Improved motor development and good long-term glycaemic control with sulfonylurea treatment in a patient with the syndrome of intermediate developmental delay, early-onset generalised epilepsy and neonatal diabetes associated with the V59M mutation in the KCNJ11 gene," *Diabetologia*, vol. 49, no. 11, pp. 2559–2563, 2006.

[49] A. T. Hattersley and F. M. Ashcroft, "Activating mutations in Kir6.2 and neonatal diabetes: new clinical syndromes, new scientific insights, and new therapy," *Diabetes*, vol. 54, no. 9, pp. 2503–2513, 2005.

[50] G. S. Sellick, K. T. Barker, I. Stolte-Dijkstra et al., "Mutations in PTF1A cause pancreatic and cerebellar agenesis," *Nature Genetics*, vol. 36, no. 12, pp. 1301–1305, 2004.

[51] D. A. Stoffers, N. T. Zinkin, V. Stanojevic, W. L. Clarke, and J. F. Habener, "Pancreatic agenesis attributable to a single nucleotide

deletion in the human IPF1 gene coding sequence," *Nature Genetics*, vol. 15, no. 1, pp. 106–110, 1997.

[52] V. Senée, C. Chelala, S. Duchatelet et al., "Mutations in GLIS3 are responsible for a rare syndrome with neonatal diabetes mellitus and congenital hypothyroidism," *Nature Genetics*, vol. 38, no. 6, pp. 682–687, 2006.

[53] R. Spiegel, A. Dobbie, C. Hartman, L. de Vries, S. Ellard, and S. A. Shalev, "Clinical characterization of a newly described neonatal diabetes syndrome caused by RFX6 mutations," *American Journal of Medical Genetics A*, vol. 155, no. 11, pp. 2821–2825, 2011.

[54] E. de Franco, C. Shaw-Smith, S. E. Flanagan et al., "GATA6 mutations cause a broad phenotypic spectrum of diabetes from pancreatic agenesis to adult-onset diabetes without exocrine insufficiency," *Diabetes*, vol. 62, no. 3, pp. 993–997, 2013.

[55] O. Rubio-Cabezas, J. N. Jensen, M. I. Hodgson et al., "Permanent neonatal diabetes and enteric anendocrinosis associated with biallelic mutations in NEUROG3," *Diabetes*, vol. 60, no. 4, pp. 1349–1353, 2011.

[56] O. Rubio-Cabezas, J. A. L. Minton, I. Kantor, D. Williams, S. Ellard, and A. T. Hattersley, "Homozygous mutations in NEUROD1 are responsible for a novel syndrome of permanent neonatal diabetes and neurological abnormalities," *Diabetes*, vol. 59, no. 9, pp. 2326–2331, 2010.

[57] C. L. Bennett, J. Christie, F. Ramsdell et al., "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3," *Nature Genetics*, vol. 27, no. 1, pp. 20–21, 2001.

[58] M. Delépine, M. Nicolino, T. Barrett, M. Golamaully, G. Mark Lathrop, and C. Julier, "EIF2AK3, encoding translation initiation factor 2-α kinase 3, is mutated in patients with Wolcott-Rallison syndrome," *Nature Genetics*, vol. 25, no. 4, pp. 406–409, 2000.

[59] F. H. Sansbury, S. E. Flanagan, J. A. L. Houghton et al., "SLC2A2 mutations can cause neonatal diabetes, suggesting GLUT2 may have a role in human insulin secretion," *Diabetologia*, vol. 55, no. 9, pp. 2381–2385, 2012.

[60] C. Shaw-Smith, S. E. Flanagan, A.-M. Patch et al., "Recessive SLC19A2 mutations are a cause of neonatal diabetes mellitus in thiamine-responsive megaloblastic anaemia," *Pediatric Diabetes*, vol. 13, no. 4, pp. 314–321, 2012.

[61] A. S. Slingerland and A. T. Hattersley, "Activating mutations in the gene encoding Kir6.2 alter fetal and postnatal growth and also cause neonatal diabetes," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 7, pp. 2782–2788, 2006.

[62] F. M. Matschinsky and P. J. Randle, "Evolution of the glucokinase glucose sensor paradigm for pancreatic ta cells," *Diabetologia*, vol. 36, no. 11, pp. 1215–1217, 1993.

[63] D. Zelent, H. Najafi, S. Odili et al., "Glucokinase and glucose homeostasis: proven concepts and new ideas," *Biochemical Society Transactions*, vol. 33, part 1, pp. 306–310, 2005.

[64] P. R. Njølstad, O. Søvik, A. Cuesta-Muñoz et al., "Neonatal diabetes mellitus due to complete glucokinase deficiency," *New England Journal of Medicine*, vol. 344, no. 21, pp. 1588–1592, 2001.

[65] P. R. Njølstad, J. V. Sagen, L. Bjørkhaug et al., "Permanent neonatal diabetes caused by glucokinase deficiency: inborn error of the glucose-insulin signaling pathway," *Diabetes*, vol. 52, no. 11, pp. 2854–2860, 2003.

[66] K. K. Osbak, K. Colclough, C. Saint-Martin et al., "Update on mutations in glucokinase (GCK), which cause maturity-onset diabetes of the young, permanent neonatal diabetes, and hyperinsulinemic hypoglycemia," *Human Mutation*, vol. 30, no. 11, pp. 1512–1526, 2009.

[67] R. Singh and E. R. Pearson, "The importance of making a genetic diagnosis of diabetes," *Canadian Journal of Diabetes*, vol. 30, no. 2, pp. 183–190, 2006.

[68] A. Stride, M. Vaxillaire, T. Tuomi et al., "The genetic abnormality in the beta cell determines the response to an oral glucose load," *Diabetologia*, vol. 45, no. 3, pp. 427–435, 2002.

[69] G. Velho, H. Blanché, M. Vaxillaire et al., "Identification of 14 new glucokinase mutations and description of the clinical profile of 42 MODY-2 families," *Diabetologia*, vol. 40, no. 2, pp. 217–224, 1997.

[70] J. L. Leahy, S. Bonner-Weir, and G. C. Weir, "Minimal chronic hyperglycemia is a critical determinant of impaired insulin secretion after an incomplete pancreatectomy," *Journal of Clinical Investigation*, vol. 81, no. 5, pp. 1407–1414, 1988.

[71] R. P. Robertson and J. S. Harmon, "Diabetes, glucose toxicity, and oxidative stress: a case of double jeopardy for the pancreatic islet β cell," *Free Radical Biology and Medicine*, vol. 41, no. 2, pp. 177–184, 2006.

[72] D. Martin, C. Bellanné-Chantelot, I. Deschamps, P. Froguel, J. J. Robert, and G. Velho, "Long-term follow-up of oral glucose tolerance test-derived glucose tolerance and insulin secretion and insulin sensitivity indexes in subjects with glucokinase mutations (MODY2)," *Diabetes Care*, vol. 31, no. 7, pp. 1321–1323, 2008.

[73] S. Ellard, F. Beards, L. I. S. Allen et al., "A high prevalence of glucokinase mutations in gestational diabetic subjects selected by clinical criteria," *Diabetologia*, vol. 43, no. 2, pp. 250–253, 2000.

[74] C. Colom and R. Corcoy, "Maturity onset diabetes of the young and pregnancy," *Best Practice & Research. Clinical Endocrinology & Metabolism*, vol. 24, no. 4, pp. 605–615, 2010.

[75] G. Spyer, A. T. Hattersley, J. E. Sykes, R. H. Sturley, and K. M. MacLeod, "Influence of maternal and fetal glucokinase mutations in gestational diabetes," *American Journal of Obstetrics and Gynecology*, vol. 185, no. 1, pp. 240–241, 2001.

[76] A. J. Chakera, V. L. Carleton, S. Ellard et al., "Antenatal diagnosis of fetal genotype determines if maternal hyperglycemia due to a glucokinase mutation requires treatment," *Diabetes Care*, vol. 35, no. 9, pp. 1832–1834, 2012.

[77] R. Singh, E. Pearson, P. J. Avery et al., "Reduced beta cell function in offspring of mothers with young-onset type 2 diabetes," *Diabetologia*, vol. 49, no. 8, pp. 1876–1880, 2006.

[78] T. L. Jetton, Y. Liang, C. C. Pettepher et al., "Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut," *Journal of Biological Chemistry*, vol. 269, no. 5, pp. 3641–3654, 1994.

[79] S. S. Fajans, G. I. Bell, and K. S. Polonsky, "Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young," *New England Journal of Medicine*, vol. 345, no. 13, pp. 971–980, 2001.

[80] M. A. Maestro, C. Cardalda, S. F. Boj, R. F. Luco, J. M. Servitja, and J. Ferrer, "Distinct roles of HNF1beta, HNF1alpha, and HNF4alpha in regulating pancreas development, beta-cell function and growth," *Endocrine Development*, vol. 12, pp. 33–45, 2007.

[81] B. M. Shields, S. Hicks, M. H. Shepherd, K. Colclough, A. T. Hattersley, and S. Ellard, "Maturity-onset diabetes of the young (MODY): how many cases are we missing?" *Diabetologia*, vol. 53, no. 12, pp. 2504–2508, 2010.

[82] C. Bellanné-Chantelot, C. Carette, J. P. Riveline et al., "The type and the position of HNF1A mutation modulate age at diagnosis of diabetes in patients with maturity-onset diabetes of the young (MODY)-3," *Diabetes*, vol. 57, no. 2, pp. 503–508, 2008.

[83] A. Stride, S. Ellard, P. Clark et al., "β-cell dysfunction, insulin sensitivity, and glycosuria precede diabetes in hepatocyte nuclear factor-1α mutation carriers," *Diabetes Care*, vol. 28, no. 7, pp. 1751–1756, 2005.

[84] B. Isomaa, M. Henricsson, M. Lehto et al., "Chronic diabetic complications in patients with MODY3 diabetes," *Diabetologia*, vol. 41, no. 4, pp. 467–473, 1998.

[85] A. Steele, A. Hattersley, and E. Pearson, "Increased all-cause and cardiovascular mortality in MODY due to HNF-1alpha mutations," *Diabet Med*, vol. 21, supplement 2, article 18, 2004.

[86] E. Pearson, J. McEneny, I. Young, and A. Hattersley, "HDL-cholesterol: differentiating between HNF-1alpha MODY and type 2 diabetes," *Diabetic Medicine*, vol. 20, supplement 2, article 15, 2003.

[87] E. R. Pearson, B. J. Starkey, R. J. Powell, F. M. Gribble, P. M. Clark, and A. T. Hattersley, "Genetic cause of hyperglycaemia and response to treatment in diabetes," *The Lancet*, vol. 362, no. 9392, pp. 1275–1281, 2003.

[88] M. Shepherd, E. R. Pearson, J. Houghton, G. Salt, S. Ellard, and A. T. Hattersley, "No deterioration in glycemic control in HNF-1alpha maturity-onset diabetes of the young following transfer from long-term insulin to sulphonylureas," *Diabetes Care*, vol. 26, no. 11, pp. 3191–3192, 2003.

[89] M. Shepherd, B. Shields, S. Ellard, O. Rubio-Cabezas, and A. T. Hattersley, "A genetic diagnosis of HNF1A diabetes alters treatment and improves glycaemic control in the majority of insulin-treated patients," *Diabetic Medicine*, vol. 26, no. 4, pp. 437–441, 2009.

[90] M. Pontoglio, D. Prié, C. Cheret et al., "HNF1α controls renal glucose reabsorption in mouse and man," *The EMBO Reports*, vol. 1, no. 4, pp. 359–365, 2000.

[91] K. R. Owen, G. Thanabalasingham, T. J. James et al., "Assessment of high-sensitivity C-reactive protein levels as diagnostic discriminator of maturity-onset diabetes of the young due to HNF1A mutations," *Diabetes Care*, vol. 33, no. 9, pp. 1919–1924, 2010.

[92] R. E. J. Besser, M. H. Shepherd, T. J. McDonald et al., "Urinary C-peptide creatinine ratio is a practical outpatient tool for identifying hepatocyte nuclear factor 1-α/hepatocyte nuclear factor 4-α maturity-onset diabetes of the young from long-duration type 1 diabetes," *Diabetes Care*, vol. 34, no. 2, pp. 286–291, 2011.

[93] E. Pearson, A. M. Steele, T. G. Barrett et al., *A Novel Cause of Hyperinsulinemic Hypoglycemia and Increased Birth Weight Is Seen in Patients with HNF-4A but Not HNF-1A Mutations*, American Diabetes Association, Orlando, Fla, USA, 2006, 66th Scientific Sessions, 1585-poster.

[94] E. L. Edghill, C. Bingham, S. Ellard, and A. T. Hattersley, "Mutations in hepatocyte nuclear factor-1β and their related phenotypes," *Journal of Medical Genetics*, vol. 43, no. 1, pp. 84–90, 2006.

[95] F. J. Iborra, H. Kimura, and P. R. Cook, "The functional organization of mitochondrial genomes in human cells," *BMC Biology*, vol. 2, article 9, 2004.

[96] R. Murphy, D. M. Turnbull, M. Walker, and A. T. Hattersley, "Clinical features, diagnosis and management of maternally inherited diabetes and deafness (MIDD) associated with the 3243A>G mitochondrial point mutation," *Diabetic Medicine*, vol. 25, no. 4, pp. 383–399, 2008.

[97] J. A. Maassen, G. M. C. Janssen, and L. M. 'T Hart, "Molecular mechanisms of mitochondrial diabetes (MIDD)," *Annals of Medicine*, vol. 37, no. 3, pp. 213–221, 2005.

[98] Y. Oka, H. Katagiri, H. Ishihara, T. Asano, T. Kobayashi, and M. Kikuchi, "β-cell loss and glucose induced signalling defects in diabetes mellitus caused by mitochondrial tRNA(Leu(UUR)) gene mutation," *Diabetic Medicine*, vol. 13, no. 6, pp. S98–S102, 1996.

[99] P. J. Guillausseau, D. Dubois-Laforgue, P. Massin et al., "Heterogeneity of diabetes phenotype in patients with 3243 bp mutation of mitochondrial DNA (Maternally Inherited Diabetes and Deafness or MIDD)," *Diabetes and Metabolism*, vol. 30, no. 2, pp. 181–186, 2004.

[100] J. A. Maassen, L. M. 'T Hart, E. van Essen et al., "Mitochondrial diabetes: molecular mechanisms and clinical presentation," *Diabetes*, vol. 53, supplement 1, pp. S103–S109, 2004.

[101] P. J. Guillausseau, P. Massin, D. Dubois-LaForgue et al., "Maternally inherited diabetes and deafness: a multicenter study," *Annals of Internal Medicine*, vol. 134, no. 9 part 1, pp. 721–728, 2001.

[102] M. Laloi-Michelin, T. Meas, C. Ambonville et al., "The clinical variability of maternally inherited diabetes and deafness is associated with the degree of heteroplasmy in blood leukocytes," *The Journal of Clinical Endocrinology & Metabolism*, vol. 94, no. 8, pp. 3025–3030, 2009.

[103] M. I. McCarthy, "Genomics, type 2 diabetes, and obesity," *New England Journal of Medicine*, vol. 363, no. 24, pp. 2339–2350, 2010.

[104] W. L. Donohue and I. Uchida, "Leprechaunism. A euphuism for a rare familial disorder," *The Journal of Pediatrics*, vol. 45, no. 5, pp. 505–519, 1954.

[105] J. Hone, D. Accili, H. Psiachou et al., "Homozygosity for a null allele of the insulin receptor gene in a patient with leprechaunism," *Human Mutation*, vol. 6, no. 1, pp. 17–22, 1995.

[106] C. Musso, E. Cochran, S. A. Moran et al., "Clinical course of genetic diseases of the insulin receptor (type A and Rabson-Mendenhall syndromes): a 30-year prospective," *Medicine*, vol. 83, no. 4, pp. 209–222, 2004.

[107] R. K. Semple, E. K. Cochran, M. A. Soos et al., "Plasma adiponectin as a marker of insulin receptor dysfunction," *Diabetes Care*, vol. 31, no. 5, pp. 977–979, 2008.

[108] T. Okada, C. W. Liew, J. Hu et al., "Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 21, pp. 8977–8982, 2007.

[109] P. D. Cruz Jr. and J. A. Hud Jr., "Excess insulin binding to insulin-like growth factor receptors: proposed mechanism for acanthosis nigricans," *Journal of Investigative Dermatology*, vol. 98, supplement 6, pp. 82S–85S, 1992.

[110] L. Poretsky, N. A. Cataldo, Z. Rosenwaks, and L. C. Giudice, "The insulin-related ovarian regulatory system in health and disease," *Endocrine Reviews*, vol. 20, no. 4, pp. 535–582, 1999.

[111] S. George, J. J. Rochford, C. Wolfrum et al., "A family with severe insulin resistance and diabetes due to a mutation in AKT2," *Science*, vol. 304, no. 5675, pp. 1325–1328, 2004.

[112] N. Kubota, T. Kubota, S. Itoh et al., "Dynamic functional relay between insulin receptor substrate 1 and 2 in hepatic insulin signaling during fasting and feeding," *Cell Metabolism*, vol. 8, no. 1, pp. 49–64, 2008.

[113] A. Garg, "Lipodystrophies," *American Journal of Medicine*, vol. 108, no. 2, pp. 143–152, 2000.

[114] A. K. Agarwal, E. Arioglu, S. de Almeida et al., "AGPAT2 is mutated in congenital generalized lipodystrophy linked to chromosome 9q34," *Nature Genetics*, vol. 31, no. 1, pp. 21–23, 2002.

[115] M. Chandalia, A. Garg, F. Vuitch, and F. Nizzi, "Clinical case seminar: postmortem findings in congenital generalized lipodystrophy," *Journal of Clinical Endocrinology and Metabolism*, vol. 80, no. 10, pp. 3077–3081, 1995.

[116] A. K. Agarwal and A. Garg, "Congenital generalized lipodystrophy: significance of triglyceride biosynthetic pathways," *Trends in Endocrinology and Metabolism*, vol. 14, no. 5, pp. 214–221, 2003.

[117] K. M. Szymanski, D. Binns, R. Bartz et al., "The lipodystrophy protein seipin is found at endoplasmic reticulum lipid droplet junctions and is important for droplet morphology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 52, pp. 20890–20895, 2007.

[118] V. A. Payne, N. Grimsey, A. Tuthill et al., "The human lipodystrophy gene BSCL2/Seipin may be essential for normal adipocyte differentiation," *Diabetes*, vol. 57, no. 8, pp. 2055–2060, 2008.

[119] A. K. Agarwal, V. Simha, E. A. Oral et al., "Phenotypic and genetic heterogeneity in congenital generalized lipodystrophy," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 10, pp. 4840–4847, 2003.

[120] Y. K. Hayashi, C. Matsuda, M. Ogawa et al., "Human PTRF mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy," *Journal of Clinical Investigation*, vol. 119, no. 9, pp. 2623–2633, 2009.

[121] A. Rajab, V. Straub, L. J. McCann et al., "Fatal cardiac arrhythmia and long-QT syndrome in a new form of congenital generalized lipodystrophy with muscle rippling (CGL4) due to PTRF-CAVIN mutations," *PLoS Genetics*, vol. 6, no. 3, Article ID e1000874, 2010.

[122] C. A. Kim, M. Delépine, E. Boutet et al., "Association of a homozygous nonsense caveolin-1 mutation with berardinelli-seip congenital lipodystrophy," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 4, pp. 1129–1134, 2008.

[123] A. Garg and A. K. Agarwal, "Caveolin-1: a new locus for human lipodystrophy," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 4, pp. 1183–1185, 2008.

[124] A. Garg, "Clinical review#: lipodystrophies: genetic and acquired body fat disorders," *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 11, pp. 3313–3325, 2011.

[125] S. Shackleton, D. J. Lloyd, S. N. J. Jackson et al., "LMNA, encoding lamin A/C, is mutated in partial lipodystrophy," *Nature Genetics*, vol. 24, no. 2, pp. 153–156, 2000.

[126] J. Rankin and S. Ellard, "The laminopathies: a clinical review," *Clinical Genetics*, vol. 70, no. 4, pp. 261–274, 2006.

[127] R. A. Hegele, H. Cao, C. Frankowski, S. T. Mathews, and T. Leff, "PPARG F388L, a transactivation-deficient mutant, in familial partial lipodystrophy," *Diabetes*, vol. 51, no. 12, pp. 3586–3590, 2002.

[128] O. Rubio-Cabezas, V. Puri, I. Murano et al., "Partial lipodystrophy and insulin resistant diabetes in a patient with a homozygous nonsense mutation in CIDEC," *EMBO Molecular Medicine*, vol. 1, no. 5, pp. 280–287, 2009.

[129] S. Gandotra, C. Le Dour, W. Bottomley et al., "Perilipin deficiency and autosomal dominant partial lipodystrophy," *The New England Journal of Medicine*, vol. 364, no. 8, pp. 740–748, 2011.

[130] J. M. Guettier, J. Y. Park, E. K. Cochran et al., "Leptin therapy for partial lipodystrophy linked to a PPAR-γ mutation," *Clinical Endocrinology*, vol. 68, no. 4, pp. 547–554, 2008.

[131] A. Y. Chong, B. C. Lupsa, E. K. Cochran, and P. Gorden, "Efficacy of leptin therapy in the different forms of human lipodystrophy," *Diabetologia*, vol. 53, no. 1, pp. 27–35, 2010.

[132] E. Danforth Jr., "Failure of adipocyte differentiation causes type II diabetes mellitus?" *Nature Genetics*, vol. 26, no. 1, article 13, 2000.

[133] S. Virtue and A. Vidal-Puig, "Adipose tissue expandability, lipotoxicity and the metabolic syndrome—an allostatic perspective," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1801, no. 3, pp. 338–349, 2010.

[134] I. S. Farooqi, "Genetic aspects of severe childhood obesity," *Pediatric Endocrinology Reviews*, vol. 3, supplement 4, pp. 528–536, 2006.

[135] C. J. Willer, E. K. Speliotes, R. J. F. Loos et al., "Six new loci associated with body mass index highlight a neuronal influence on body weight regulation," *Nature Genetics*, vol. 41, no. 1, pp. 25–34, 2009.

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Research Article

Paradoxical Glucose-Sensitizing yet Proinflammatory Effects of Acute ASP Administration in Mice

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Acylation stimulating protein (ASP) is an adipokine derived from the immune complement system, which stimulates fat storage and is typically increased in obesity, type 2 diabetes, and cardiovascular disease. Using a diet-induced obesity (DIO) mouse model, the acute effects of ASP on energy metabolism and inflammatory processes *in vivo* were evaluated. We hypothesized that ASP would specifically exert proinflammatory effects. C57Bl/6 wild-type mice were put on a high-fat-high-sucrose diet for 12 weeks. Mice were then subjected to both glucose and insulin tolerance tests, each manipulation being preceded by recombinant ASP or vehicle (control) bolus injection. ASP supplementation increased whole-body glucose excursion, and this was accomplished with reduced concomitant insulin levels. However, ASP did not directly alter insulin sensitivity. ASP supplementation induced a proinflammatory phenotype, with higher levels of cytokines including IL-6 and TNF-α in plasma and in adipose tissue, liver, and skeletal muscle mRNA. Additionally, ASP increased M1 macrophage content of these tissues. ASP exerted a direct concentration-dependent role in the migration and M1 activation of cultured macrophages. Altogether, the *in vivo* and *in vitro* experiments demonstrate that ASP plays a role in both energy metabolism and inflammation, with paradoxical whole-body glucose-sensitizing yet proinflammatory effects.

1. Introduction

The past decades of obesity research have seen a change of paradigm. Adipose tissue was once labeled as a passive storage organ, while obesity comorbidities were predominantly linked with energy metabolism disorders. Currently, it is widely recognized that adipose tissue also plays a major endocrine role and that progressive metabolic diseases such as type 2 diabetes and atherosclerosis manifest a strong immune component. Moreover, molecules secreted by the expanding adipose tissue modulate systemic inflammation and contribute heavily to the pathology of the aforementioned diseases [1].

Acylation stimulating protein (ASP), identical to C3adesArg, is a protein generated through the alternative complement pathway of the immune system [2]. C3a/C3adesArg is rapidly produced during acute immunological responses such as bacterial infection, which triggers the classical or lectin pathways of the complement system. However, in the absence of acute immune response, spontaneous activation of the alternative pathway of complement, mediated through adipsin, factor B, and complement C3 convertase, leads to the generation of C3a. C3a is rapidly desarginated by carboxypeptidase B or N to generate C3adesArg, termed ASP based on its specific metabolic effects. ASP acts through its only known receptor, C5L2. This 7-transmembrane receptor is widely expressed, with expression in tissues such as liver, muscle, adipose tissue, immune cells, and brain, and C5L2 shares significant homology with other complement receptors such as C3a receptor and C5a receptor [3].

In vitro, the functional role of ASP/C3adesArg has been extensively studied on adipocytes. Its primary effect is the

promotion of fat storage, accomplished by increasing diacylglycerol acyltransferase activity [4] and GLUT4 translocation [5, 6], which drive fatty acid and glucose uptake into cells and provide the substrates for triglyceride synthesis. These effects have been shown to be independent and additive to those of insulin [7]. In adipocytes, ASP action is mediated through activation of PI3kinase, phospholipases C and A2, Akt phosphorylation, and protein kinase C [8].

The presence of C5L2 is required for ASP response in adipocytes [9–12], but in other cell types, the interaction of ASP with C5L2 remains controversial. However, C5a is also a ligand for both C5L2 and C5aR [13–15]. While C3a binds C3a receptor (C3aR), initiating a potent proinflammatory signaling cascade, C3adesArg does not bind C3aR, and it has canonically been considered as an inert fragment, indicative of complement system activation, and subsequent proteolytic cleavage of C3a [16]. However, C3adesArg has also been shown to be implicated in several immune processes, with less potency than C3a [17–20]. For example, C3adesArg retains indirect chemotactic properties on hematopoietic cells [19] and can also induce cytokine secretion by cultured macrophages and modulate liver regeneration [20].

ASP has been shown to possess a metabolic role in both humans and rodents. Acute postprandial production of ASP within the adipose tissue bed has been demonstrated in human studies, and this acute production directly correlates with dietary fatty acid uptake into adipose tissues [21, 22]. By contrast, ASP levels are chronically increased in obesity, type 2 diabetes, and cardiovascular diseases in humans [23], and higher circulating levels of fasting ASP in humans are associated with a delayed clearance of postprandial triglyceride [24]. This suggests that acute ASP responses may be very different from the metabolic profile associated with chronically elevated ASP, and the exact consequences of acute versus chronic ASP in these diseases remain unclear.

The aim of the present study was to assess the effects of acute ASP administration on *in vivo* glucose metabolism and rapid inflammatory responses in a diet-induced obesity (DIO) mouse model. We hypothesized that ASP could modulate both adipose inflammation and alter systemic glucose disposal.

2. Materials and Methods

2.1. Animals. Male C57Bl/6 wild-type mice were obtained through breeding in our internal colony. All mice were individually housed in a sterile barrier facility with a 12 h light 12 h dark cycles. At 8 weeks, mice were placed on high-fat-high-sucrose (HF/HS) diet (58% kcal fat D12331; Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks. All mice were weighed and randomly separated into two groups of equal body weight at week 11. Throughout the study, treated mice received a total of three ASP doses (high physiological) intraperitoneally (1.5 μg/g body weight), and control mice received three PBS (vehicle) injections at the following times: (i) before glucose tolerance test (GTT) early week 11, (ii) ITT late week 11, and (iii) GTT sacrifice week 12, with a minimum of 5 days recovery between each injection. Blood was collected through cardiac puncture, and tissues were harvested and immediately frozen in liquid nitrogen. Tissues were subsequently transferred to −80°C and stored for further analysis. All protocols were approved and were conducted in accordance with the Canadian Council of Animal Care (CCAC) guidelines and approved by the Laval University Animal Care Committee.

2.2. Recombinant ASP. Recombinant human ASP was prepared as previously described, using a Ni^{2+}-Sepharose column for initial purification via binding to the 6XHis-tag sequence at the amino-terminal, followed by HPLC purification as described in detail elsewhere [25]. No denaturing agents were used at any step in the purification to avoid ASP inactivation. Recombinant ASP purity was assessed with mass spectrometry and was endotoxin-free as evidenced by the Limulus amebocyte lysate (LAL) endotoxin assay (Lonza, Walkersville, MD).

2.3. Plasma Analysis. Blood was collected through cardiac puncture. Plasma triglycerides, non-esterified fatty acids (NEFA), and glucose were measured using colorimetric enzymatic kits as follows: plasma triglyceride (Roche Diagnostics, Richmond, VA, USA), NEFA (Wako Chemicals, Richmond, VA, USA), and glucose (Sigma, Saint Louis, MO, USA). IL-6, IL-10, G-CSF, GM-CSF, KC, MCP-1, MIP-1α, TNF-α, insulin, leptin, and PAI-1 were measured using suspension bead array immunoassay kits following the manufacturer's specifications (Bio-Plex Pro Mouse Cytokine Assay 23-plex and Bio-Plex Pro Mouse Diabetes Assay 8-plex, Biorad, Mississauga, ON, Canada) on a Bio-Plex series 100 instrument (BioRad, Mississauga, ON, Canada), and information on intra- and interassay CVs IS provided by the manufacturer and is freely available.

2.4. Glucose and Insulin Tolerance Tests. A glucose tolerance test (GTT) was performed on mice (at week 11 of their diet) after an overnight fast. Following an intraperitoneal glucose injection (2 mg/g body weight) supplemented with/without ASP (1.5 μg/g body weight), blood samples were taken at 0, 15, 30, 60, and 90 min. Mice were allowed 5 days to recover. An insulin tolerance test (ITT) was then performed following a 4-hour fast. Blood samples were taken at 0, 15, 30, 60, and 90 min after an intraperitoneal insulin injection (1 mU/g of body weight) supplemented with/without ASP (1.5 μg/g body weight) as indicated. Glucose was measured using a colorimetric enzymatic kit (Sigma, Saint Louis, MO, USA), while insulin was measured using a RIA kit (Linco, St. Charles, MO, USA), and information on intra- and interassay CVs is provided by the manufacturer and is freely available.

2.5. Tissue Glucose Uptake. At the end of the 12-week diet protocol, overnight-fasted mice received an intraperitoneal injection of glucose (2 mg/g body weight) supplemented with radioactive deoxyglucose (0.5 μCi (^{3}H) deoxyglucose/g of body weight) and ASP (1.5 μg/g body weight) and were euthanized exactly 2 hours later. Radioactive glucose uptake in visceral adipose tissue, muscle, and liver was evaluated using (^{3}H) deoxyglucose tracer uptake in tissue homogenates. Raw

Table 1: Real-time qPCR primers.

F4-80 forward	CTTTGGCTATGGGCTTCCAGTC
F4-80 reverse	GCAAGGAGGACAGAGTTTATCGTG
CD11c forward	CTGGATAGCCTTTCTTCTGCTG
CD11c reverse	GCACACTGTGTCCGAACTC
CD163 forward	GGGTCATTCAGAGGCACACTG
CD163 reverse	CTGGCTGTCCTGTCAAGGCT
IL-6 forward	GAGGATACCACTCCCAACAGACC
IL-6 reverse	AAGTGCATCATCGTTGTTCATACA
KC forward	TCTCCGTTACTTGGGGACAC
KC reverse	CCACACTCAAGAATGGTCGC
MCP-1 forward	ATTGGGATCATCTTGCTGGT
MCP-1 reverse	CCTGCTGTTCACAGTTGCC
MIP-1α forward	GTGGAATCTTCCGGCTGTAG
MIP-1α reverse	ACCATGACACTCTGCAACCA
PAI-1 forward	GCCAGGGTTGCACTAAACAT
PAI-1 reverse	GCCTCCTCATCCTGCCTAA
TNF-α forward	CATCTTCTCAAAATTCGAGTGACAA
TNF-α reverse	TGGGAGTAGACAAGGTACAACCC

Quantitative real-time PCR primer sequences used in evaluation of expression.

dpm results were corrected for total protein content (muscle and liver) or weight (adipose tissue) and are expressed as relative deoxyglucose uptake.

2.6. Tissue Lipid. Adipose tissue, liver and muscle triglycerides, and NEFA were extracted from tissues using heptane : isopropanol (3 : 2). The organic extract was transferred, while the remaining tissue (liver and muscle) was air dried, dissolved in 0.3 N NaOH, and assessed for protein content using the Bradford method (Bio-Rad, Mississauga, ON, Canada). Organic extracts were lyophilized, and lipids were redissolved in 10% Triton X-100 aqueous solution. Triglycerides and NEFA were measured using commercial colorimetric kits as described above. Results are expressed as μmoles of triglycerides or NEFA per gram (g) of protein.

2.7. Real-Time Quantitative PCR. Tissue or cell mRNA was extracted, purified, and reverse transcribed into cDNA using RNeasy Mini Kits or RNeasy Lipid Tissue Mini Kits and QuantiTect Reverse Transcription Kits (Qiagen, Gaithersburg, MD, USA). mRNA for *F4-80*, *CD11c*, *CD163*, *IL-6*, *KC*, *MCP-1*, *MIP-1α*, *PAI-1*, and *TNF-α* was quantified using custom primers (Table 1). Relative gene expression was calculated and corrected using *GAPDH* (QuantiTect Primer Assay, Qiagen, Gaithersburg, MD, USA) as housekeeping gene. All procedures followed the manufacturer's instructions and minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [26].

2.8. Macrophage Migration. Macrophage migration was assessed using QCM Chemotaxis 5 μM 24-well cell migration assay (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Briefly, confluent RAW 264.7 cells were harvested and resuspended in serum-free media, and 1×10^6 cells were added to each insert. Adipocyte-conditioned media was obtained by incubating 3T3-L1 differentiated adipocytes overnight in serum-free DMEM/F12. 3T3-L1-conditioned media or serum-free medium control was then added to the lower chambers, with or without ASP (50 nM or 200 nM). Cells were incubated for 20 hours at 37°C in a CO_2 incubator. Macrophages that migrated through the membrane were quantified following the manufacturer's instructions.

2.9. Macrophage Polarization. RAW 264.7 cells were seeded in 25 cm^2 flasks (1×10^6 cells per flask) in DMEM/F12 with 10% FBS medium. Cells were incubated for 24 h to permit attachment and cultured to reach 80% confluence. Flasks were then subjected to a combination of a polarization treatment with/without a fixed ASP concentration. Polarization treatments were as follows: *Control treatment* consisted of serum-free media; *M1-polarization treatment* consisted of 100 ng/mL LPS and 100 ng/mL of IFN-γ; *M2-polarization treatment* consisted of 10 ng/mL of IL-4 and 10 ng/mL of IL-13. Concentrations of ASP used in conjunction with each treatment were 0 nM (baseline), 50 nM (low physiological), and 200 nM (high physiological). Cells were incubated 6 hours at 37°C in a CO_2 incubator. After incubation, cells were immediately lysed, and mRNA was extracted.

2.10. Statistical Analysis. Results are expressed as mean ± SEM. Groups were compared using the appropriate statistical test, either one-way ANOVA with linear trend post hoc test, two-way ANOVA with Student-Newman-Keuls post hoc test, or t-test using Prism 5.0 software (GraphPad, CA, USA). Statistical significance was set as $P < 0.05$, where $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ and where $0.05 < P < 0.1$ was considered a tendency.

3. Results

3.1. ASP Injections Did not Alter Body Weight or Food Intake. Mice were injected with high-physiological doses of ASP at the indicated time periods, as described in the methods. No change in body weight or food intake between ASP-treated animals and controls was recorded during the 12-week treatment period.

3.2. ASP Alters Substrate Partitioning. An intraperitoneal glucose tolerance test (GTT) with/without ASP was performed. The recombinant ASP dose was chosen to represent a high-physiological concentration of endogenous ASP, such as seen in severely obese individuals [22]. Glucose and insulin levels were monitored throughout a 90-minute timeframe after the injection (Figure 1). A more efficient glucose clearance was achieved with acute ASP supplementation (Figure 1(a), $P < 0.05$). Concomitant insulin levels showed a statistical tendency to be lower in the ASP-treated mice (Figure 1(b), $P = 0.08$). In order to assess if ASP acutely influenced insulin sensitivity, an intraperitoneal insulin tolerance test (ITT) was performed in the same animals, supplemented or not with ASP. No difference in insulin-mediated glucose response was

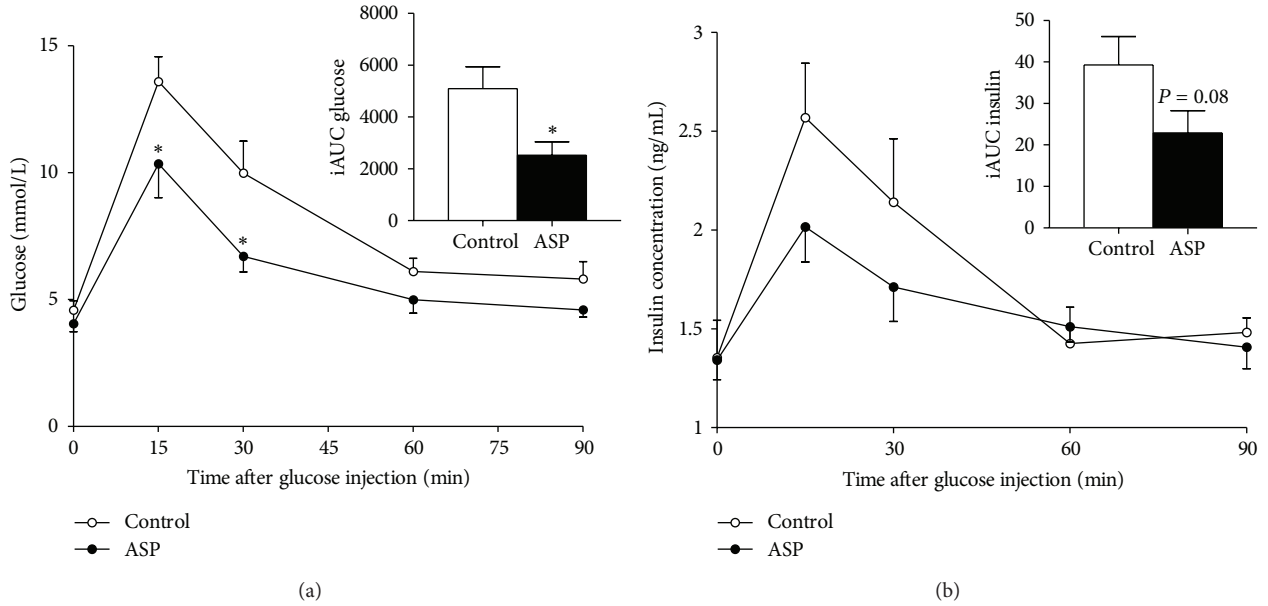

Figure 1: Glucose excursion is altered by ASP. Glucose with/without ASP was administered, and plasma glucose and insulin were measured over 90 minutes. Glucose levels are shown in (a) and concomitant insulin levels in (b). Incremental area under the curve for each graph is shown in the insert. Data are presented for glucose (mmol/L, (a)) and insulin (ng/mL, (b)). Results are expressed as mean ± SEM where differences versus controls (vehicle) are expressed as $^{*}P < 0.05$, with $n = 7$-8 per group.

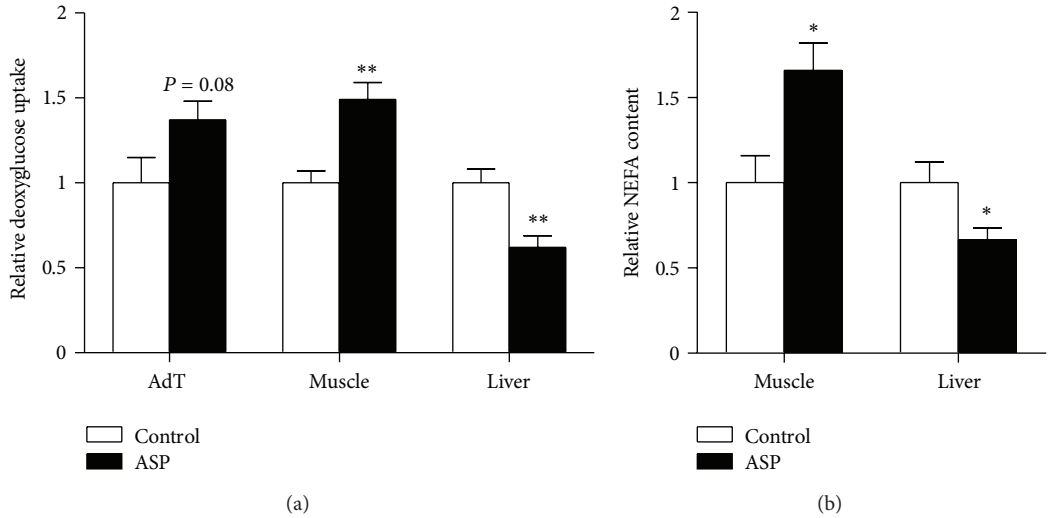

Figure 2: ASP affects substrate partitioning. Relative ^{3}H-deoxyglucose uptake in adipose tissue (AdT), liver and skeletal muscle is shown in (a). Relative nonesterified fatty acid content of liver, and skeletal muscle is shown in (b). Results are expressed as relative absorption or content compared with controls (vehicle) which are set as 1.0. Results are expressed as mean ± SEM where differences versus controls (vehicle) are expressed as $^{*}P < 0.05$ and $^{**}P < 0.01$, with $n = 7$-8 per group.

detected in mice treated acutely with/without ASP (data not shown).

A GTT with (^{3}H) deoxyglucose supplemented with/without ASP was used to evaluate ASP effects on glucose uptake in specific tissues. Acute ASP treatment altered glucose uptake in a tissue-specific fashion; tissues relying mostly on active transport showed increased or tended to increase glucose uptake (adipose tissue, $P = 0.08$, and skeletal muscle, $P < 0.01$), while the liver, relying mainly on passive transport, exhibited lower glucose uptake ($P < 0.01$), as shown in Figure 2(a).

Acute ASP treatment did not alter muscle or liver triglyceride (data not shown). However, cellular NEFA content was increased in skeletal muscle ($P < 0.05$) and reduced in liver ($P < 0.05$), as seen in Figure 2(b). Circulating NEFA was decreased as well ($P < 0.05$) (Table 2).

Table 2: Plasma lipids and cytokines.

Cytokine (unit)	Control ($n = 8$)	ASP ($n = 7$)
TG (mmol/L)	1.2 ± 0.2	1.1 ± 0.1
NEFA (mmol/L)	0.76 ± 0.07	0.53 ± 0.04*
IL-6 (pg/mL)	1.8 ± 0.9	22.8 ± 3.7***
IL-10 (pg/mL)	6.8 ± 2.3	18.6 ± 4.8$^{\#}$
G-CSF (pg/mL)	38.8 ± 7.7	70.2 ± 12.9$^{\#}$
GM-CSF (pg/mL)	14.3 ± 2.5	29.3 ± 5.9*
KC (pg/mL)	65.3 ± 12.2	403.4 ± 95.1**
MIP-1α (pg/mL)	3.2 ± 0.7	6.76 ± 1.2*
TNF-α (pg/mL)	39.6 ± 4.4	105.0 ± 24.1*
PAI-1 (ng/mL)	827 ± 167	1382 ± 84**
Leptin (μg/mL)	16.1 ± 4.0	6.5 ± 2.2*

Plasma characteristics of mice, with/without ASP injection. Plasma lipids and cytokines were compared by t-tests. Results are expressed as mean ± sem where $^{\#}P = 0.08$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

3.3. ASP Increased Plasma Cytokine Concentrations and Altered Liver and Adipose Tissue Cytokine Expression. The ASP bolus also induced acute changes in adipokine and cytokine profiles. Plasma leptin was reduced, while several inflammation-related cytokines including IL-6, IL-10, PAI-1, TNF-α, KC, and MIP-1α were significantly increased (Figure 3(a), Table 2). mRNA levels for these same cytokines were evaluated in liver and adipose tissue, with increased expression of IL-6, KC, TNF-α, and MCP-1 in both tissues and increased MIP-1α and PAI-1 in liver (Figure 3(a)). A global inflammatory phenotype is clearly induced by acute ASP injection in obese mice, mediated by both adipose tissue and liver changes.

3.4. Macrophage Infiltration and Polarization Is Influenced by ASP. Macrophage infiltration and polarization were evaluated in adipose tissue, skeletal muscle, and liver. As shown in Figure 3(b), macrophage marker F4/80 was not significantly altered by ASP treatment, although it tended to increase in the skeletal muscle ($P = 0.07$). No change was seen in anti-inflammatory M2 polarization marker (CD163). However, in all tissues, proinflammatory M1 polarization marker was significantly higher or tended to increase (adipose tissue, $P < 0.05$, skeletal muscle, $P = 0.07$, liver, $P = 0.10$), suggesting a preferential activation of infiltrating macrophages to an M1 phenotype with acute ASP treatment. In order to further investigate the direct role of ASP in the physiological changes observed in mice, *in vitro* effects of ASP on macrophage migration and polarization in RAW 264.7 macrophages were evaluated. ASP showed dose-dependent chemoattractant migration properties when incubated alone with macrophages (Figure 4(a)), and these effects were further additive to those of 3T3-L1 adipocyte-conditioned media. ASP by itself had very limited capacity to induce macrophage M1 or M2 phenotype polarization (Figures 4(b) and 4(c)). However, RAW 264.7 macrophages treated with INF-γ and LPS to stimulate M1 polarization, as assessed by CD11c mRNA expression (Figure 4(b)), showed further M1 polarization with ASP treatment, in a dose-dependent fashion (Figure 4(b)). By contrast, while treatment with IL-4 and IL-13 increased M2 polarization, as assessed through CD163 mRNA expression (Figure 4(c)), ASP did not further enhance this effect.

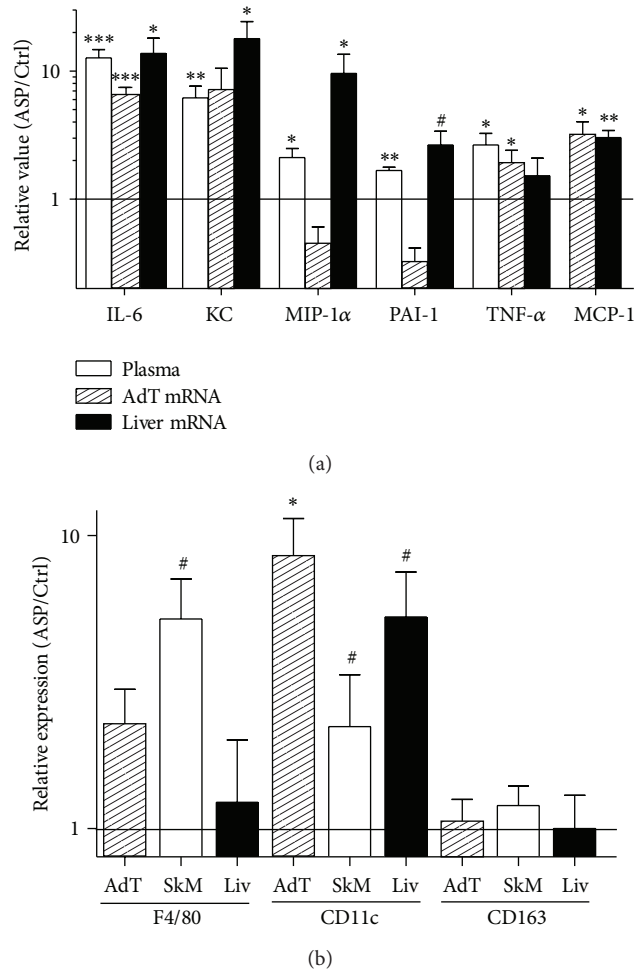

Figure 3: ASP induces proinflammatory effects *in vivo*. Relative plasma levels, adipose tissue, and liver mRNA content of several cytokines are shown in (a), for mice that were injected with ASP as compared to controls (PBS as vehicle). For the same animals, relative mRNA levels of total macrophages marker (F4/80), M1 macrophages marker (CD11c), and M2 macrophage marker (CD163) within adipose tissue, liver, and muscle are shown in (b). Results are expressed as relative concentration or expression compared with controls (vehicle) which are set as 1.0. Results are expressed as mean ± SEM where differences versus controls are expressed as $^{\#}P = 0.08$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, with $n = 7$-8 per group.

4. Discussion

In the present study, the inflammatory role of acute high-physiological ASP administration in a context of established obesity was evaluated. Previous studies have focused either on the metabolic role of ASP in adipocytes or the immune role of ASP/C3adesArg in immune cells. This study examines a broader, more integrative physiological role of ASP. This study

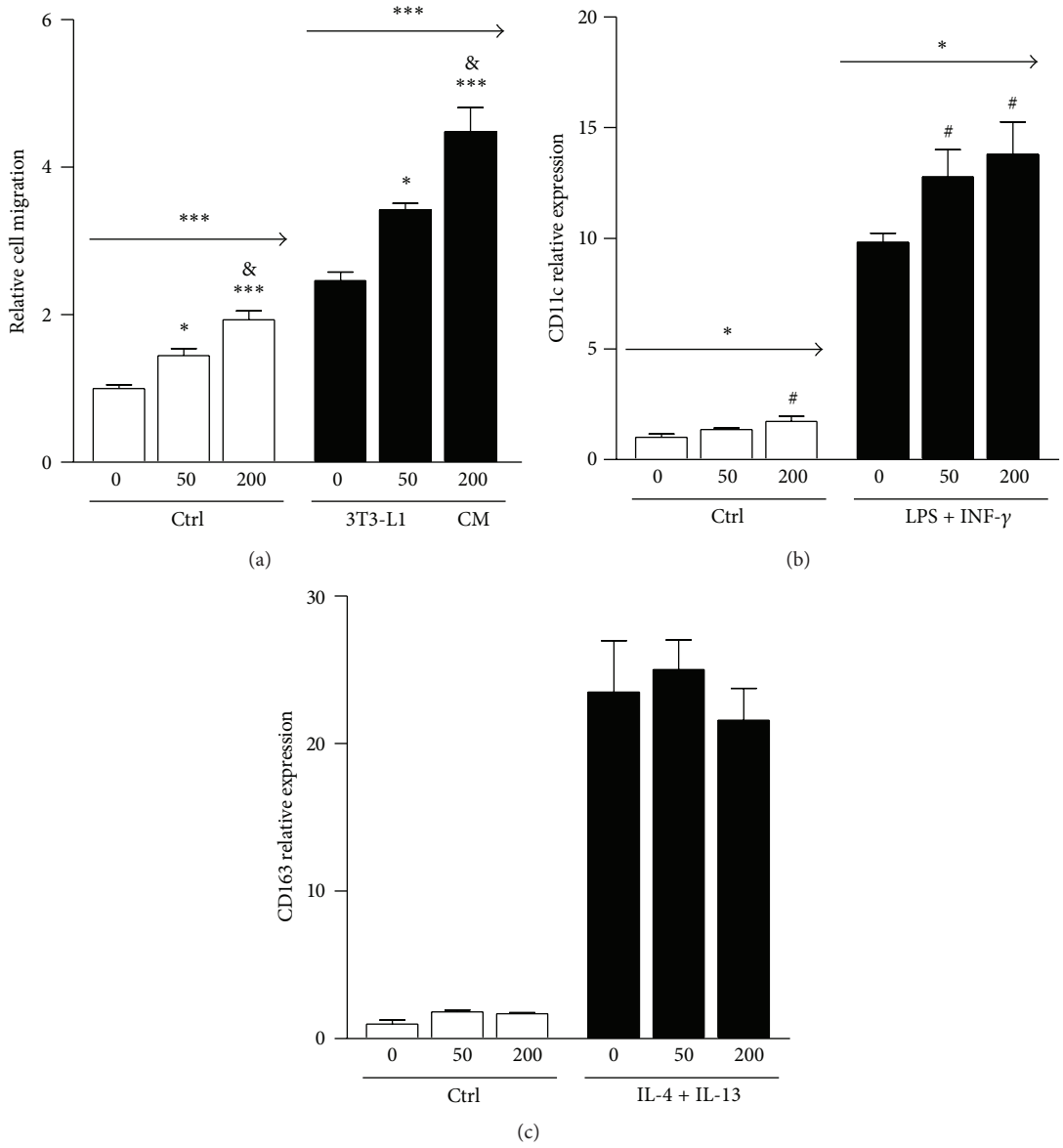

FIGURE 4: ASP induces proinflammatory effects *in vitro*. In (a), *in vitro* chemoattractive effects of three concentrations of ASP combined or not with adipocyte conditioned media on macrophages are shown. ASP effects on macrophage polarization to M1 phenotype are shown using M1 macrophage marker CD11c in (b) while ASP effects on M2 polarization are shown using M2 macrophage marker CD163 in (c). Results are expressed as relative expression or migration compared with Controls. Results are expressed as mean ± SEM where differences versus Control are expressed as $^{\#}P = 0.08$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, with $n = 4$. & are used in (a) to indicate a $P < 0.05$ significant difference in between 200 nM and 50 nM ASP concentration effect. Arrows are used to indicate a significant linear trend by ANOVA analysis where $^{*}P < 0.05$ and $^{***}P < 0.001$.

clearly demonstrated that ASP/C3adesArg acutely enhances glucose-insulin clearance mechanisms in spite of its acute paradoxical *in vivo* and *in vitro* proinflammatory effects.

One advantage of the current study is that acute ASP effects rather than chronic effects were evaluated. In previous studies, effects of chronic administration of ASP were evaluated in either C3KO or wild-type mice [27, 28], demonstrating effects on decreased insulin sensitivity, weight gain, and lipid/glucose metabolism. However, it is difficult to establish whether these effects are direct or indirect or a consequence of resistance induced by chronic stimulation. Similar concerns are an issue in knockout mice models where the proinflammatory effects [27, 29] may result from indirect disruptions in the immune system. Specifically, C3KO mice lack ASP but also lack an active complement system; C5L2KO mice lack ASP/C5L2 interaction but also lack C5a/C5L2 interaction which could disrupt the balance in C5a/C5aR-mediated effects.

The results shown in this study are not consequent to changes in body weight or food intake, which could have

been confounding factors. We can therefore directly relate the changes observed to acute bolus ASP-mediated effects. The dose of ASP used was high physiological-pathological, as it theoretically could increase circulating ASP levels in obese mice by up to tenfold (from 400 nM to 4,2 μM) and would mimic the high concentration of ASP generated following an acute immune activation of the complement system or seen in obese individuals. The most striking results obtained are the rapid, global increases in plasma inflammatory cytokines and the M1 polarization of tissue macrophages upon acute ASP treatment. We show additional *in vitro* evidence that supports a direct role for ASP in macrophage recruitment and M1 activation that is additive to classic cytokine effects. Notwithstanding the demonstrated proinflammatory ASP effects, acute ASP enhances plasma glucose excursion in DIO mice, with increased uptake into skeletal muscle and adipose tissue and reduced liver glucose uptake, and this effect is mediated in the presence of lower circulating insulin levels. While this could point towards increased insulin sensitivity, we also demonstrated that a single acute dose of ASP does not directly alter insulin sensitivity. We therefore speculate that the known *in vitro* activating effect of ASP on GLUT4 translocation in adipocyte and muscle cells [6] could explain the increase in muscle and adipose tissue glucose uptake, while passive concentration-dependent uptake in the liver, relying mostly on GLUT2 transporters, would indirectly be lowered.

Our study, while demonstrating intriguing novel properties of ASP, is also consistent with previously published results. Chronic ASP supplementation decreased plasma NEFA, as in the present study [28]. An acute ASP bolus, on the other hand, has been shown to increase glucose excursion following a fat load [30], while C5L2$^{-/-}$ (ASP receptor) deficient mice showed delayed glucose clearance [12]. Additionally, the potential chemoattractive role proposed for ASP [19, 20] has been demonstrated specifically in this study. He et al. also showed that high levels of ASP increased TNF-α and IL-6 liver expression [20]. We demonstrate here that this phenotype is also present in other tissues as well as in the circulation and extends to other major cytokines. By contrast, leptin levels were acutely decreased by ASP bolus. This effect could be potentially linked with the reduction of insulin secretion, a well-known stimulator of leptin release.

Inflammation and macrophage recruitment are inherent components of several biological systems related to tissue injury and infections and are beneficial in the short term. At the same time, the capacity of immune cells to exert their respective functions is modulated by their metabolic status. To label ASP acute effects as deleterious because of the proinflammatory capacities is likely an oversimplification of a potentially complex process. Certain cytokines exhibit both proinflammatory and glucose-sensitizing effects, while others have dual roles (that are either concentration dependent or time dependent) in immunity. Adiponectin is a well-known adipokine that improves insulin sensitivity but has been shown to exert both anti-inflammatory and proinflammatory effects, depending on the context [31]. IL-6 is another prime example of a cytokine with paradoxical effects; IL-6 increases glucose excursion through a short-term insulin-sensitizing process in spite of its role in inflammation [32, 33]. Long-term exposure to IL-6 may then, paradoxically, induce insulin resistance [34].

Humans with obesity, type 2 diabetes, and cardiovascular disease have been shown to exhibit higher chronic plasma ASP levels [2]. These morbidities are associated with macrophage infiltration and M1 polarization within several tissues. However, even in the absence of chronically increased ASP, adipose tissue acutely increases local ASP production postprandially [21, 22]. Thus, the response to an acute increase versus chronically elevated levels may differ and may depend on the concomitant diet (such as high-fat and high-sucrose diet). While it has been suggested that C3adesArg is simply an inactive immune by-product representative of complement activation, we clearly demonstrate in this study a direct role of ASP within these inflammatory processes. The relative contribution of acute ASP-stimulated inflammation in obesity-related diseases remains to be investigated. As with several signaling pathways in obesity, it could be that chronic substrate overload leads to excessive activation of the ASP signaling pathway, eliciting a deleterious response that could exacerbate the development of comorbidities through a low-grade proinflammatory phenotype transition [3].

5. Conclusion

The present study sheds new light on the dual role of ASP in energy metabolism and inflammation. ASP exhibits both substrate storage and proinflammatory properties that have been strongly associated with several pathologies. Effectively, neutralizing a hormone that can be both produced locally and acts locally represents a strong pharmaceutical challenge. A TNF-α inhibitor, for example, failed at altering insulin sensitivity in a human cohort [35]. Additional studies on C5L2, the only known receptor for ASP, are needed to better understand ASP-C5L2 signal transduction and to assess the potential of C5L2 as a pharmaceutical target in obesity and metabolic syndrome.

Abbreviations

ASP: Acylation stimulating protein
C3aR: Complement C3a receptor
DIO: Diet-inducedobesity
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
GTT: Glucose tolerance test
HF/HS: High-fat/high-sucrose
ITT: Insulin tolerance test
KC: Keratinocyte-derived chemokine
MCP-1: Monocyte chemoattractant protein-1
MIP-1α: Macrophage inflammatory protein-1α
NEFA: Nonesterified fatty acids
PAI-1: Plasminogen activator inhibitor-1
TG: Triglyceride.

Conflict of Interests

The authors have no conflict of interests to declare.

Author's Contribution

A. Fisette designed the study, acquired data, analyzed results, and wrote the paper. P. Poursharifi acquired data and reviewed paper. K. Oikonomopoulou acquired data and reviewed paper. M. N. Munkonda acquired data and reviewed paper. M. Lapointe generated recombinant ASP and reviewed paper. K. Cianflone designed the study and reviewed paper.

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References

[1] E. Maury and S. M. Brichard, "Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome," *Molecular and Cellular Endocrinology*, vol. 314, no. 1, pp. 1–16, 2010.

[2] K. Cianflone, Z. Xia, and L. Y. Chen, "Critical review of acylation-stimulating protein physiology in humans and rodents," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1609, no. 2, pp. 127–143, 2003.

[3] A. Fisette and K. Cianflone, "The ASP and C5L2 pathway: another bridge between inflammation and metabolic homeostasis," *Clinical Lipidology*, vol. 5, no. 3, pp. 367–377, 2010.

[4] Z. Yasruel, K. Cianflone, A. D. Sniderman, M. Rosenbloom, M. Walsh, and M. A. Rodriguez, "Effect of acylation stimulating protein on the triacylglycerol synthetic pathway of human adipose tissue," *Lipids*, vol. 26, no. 7, pp. 495–499, 1991.

[5] M. Maslowska, A. D. Sniderman, R. Germinario, and K. Cianflone, "ASP stimulates glucose transport in cultured human adipocytes," *International Journal of Obesity*, vol. 21, no. 4, pp. 261–266, 1997.

[6] Y. Tao, K. Cianflone, A. D. Sniderman, S. P. Colby-Germinario, and R. J. Germinario, "Acylation-stimulating protein (ASP) regulates glucose transport in the rat L6 muscle cell line," *Biochimica et Biophysica Acta—Lipids and Lipid Metabolism*, vol. 1344, no. 3, pp. 221–229, 1997.

[7] V. Van Harmelen, S. Reynisdottir, K. Cianflone et al., "Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin," *Journal of Biological Chemistry*, vol. 274, no. 26, pp. 18243–18251, 1999.

[8] M. Maslowska, H. Legakis, F. Assadi, and K. Cianflone, "Targeting the signaling pathway of acylation stimulating protein," *Journal of Lipid Research*, vol. 47, no. 3, pp. 643–652, 2006.

[9] D. Kalant, S. Cain, M. Maslowska, A. Sniderman, K. Cianflone, and P. Monk, "The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg(77)/acylation-stimulating protein," *Journal of Biological Chemistry*, vol. 278, no. 11, pp. 123–129, 2003.

[10] D. Kalant, R. MacLaren, W. Cui et al., "C5L2 is a functional receptor for acylation-stimulating protein," *Journal of Biological Chemistry*, vol. 280, no. 25, pp. 23936–23944, 2005.

[11] W. Cui, M. Simaan, S. Laporte, R. Lodge, and K. Cianflone, "C5a- and ASP-mediated C5L2 activation, endocytosis and recycling are lost in S323I-C5L2 mutation," *Molecular Immunology*, vol. 46, no. 15, pp. 3086–3098, 2009.

[12] S. Paglialunga, P. Schrauwen, C. Roy et al., "Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in C5L2 knockout mice," *Journal of Endocrinology*, vol. 194, no. 2, pp. 293–304, 2007.

[13] N. P. Gerard and C. Gerard, "The chemotactic receptor for human C5a anaphylatoxin," *Nature*, vol. 349, no. 6310, pp. 614–617, 1991.

[14] P. N. Monk, A. M. Scola, P. Madala, and D. P. Fairlie, "Function, structure and therapeutic potential of complement C5a receptors," *British Journal of Pharmacology*, vol. 152, no. 4, pp. 429–448, 2007.

[15] N. J. Chen, C. Mirtsos, D. Suh et al., "C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a," *Nature*, vol. 446, no. 7132, pp. 203–207, 2007.

[16] V. A. Bokisch and H. J. Müller-Eberhard, "Anaphylatoxin inactivator of human plasma: its isolation and characterization as a carboxypeptidase," *Journal of Clinical Investigation*, vol. 49, no. 12, pp. 2427–2436, 1970.

[17] N. Haeffner-Cavaillon, J. M. Cavaillon, M. Laude, and M. D. Kazatchkine, "C3a(C3adesArg) induces production and release of interleukin 1 by cultured human monocytes," *Journal of Immunology*, vol. 139, no. 3, pp. 794–799, 1987.

[18] T. Takabayashi, E. Vannier, B. D. Clark et al., "A new biologic role for C3a and C3a desArg: regulation of TNF-α and IL-1β synthesis," *Journal of Immunology*, vol. 156, no. 9, pp. 3455–3460, 1996.

[19] M. Honczarenko, B. Lu, A. Nicholson-Weller, N. P. Gerard, L. E. Silberstein, and C. Gerard, "C5L2 receptor is not involved in C3a/C3a-desArg-mediated enhancement of bone marrow hematopoietic cell migration to CXCL12 [7]," *Leukemia*, vol. 19, no. 9, pp. 1682–1683, 2005.

[20] S. He, C. Atkinson, F. Qiao, K. Cianflone, X. Chen, and S. Tomlinson, "A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice," *Journal of Clinical Investigation*, vol. 119, no. 8, pp. 2304–2316, 2009.

[21] J. Saleh, L. K. M. Summers, K. Cianflone, B. A. Fielding, A. D. Sniderman, and K. N. Frayn, "Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue *in vivo* in the postprandial period," *Journal of Lipid Research*, vol. 39, no. 4, pp. 884–891, 1998.

[22] D. Kalant, S. Phélis, B. A. Fielding, K. N. Frayn, K. Cianflone, and A. D. Sniderman, "Increased postprandial fatty acid trapping in subcutaneous adipose tissue in obese women," *Journal of Lipid Research*, vol. 41, no. 12, pp. 1963–1968, 2000.

[23] A. Onat, G. Can, R. Rezvani, and K. Cianflone, "Complement C3 and cleavage products in cardiometabolic risk," *Clinica Chimica Acta*, vol. 412, no. 13-14, pp. 1171–1179, 2011.

[24] K. Cianflone, R. Zakarian, C. Couillard, B. Delplanque, J. P. Despres, and A. Sniderman, "Fasting acylation-stimulating protein is predictive of postprandial triglyceride clearance," *Journal of Lipid Research*, vol. 45, no. 1, pp. 124–131, 2004.

[25] W. Cui, M. Lapointe, D. Gauvreau, D. Kalant, and K. Cianflone, "Recombinant C3adesArg/acylation stimulating protein (ASP) is highly bioactive: a critical evaluation of C5L2 binding and 3T3-L1 adipocyte activation," *Molecular Immunology*, vol. 46, no. 16, pp. 3207–3217, 2009.

[26] S. A. Bustin, V. Benes, J. A. Garson et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments," *Clinical Chemistry*, vol. 55, no. 4, pp. 611–622, 2009.

[27] M. N. Munkonda, M. Lapointe, P. Miegueu et al., "Recombinant acylation stimulating protein administration to C3(-/-) mice increases insulin resistance via adipocyte inflammatory mechanisms," *PLoS One*, vol. 7, Article ID e46883, 2012.

[28] S. Paglialunga, A. Fisette, M. Munkonda, Y. Gao, D. Richard, and K. Cianflone, "The effects of acylation stimulating protein supplementation VS antibody neutralization on energy expenditure in wildtype mice," *BMC Physiology*, vol. 10, no. 1, article 4, 2010.

[29] A. Fisette, M. N. Munkonda, K. Oikonomopoulou, S. Paglialunga, J. D. Lambris, and K. Cianflone, "C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice," *Immunobiology*, vol. 218, no. 1, pp. 127–133, 2012.

[30] I. Murray, A. D. Sniderman, and K. Cianflone, "Enhanced triglyceride clearance with intraperitoneal human acylation stimulating protein in C57BL/6 mice," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 277, no. 3, pp. E474–E480, 1999.

[31] M. Y. Jung, H. S. Kim, H. J. Hong, B. S. Youn, and T. S. Kim, "Adiponectin induces dendritic cell activation via PLCγ/JNK/NF-κB pathways, leading to Th1 and Th17 polarization," *The Journal of Immunology*, vol. 188, pp. 2592–2601, 2012.

[32] B. K. Pedersen, M. A. Febbraio, and R. A. Mooney, "Interleukin-6 does/does not have a beneficial role in insulin sensitivity and glucose homeostasis," *Journal of Applied Physiology*, vol. 102, no. 2, pp. 814–819, 2007.

[33] R. A. Mooney, "Counterpoint: interleukin-6 does not have a beneficial role in insulin sensitivity and glucose homeostasis," *Journal of Applied Physiology*, vol. 102, no. 2, pp. 816–819, 2007.

[34] J. J. Senn, P. J. Klover, I. A. Nowak, and R. A. Mooney, "Interleukin-6 induces cellular insulin resistance in hepatocytes," *Diabetes*, vol. 51, no. 12, pp. 3391–3399, 2002.

[35] L. E. Bernstein, J. Berry, S. Kim, B. Canavan, and S. K. Grinspoon, "Effects of etanercept in patients with the metabolic syndrome," *Archives of Internal Medicine*, vol. 166, no. 8, pp. 902–908, 2006.

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Research Article

Glucagon-Like Peptide-1 Triggers Protective Pathways in Pancreatic Beta-Cells Exposed to Glycated Serum

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Advanced glycation end products (AGEs) might play a pathophysiological role in the development of diabetes and its complications. AGEs negatively affect pancreatic beta-cell function and the expression of transcriptional factors regulating insulin gene. Glucagon-like peptide-1 (GLP-1), an incretin hormone that regulates glucose homeostasis, might counteract the harmful effects of AGEs on the beta cells in culture. The aim of this study was to identify the intracellular mechanisms underlying GLP-1-mediated protection from AGE-induced detrimental activities in pancreatic beta cells. HIT-T15 cells were cultured for 5 days with glycated serum (GS, consisting in a pool of AGEs), in the presence or absence of 10 nmol/L GLP-1. After evaluation of oxidative stress, we determined the expression and subcellular localization of proteins involved in maintaining redox balance and insulin gene expression, such as nuclear factor erythroid-derived 2 (Nrf2), glutathione reductase, PDX-1, and MafA. Then, we investigated proinsulin production. The results showed that GS increased oxidative stress, reduced protein expression of all investigated factors through proteasome activation, and decreased proinsulin content. Furthermore, GS reduced ability of PDX-1 and MafA to bind DNA. Coincubation with GLP-1 reversed these GS-mediated detrimental effects. In conclusion, GLP-1, protecting cells against oxidants, triggers protective intercellular pathways in HIT-T15 cells exposed to GS.

1. Introduction

Pancreatic beta cell dysfunction is a key pathophysiological target in diabetes mellitus [1–3]. The concept that glucose via glycation as well as glucotoxicity is one of the main damaging molecules is widely accepted [4, 5]. Furthermore, hyperglycemia increases the production of AGEs, a group of compounds derived from the nonenzymatic reaction between reducing sugars and proteins, lipids, and DNA [6]. It is well known that a long-lasting deleterious effect of hyperglycemia persists independently of the level of glucose [7–9]. This "memory" might be explained by the persistent overproduction of reactive oxygen species (ROS) directly induced by AGEs via the activation of their receptors [10]. Furthermore, the increase in pancreatic beta-cell responsiveness to oxidants [11, 12] might result in a decreased nuclear availability of the regulators of insulin promoters PDX-1 (pancreatic and duodenal homeobox-1) and MafA (v-maf musculoaponeurotic fibrosarcoma oncogene homologue A) [13–16]. Recently, we also showed that exposure of pancreatic beta-cells to AGEs decreased glutathione (GSH) availability and negatively affected expression and subcellular localization of PDX-1 [11, 16]. Since GSH is a pivotal antioxidant factor [17] regulated via the new synthesis of GSH from GSSG (glutathione disulphide) by glutathione reductase (GSR), we also focused on these molecular mechanisms. It has been reported that both GSH synthesis and GSR expression are regulated by nuclear factor erythroid 2 p45-related factor 2 (Nrf2), a basic leucine zipper transcription factor that in response to oxidative stress translocates to the nucleus

Table 1: DNA sequences of the sense and antisense primers for RT-PCR analysis and cycler conditions.

Gene	Accession number (GeneBank)	Primer sequences	Product size (bp)	Annealing temperature (°C)	Number of cycles
Preproinsulin	XM_003508080.1	5′-*CTTTTGTCAAACAGCACCTTTGTG*-3′ *(sense)* 5′-*TCAGTTGCAGTAGTTCTCCAGTTGG*-3′ *(antisense)*	263	48	25
PDX-1	XM_003496658.1	5′-*CAGCTCCCTTTCCCGTGG A*-3′ *(sense)* 5′-*GAGCATCACTGCCAGCTCCA*-3′ *(antisense)*	204	55	28
Nrf2	XM_003498316.1	5′-*CTCACTGGATAAAGAAGTC*-3′ *(sense)* 5′-*CCGCCCAGAAGTTCAGAGA*-3′ *(antisense)*	240	60	40
β-Actin	NM_031144.2	5′-*AAGAGAAGCTGTGCTATGTTGC*-3′ *(sense)* 5′-*CCTTGATCTTCATGGTGCTAGG*-3′ *(antisense)*	324	48	25

PDX-1, Pancreatic and duodenal homeobox 1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2.

and binds to antioxidant-response elements (AREs) in the promoters of target genes [18, 19]. Interestingly, it has been also reported that Nrf2 is upregulated by analogues of glucagon-like peptide-1 (GLP-1) [20]. Given the potential regulatory activity of GLP-1 (an incretin hormone that participates to glucose homeostasis [21]), the aim of the present study was to identify the potential protective pathways triggered by GLP-1 to counteract pancreatic beta-cell dysfunction mediated by glycated serum (GS).

2. Materials and Methods

2.1. Cell Culture and Stimulation. The hamster pancreatic beta-cell line, HIT-T15, was purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were grown in RPMI 1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 100 IU penicillin-G, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Culture media were replaced every 2 days. When cells reached confluence, they were passaged after trypsin-EDTA detachment and seeded in multiwell plates for various experiments. Then, HIT-T15 cells were plated in 6-well dishes (7×10^5 cells per well) and incubated for 5 days in media containing GS in the presence or absence of 10 nmol/L GLP-1. The investigation protocol was conformed to the principles outlined in the Declaration of Helsinki.

2.2. Preparation of GS. GS was prepared by incubation at 37°C for 7 days of heat-inactivated FBS with 50 mmol/L ribose. The serum was then extensively dialyzed against 0.1 M PBS, pH 7.4, to remove surplus sugar. Aliquots of FBS were processed in the same way but without ribose (NGS) and were used for standard culture (CTR). Content of pentosidine, a well-known marker of glycoxidative stress, was evaluated by HPLC and used as a measure of proteins' glycation [22]. The concentration of pentosidine in the experimental medium containing GS ranged between 1.5 and 4×10^5 pmol/l, which corresponds to the plasma levels of pentosidine detected in the plasma of diabetic patients [23].

2.3. Reactive Oxygen Species Detection. Intracellular reactive oxygen species (ROS) level was measured using the cell-permeable fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Milan, Italy). In brief, cells were seeded into 6-well culture plates at 7×10^5 cells/well and treated for 5 days with GS with or without GLP-1 then washed twice with Hank's Buffered Salt Solution (HBSS) and incubated with fresh DCFH-DA (10 μM) in HBSS for 30 min at 37°C in 5% CO_2. DCFH-DA stock solution (20.5 mM) was prepared in DMSO and stored at −20°C for maximum one month. After that, cells were washed twice in HBSS, and wells were filled with 1 mL HBSS before fluorescence acquisition in a plate reader (TECAN InfinitePro200) (Ex: λ485/Em: λ535 nm). Fluorescent emission was normalized to total protein content.

2.4. Reverse Transcriptase Polymerase Reaction. Total RNA was extracted from HIT-T15 with RNeasy kit (QIAGEN s.r.l., Milan, Italy) according to manufacturer's instructions. The RNA concentrations were determined spectrophotometrically and equal quantities of total RNA were used from different samples. One microgram of RNA was reverse transcripted to cDNA using GoScript Reverse Transcription System (PROMEGA ITALIA, Milan, Italy) and then amplified by PCR. As reported in Table 1 all the samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles (28 cycles for Actin, Preproinsulin and PDX-1; 40 cycles for Nrf2). PCR products were electrophoresed onto a 1.5% agarose gel containing EuroSafe Nucleic Acid Stain (EUROCLONE S.p.A, Milan, Italy) and visualized under UV light. The relative intensities of the bands were quantified by densitometric analysis.

2.5. Cell Lysis and Subcellular Fractionation. At the end of the experiments, a set of HIT-T15 cells were lysed in RIPA buffer (50 mmol/L Tris HCl pH 7.5, 150 mmol/L NaCl, 1% NP40, 0.1% SDS), supplemented with protease and phosphatase inhibitors. Another set of HIT-T15 cells was processed for subcellular fractionation using the Subcellular Protein Fractionation Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, various cellular compartments were isolated by sequential addition of different extraction buffers to the cell pellet. Each subcellular fraction was collected after centrifugation and stored at −80°C. Nuclear soluble and chromatin-bound protein

extracts obtained from each experimental condition were used for immunoblot analysis. Protein concentration of each sample was determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

2.6. Immunoblot. Thirty micrograms of total cell lysate were separated on an SDS-PAGE and transferred onto nitrocellulose. Filters were blocked in 5% BSA and incubated overnight at 4°C with primary specific antibodies (against: GSR, Nrf2, and β-Actin from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA; against: PDX-1 from Millipore, Billerica, MA, USA; against MafA from Bethyl Lab.). Secondary specific horseradish-peroxidase-linked antibodies were added for 1 h at room temperature. Bound antibodies were detected using an enhanced chemiluminescence lighting system (Luminata Classico, Millipore, Billerica, MA, USA), according to manufacturer's instructions. Bands of interest were quantified by densitometry using the NIH program ImageJ. To verify equal loading of the proteins, membranes were stripped again, reblocked, and reprobed to detect β-actin. Values of proteins of interest were normalized to total amounts of β-actin and expressed as percentages of NGS control (defined as 100%).

2.7. siRNA. For the GLP-1R siRNA experiment a pool of 4 prevalidated siRNAsdesigned for hamster GLP-1R were used (Riboxx GmbH, Radebeul, Germany). HIT-T15 cells were seeded in 12-well plates in culture medium without antibiotics and grown overnight to reach 40% confluency. The next day, FECT-siRNA complexes were prepared according to manufacturer's instructions. Cells were transfected with 20 nmol/L GLP-1RsiRNA or control siRNA (which correspond to a nontargetting 23-nucleotide siRNA designed as negative control) for 24 h before switching to fresh culture medium. Forty-eight hours after transfection cells were incubated for 5 days in media containing GS and GLP-1. We tested GLP-1R protein level by immunoblotting. Transfection results in 50% knockdown of GLP-1R at 48 h, and 7 days after transfection.

2.8. Proinsulin Content. To evaluate proinsulin content another set of cells, grown in the same conditions, was washed twice with PBS, pH 7.4, at 0°C, extracted with acid/ethanol (0.15 mol/L HCl in 75% ethanol in H_2O) for 16 h at 0°C, then centrifuged at 15,000 ×g at 4°C. Supernatants were collected and stored at −20°C until the proinsulin determination was performed by ELISA (Mercodia AB, Uppsala, Sweden). The results were normalized to total protein concentration.

2.9. Statistical Analysis. The results are representative of at least 3 experiments. All analyses were carried out with the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean±SE and then analysed using Student's t-test. P value <0.05 was considered as statistically significant.

3. Results

3.1. GLP-1 Reduces GS-Mediated ROS Release. Exposure of HIT-T15 cells to GS significantly increased (by 1.5-fold) the release of ROS as compared to control (CTR). Coincubation with GLP-1 abrogated GS-mediated ROS production (Figure 1).

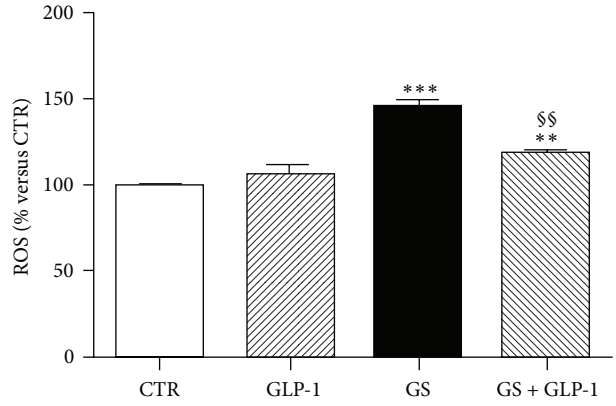

Figure 1: GLP-1 abrogates AGE-induced intracellular ROS production. After treatment for 5 days in standard medium (CTR) or in medium containing AGEs (GS) in the presence or absence of 10 nmol/L GLP-1, HIT-T15 cells were prelabeled with DCFH-DA for 30 min and fluorescence was analyzed. Data were expressed as the mean ± SE of four independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus CTR; $^{§§}P < 0.01$ versus GS.

3.2. GLP-1 Restores Nrf2 Protein Levels in Pancreatic Beta-Cells Exposed to GS. We have recently shown that incubation with GS alters oxidative stress and the availability of the reduced form of glutathione (GSH) in the same culture model of pancreatic beta-cells [11]. Since lower levels of GSH were found in mice lacking the transcriptional repressor Nrf2 [24], which is implicated in the regulation of detoxification enzymes [25], we investigated whether Nrf2 expression (both mRNA and protein) was affected by GS and/or GLP-1. mRNA expression of Nrf2 was not affected by the incubation with GS in the presence or absence of GLP-1 (Figures 2(a) and 2(b)). However, GLP-1 significantly upregulated the protein expression of Nrf2 (Figures 2(c) and 2(d)) in cells cultured with GS for 5 days. Since Nrf2 has been shown to maintain GSH homeostasis by upregulating the expression of GSR [19], we further investigated whether GS and GLP-1 might affect expression of GSR. Our results showed that GS did not affect GSR protein expression (Figures 3(a) and 3(b)). On the other hand, GLP-1 increased GSR protein expression in both control and GS-treated cells. However, the statistical analysis revealed a significant increase only for control cells (Figures 3(a) and 3(b)).

3.3. GLP-1 Restores MafA and PDX-1 Levels in Pancreatic Beta-Cells Exposed to GS. Oxidative stress-induced damage has been shown as related to the downregulation of the endocrine transcription factors MafA and PDX-1 [26]. Figure 4 showed that a glycated environment (GS) significantly decreased MafA expression. Coincubation with GLP-1 to the GS restored MafA protein expression (Figures 4(a) and 4(b)). GLP-1 also improved

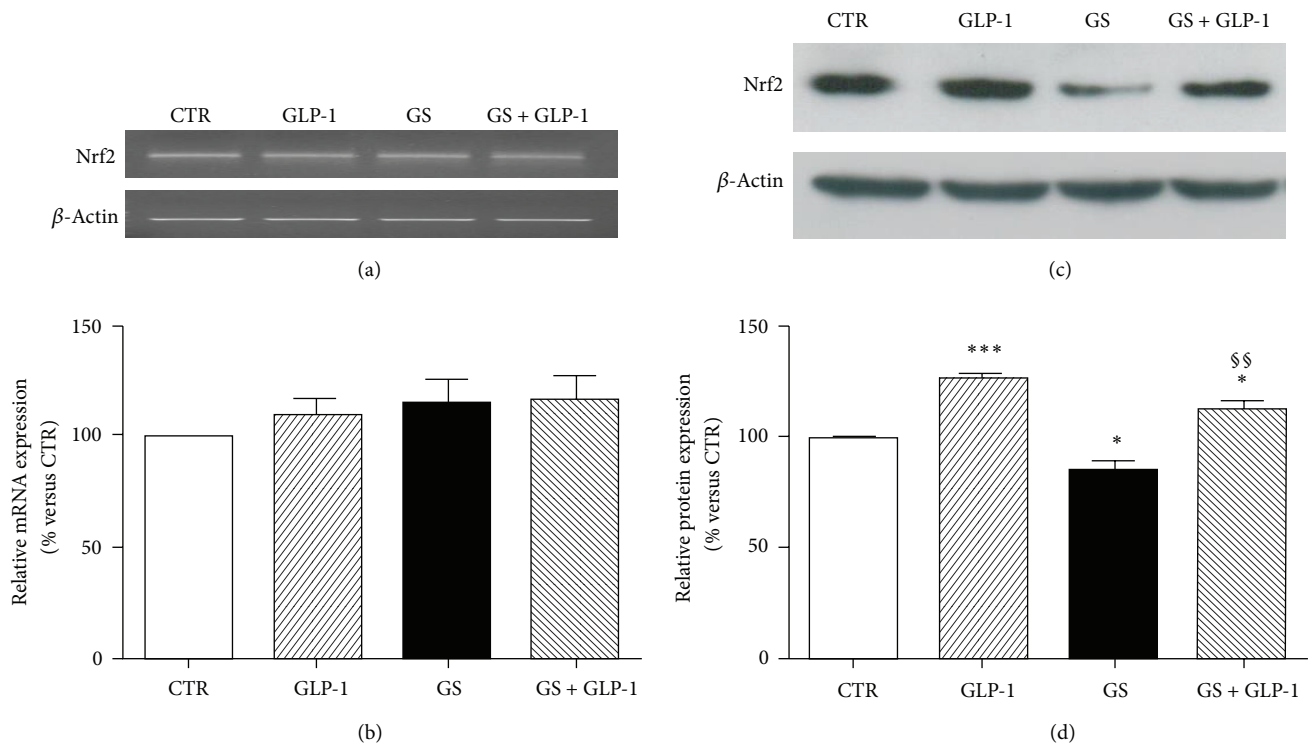

Figure 2: GLP-1 restores Nrf2 protein levels in HIT-T15 cells cultured in the presence of GS. (a)-(b) Semiquantitative RT-PCR of Nrf2 mRNA expression in HIT-T15 cells cultured 5 days in standard medium (CTR) or in medium containing AGEs (GS) in the presence or absence of 10 nmol/L GLP-1. (a) Representative agarose gel of three different experiments. (b) Quantification of densitometries of agarose gel bands. Data were represented as mean±SE of percentage change from CTR level (100%) ($n = 3$). (c)-(d) Western blot analysis of Nrf2 protein intracellular expression in HIT-T15 cells cultured in the same conditions as mRNA experiments. (c) Representative western blot of three different experiments. (d) Quantification of densitometries of western blot bands. Data were expressed as mean ± SE of fold induction relative to β-actin ($n = 3$). $^{*}P < 0.05$ versus CTR; $^{***}P < 0.001$ versus CTR; $^{§§}P < 0.01$ versus GS.

the downregulation of PDX-1 induced by GS (Figure 5). Although no statistically significant effect was observed at mRNA expression level (Figures 5(a) and 5(b)), GLP-1 completely reverted the GS-mediated reduction of PDX-1 at protein level (Figures 5(c) and 5(d)). The discrepancy between gene and protein expression observed in the results shown above suggests the involvement of a posttranslational mechanism. The ubiquitin-proteasome system is the main mechanism degrading intracellular altered proteins and it might be altered by AGEs [27]. To verify this hypothesis, we evaluated the levels of PDX-1 and MafA in HIT-T15 cells treated for the last 15 h in the presence or absence of the proteasome inhibitor MG-132. The low concentration of MG-132 (150 nmol/L) and reduced time of exposure (15 h) were selected to avoid cytotoxicity [28]. As shown in Figure 6, treatment of cells with MG-132 induced accumulation of PDX-1 and MafA within the cells. Furthermore, the nuclear distribution of MafA and PDX-1 was also investigated (Figure 7). We found that GS did not affect distribution of MafA in the nuclear soluble compartment but reduced the immunoreactivity of MafA in the chromatin-bound fractions (Figures 7(c) and 7(d)). GLP-1 increased both nuclear soluble and chromatin-bound fraction of MafA in cells cultured with GS (Figure 7). Stimulation with GS reduced the presence of PDX-1 in the chromatin-bound fractions as compared to control CTR (Figures 7(c) and 7(d)). Coincubation with GLP-1 selectively increased immunoreactivity of PDX-1 in the chromatin-bound fractions of GS-cultured cells (Figures 7(c) and 7(d)).

3.4. GLP-1 Reverses GS-Induced Reduction of Proinsulin Production in Pancreatic Beta-Cells. We recently demonstrated that exposure to GS decreased insulin content in HIT-T15 cells [12, 29]. To assess whether the GLP-1-mediated improvements on expression and subcellular localization of MafA and PDX-1 were associated with the increase in proinsulin production, we evaluated preproinsulin gene expression and proinsulin content. GS did not affect preproinsulin mRNA expression (Figures 8(a) and 8(b)). However, incubation with GS decreased proinsulin content (Figure 8(c)). GLP-1 significantly increased proinsulin content in HIT-T15 cells treated in standard condition (CTR) or in the presence of GS (Figure 8(c)).

3.5. Loss of GLP-1R Expression Abrogates GLP-1-Mediated Effects. To confirm that GLP-1-mediated amelioration of GS-induced damage is mediated through the interaction with its transmembrane receptor, the expression of GLP-1R was downregulated by siRNA. Expression of GLP-1R

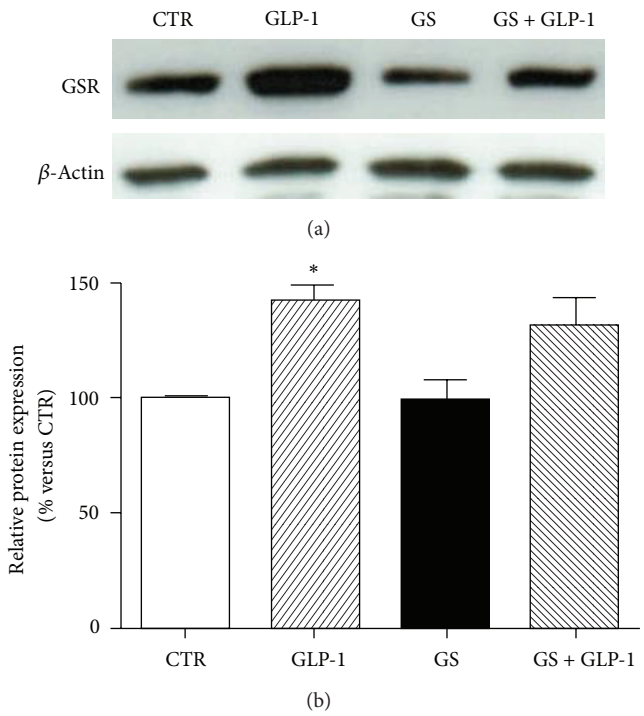

Figure 3: GLP-1 does not affect GSR expression in HIT-T15 cells cultured with AGEs. (a) Representative western blot of three different experiments. (b) Quantification of densitometries of western blot bands. Data were expressed as mean ± SE of fold induction relative to β-actin ($n = 3$). $^{*}P < 0.05$ versus CTR.

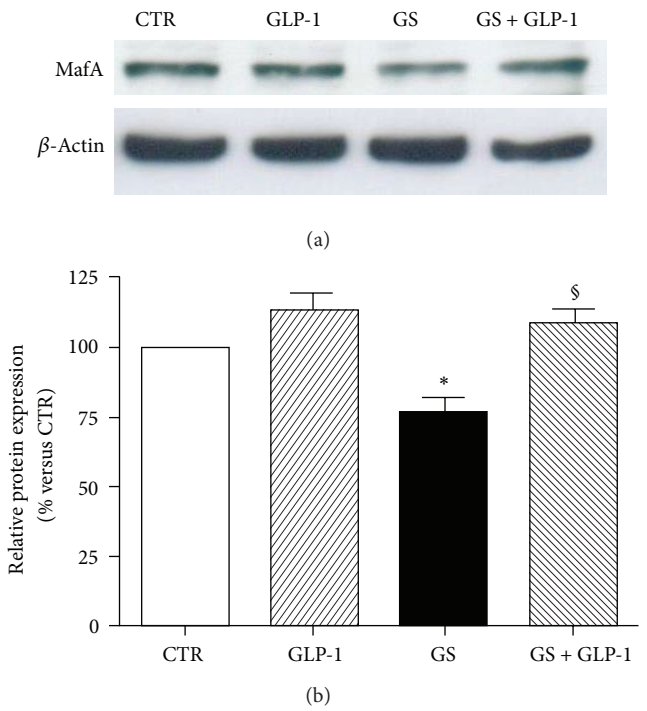

Figure 4: GLP-1 increases MafA protein expression levels in HIT-T15 cells cultured in the presence of GS. (a) Representative western blot of three different experiments. (b) Quantification of densitometries of western blot bands. Data were expressed as mean ± SE of fold induction relative to β-actin ($n = 3$). $^{*}P < 0.05$ versus CTR; $^{\S}P < 0.05$ versus GS.

was analyzed at the beginning (48 hours after transfection) and at the end of the treatment (7 days after transfection). As shown in Figures 9(a) and 9(b), transfection of HIT-T15 cells with GLP-1R siRNA results in more than 50% knockdown of protein content up to 7 days after transfection. Then, we investigated the beneficial effects induced by GLP-1 on the expression of PDX-1 and MafA. The results showed that downregulation of GLP-1R expression prevented the protective effects of GLP-1 on GS-induced depletion of PDX-1 and MafA (Figure 9(c)).

4. Discussion

We stimulated cells in the presence of GS (consisting in a pool of AGEs), showing that the detrimental effects of GS on the pancreatic beta-cell line HIT-T15 are due to the deprivation of proteins involved in the maintenance of the redox balance, and to the downregulation of transcription factors of the insulin gene. Furthermore, we showed that GLP-1 was able to counteract AGE-mediated dysfunction, restoring selective pathways regulating proinsulin production in pancreatic beta-cells.

We previously reported that AGE-induced pancreatic beta cell dysfunction was associated with an increase in oxidative stress [11, 12]. In the present paper, we found that GS increased ROS production and reduced Nrf2 expression. These findings suggest that AGE-induced damage in pancreatic beta-cells is not only due to ROS overproduction, but also to an increased susceptibility to oxidative stress. Furthermore, we showed that GLP-1 upregulated the protein expression of Nrf2 and GSR (the enzyme converting GSSG to GSH), thus resulting in an improvement of the antioxidant response. Since we recently showed that AGEs increased GSSG and decreased GSH levels in pancreatic beta-cells, it could be possible that GLP-1-mediated protection might prevent redox imbalance increasing GSH availability [11]. In addition, the molecular pathway responsible for GLP-1-mediated increase of GSH levels might be related to the upregulation of Nrf2 expression. The production of ROS has been often associated with the decrease in the nuclear availability of MafA and PDX-1, two transcription factors, which synergistically activate the insulin gene promoter [30]. Importantly, it has been reported that AGEs reduce insulin synthesis in pancreatic beta-cells by decreasing expression of PDX-1 through repression of PDX-1 protein expression at the posttranslational level [29, 31]. According to these findings, we showed that GS did not affect mRNA expression level of PDX-1 but decreased the intracellular protein. Similar results were showed also for MafA protein levels. We found that the pharmacological inhibition of proteasome resulted in the prevention of PDX-1 and MafA depletion in cells cultured in the presence of GS, confirming that AGEs may reduce PDX-1 and MafA protein expression

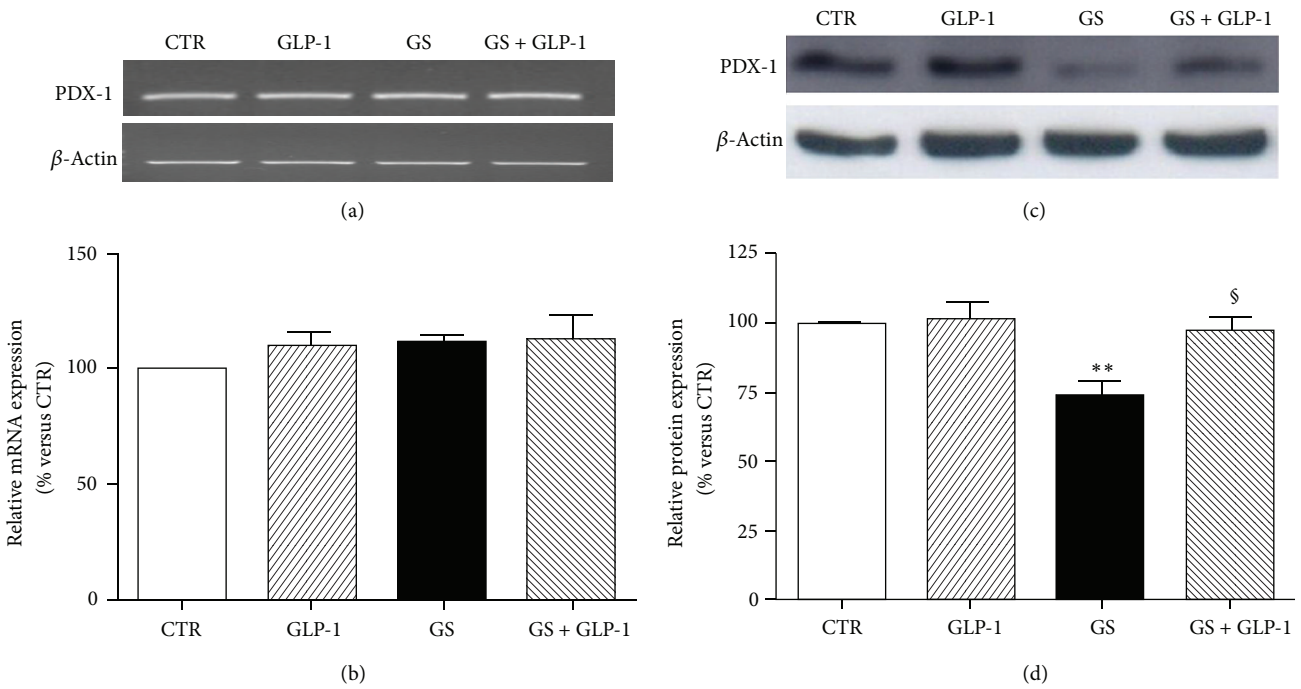

Figure 5: GLP-1 restores PDX-1 protein levels in HIT-T15 cells cultured in the presence or absence of AGEs. (a)-(b) Semiquantitative RT-PCR for PDX-1 mRNA expression in HIT-T15 cells cultured 5 days in standard medium (CTR) or in medium containing AGEs (GS) in the presence or absence of 10 nmol/L GLP-1. (a) Representative agarose gel of three different experiments. (b) Quantification of densitometries of agarose gel bands. Data were represented as percentage change from CTR level (100%) ($n = 3$). (c)-(d) Western blot analysis for PDX-1 protein intracellular expression on HIT-T15 cells, cultured in the same conditions of mRNA experiments. (c) Representative western blot of three different experiments. (d) Quantification of densitometries of western blot bands. Data were expressed as mean ± SE of fold induction relative to β-actin ($n = 3$). $^{**}P < 0.01$ versus CTR; $^{\S}P < 0.05$ versus GS.

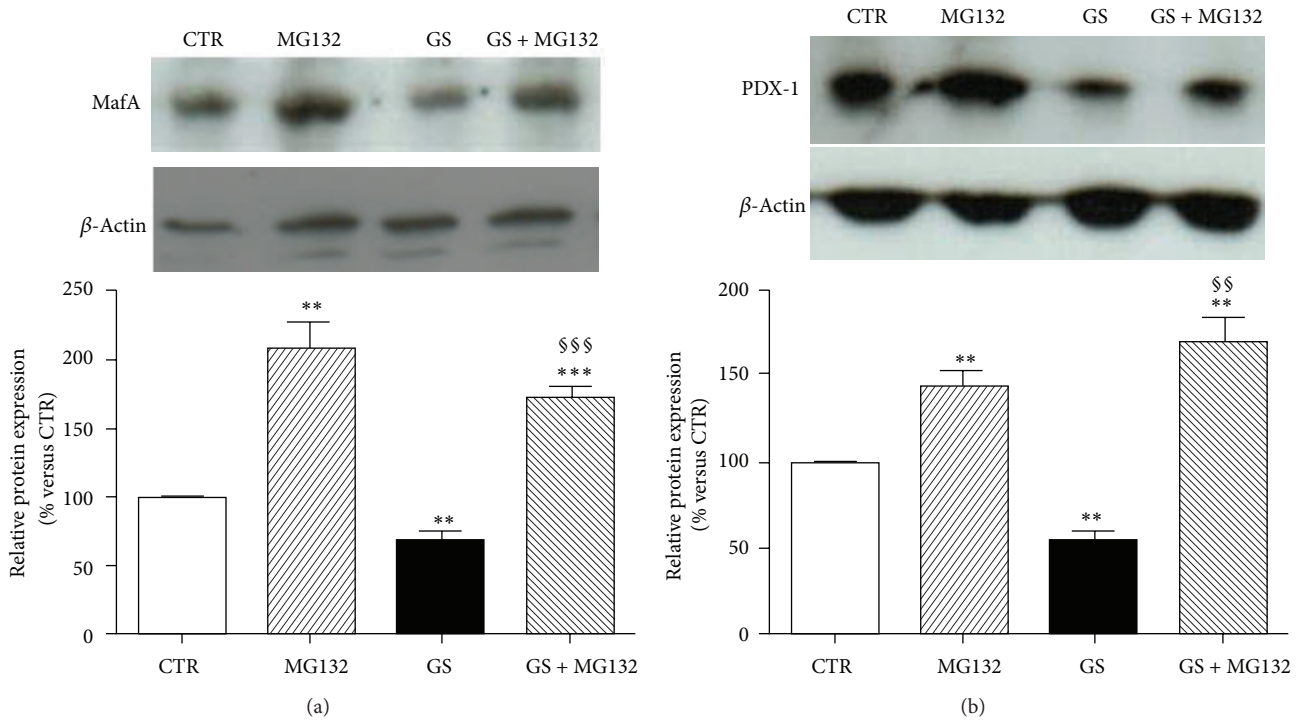

Figure 6: Pharmacological inhibition of proteasome increased accumulation of MafA and PDX-1. HIT-T15 cells were cultured for the last 15 h in the presence or absence of the proteasome inhibitor MG-132, and whole cell extracts were analyzed by western blotting using specific antibodies against PDX-1 and MafA. Actin amounts were analyzed as a loading control. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus CTR or versus the same condition without MG132.

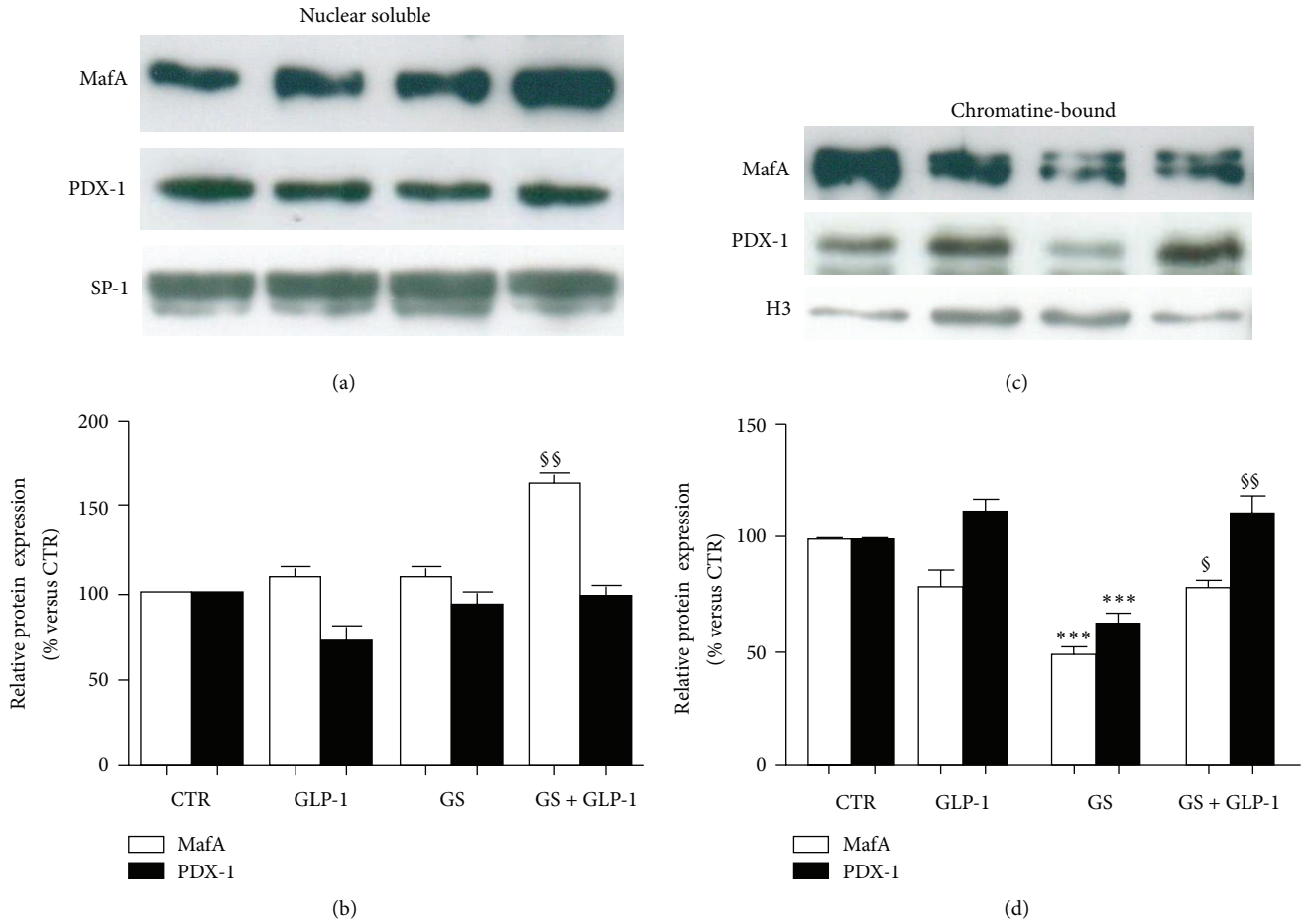

Figure 7: Effects of AGEs on nuclear distribution of MafA and PDX-1. Western blot analysis of nuclear soluble (a-b) and chromatin-bound (c-d) localization of MafA and PDX-1. (a) Representative western blot of three different experiments. SP-1 amounts were analyzed as a loading control. (b) Quantification of densitometries of western blot bands. Data were represented as percentage change from CTR level (100%) ($n = 3$). (c) Representative western blot of three different experiments. Histone H3 (H3) amounts were analyzed as a loading control. (d) Quantification of densitometries of western blot bands. Data were represented as percentage change from NGS level (100%) ($n = 3$). $^{***}P < 0.001$ versus CTR; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ versus GS.

targeting proteasome degradation. Furthermore, our results indicate that GS also decreased the ability of these transcriptional factors to bind DNA. Indeed, we found that GS decreased the immunoreactivity of these transcription factors in the chromatin-bound fraction, suggesting that, although they still localized in the nucleus, their activity might be reduced. These results are in accordance with those of Poitout and coworkers, reporting that glucotoxicity and lipotoxicity inhibited insulin expression through reduction of both DNA-binding activity and protein levels of MafA and PDX-1 [32]. Since it has been reported that insulin gene expression can be regulated by epigenetic mechanisms and that methylation of the insulin promoter negatively correlates with insulin gene expression [33], our results suggest that AGEs may alter DNA making it less accessible to transcription factors of the insulin gene. Despite these detrimental GS-mediated effects on MafA and PDX-1, we did not observe any reduction in preproinsulin mRNA expression levels. These results suggest that the residual binding activity of MafA and PDX-1 might be sufficient for maintaining acceptable levels of insulin gene transcription. Similarly to PDX-1, the discrepancy between conserved mRNA expression and decreased proinsulin content suggests the involvement of a posttranslational mechanism. Given a high secretory demand, the endoplasmic reticulum (ER) is very well developed and highly active in pancreatic β-cell. This also likely increases the susceptibility of these cells to ER stressors, which might produce signals mediating glucose-induced impairment of cell function and death. Experimental evidence indicated a role of ER stress in the progressive reduction of insulin secretion [34–36]. Since GLP-1R ligands have been described to potentiate the expression of proteins involved in the response to ER stress [37], GLP-1/GLP-1R might directly trigger ER protective pathways restoring proinsulin content. Importantly, we showed that coincubation with GLP-1 selectively restored these insulin-related transcriptional pathways. Indeed, GLP-1 treatment was also associated with an improvement in the binding

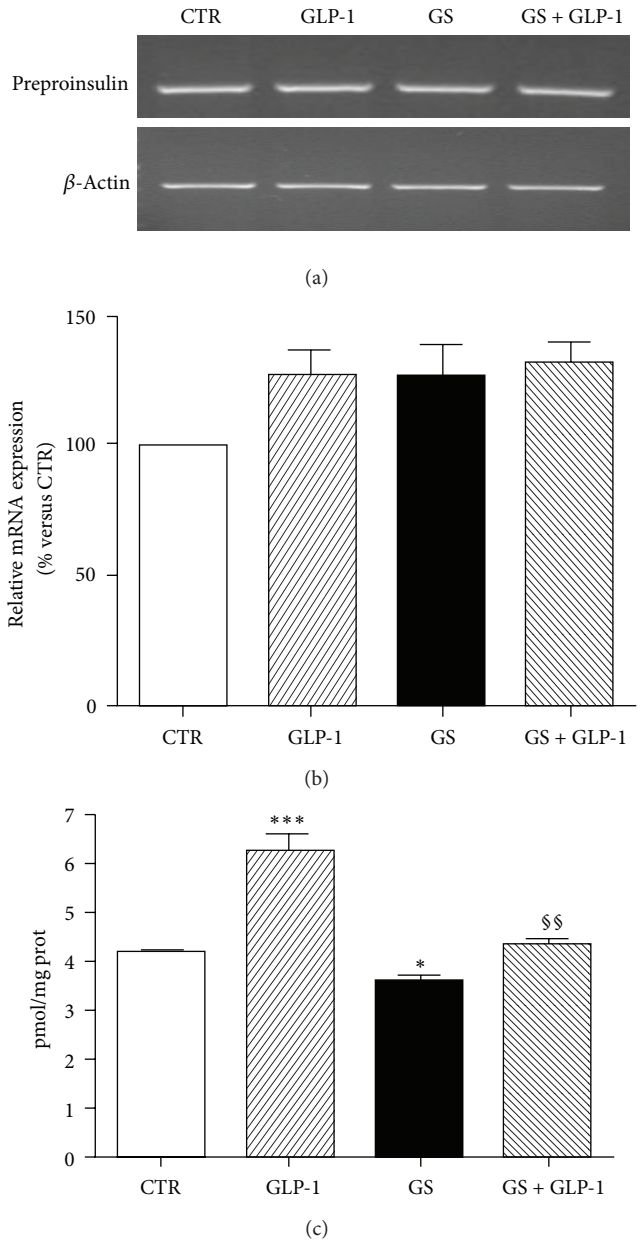

Figure 8: GLP-1 restores proinsulin content in HIT-T15 cells. (a) Semiquantitative RT-PCR for preproinsulin. Representative agarose gel of three different experiments. (b) Quantification of densitometries of agarose gel bands. Data are represented as percentage change from CTR level (100%) ($n = 3$). (c) Intracellular proinsulin was measured after acidified ethanol extraction. Data were expressed as mean ± SE of at least 3 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus CTR; $^{§§}P < 0.01$ versus GS.

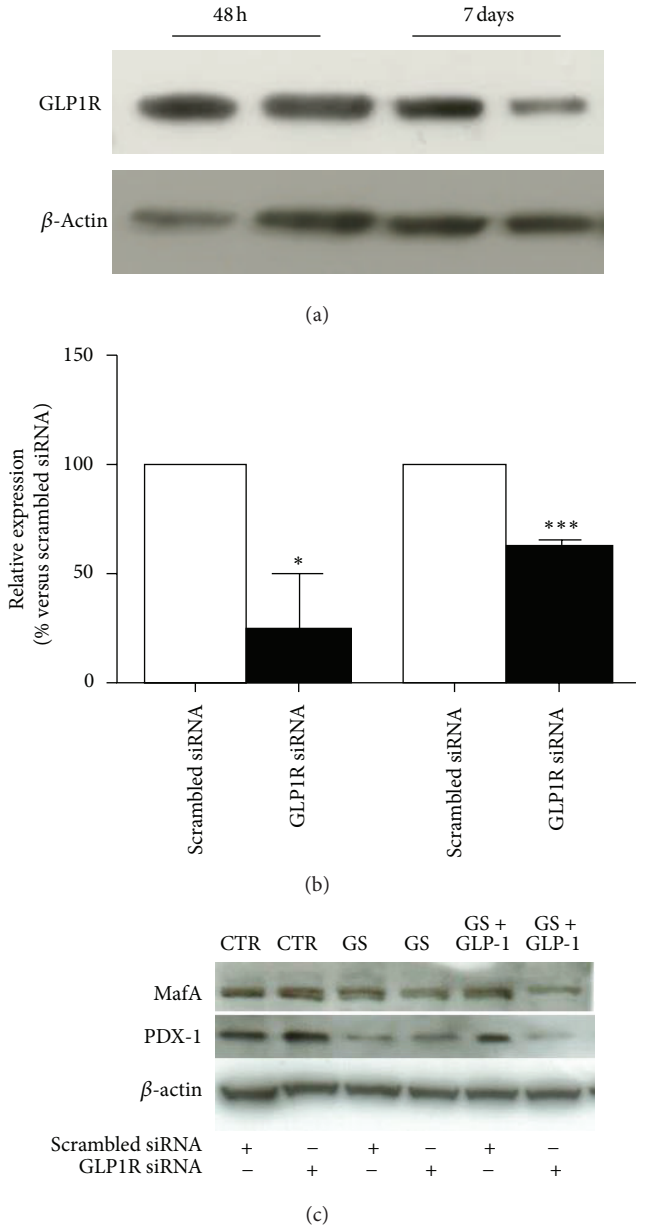

Figure 9: GLP-1R downregulation in HIT-T15 cells. HIT-T15 cells were transfected with specific siRNA for GLP-1R (GLP-1R-siRNA) or a random sequence (scrambled-siRNA). (a) Expression of GLP-1R was analyzed 48 hours and 7 days after transfection. (b) Transfection of HIT-T15 cells with GLP-1R siRNA results in more than 50% knockdown of GLP-1R expression. (c) Five days after treatment with GS, cells were lysed and immunoblotted with specific antibodies against MafA, PDX-1, and β-actin proteins. Blots are representative of three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus scrambled siRNA.

to the chromatin of these transcription factors. Given the silencing experiments, GLP-1-mediated beneficial effects were due to its binding to GLP-1R on cell surface. These results demonstrated that GLP-1 via its transmembrane receptor might ameliorate the DNA accessibility to the protective transcriptional factors, thus reversing GS-induced pancreatic beta-cell injury.

Although HIT-T15 cells are among the most widely used insulin-secreting cell lines and might provide valuable information about both physiological and pathophysiological processes [38], their use represents a limitation of this study. In fact, the use of a cell line represents a simplified in vitro model that is characterized by low variation between

experiments and potentially different from in vivo pancreatic islets or primary beta-cell pathophysiology. Another limitation might be represented by the use of GS that might contain artificial AGEs due to the preparation method. These points render our conclusions as highly speculative and potentially might not reflect the in vivo human pathophysiology.

5. Conclusions

We provided evidence that GLP-1 protects against the detrimental effects of GS on insulin production in the HIT-T15 pancreatic beta-cell line. GLP-1 has been shown to potently counteract ROS release and prevent transcriptional factor level reduction as well as pancreatic beta cell dysfunction. GLP-1 might be considered as a potential antagonist of the acceleration of pancreatic beta-cell deterioration in the presence of GS.

Glossary

AGEs:	Advanced glycation end products
GLP-1:	Glucagon-like peptide-1
GLP-1R:	Glucagon-like peptide-1 receptor
PDX-1:	Pancreatic and duodenal homeobox-1
MafA:	v-Maf musculoaponeurotic fibrosarcoma oncogene homologue A
GSH:	Glutathione
GSSG:	Glutathione disulfide
Nrf2:	Nuclear factor erythroid-derived 2
GSR:	Glutathione reductase
FBS:	Foetal bovine serum
GS:	Glycated serum
NGS:	Nonglycated serum
PBS:	Phosphate saline buffer
RT-PCR:	Reverse transcriptase polymerase reaction
siRNA:	Short-interfering RNA.

Conflict of Interests

The authors have no conflict of interests to disclose.

Acknowledgments

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References

[1] B. L. Wajchenberg, "β-cell failure in diabetes and preservation by clinical treatment," *Endocrine Reviews*, vol. 28, no. 2, pp. 187–218, 2007.

[2] M. Stumvoll, B. J. Goldstein, and T. W. van Haeften, "Type 2 diabetes: principles of pathogenesis and therapy," *The Lancet*, vol. 365, no. 9467, pp. 1333–1346, 2005.

[3] Y. Lin and Z. Sun, "Current views on type 2 diabetes," *Journal of Endocrinology*, vol. 204, no. 1, pp. 1–11, 2010.

[4] R. P. Robertson, J. Harmon, P. O. Tran, Y. Tanaka, and H. Takahashi, "Glucose toxicity in β-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection," *Diabetes*, vol. 52, no. 3, pp. 581–587, 2003.

[5] C. E. Gleason, M. Gonzalez, J. S. Harmon, and R. Paul Robertson, "Determinants of glucose toxicity and its reversibility in the pancreatic islet β-cell line, HIT-T15," *American Journal of Physiology*, vol. 279, no. 5, pp. E997–E1002, 2000.

[6] R. Singh, A. Barden, T. Mori, and L. Beilin, "Advanced glycation end-products: a review," *Diabetologia*, vol. 44, no. 2, pp. 129–146, 2001.

[7] A. Ceriello, M. A. Ihnat, and J. E. Thorpe, "The "Metabolic memory": is more than just tight glucose control necessary to prevent diabetic complications?" *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 2, pp. 410–415, 2009.

[8] J. Drzewoski, J. Kasznicki, and Z. Trojanowski, "The role of "metabolic memory" in the natural history of diabetes mellitus," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 119, no. 7-8, pp. 493–500, 2009.

[9] S. Roy, R. Sala, E. Cagliero, and M. Lorenzi, "Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 1, pp. 404–408, 1990.

[10] M. A. Ihnat, J. E. Thorpe, C. D. Kamat et al., "Reactive oxygen species mediate a cellular "memory" of high glucose stress signalling," *Diabetologia*, vol. 50, no. 7, pp. 1523–1531, 2007.

[11] A. Puddu, D. Storace, A. Durante, P. Odetti, and G. L. Viviani, "Glucagon-like peptide-1 counteracts the detrimental effects of Advanced Glycation End-Products in the pancreatic beta cell line HIT-T 15," *Biochemical and Biophysical Research Communications*, vol. 398, no. 3, pp. 462–466, 2010.

[12] G. Luciano Viviani, A. Puddu, G. Sacchi et al., "Glycated fetal calf serum affects the viability of an insulin-secreting cell line in vitro," *Metabolism*, vol. 57, no. 2, pp. 163–169, 2008.

[13] H. Wang, T. Brun, K. Kataoka, A. J. Sharma, and C. B. Wollheim, "MAFA controls genes implicated in insulin biosynthesis and secretion," *Diabetologia*, vol. 50, no. 2, pp. 348–358, 2007.

[14] H. Kaneto, T. Miyatsuka, D. Kawamori et al., "PDX-1 and MafA play a crucial role in pancreatic β-cell differentiation and maintenance of mature β-cell function," *Endocrine Journal*, vol. 55, no. 2, pp. 235–252, 2008.

[15] H. Kaneto, T. A. Matsuoka, T. Miyatsuka et al., "PDX-1 functions as a master factor in the pancreas," *Frontiers in Bioscience*, vol. 13, pp. 6406–6420, 2008.

[16] H. Kaneto, T. A. Matsuoka, S. Kawashima et al., "Role of MafA in pancreatic β-cells," *Advanced Drug Delivery Reviews*, vol. 61, no. 7-8, pp. 489–496, 2009.

[17] S. C. Lu, "Regulation of glutathione synthesis," *Molecular Aspects of Medicine*, vol. 30, no. 1-2, pp. 42–59, 2009.

[18] L. M. Aleksunes and J. E. Manautou, "Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease," *Toxicologic Pathology*, vol. 35, no. 4, pp. 459–473, 2007.

[19] C. J. Harvey, R. K. Thimmulappa, A. Singh et al., "Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress," *Free Radical Biology and Medicine*, vol. 46, no. 4, pp. 443–453, 2009.

[20] M. H. Noyan-Ashraf, M. Abdul Momen, K. Ban et al., "GLP-1R agonist liraglutide activates cytoprotective pathways and improves outcomes after experimental myocardial infarction in mice," *Diabetes*, vol. 58, no. 4, pp. 975–983, 2009.

[21] L. L. Baggio and D. J. Drucker, "Biology of Incretins: GLP-1 and GIP," *Gastroenterology*, vol. 132, no. 6, pp. 2131–2157, 2007.

[22] D. R. Sell, R. H. Nagaraj, S. K. Grandhee et al., "Pentosidine: a molecular marker for cumulative damage to proteins in diabetes, aging, and uremia," *Diabetes/Metabolism Reviews*, vol. 7, no. 4, pp. 239–251, 1991.

[23] S. Sugiyama, T. Miyata, Y. Ueda et al., "Plasma levels of pentosidine in diabetic patients: an advanced glycation end product," *Journal of the American Society of Nephrology*, vol. 9, no. 9, pp. 1681–1688, 1998.

[24] J. Y. Chan and M. Kwong, "Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein," *Biochimica et Biophysica Acta*, vol. 1517, no. 1, pp. 19–26, 2000.

[25] B. Benassi, M. Fanciulli, F. Fiorentino et al., "c-Myc phosphorylation is required for cellular response to oxidative stress," *Molecular Cell*, vol. 21, no. 4, pp. 509–519, 2006.

[26] S. Shao, Z. Fang, X. Yu, and M. Zhang, "Transcription factors involved in glucose-stimulated insulin secretion of pancreatic beta cells," *Biochemical and Biophysical Research Communications*, vol. 384, no. 4, pp. 401–404, 2009.

[27] M. A. Queisser, D. Yao, S. Geisler et al., "Hyperglycemia impairs proteasome function by methylglyoxal," *Diabetes*, vol. 59, no. 3, pp. 670–678, 2010.

[28] S. Costes, B. vandewalle, C. Tourrel-Cuzin et al., "Degradation of cAMP-responsive element-binding protein by the ubiquitin-proteasome pathway contributes to glucotoxicity in β-cells and human pancreatic islets," *Diabetes*, vol. 58, no. 5, pp. 1105–1115, 2009.

[29] A. Puddu, D. Storace, P. Odetti, and G. L. Viviani, "Advanced glycation end-products affect transcription factors regulating insulin gene expression," *Biochemical and Biophysical Research Communications*, vol. 395, no. 1, pp. 122–125, 2010.

[30] L. Zhao, M. Guo, T. A. Matsuoka et al., "The islet β cell-enriched MafA activator is a key regulator of insulin gene transcription," *Journal of Biological Chemistry*, vol. 280, no. 12, pp. 11887–11894, 2005.

[31] T. Shu, Y. Zhu, H. Wang, Y. Lin, Z. Ma, and X. Han, "Ages decrease insulin synthesis in pancreatic β-cell by repressing pdx-1 protein expression at the post-translational level," *PLoS ONE*, vol. 6, no. 4, Article ID e18782, 2011.

[32] V. Poitout, D. Hagman, R. Stein, I. Artner, R. P. Robertson, and J. S. Harmon, "Regulation of the insulin gene by glucose and fatty acids," *Journal of Nutrition*, vol. 136, no. 4, pp. 873–876, 2006.

[33] B. T. Yang, T. A. Dayeh, C. L. Kirkpatrick et al., "Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA1c levels in human pancreatic islets," *Diabetologia*, vol. 54, no. 2, pp. 360–367, 2011.

[34] C. J. Rhodes, "Type 2 diabetes—a matter of β-cell life and death?" *Science*, vol. 307, no. 5708, pp. 380–384, 2005.

[35] F. Ovalle and D. S. H. Bell, "Effect of rosiglitazone versus insulin on the pancreatic β-cell function of subjects with type 2 diabetes," *Diabetes Care*, vol. 27, no. 11, pp. 2585–2589, 2004.

[36] T. A. Buchanan, A. H. Xiang, R. K. Peters et al., "Preservation of pancreatic β-cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk Hispanic women," *Diabetes*, vol. 51, no. 9, pp. 2796–2803, 2002.

[37] B. Yusta, L. L. Baggio, J. L. Estall et al., "GLP-1 receptor activation improves β cell function and survival following induction of endoplasmic reticulum stress," *Cell Metabolism*, vol. 4, no. 5, pp. 391–406, 2006.

[38] M. Skelin, M. Rupnik, and A. Cencic, "Pancreatic beta cell lines and their applications in diabetes mellitus research," *ALTEX*, vol. 27, no. 2, pp. 105–113, 2010.

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Review Article

Role of Mitogen-Activated Protein Kinase Pathways in Multifactorial Adverse Cardiac Remodeling Associated with Metabolic Syndrome

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Metabolic syndrome has been widely associated with an increased risk for acute cardiovascular events. Emerging evidence supports metabolic syndrome as a condition favoring an adverse cardiac remodeling, which might evolve towards heart dysfunction and failure. This pathological remodeling has been described to result from the cardiac adaptive response to clinical mechanical conditions (such as hypertension, dyslipidemia, and hyperglycemia), soluble inflammatory molecules (such as cytokines and chemokines), as well as hormones (such as insulin), characterizing the pathophysiology of metabolic syndrome. Moreover, these cardiac processes (resulting in cardiac hypertrophy and fibrosis) are also associated with the modulation of intracellular signalling pathways within cardiomyocytes. Amongst the different intracellular kinases, mitogen-activated protein kinases (MAPKs) were shown to be involved in heart damage in metabolic syndrome. However, their role remains controversial. In this paper, we will discuss and update evidence on MAPK-mediated mechanisms underlying cardiac adverse remodeling associated with metabolic syndrome.

1. Introduction

The prevalence of metabolic syndrome is rapidly increasing in the western world [1]. Metabolic syndrome has been defined as a cluster of multiple disorders including insulin resistance, abdominal obesity, dyslipidemia, increased blood pressure, hypercholesterolemia, and proinflammatory state [2]. Several definitions have been historically proposed during the last decades, also including oxidative stress, leptin resistances and endothelial dysfunction as key pathophysiological mechanisms contributing to the increase of cardiovascular risk that affect metabolic syndrome patients [2, 3]. Considering these paradigms, it is clear that the metabolic syndrome is a fully heterogeneous construction, raising important scientific limitations for meta-analyses of clinical investigations. Although some common components (such as dyslipidemia, hypertension, and hyperglycemia) are recurrent in the different definitions [4], these other disorders might represent an important variable in the analysis of different cohorts. Several conditions included in the metabolic syndrome have been shown as strongly associated with an acceleration of atherogenesis and an increased incidence of acute ischemic events [1]. In addition, some processes (mainly systemic insulin resistance and inflammation) have been proposed to contribute to physiological organ remodelling and pathological damage in metabolic syndrome. In particular, different chronic adverse heart remodeling and the development of liver steatosis have been widely described [5, 6]. In this paper, we will focus on the pathophysiology of heart remodelling and damage during metabolic syndrome. Metabolism syndrome patients affected

by diabetes are associated with altered myocardial substrate metabolism, which has emerged as an important contributor to the development of cardiomyopathy [7]. In diabetes and concomitant metabolic syndrome, an increased cardiac fatty acid metabolism and reduced glucose metabolism have been reported [7]. Although initially profitable, the rate of fatty acid uptake reaches a point where it exceeds the rate of fatty acid oxidation, thereby promoting the accumulation of lipids, resulting in lipotoxicity and associated cardiac dysfunction [8]. This leads to some complications, such as cardiac hypertrophy, which is identified as a cardiac pathological remodeling. However, cardiac remodeling does not necessarily refer to pathological adaptation of the myocardium. Indeed, short-term compensatory mechanisms are beneficial for the heart because it adapts cardiac output to physiological or pathological loading conditions such as exercise, hypertension, or aortic stenosis. In contrast, sustained overload leads to maladaptive and detrimental remodeling, as reported in detail by Buckberg and coworkers [9]. Altogether, these studies suggest that cardiac remodeling in metabolic syndrome depends on the different component disorders and might not be a disease *per se* but rather an adaptive response.

Several intracellular signaling pathways, continuously sensing the extracellular stimuli and modulating the different intracellular responses, have been investigated to characterize their role in cardiomyocyte modifications and potential injury associated with metabolic syndrome. Mitogen-activated protein kinases (MAPKs) are cytosolic signaling proteins that become activated after specific phosphorylation [10]. In response to wide extracellular stimuli, MAPKs have been shown to modulate various cellular processes, such as cell growth and cell size regulation [11]. Although not specifically performed in models of metabolic syndrome, *in vitro* studies using isolated cardiomyocytes have shown that MAPKs might be involved in cardiac hypertrophy via three traditional phases: (i) the activation of specific transmembrane proteins; (ii) intracellular signal transduction; (iii) the activation of cytosolic and nuclear events [12]. Since cardiac hypertrophy is characterized by increased cell size, it has been suggested that MAPKs might play a critical role in cardiac remodeling in hypertensive patients with metabolic syndrome. This appears to be achieved by modulating the activity of numerous transcription factors that target specific genes involved in structural response of the myocardium. In this paper, we will provide an overview on the role of MAPKs in the adverse cardiac remodeling that is associated with metabolic syndrome.

2. Different Structural Adverse Cardiac Remodeling in Metabolic Syndrome

Diagnostic criteria of metabolic syndrome (as indicated by ATP III classification [24]) have been reported to independently predict the development of diastolic dysfunction and cardiac hypertrophy [13, 19, 20] (Table 1). Cardiac hypertrophy is commonly defined as an increase in heart size or more particularly as an increase in ventricular size with or without increased wall thickness relative to body size [25]. This cardiac modification was shown to be a common alteration in subjects with different stages of obesity, which is one of the central features in metabolic syndrome [15, 16, 26–28]. These studies have used noninvasive methods such as echocardiography and magnetic resonance imaging (MRI) to assess cardiac adaptations in obese patients. The results indicate that obesity is associated with a high prevalence of cardiac hypertrophy, characterized by an enhancement of left ventricular cavity size as well as wall thickness. Moreover, it has been observed that wall thickness was increased to greater extent than left ventricular cavity size, revealing a concentric instead of eccentric cardiac hypertrophy. Indeed, computed tomography and MRI demonstrated that fat tissue deposits are well detectable within the heart of obese subjects and commonly accumulate anterior to the right ventricle [29–31]. Although noninvasive methods are useful to characterize cardiac size, structure, and function, they have limitations; in that they do not allow analyzing the biochemical composition of the hypertrophic heart in metabolic syndrome. Interestingly, postmortem studies confirmed the presence of cardiac hypertrophy and cardiac fat tissue deposits in obese patients [17, 18]. The amount of epicardial fat has been reported to be correlated with both visceral fat and the severity of ventricular hypertrophy [14]. Several studies have been conducted in animal models to better understand the cardiac adaptation in diabetes [32, 33]. Diabetes was associated with an increase in left ventricular internal dimension during diastole (LVIDD) and systole (LVIDS) in rats [34]. This adverse cardiac remodeling was independent of hypertension [34]. These results are consistent with a previous study, demonstrating that diabetic rats develop an eccentric left ventricular hypertrophy associated with a decreased cardiac systolic function, and related to impaired collagen turnover [35].

The Prospective Cardiovascular Münster (PROCAM) study, recruiting a cohort of 2754 males aged 40–65 years over a four-year period, showed that patients, who had either hypertension or diabetes, had a 2.5-fold increased risk of cardiovascular morbidity. However, when developing both, patients had an eightfold increase in cardiovascular risk. This multiplicative relevance was confirmed by a twentyfold increase of cardiovascular risk in patients with concomitant diabetes mellitus, hypertension, and abnormal lipid profile [36]. Since the metabolic syndrome might include all these disorders by definition, it is easy to understand that the alterations of an atherosclerotic targeted organ, such as the myocardium, might result from both acute and chronic ischemia. However, also in the absence of traditional atherosclerotic complications, the measurement of diastolic function worsened progressively during metabolic syndrome [13], indicating an adverse cardiac remodeling independent of cardiac necrosis and potentially related to the soluble mediators increased during the disease. In line with these results, different studies have confirmed the development of left ventricular diastolic dysfunction in subjects presenting metabolic syndrome [20, 21, 37, 38].

Interestingly, hypertensive patients with concomitant metabolic syndrome have been shown to present increased

Table 1: Different disorders in metabolic syndrome are associated with cardiac structural and functional changes.

Metabolic syndrome characteristic	Adverse cardiac remodelling	Reference	Method of assessment
Obesity	Obese women have higher end-diastolic septal and posterior wall thickness, left ventricle mass, and relative wall thickness than nonobese	[13]	Echocardiography and tissue Doppler imaging
	Uncomplicated severe obesity is associated with adapted and appropriate changes in cardiac structure and function	[14]	Echocardiography
	Reduced left ventricle systolic and diastolic function and increased myocardial reflectivity characterize obese patients as compared to referents	[15]	Transthoracic echocardiography, myocardial Doppler-derived systolic and early diastolic velocity, strain and strain rate imaging, and tissue characterization with cyclic variation and calibrated integrated backscatter
Diabetes	Diabetes, fasting glucose, and fasting insulin levels are associated with left ventricular hypertrophy	[16]	Echocardiography and laboratory testing
	Increased heart size in obese men	[17]	Autopsy
	Postmortem analysis of obese patients that died from gastric bypass complication revealed cardiac hypertrophy	[18]	Autopsy
Hypertension	Left ventricle mass is positively associated with the number of metabolic risk factors in normotensive and hypertensive participants	[19]	Echocardiography
Metabolic syndrome (defined as a cluster of all previously cited disorders [2])	Increased left ventricular mass and reduce left ventricular relaxation	[20]	Echocardiography was used to assess pulse-wave Doppler and tissue Doppler imaging
	Ventricular diastolic dysfunction, mean left ventricular mass, and left ventricular diameter significantly increase with the number of features of the metabolic syndrome	[21]	Structured clinical interview with a physician, ECG and a transthoracic M-mode, and 2D echocardiogram
	High levels of IL-6 that could be observed in metabolic syndrome induce cardiac fibrosis	[22]	Blood-perfused isolated heart
	Cardiotrophin-1 treatment, mimicking the upregulated level found in metabolic syndrome, induces cardiac fibrosis	[23]	Echocardiography, Doppler, and echo tracking device and *ex vivo* approach

left ventricular mass and wall thickness as compared to patients exclusively affected by hypertension [39]. Furthermore, the authors showed that metabolic syndrome might induce an adverse cardiac remodeling via different pathophysiological mechanisms with a multiplicative effect. Metabolic syndrome patients had not only abnormal diastolic left ventricular relaxation, but also increased cardiac hypertrophy [40]. Cardiac hypertrophy predisposes individuals to cardiac arrhythmias, congestive heart failure, and diastolic dysfunction [41]. Consistently, several evidences have revealed a positive association between the metabolic syndrome and the severity of left ventricular hypertrophy [39, 40, 42]. Indeed, metabolic syndrome induces abnormal loading, which may favor the left ventricular hypertrophy. Since pathological cardiac hypertrophy has been associated with sudden cardiac death, heart failure, and stroke [43], it was proposed that this cardiac alteration might further increase cardiovascular risk in metabolic syndrome [44].

Increased myocardial fibrosis and stiffness have been also observed in animal models of obesity and metabolic syndrome [45]. Since collagen and fibrosis determine tissue compliance, cardiac deposition of this protein might promote left ventricular diastolic dysfunction and negatively affect diastolic function [46, 47]. Several other molecular mechanisms (i.e., insulin resistance aggravating asymptomatic myocardial inflammation and association between visceral obesity and myocardial adiposity) have also been suggested to potentially induce such cardiac structural alterations [48, 49].

Most importantly, the renin-angiotensin-aldosterone (RAA) system might also be involved cardiac fibroblast proliferation and collagen synthesis, thereby increasing cardiac fibrosis [50]. The active role for RAA system was confirmed by the finding that pharmacologic inhibition of this pathway ameliorates heart failure [51, 52], also in metabolic syndrome patients [53]. Thus, these studies suggest that RAA system could be a critical player underlying cardiac modifications in hypertensive metabolic syndrome patients.

3. Pathophysiological Mediators of Adverse Cardiac Remodeling in Metabolic Syndrome

Cardiac structural remodeling in metabolic syndrome might be due to different pathological triggers. Hypertrophic growth accompanies heterogeneous metabolic syndrome disorders, including not only diabetes and hypertension, but also coronary heart disease and ischemic cardiac remodeling. Mechanical alterations have been classically described as major causes inducing an adverse cardiac remodelling [54]. However, metabolic syndrome has been associated with left ventricular hypertrophy [40] independently of ischemic cardiac remodeling. Given the upregulation of several hormones and cytokines in metabolic syndrome patients [55], a potential role on cardiac remodeling has been proposed for these molecules [56]. In particular, a hormonal and inflammatory dysregulation might contribute to the development of cardiac hypertrophy and fibrosis [55]. For instance, increased aldosterone plasma levels in patients with metabolic syndrome [57, 58] might be directly associated with the development of left ventricular hypertrophy [59] or cardiac fibrosis [60]. Although clinical studies have reported that aldosterone induces left ventricular hypertrophy [61], the mechanism by which aldosterone promotes cardiac hypertrophy remains unclear. Okoshi and coworkers showed that aldosterone directly induced cardiac hypertrophy and atrial natriuretic peptide (ANP) mRNA expression (a molecular marker of cardiac hypertrophy) in neonatal rat ventricular myocytes [62]. These results were accompanied by enhanced activation of ERK1/2- and JNK-mediated pathways. This critical role of the ERK pathway in the development of cardiac hypertrophy (in response to endothelin-1) was also confirmed by the *in vitro* abrogation of cardiomyocyte hypertrophy in the presence of the pharmacological inhibitor of ERKs [63, 64].

On the other hand, aldosterone has been shown as a potent inducer of cardiac fibrosis [60, 65, 66]. Therefore, emerging evidence indicates a crucial role for aldosterone in maladaptive cardiac remodeling in metabolic syndrome [67, 68].

Considering the hypothesized inflammatory etiology of the metabolic syndrome [69], it was proposed that elevated circulating levels of cytokines, adipocytokines, and chemokines might actively regulate cardiac remodeling and via the activation of inflammatory signaling pathways [70–72]. Amongst several mediators, tumor necrosis factor (TNF) and interleukin-6 (IL-6) were shown to promote both insulin resistance [73] and cardiac hypertrophy [74], suggesting that inflammation in metabolic syndrome might be a central feature in atherogenesis as well as in cardiac hypertrophy. In particular, TNF expression, which was shown to be increased in response to pressure overload in the adult heart [75], might be one of the most important mediators of cardiac hypertrophy in metabolic syndrome with hypertension [76]. The molecular pathways potentially involved in metabolic syndrome-induced cardiac hypertrophy have been only partially investigated. To summarize, mechanical stress remains the main responsible of adverse cardiac remodelling in metabolic syndrome, such as hypertension. However, considering the potential dysregulation of inflammatory and hormonal systems, the cardiac pathophysiology in metabolic syndrome might be partially influenced also by these soluble molecules. In the following sections, we will review the pathophysiological role of MAPKs in cardiac remodeling in a general context and also particularly in metabolic syndrome.

4. Role of MAPK in Cardiac Remodeling

4.1. Extracellular Signal Regulated Kinases (ERKs). One of the most studied MAPK pathways is the Ras/Raf/ERKs pathway. Extracellular stimuli such as stress or hormones activate diverse receptors at the cell surface, driving intracellular recruitment and activation of the guanosine triphosphate (GTP) small G-protein (Ras). This, in turn, induces Raf-1 kinase translocation to the plasma membrane and Raf-1-mediated phosphorylation of MEK proteins (MEK1 and 2). Thereafter, ERKs are activated by MEKs and regulate a large number of nuclear and cytosolic proteins [77] that directly modulate numerous intracellular processes.

For instance, pressure overload was shown to influence ERK-mediated intracellular signaling as well as extracellular matrix deposition within the heart [78]. In response to chronic pressure overload, cardiomyocytes start to grow leading to heart enlargement and hypertrophy. Pressure overload induced by transverse aortic constriction (TAC) in rodents was shown to mediate hypertrophic effect through ERK activation [79]. In addition, Esposito and coworkers showed that TAC procedure is associated with the activation of all three major MAPKs (ERK1/2, p38 MAPK, and JNK) in mice [80]. Consistent with the animal studies, clinical researches reported increased cardiac activation of ERK1/2, JNK, and p38 MAPK in failing human hypertrophic hearts [81]. In line with these results, ERK1/2 activation has been shown as a key element directly promoting cardiac hypertrophy [26, 82].

It was proposed that ERK1/2 induces the activation of various transcription factors by modulating their phosphorylation level, hence leading to hypertrophy. Indeed, using a model of phenylephrine-induced cardiac myocyte hypertrophy, Babu and coworkers revealed that ERK1/2 pathway is involved in Elk-1 upregulation in a model of phenylephrine-mediated hypertrophy [83]. Therefore, these studies suggest a central role for ERK in the pathophysiological development of cardiac hypertrophy. The molecular mechanisms downstream of this pathway remains to be clearly defined.

4.2. Janus Kinases (JNKs). The hypertrophic effects of JNKs are still controversial. Wang and coworkers showed that

specific activation of the JNK pathway was associated with hypertrophy in neonatal cardiomyocytes overexpressing MEK7 [84]. In line with these results, transfection of ventricular myocytes with MEK1 (a MAPK-activating JNK) leads to cardiac hypertrophy via JNK activation [85]. By contrast, transgenic mice selectively overexpressing MEK7 in the cardiac tissue were not shown to develop cardiac hypertrophy despite JNK1 and JNK2 upregulation [86]. Although these mice died from congestive heart failure, they had normal cardiomyocytes size and they did not develop ventricular hypertrophy. Importantly, mice overexpressing MEK7 also presented diastolic dysfunction and paradoxically high levels of ANF mRNA, which is considered a marker for cardiac hypertrophy [86]. The reduction in connexin 43 and gap junctions between ventricular cardiomyocytes might explain the absence of hypertrophy in the presence of increased ANF expression [86]. Also in the case of JNK, the exact mechanisms through which it regulates cardiac hypertrophy remains poorly determined.

4.3. p38 MAPK. In addition to JNK and ERK1/2, p38 MAPK was also intensively investigated. There are at least four isoforms of p38 MAPK that have been involved in cardiac remodeling and inflammation [87]. This signaling pathway is predominantly involved in the inflammatory response, and it can be activated by proinflammatory cytokines, chemokines, and hormones [88–90]. MEK 3, 4, and MEK 6 are the upstream kinases that directly activate p38MAPK [82]. Several downstream transcription factors have been identified as potential p38MAPK substrates, including activating transcription factor-1 (ATF-1), ATF-2, Elk-1, serum response factor (SRF), growth arrest, and myocyte enhance factor 2C (MEF 2C) [91–93]. Since a large amount of studies has been performed *in vitro* in neonatal rat cardiomyocytes, the role of p38 MAPK in cardiac clinical modifications remains to be confirmed. Nevertheless, p38 MAPK has been implicated in the regulation of both cardiac growth and hypertrophy [94]. Indeed, the inhibition of the p38 MAPK pathway via pharmacological inhibitors or adenovirus blunted the hypertrophic effect associated with p38 MAPK activation [94–96]. These hypertrophic effects of p38 MAPK phosphorylation in cardiomyocytes are also supported by another study where specific activation via adenovirus in ventricular muscle cells induced cardiac hypertrophy [84].

By contrast, Choukroun and coworkers have shown that p38 MAPK is not required for agonist-induced hypertrophy in cardiomyocytes [98]. To determine the role of the p38 MAPK pathway in the response to endothelin-1 (ET-1, a hypertrophic agent), neonatal rat cardiomyocytes were concomitantly treated with ET-1 and the selective p38 MAPK inhibitor, SB203580. The results revealed that this inhibitor had no effect on ET-1-induced hypertrophy, hence suggesting that p38 MAPK activation may not be required during cardiac cell hypertrophy [98]. Since these preliminary studies present some limitations (due to the use of the cardiomyocyte model [immature neonatal cells] and the modest specificity of pharmacological inhibitor, that can inhibit also other intracellular pathways), a genetic approach was also performed. Transgenic mice specifically overexpressing MEK 3 and MEK 6 (upstream activators of p38 MAPK) in the heart do not exhibit cardiac hypertrophy, despite the development of ventricular wall thinning and premature death with signs of congestive heart failure [99].

Taken together, these basic research studies suggest that p38 MAPK, ERKs, and JNKs may all be involved in promoting cardiac hypertrophy (Figure 1). In particular, p38 MAPK activation might also contribute to cardiac fibrosis, while ERK and JNK activation might promote cardiomyocyte growth and defects in gap junctions, respectively. The activation of a single MAPK-mediated cascade might not be sufficient to determine a clinically relevant adverse cardiac remodeling.

5. Potential Role of MAPK in Metabolic Syndrome-Related Cardiac Adverse Remodeling

As suggested above, the alterations in cardiac MAPK activation might be induced in metabolic syndrome by insulin resistance and abnormal inflammation. The association between these molecular dynamics was so relevant that an altered MAPK activation pattern might also be a potential cause of hyperinsulinemia [100]. This pathophysiological role of MAPK in metabolic syndrome-induced heart remodeling was confirmed for JNK activation that was associated with both developments of insulin resistance and cardiac hypertrophy in metabolic syndrome [82, 101]. Assessing the role of ERKs in metabolic syndrome cardiac remodeling is much more complicated since ERK2-knockout mice are not viable [102, 103]. On the other hand, ERK1 knockout mice are viable and fertile. Thus, ERK1-deficient mice were investigated, and they were shown to be protected from diet-induced obesity and insulin resistance [104]. Nonetheless, mice lacking the ERK1 negative regulator p62 presented altered metabolism with increased adipogenesis, reduced energy expenditure, and reduced insulin sensitivity [105]. However, these animals were not investigated on concomitant cardiac remodeling and might be considered a good model to assess metabolic syndrome adverse heart remodeling.

Inflammatory mediators such as TNF are elevated in metabolic syndrome [106]. Condorelli and coworkers showed that Akt and the JNK MAPK mediate TNF-induced hypertrophy in cultured cardiomyocytes [107]. TNF-mediated eccentric cardiac hypertrophy in response to intermittent hypoxia was shown to be mediated by ERK and STAT3 activation in adult rat myocardium [97]. On the other hand, IL-6 levels might also be involved in the determinism of cardiac hypertrophy [22]. Consistent with this hypothesis, Hirota and coworkers demonstrated a concomitant overexpression of both IL-6 and IL-6 receptor in a mouse model of cardiac hypertrophy [108]. Although these observations suggest a potential role for IL-6 in metabolic syndrome, the potential activation of intracellular signaling pathways by this cytokine remains unclear. Cardiotrophin-1 (CT-1), a newly discovered member of the IL-6 family and a key regulator of metabolism were also reported to be upregulated

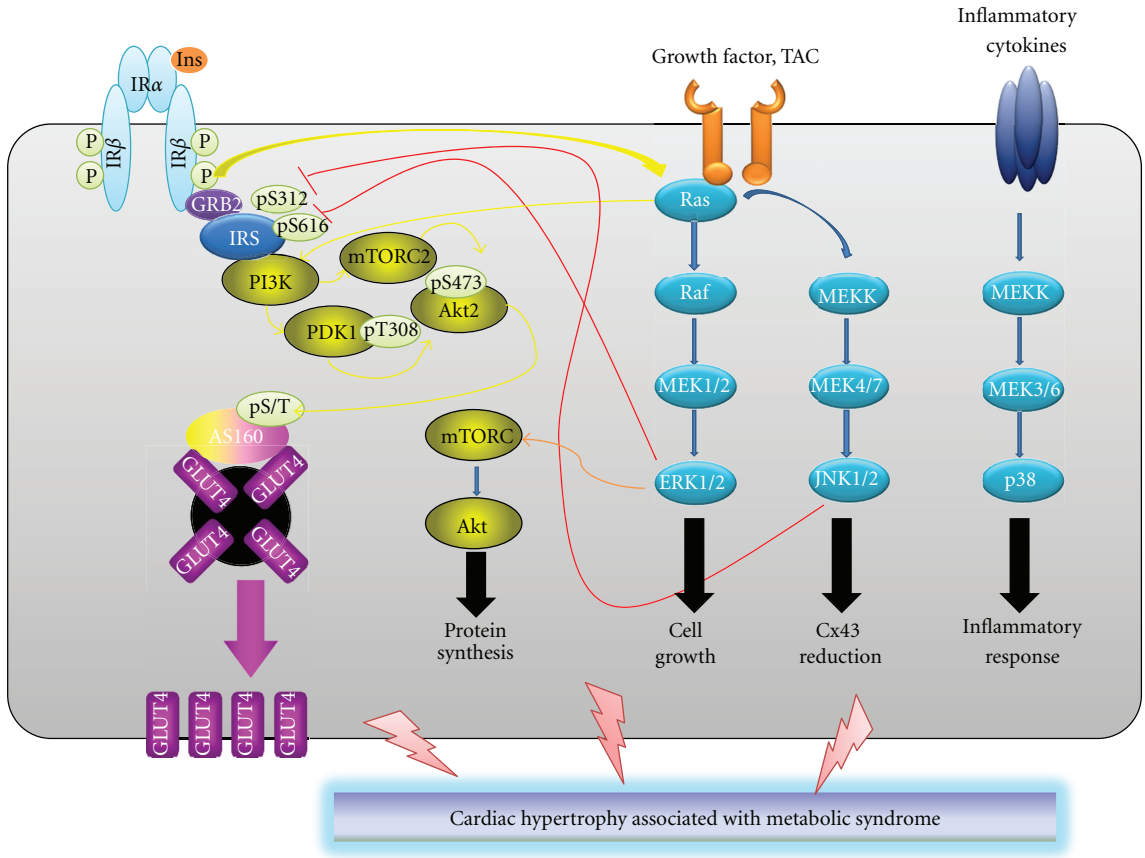

Figure 1: Intracellular pathways mediating hypertrophic cardiac remodelling in metabolic syndrome. Intracellular pathways mediated by phosphatidylinositol 3-kinase (PI3-K) activation play a pivotal role in insulin-mediated glucose transport in cardiomyocytes. Metabolic syndrome is associated with impaired intracellular signaling that could be activated by various stimuli, including inflammatory cytokines. These pathways, mainly dependent on MAPK activation (composed of extracellular signal-regulated kinase [ERK], c-Jun N-terminal kinase [JNK], and p38 MAPK), could be also triggered by chirurgical manipulation (such as transverse aortic constriction [TAC]). MAPK activation might be considered as a critical mechanism aggravating cardiac hypertrophy as well as cardiomyocyte insulin resistance in metabolic syndrome.

in metabolic syndrome [109, 110]. This cytokine was shown to favor cardiac hypertrophy and fibrosis in mice [23]. Thus, CT-1 might also underlie adverse remodeling in metabolic syndrome and thus represent an attractive pathophysiological target.

Although evidence for the role of p38 MAPK in metabolic syndrome is still lacking (except for some animal studies in diabetic fibrotic cardiomyopathy) [111], enhanced MAPK signaling was shown to increase both insulin sensitivity and promote hypertrophic cardiac remodeling (Table 2). Thus, these proteins might represent a promising target to improve metabolic, inflammatory, and cardiac pathophysiology in metabolic syndrome.

6. Conclusions

In the past decades, relevant progresses have been made in the attempt to define the role of MAPKs in metabolic syndrome and in its clinical manifestations, including heart adverse pathophysiological remodelling. The heart structure has been described to be directly influenced by the mechanical stress, characterizing certain components of the metabolic syndrome (such as hypertension). Importantly, this adverse cardiac remodelling might be partially regulated by elevated hormones and inflammatory cytokines. MAPKs might represent the final pathways commonly activated within cardiomyocytes by both mechanical and soluble determinants. Despite some limitations due to animal and *in vitro* models, MAPK (mainly JNK and ERK) activation in the peripheral organs (including the heart) in metabolic syndrome was shown to induce insulin resistance and to increase inflammation. On the other hand, although the effect of p38 MAPK and JNK phosphorylation remains controversial, mounting evidence indicates ERK1/2 as responsible for promoting cardiomyocyte growth. We believe that MAPKs might be considered as potential therapeutic targets for drugs aimed to improve metabolic and cardiac dysfunctions in metabolic syndrome. Indeed several inhibitors of MAPK signaling proteins are currently available. Some of them are already being tested in clinical trials for oncological disorders and may theoretically

Table 2: Role of MAPK activation in metabolic syndrome-associated cardiac hypertrophy.

Author and reference number	Year	Experimental model	MAPK phosphorylation	Cardiac remodeling
Okoshi et al. [62]	2004	Primary cultures of neonatal rat cardiomyocytes	Increased P-ERK1/2	Aldosterone induces hypertrophy through ERK1/2 activation
Yue et al. [63]	2000	Primary cultures of neonatal rat cardiomyocytes	Increased P-ERK1/2	ERK1/2 activation mediates endothelin-1- and phenylephrine-induced cardiac hypertrophy
Wang and Proud [64]	2002	Adult rat ventricular cardiomyocytes		
Chen et al. [97]	2007	Rat myocardium	Increased P-ERK5; P-STAT-3; P-p38 MAPK	Long-term intermittent hypoxia is associated to induced cardiac hypertrophy through the activation of MAPK pathways
Takeishi et al. [79]	2001	Guinea pigs	Increased P-ERK1/2, P-p38 MAPK	Chronic pressure-overload and acute mechanical stretch-induced cardiac hypertrophy is mediated by ERK1/2 and p38 MAPK activation
Esposito et al. [80]	2010	Mice left ventricle and white blood cells from mice and hypertensive patients with controlled blood pressure values	Increased P-ERK1/2, P-p38 MAPK, P-JNK	MAPKs are sensors of pressure overload
Rose et al. [82]	1998	Primary cultures of neonatal rat cardiomyocytes	Increased P-JNK and P-p38 MAPK	JNK activation induces hypertrophy, while concomitant activation of p38 MAPK and JNK inhibits hypertrophic response
Wang et al. [84]	1998	Primary cultures of neonatal rat cardiomyocytes	Increased P-JNK and P-p38 MAPK	JNK induces hypertrophy, while concomitant activation of p38 MAPK and JNK fails to promote hypertrophic response
Zechner et al. [96]	1997	Primary cultures of neonatal rat cardiomyocytes	Increased P-p38 MAPK	Transfection of the cell with constructs activating MAPKs revealed a central role of p38 MAPK activation in cardiac hypertrophy
Choukroun et al. [98]	1998	Primary cultures of neonatal rat cardiomyocytes	Increased SAPK and not P-ERK	In contrast to SAPK, ERK activation is not required for hypertrophic response induced by endothelin

find application also for the treatment or prevention of heart dysfunction in metabolic syndrome. Alternatively, strategies targeting downstream effectors/transcription factors in these cascades could also be a viable therapeutic option.

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References

[1] B. Isomaa, P. Almgren, T. Tuomi et al., "Cardiovascular morbidity and mortality associated with the metabolic syndrome," *Diabetes Care*, vol. 24, no. 4, pp. 683–689, 2001.

[2] R. H. Eckel, S. M. Grundy, and P. Z. Zimmet, "The metabolic syndrome," *The Lancet*, vol. 365, no. 9468, pp. 1415–1428, 2005.

[3] J. A. Armitage, I. Y. Khan, P. D. Taylor, P. W. Nathanielsz, and L. Poston, "Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals?" *Journal of Physiology*, vol. 561, no. 2, pp. 355–377, 2004.

[4] F. Montecucco and F. Mach, "Update on therapeutic strategies to increase adiponectin function and secretion in metabolic syndrome," *Diabetes, Obesity and Metabolism*, vol. 11, no. 5, pp. 445–454, 2009.

[5] H. von Bibra and M. St John Sutton, "Impact of diabetes on postinfarction heart failure and left ventricular remodeling," *Current Heart Failure Reports*, vol. 8, pp. 242–251, 2011.

[6] M. Roden, "Mechanisms of Disease: hepatic steatosis in type 2 diabetes—pathogenesis and clinical relevance," *Nature Clinical Practice Endocrinology and Metabolism*, vol. 2, no. 6, pp. 335–348, 2006.

[7] G. D. Lopaschuk, "Metabolic abnormalities in the diabetic heart," *Heart Failure Reviews*, vol. 7, no. 2, pp. 149–159, 2002.

[8] L. S. Szczepaniak, R. L. Dobbins, G. J. Metzger et al., "Myocardial triglycerides and systolic function in humans: in vivo evaluation by localized proton spectroscopy and cardiac imaging,"

Magnetic Resonance in Medicine, vol. 49, no. 3, pp. 417–423, 2003.

[9] G. Buckberg, J. I. E. Hoffman, A. Mahajan, S. Saleh, and C. Coghlan, "Cardiac mechanics revisited: the relationship of cardiac architecture to ventricular function," *Circulation*, vol. 118, no. 24, pp. 2571–2587, 2008.

[10] Y. Zhang and C. Dong, "Regulatory mechanisms of mitogen-activated kinase signaling," *Cellular and Molecular Life Sciences*, vol. 64, no. 21, pp. 2771–2789, 2007.

[11] W. Zhang and H. T. Liu, "MAPK signal pathways in the regulation of cell proliferation in mammalian cells," *Cell Research*, vol. 12, no. 1, pp. 9–18, 2002.

[12] M. A. Hefti, B. A. Harder, H. M. Eppenberger, and M. C. Schaub, "Signaling pathways in cardiac myocyte hypertrophy," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 11, pp. 2873–2892, 1997.

[13] L. R. Peterson, A. D. Waggoner, K. B. Schechtman et al., "Alterations in left ventricular structure and function in young healthy obese women: assessment by echocardiography and tissue Doppler imaging," *Journal of the American College of Cardiology*, vol. 43, no. 8, pp. 1399–1404, 2004.

[14] G. Iacobellis, M. C. Ribaudo, G. Leto et al., "Influence of excess fat on cardiac morphology and function: study in uncomplicated obesity," *Obesity Research*, vol. 10, no. 8, pp. 767–773, 2002.

[15] C. Y. Wong, T. O'Moore-Sullivan, R. Leano, N. Byrne, E. Beller, and T. H. Marwick, "Alterations of left ventricular myocardial characteristics associated with obesity," *Circulation*, vol. 110, no. 19, pp. 3081–3087, 2004.

[16] E. Avelar, T. V. Cloward, J. M. Walker et al., "Left ventricular hypertrophy in severe obesity: interactions among blood pressure, nocturnal hypoxemia, and body mass," *Hypertension*, vol. 49, no. 1, pp. 34–39, 2007.

[17] M. L. Kortelainen, "Myocardial infarction and coronary pathology in severely obese people examined at autopsy," *International Journal of Obesity*, vol. 26, no. 1, pp. 73–79, 2002.

[18] P. M. Cummings, B. H. Le, and M. B. S. Lopes, "Postmortem findings in morbidly obese individuals dying after gastric bypass procedures," *Human Pathology*, vol. 38, no. 4, pp. 593–597, 2007.

[19] G. de Simone, V. Palmieri, J. N. Bella et al., "Association of left ventricular hypertrophy with metabolic risk factors: the HyperGEN study," *Journal of Hypertension*, vol. 20, no. 2, pp. 323–331, 2002.

[20] L. D. L. Fuentes, A. L. Brown, S. J. Mathews et al., "Metabolic syndrome is associated with abnormal left ventricular diastolic function independent of left ventricular mass," *European Heart Journal*, vol. 28, no. 5, pp. 553–559, 2007.

[21] A. Azevedo, P. Bettencourt, P. B. Almeida et al., "Increasing number of components of the metabolic syndrome and cardiac structural and functional abnormalities—cross-sectional study of the general population," *BMC Cardiovascular Disorders*, vol. 7, article 17, 2007.

[22] G. C. Meléndez, J. L. McLarty, S. P. Levick, Y. Du, J. S. Janicki, and G. L. Brower, "Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats," *Hypertension*, vol. 56, no. 2, pp. 225–231, 2010.

[23] N. Lopez-Andres, A. Rousseau, R. Akhtar, L. Calvier, C. Inigo, C. Labat et al., "Cardiotrophin 1 is involved in cardiac, vascular, and renal fibrosis and dysfunction," *Hypertension*, vol. 60, pp. 563–573, 2012.

[24] S. M. Grundy, H. B. Brewer, J. I. Cleeman, S. C. Smith, and C. Lenfant, "Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition," *Circulation*, vol. 109, no. 3, pp. 433–438, 2004.

[25] R. D. Hannan, A. Jenkins, A. K. Jenkins, and Y. Brandenburger, "Cardiac hypertrophy: a matter of translation," *Clinical and Experimental Pharmacology and Physiology*, vol. 30, no. 8, pp. 517–527, 2003.

[26] K. L. Peterson, "Pressure overload hypertrophy and congestive heart failure: where is the "Achilles' Heel"?" *Journal of the American College of Cardiology*, vol. 39, no. 4, pp. 672–675, 2002.

[27] S. R. Heckbert, W. Post, G. D. N. Pearson et al., "Traditional Cardiovascular Risk Factors in Relation to Left Ventricular Mass, Volume, and Systolic Function by Cardiac Magnetic Resonance Imaging. The Multiethnic Study of Atherosclerosis," *Journal of the American College of Cardiology*, vol. 48, no. 11, pp. 2285–2292, 2006.

[28] G. Iacobellis, M. C. Ribaudo, A. Zappaterreno, C. V. Iannucci, U. Di Mario, and F. Leonetti, "Adapted changes in left ventricular structure and function in severe uncomplicated obesity," *Obesity Research*, vol. 12, no. 10, pp. 1616–1621, 2004.

[29] H. Tandri, E. Castillo, V. A. Ferrari et al., "Magnetic resonance imaging of arrhythmogenic right ventricular dysplasia. Sensitivity, specificity, and observer variability of fat detection versus functional analysis of the right ventricle," *Journal of the American College of Cardiology*, vol. 48, no. 11, pp. 2277–2284, 2006.

[30] D. K. Tansey, Z. Aly, and M. N. Sheppard, "Fat in the right ventricle of the normal heart," *Histopathology*, vol. 46, no. 1, pp. 98–104, 2005.

[31] E. Kim, Y. H. Choe, B. K. Han et al., "Right ventricular fat infiltration in asymptomatic subjects: observations from ECG-gated 16-slice multidetector CT," *Journal of Computer Assisted Tomography*, vol. 31, no. 1, pp. 22–28, 2007.

[32] P. K. Mazumder, B. T. O'Neill, M. W. Roberts et al., "Impaired cardiac efficiency and increased fatty acid oxidation in insulin-resistant ob/ob mouse hearts," *Diabetes*, vol. 53, no. 9, pp. 2366–2374, 2004.

[33] J. Buchanan, P. K. Mazumder, P. Hu et al., "Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity," *Endocrinology*, vol. 146, no. 12, pp. 5341–5349, 2005.

[34] D. Landau, C. Chayat, N. Zucker et al., "Early blood pressure-independent cardiac changes in diabetic rats," *Journal of Endocrinology*, vol. 197, no. 1, pp. 75–83, 2008.

[35] E. Bollano, E. Omerovic, H. Svensson, F. Waagstein, and M. Fu, "Cardiac remodeling rather than disturbed myocardial energy metabolism is associated with cardiac dysfunction in diabetic rats," *International Journal of Cardiology*, vol. 114, no. 2, pp. 195–201, 2007.

[36] G. Assmann, "Dyslipidaemia and global cardiovascular risk: clinical issues," *European Heart Journal, Supplement*, vol. 8, pp. F40–F46, 2006.

[37] M. Barylski, J. Ciecwierz, M. Banach, R. Irzmanski, L. Pawlicki, and J. Kowalski, "Left ventricular diastolic dysfunction in subjects with metabolic syndrome," *Atherosclerosis Supplements*, vol. 9, article 232, 2008.

[38] D. Penjaskovic, D. Sakac, J. Dejanovic, R. Zec, N. Zec Petkovic, and A. Stojsic Milosavljevic, "Left ventricular diastolic dysfunction in patients with metabolic syndrome," *Medicinski Pregled*, vol. 65, pp. 18–22, 2012.

[39] G. Mulè, E. Nardi, S. Cottone et al., "Influence of metabolic syndrome on hypertension-related target organ damage," *Journal of Internal Medicine*, vol. 257, no. 6, pp. 503–513, 2005.

[40] M. Chinali, R. B. Devereux, B. V. Howard et al., "Comparison of cardiac structure and function in American Indians with and without the metabolic syndrome (the Strong Heart Study)," *American Journal of Cardiology*, vol. 93, no. 1, pp. 40–44, 2004.

[41] P. M. Okin, R. B. Devereux, S. Jern et al., "Regression of electrocardiographic left ventricular hypertrophy during antihypertensive treatment and the prediction of major cardiovascular events," *Journal of the American Medical Association*, vol. 292, no. 19, pp. 2343–2349, 2004.

[42] G. Schillaci, M. Pirro, G. Vaudo et al., "Metabolic syndrome is associated with aortic stiffness in untreated essential hypertension," *Hypertension*, vol. 45, no. 6, pp. 1078–1082, 2005.

[43] D. G. Krauser and R. B. Devereux, "Ventricular hypertrophy and hypertension: prognostic elements and implications for management," *Herz Kardiovaskuläre Erkrankungen*, vol. 31, no. 4, pp. 305–316, 2006.

[44] G. de Simone, R. B. Devereux, M. Chinali et al., "Metabolic syndrome and left ventricular hypertrophy in the prediction of cardiovascular events: the Strong Heart Study," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 19, no. 7, p. 520, 2009.

[45] Z. L. Li, J. R. Woollard, B. Ebrahimi, J. A. Crane, K. L. Jordan, A. Lerman et al., "Transition from obesity to metabolic syndrome is associated with altered myocardial autophagy and apoptosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, pp. 1132–1141, 2012.

[46] B. Swynghedauw, "Molecular mechanisms of myocardial remodeling," *Physiological Reviews*, vol. 79, no. 1, pp. 215–262, 1999.

[47] X. Y. Zhu, E. Daghini, M. Rodriguez-Porcel et al., "Redox-sensitive myocardial remodeling and dysfunction in swine diet-induced experimental hypercholesterolemia," *Atherosclerosis*, vol. 193, no. 1, pp. 62–69, 2007.

[48] E. D. Abel, S. E. Litwin, and G. Sweeney, "Cardiac remodeling in obesity," *Physiological Reviews*, vol. 88, no. 2, pp. 389–419, 2008.

[49] J. B. Meigs, M. K. Rutter, L. M. Sullivan, C. S. Fox, R. B. D'Agostino, and P. W. F. Wilson, "Impact of insulin resistance on risk of type 2 diabetes and cardiovascular disease in people with metabolic syndrome," *Diabetes Care*, vol. 30, no. 5, pp. 1219–1225, 2007.

[50] K. T. Weber and C. G. Brilla, "Pathological hypertrophy and cardiac interstitium: fibrosis and renin-angiotensin-aldosterone system," *Circulation*, vol. 83, no. 6, pp. 1849–1865, 1991.

[51] A. Sato and T. Saruta, "Aldosterone escape during angiostensin-converting enzyme inhibitor therapy in essential hypertensive patients with left ventricular hypertrophy," *Journal of International Medical Research*, vol. 29, no. 1, pp. 13–21, 2001.

[52] A. D. Struthers, "The clinical implications of aldosterone escape in congestive heart failure," *European Journal of Heart Failure*, vol. 6, no. 5, pp. 539–545, 2004.

[53] W. Kosmala, M. Przewlocka-Kosmala, H. Szczepanik-Osadnik, A. Mysiak, T. O'Moore-Sullivan, and T. H. Marwick, "A randomized study of the beneficial effects of aldosterone antagonism on LV function, structure, and fibrosis markers in metabolic syndrome," *Cardiovascular Imaging*, vol. 4, pp. 1239–1249, 2011.

[54] L. H. Opie, P. J. Commerford, B. J. Gersh, and M. A. Pfeffer, "Controversies in ventricular remodelling," *The Lancet*, vol. 367, no. 9507, pp. 356–367, 2006.

[55] E. J. Gallagher, D. Leroith, and E. Karnieli, "Insulin resistance in obesity as the underlying cause for the metabolic syndrome," *Mount Sinai Journal of Medicine*, vol. 77, no. 5, pp. 511–523, 2010.

[56] H. V. Lin and D. Accili, "Hormonal regulation of hepatic glucose production in health and disease," *Cell Metabolism*, vol. 14, no. 1, pp. 9–19, 2011.

[57] M. Bochud, J. Nussberger, P. Bovet et al., "Plasma aldosterone is independently associated with the metabolic syndrome," *Hypertension*, vol. 48, no. 2, pp. 239–245, 2006.

[58] D. A. Calhoun and K. Sharma, "The role of aldosteronism in causing obesity-related cardiovascular risk," *Cardiology Clinics*, vol. 28, no. 3, pp. 517–527, 2010.

[59] A. H. El-Gharbawy, V. S. Nadig, J. Morley Kotchen et al., "Arterial pressure, left ventricular mass, and aldosterone in essential hypertension," *Hypertension*, vol. 37, no. 3, pp. 845–850, 2001.

[60] C. G. Brilla, L. S. Matsubara, and K. T. Weber, "Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism," *Journal of Molecular and Cellular Cardiology*, vol. 25, no. 5, pp. 563–575, 1993.

[61] K. Matsumura, K. Fujii, H. Oniki, M. Oka, and M. Iida, "Role of aldosterone in left ventricular hypertrophy in hypertension," *American Journal of Hypertension*, vol. 19, no. 1, pp. 13–18, 2006.

[62] M. P. Okoshi, X. Yan, K. Okoshi et al., "Aldosterone directly stimulates cardiac myocyte hypertrophy," *Journal of Cardiac Failure*, vol. 10, no. 6, pp. 511–518, 2004.

[63] T. L. Yue, J. L. Gu, C. Wang et al., "Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy," *Journal of Biological Chemistry*, vol. 275, no. 48, pp. 37895–37901, 2000.

[64] L. Wang and C. G. Proud, "Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes," *Circulation Research*, vol. 91, no. 9, pp. 821–829, 2002.

[65] V. Robert, N. Van Thiem, S. L. Cheav, C. Mouas, B. Swynghedauw, and C. Delcayre, "Increased cardiac types I and III collagen mRNAs in aldosterone-salt hypertension," *Hypertension*, vol. 24, no. 1, pp. 30–36, 1994.

[66] M. Young, M. Fullerton, R. Dilley, and J. Funder, "Mineralocorticoids, hypertension, and cardiac fibrosis," *Journal of Clinical Investigation*, vol. 93, no. 6, pp. 2578–2583, 1994.

[67] J. R. Sowers, "Metabolic risk factors and renal disease," *Kidney International*, vol. 71, no. 8, pp. 719–720, 2007.

[68] J. R. Sowers, A. Whaley-Connell, and M. Epstein, "Narrative review: the emerging clinical implications of the role of aldosterone in the metabolic syndrome and resistant hypertension," *Annals of Internal Medicine*, vol. 150, no. 11, pp. 776–783, 2009.

[69] F. Emanuela, M. Grazia, R. Marco de, L. Maria Paola, F. Giorgio, and B. Marco, "Inflammation as a link between obesity and metabolic syndrome," *Journal of Nutrition and Metabolism*, vol. 2012, Article ID 476380, 2012.

[70] P. Sartipy and D. J. Loskutoff, "Monocyte chemoattractant protein 1 in obesity and insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7265–7270, 2003.

[71] K. E. Wellen and G. S. Hotamisligil, "Inflammation, stress, and diabetes," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1111–1119, 2005.

[72] G. S. Hotamisligil, N. S. Shargill, and B. M. Spiegelman, "Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance," *Science*, vol. 259, no. 5091, pp. 87–91, 1993.

[73] S. E. Shoelson, J. Lee, and A. B. Goldfine, "Inflammation and insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1793–1801, 2006.

[74] M. N. Sack, R. M. Smith, and L. H. Opie, "Tumor necrosis factor in myocardial hypertrophy and ischaemia—an anti-apoptotic perspective," *Cardiovascular Research*, vol. 45, no. 3, pp. 688–695, 2000.

[75] S. R. Kapadia, H. Oral, J. Lee, M. Nakano, G. E. Taffet, and D. L. Mann, "Hemodynamic regulation of tumor necrosis factor-α gene and protein expression in adult feline myocardium," *Circulation Research*, vol. 81, no. 2, pp. 187–195, 1997.

[76] K. Nakamura, K. Fushimi, H. Kouchi et al., "Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotensin II," *Circulation*, vol. 98, no. 8, pp. 794–799, 1998.

[77] C. Widmann, S. Gibson, M. B. Jarpe, and G. L. Johnson, "Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human," *Physiological Reviews*, vol. 79, no. 1, pp. 143–180, 1999.

[78] J. Heineke and J. D. Molkentin, "Regulation of cardiac hypertrophy by intracellular signalling pathways," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 8, pp. 589–600, 2006.

[79] Y. Takeishi, Q. Huang, J. I. Abe et al., "Src and multiple MAP kinase activation in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 9, pp. 1637–1648, 2001.

[80] G. Esposito, C. Perrino, G. G. Schiattarella et al., "Induction of mitogen-activated protein kinases is proportional to the amount of pressure overload," *Hypertension*, vol. 55, no. 1, pp. 137–143, 2010.

[81] S. Haq, G. Choukroun, H. Lim et al., "Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure," *Circulation*, vol. 103, no. 5, pp. 670–677, 2001.

[82] B. A. Rose, T. Force, and Y. Wang, "Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale," *Physiological Reviews*, vol. 90, no. 4, pp. 1507–1546, 2010.

[83] G. J. Babu, J. M. Lalli, M. A. Sussman, J. I. Sadoshima, and M. Periasamy, "Phosphorylation of Elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy," *Journal of Molecular and Cellular Cardiology*, vol. 32, no. 8, pp. 1447–1457, 2000.

[84] Y. Wang, B. Su, V. P. Sah, J. H. Brown, J. Han, and K. R. Chien, "Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells," *Journal of Biological Chemistry*, vol. 273, no. 20, pp. 5423–5426, 1998.

[85] M. A. Bogoyevitch, J. Gillespie-Brown, A. J. Ketterman et al., "Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart: p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion," *Circulation Research*, vol. 79, no. 2, pp. 162–173, 1996.

[86] B. G. Petrich, B. C. Eloff, D. L. Lerner et al., "Targeted activation of c-Jun N-terminal kinase in vivo induces restrictive cardiomyopathy and conduction defects," *Journal of Biological Chemistry*, vol. 279, no. 15, pp. 15330–15338, 2004.

[87] S. Frantz, T. Behr, K. Hu et al., "Role of p38 mitogen-activated protein kinase in cardiac remodelling," *British Journal of Pharmacology*, vol. 150, no. 2, pp. 130–135, 2007.

[88] K. Ono and J. Han, "The p38 signal transduction pathway Activation and function," *Cellular Signalling*, vol. 12, no. 1, pp. 1–13, 2000.

[89] A. Waehre, B. Halvorsen, A. Yndestad et al., "Lack of chemokine signaling through CXCR5 causes increased mortality, ventricular dilatation and deranged matrix during cardiac pressure overload," *PLoS ONE*, vol. 6, no. 4, Article ID e18668, 2011.

[90] G. Lacerda-Miranda, V. M. Soares, A. K. G. Vieira, J. G. Lessa, A. C. S. Rodrigues-Cunha, E. Cortez et al., "Ghrelin signaling in heart remodeling of adult obese mice," *Peptides*, vol. 35, pp. 65–73, 2012.

[91] A. Besnard, B. Galan-Rodriguez, P. Vanhoutte, and J. Caboche, "Elk-1 a transcription factor with multiple facets in the brain," *Frontiers in Neuroscience*, vol. 5, article 35, 2011.

[92] J. Darragh, A. Soloaga, V. A. Beardmore et al., "MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling," *Biochemical Journal*, vol. 390, no. 3, pp. 749–759, 2005.

[93] J. Han and J. D. Molkentin, "Regulation of MEF2 by p38 MAPK and its implication in cardiomyocyte biology," *Trends in Cardiovascular Medicine*, vol. 10, no. 1, pp. 19–22, 2000.

[94] Q. Liang and J. D. Molkentin, "Redefining the roles of p38 and JNK signaling in cardiac hypertrophy: dichotomy between cultured myocytes and animal models," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 12, pp. 1385–1394, 2003.

[95] S. Nemoto, Z. Sheng, and A. Lin, "Opposing effects of Jun kinase and p38 mitogen-activated protein kinases on cardiomyocyte hypertrophy," *Molecular and Cellular Biology*, vol. 18, no. 6, pp. 3518–3526, 1998.

[96] D. Zechner, D. J. Thuerauf, D. S. Hanford, P. M. McDonough, and C. C. Glembotski, "A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression," *Journal of Cell Biology*, vol. 139, no. 1, pp. 115–127, 1997.

[97] L. M. Chen, W. W. Kuo, J. J. Yang et al., "Eccentric cardiac hypertrophy was induced by long-term intermittent hypoxia in rats," *Experimental Physiology*, vol. 92, no. 2, pp. 409–416, 2007.

[98] G. Choukroun, R. Hajjar, J. M. Kyriakis, J. V. Bonventre, A. Rosenzweig, and T. Force, "Role of the stress-activated protein kinases in endothelin-induced cardiomyocyte hypertrophy," *Journal of Clinical Investigation*, vol. 102, no. 7, pp. 1311–1320, 1998.

[99] P. Liao, D. Georgakopoulos, A. Kovacs et al., "The in vivo role of p38 MAP kinases in cardiac remodeling and restrictive cardiomyopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 21, pp. 12283–12288, 2001.

[100] F. Montecucco, S. Steffens, and F. Mach, "Insulin resistance: a proinflammatory state mediated by lipid-induced signaling dysfunction and involved in atherosclerotic plaque instability," *Mediators of Inflammation*, vol. 2008, Article ID 767623, 2008.

[101] Y. Wang, "Mitogen-activated protein kinases in heart development and diseases," *Circulation*, vol. 116, no. 12, pp. 1413–1423, 2007.

[102] Y. Satoh, S. Endo, T. Ikeda et al., "Extracellular signal-regulated kinase 2 (ERK2) knockdown mice show deficits in long-term memory; ERK2 has a specific function in learning and memory," *Journal of Neuroscience*, vol. 27, no. 40, pp. 10765–10776, 2007.

[103] L. F. Bélanger, S. Roy, M. Tremblay et al., "MEK2 is dispensable for mouse growth and development," *Molecular and Cellular Biology*, vol. 23, no. 14, pp. 4778–4787, 2003.

[104] F. Bost, M. Aouadi, L. Caron et al., "The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis," *Diabetes*, vol. 54, no. 2, pp. 402–411, 2005.

[105] S. J. Lee, P. T. Pfluger, J. Y. Kim et al., "A functional role for the p62-ERK1 axis in the control of energy homeostasis and adipogenesis," *EMBO Reports*, vol. 11, no. 3, pp. 226–232, 2010.

[106] R. Monteiro and I. Azevedo, "Chronic inflammation in obesity and the metabolic syndrome," *Mediators of Inflammation*, vol. 2010, Article ID 289645, 2010.

[107] G. Condorelli, C. Morisco, M. V. G. Latronico et al., "TNF-α signal transduction in rat neonatal cardiac myocytes: definition of pathways generating from the TNF-α receptor," *FASEB Journal*, vol. 16, no. 13, pp. 1732–1737, 2002.

[108] H. Hirota, K. Yoshida, T. Kishimoto, and T. Taga, "Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 11, pp. 4862–4866, 1995.

[109] M. J. Moreno-Aliaga, N. Perez-Echarri, B. Marcos-Gomez, E. Larequi, F. J. Gil-Bea, B. Viollet et al., "Cardiotrophin-1 is a key regulator of glucose and lipid metabolism," *Cell Metabolism*, vol. 14, pp. 242–253, 2011.

[110] C. Natal, M. A. Fortuño, P. Restituto et al., "Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome," *American Journal of Physiology*, vol. 294, no. 1, pp. E52–E60, 2008.

[111] R. A. Thandavarayan, K. Watanabe, M. Ma et al., "Dominant-negative p38α mitogen-activated protein kinase prevents cardiac apoptosis and remodeling after streptozotocin-induced diabetes mellitus," *American Journal of Physiology*, vol. 297, no. 3, pp. H911–H919, 2009.