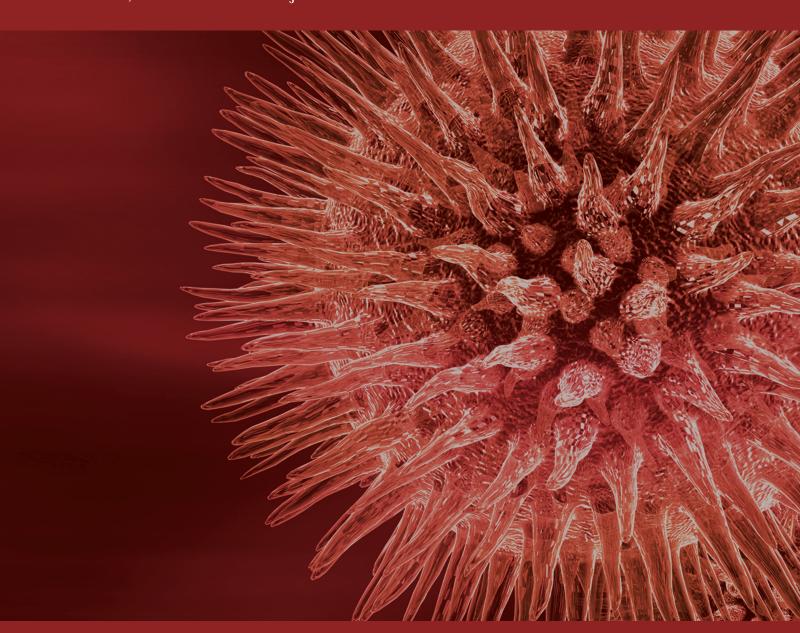
Molecular Mechanisms Involved in Inflammation and Insulin Resistance in Chronic Diseases and Possible Interventions

Guest Editors: Sandro Massao Hirabara, Hilton Kenji Takahashi, Ji An Pan, and Renata Gorjão



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Editorial

Molecular Mechanisms Involved in Inflammation and Insulin Resistance in Chronic Diseases and Possible Interventions

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Received 16 September 2012; Accepted 16 September 2012

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Inflammation and insulin resistance are present in several chronic diseases, including obesity, type 2 diabetes mellitus, metabolic syndrome, cancer, and cardiovascular diseases. Recent studies showed a close relationship between these two conditions, although the precise mechanisms are not completely understood. This special issue aimed to join original research and review articles related to the involvement of inflammation and/or insulin resistance with the development of chronic diseases as well as the interaction between these two factors and possible interventions based on important molecular targets. This issue was interested in articles that explore molecular aspects of inflammatory pathways, insulin resistance, and the crosstalk between them, in humans and in cell and animal models. Moreover, we accepted studies that explored interventions based on molecular targets for preventing or treating correlated disorders and advances for a better characterization and understanding of the mechanisms and mediators involved with the different inflammatory and insulin resistance conditions, addressing biotechnological studies for the development of new potential therapies and interventions. The potential topics for this special issue included (a) molecular basis of inflammation and insulin resistance; (b) crosstalk between inflammatory pathways and insulin signaling; (c) cell and animal models to test and understand the role of inflammation and insulin resistance as well as the interaction of these two factors on the development of chronic diseases; (d) interaction of inflammation and/or insulin resistance in human pathology

conditions; (e) factors leading to inflammation and/or insulin resistance in models of chronic diseases; (f) identification of new biomarkers of chronic inflammation and insulin resistance in different models; (g) identification of new molecular targets for reducing inflammation and insulin resistance in chronic disease models; (h) new interventions for preventing or reducing insulin resistance and inflammation based on molecular targets in signaling pathways.

In the review article from S. Hirabara et al., the authors discussed the recent findings concerning insulin resistance and inflammation, the relationship between these two factors, possible mechanisms involved, and potential interventions for the disorders related. Authors pointed the advances in the field and new biotechnological tools and methodologies that have aided to understand these processes. Discovery and identification of new biomarkers involved with the development of chronic diseases characterized by increased inflammation and insulin resistance, as well as their relevance in the comprehension of interaction of these processes, were also discussed and will allow the study of treatment or prevention for related disorders.

L. Masi et al. characterized the effects of sunflower oil supplementation on insulin resistance and inflammation in mice submitted to high-fat diet (HFD). Briefly, the authors observed that the sunflower oil supplementation induces proinflammatory responses in macrophages and insulin-sensitive peripheral tissues, as well as insulin resistance. These responses were observed in control mice

and pronounced in mice submitted to HFD. Interestingly, although sunflower oil supplementation was able to improve dislipidemia in mice fed with HFD, it increased inflammatory condition and insulin resistance state induced by the diet.

P. Li et al. studied gene variants of mitofusin-2 (MFN-2) in subjects with type 2 diabetes mellitus. This gene is involved in the mitochondrial fusion, regulating the morphology and distribution of this organelle, especially in cells and tissues with high demand of energy, including skeletal muscle and heart. Authors found a relationship between MFN-2 gene polymorphisms and type 2 diabetes mellitus, as well as between some MFN-2 gene variants and two other gene polymorphisms: peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) and estrogen-related receptor- α (ESRRA) genes, which are important transcriptional factors that also regulate mitochondrial biogenesis and oxidative capacity. These findings point MFN-2 gene as a new potential target for further studies aiming at preventive or therapeutic interventions.

In the study of G. Rodrigues et al., authors investigated the effects of the treatment with aqueous extract from *Croton cajucara* plant on hepatic oxidative stress in streptozotocin-induced diabetic rats. *Croton cajucara* is a Brazilian plant rich in several compounds, including flavonoids, coumarins, and alkaloids, that has been suggested to have important antioxidant effects, being used in various diseases, including *diabetes mellitus*, hypercholesterolemia, diarrhea, malaria, fever, gastrointestinal, renal, and hepatic disorders. The results found in this study suggest that the treatment with *Croton cajucara* aqueous extract is effective in decreasing hepatic oxidative stress and cell damage in streptozotocin-induced diabetic rats, indicating for the potential therapeutic use of this plant in related diseases.

M.-Liao et al. reviewed the role of insulin resistance in the development of chronic kidney disease and the potential mechanisms involved. They discuss how different factors, including chronic inflammation, cellular and endoplasmic reticulum oxidative stress, decreased serum erythropoietin, elevated plasma adipokine and fetuin-A, and vitamin D deficiency, can interfere in insulin sensitivity, contributing to the insulin resistance establishment and consequently to the development of chronic kidney disease.

C.-Sung et al. discussed the role of vitamin D on insulin resistance in several pathological conditions, including diabetes mellitus, hypertension, and cardiovascular diseases. Mechanisms for vitamin D deficiency associated with inherited gene polymorphisms are also presented, and its relevance in the immunoregulatory function and insulin resistance development is addressed. Although important advances in the field have been reached, the underlying mechanisms involved in these processes require further investigation.

G. Zhang et al., studying white rabbits, modified a protocol for the development of a new model of human atherosclerosis and vulnerable plaque, allowing a novel approach for related studies. Several markers of the disease were used in order to validate the model, including structural alterations, triacylglycerol and LDL-cholesterol serum

levels, inflammatory parameters, and oxidative stress. This pattern of atherosclerosis for rabbits is very close to that in humans with several advantages when compared to other animal models, showing potential applicability and clinical relevance.

C. Leandro et al. focused on gestational insulin resistance and low insulin secretion induced by undernutrition during pregnancy. They found that low-protein diet during gestation impairs the glucose-stimulated insulin secretion and glucose tolerance. In contrast, a program of pregestational and gestational moderate physical training improved the glucose-stimulated insulin secretion and partially prevented the effects of perinatal undernutrited rats. Thus, well-controlled programs of moderate physical training, which have well-characterized systemic anti-inflammatory effects, starting at early pregnancy, are a potential tool for preventing insulin resistance and gestational *diabetes mellitus* during late pregnancy, as well as for increasing insulin secretion stimulated by glucose and avoiding deleterious effects on the offspring.

A. Alfadda and R. Sallam discussed in their review article the biological functions of reactive oxygen species (ROS) in several physiological and pathological processes. The importance of physiological ROS production on normal vascular diameter regulation, cellular oxygen sensing, immune system function, skeletal muscle physiology, cellular signaling pathways, and gene expression control is presented. On the other hand, the effects of excessive ROS production on the development of diseases, including cancer, *diabetes mellitus*, obesity, chronic inflammation, cardiovascular diseases, and metabolic syndrome are also discussed. This review provides recent findings and advances in this area for understanding the functions of ROS on different targets during various conditions in health and disease.

Acknowledgments

The editors of the special issue "molecular mechanisms involved in inflammation and insulin resistance in chronic diseases and possible interventions" on Journal of Biomedicine and Biotechnology (JBB) thank the JBB Editorial Office and all authors for providing important contributions in this issue, helping to understand the molecular mechanisms involved in these conditions and addressing further research in this field. The contributions in this issue discuss novel insights, potential treatment interventions, and new molecular targets involved in the establishment of insulin resistance and inflammatory state as well as the interaction between these two factors.

Renata Gorjão Hilton Kenji Takahashi Ji An Pan Sandro Massao Hirabara Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 379024, 16 pages doi:10.1155/2012/379024

Review Article

Molecular Targets Related to Inflammation and Insulin Resistance and Potential Interventions

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Received 6 July 2012; Revised 13 September 2012; Accepted 13 September 2012

Academic Editor: Hartmut Jaeschke

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Inflammation and insulin resistance are common in several chronic diseases, such as obesity, type 2 *diabetes mellitus*, metabolic syndrome, cancer, and cardiovascular diseases. Various studies show a relationship between these two factors, although the mechanisms involved are not completely understood yet. Here, we discuss the molecular basis of insulin resistance and inflammation and the molecular aspects on inflammatory pathways interfering in insulin action. Moreover, we explore interventions based on molecular targets for preventing or treating correlated disorders, advances for a better characterization, and understanding of the mechanisms and mediators involved in the different inflammatory and insulin resistance conditions. Finally, we address biotechnological studies for the development of new potential therapies and interventions.

1. Crosstalk between Inflammatory Pathways and Insulin Signaling

1.1. Mechanism of Insulin Action. Insulin receptor is a tetramer protein composed by two extracellular α subunits and two transmembrane β subunits. The α subunits have a binding site to insulin while the β subunits contain an intrinsic tyrosine kinase activity towards intracellular side. Insulin binding to the α subunit leads to a conformational change and activation of the β subunit, resulting in tyrosyl autophosphorylation of the insulin receptor. After being activated and phosphorylated, several intracellular docking proteins bind to the insulin receptor and are also tyrosyl phosphorylated, including insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) [1, 2], Src homology collagen (SHC), and associated protein substrate (APS) [3]. IRS proteins are the major and better characterized proteins involved in insulin signaling. These proteins activate several signaling pathways involved in the regulation of important cellular events such as glucose uptake and metabolism, protein synthesis, gene expression, cell survival, growth, development, and differentiation [4-6]. IRS proteins are phosphorylated on

various tyrosine residues of the C-terminal region, generating specific sites for binding of proteins containing Src homoly-2 (SH2) domains, including phosphatidylinositol-3 kinase (PI-3K), Nck, and Grb-2. PI-3K is composed by a catalytic subunit (p110) and a regulatory subunit (p85). This kinase is an important signaling molecule, mediating metabolic effects of the insulin. Binding of p85 subunit to phosphorylated tyrosine residues of IRS proteins leads to activation of the catalytic activity of p110 subunit and subsequent increase in the generation of phosphatidylinositol 3,4-bisphosphate (PIP2) and phosphatidylinositol 3,4,5trisphosphate (PIP3) content. Downstream proteins from PI3 K pathway comprehend several serine/threonine kinases, for example, phosphoinositide-dependent protein kinase-1 (PDK-1), protein kinase B (PKB, also known as Akt), protein kinase C (PKC), p70 S6 kinase (p70S6K), and glycogen synthase kinase-3 (GSK-3). These kinases are involved in the most important biological effects induced by insulin, such as translocation of glucose transporter-4 (GLUT-4) from intracellular vesicles to plasma membrane, glycogen and protein synthesis, antiapoptotic effects, and gene expression (Figure 1) [7–11].

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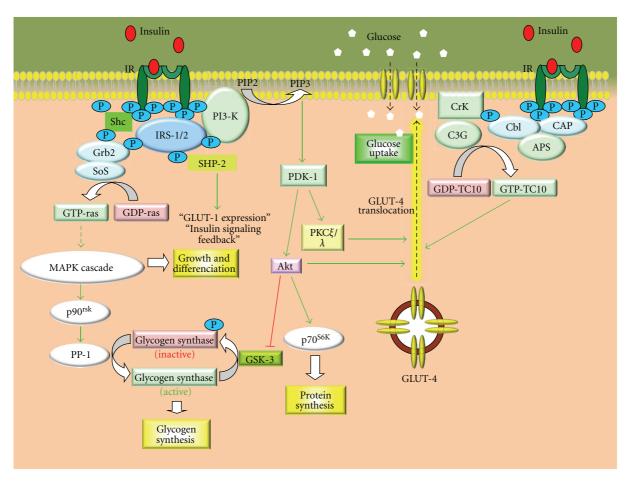


FIGURE 1: Summary of the main insulin signaling pathways. GLUT-1 and -4: glucose transporter-1 and -4; Grb-2: growth receptor binding-2; GSK-3: glycogen synthase kinase-3; IR: insulin receptor; IRS-1 and -2: insulin receptor substrate-1 and -2; MAPK: mitogen-activated protein kinase; PDK-1: phosphoinositide-dependent kinase-1; PIP2: phosphatidyl-inositol diphosphate; PI3: phosphatidyl-inositol triphosphate; P: phosphate; PKC: protein kinase C; PP-1: phosphoprotein phosphatase-1; p70S6 K: protein 70 S6 kinase; p90rsk: protein 90 ribosomal S6 kinase; Shc: Src homology collagen; SHP-2: phosphatase with Src homology 2 domain; SoS: Son of Sevenless.

Other signaling pathways involved in the glucose uptake induced by insulin start with the recruitment of APS to the activated insulin receptor and subsequent association and tyrosine phosphorylation of Cbl, which interacts with Cbl associated protein (CAP) through an SH3 domain and with flotillin, a constituent of lipid raft, through a sorbin domain. The complex CrkII/C3G then binds to the phosphorylated tyrosine residues of Cbl, activating the C3G activity that exchanges GDP for GTP of TC10, a small G-protein that belongs to the Rho family. After being activated, TC10 participates in the GLUT-4 translocation (Figure 1) [12–16].

Mitogen-activated protein kinase (MAPK) cascade starts with the association of Shc to insulin receptor, binding of Grb-2 to Shc or to IRS-1, and formation of the Grb-2/SoS (Son of Sevenless) in the plasma membrane [17–19]. This complex leads to the activation of c-Ras and raf, starting the MAPK cascade [20]. MAPK pathway is involved in the differentiation, cell growth, and development induced by insulin [21], as well as some metabolic effects, as glycogen synthesis and GLUT-4 translocation to plasma membrane (Figure 1) [22–24]. However, this cascade is not enough or even required to this later effect [25].

Disturbances in several proteins involved in the insulin signaling pathways have been found in different conditions of insulin resistance, including obesity, type 2 *diabetes mellitus*, metabolic syndrome, cardiovascular diseases, inflammatory disorders, and cancer [26–28]. Here, we will discuss possible mechanisms involved in the development of insulin resistance related to inflammatory processes.

1.2. Molecular Basis of Insulin Resistance. Insulin resistance occurs when the insulin-sensitive tissues, mainly skeletal muscle, adipose tissue, and liver, lose the ability to respond properly to the hormone [29, 30]. It is associated with several chronic diseases, especially those linked to obesity, such as type 2 diabetes mellitus, metabolic syndrome, dyslipidemias, cardiovascular diseases, cancer, and neurodegenerative diseases [31–33]. However, the precise mechanisms involved in insulin resistance are not fully understood yet [34–37]. Several factors have been proposed to participate in the development of insulin resistance, including increased plasma-free fatty acid level, subclinical chronic inflammation, oxidative and nitrative stress, altered gene expression, and mitochondrial dysfunction [29, 37].

Since free fatty acids are elevated in obesity and related diseases, these metabolites have been proposed to be responsible for the impairment in the insulin action, but the mechanisms are not completely known yet [38, 39]. High availability of fatty acids, specially long-chain saturated fatty acids, results in the establishment of insulin resistance in liver, skeletal muscle, and adipose tissue [40, 41]. Various hypothesis have been proposed to explain the insulin resistance induced by saturated fatty acids, including Randle cycle, oxidative stress, modulation of gene transcription, inflammation, accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), and mitochondrial dysfunction [42–47] (for review, see Martins et al. [37]).

A chronic state of inflammation in the insulin responsive tissues is a major contributor to insulin resistance in obesity and related diseases. Thus, a crosstalking between inflammation and insulin resistance has been suggested by several authors. However, the precise mechanism as well as the mediators involved in this interaction is not completely defined yet. In this paper, we discuss how inflammatory signaling pathways impair insulin signaling (see below).

Intracellular redox balance is a finely regulated process that involves several generating pathways and degrading systems. Physiologically, ROS participate in important biological responses, but accumulation of these molecules leads to oxidative stress condition [48]. ROS are highly oxidant molecules that can oxidize various intracellular components, including membrane phospholipids, proteins, and DNA [49, 50]. Usually, these reactions cause cellular damage, reducing the function of oxidized biomolecules. In insulin resistance, increased ROS production and/or decreased ROS degradation is observed, leading to an oxidative stress condition [51] and activation of signaling pathways related to stress. There is evidence that oxidative stress is also involved in muscle disorders, contributing to the insulin resistance process. Transgenic mice expressing human ubiquitin protein E3 ligase, a protein that binds and promotes degradation of superoxide dismutase-1, resulting in reduced superoxide degradation and consequently oxidative stress, present muscle dysfunction (atrophy and sclerosis) [52].

Activation of signaling pathways to stress has been suggested to participate in the development of insulin resistance by impairing the signaling by this hormone. Several serine/ threonine kinases activated by oxidative stress pathways, including JNK, PKC, GSK-3, NF-kB, and p38 MAPK, have been suggested to impair insulin signaling pathways [53, 54], as described below.

Expression of several genes is also altered in insulin resistance conditions. For example, expression of genes involved in lipid and glucose metabolism, insulin signaling, inflammation, redox balance, and mitochondrial function is modified, suggesting that these processes participate in the pathophysiology of insulin resistance [55–57]. Disturbed mitochondrial function has been suggested to have a central role in these alterations, since this organelle participates in all these processes (for review, see Martins et al. [37]).

1.3. Molecular Basis of Inflammation. Inflammation is a coordinated process evoked by the tissues in response to

noxious stimuli or conditions including the presence of infection, tissue injury, or malfunction. The inflammatory response is activated by molecules released by microorganisms including microbial associated molecular patterns (MAMPs) such as lipopolysaccharide, flagellin, and peptidoglycans or produced/released by host cells including intracellular components, the so-called damage-associated molecular patterns (DAMPs), of which, HMGB1, DNA, and nucleotides are part. These inducers of inflammation bind to their respective receptors and activate biological responses by the resident cells, mainly, macrophages and mast cells. These cells act directly or indirectly on the vasculature and on leukocytes to induce, among other effects, the migration of leukocytes and extravasations of plasma proteins to the tissues [58].

Several receptors have been demonstrated to act as cell sensors of damage or infection. Examples of proteins with this function include the receptors of the toll-like (TLRs) family, the C-type lectin receptors, the purinergic and advanced glycation endproducts receptors (RAGE), and the intracellular nucleotide oligomerization domain (NOD), and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs). After recognizing their ligands, several downstream pathways including c-Jun NH(2)-terminal kinase (JNK) and I κ B kinase complex (IKK) are activated, resulting in changes on transcription factors activity and expression of proteins such as cytokines, enzymes, chemokines, adhesion molecules, and amplification of the inflammatory response.

The activation of the inflammatory pathways described above is a hallmark of obesity, and it has been associated with the development of insulin resistance, atherosclerosis, and other tissue dysfunctions that are secondary to fat accumulation. An increased production of inflammatory mediators and activation of inflammatory pathways in several tissues including adipose tissue (AT), liver, pancreas, skeletal muscle, and hypothalamus are present in obese individuals [59] and define a subclinical inflammatory process also known as "meta-inflammation" (metabolically induced inflammation) [60].

In opposition to other inflammatory conditions, little information is available regarding the inducers and sensors involved in obesity-associated inflammation. In this respect, some hypotheses to explain the initial activation/recruitment of leukocyte to the tissues (mainly, AT) have been proposed. The release of DAMPs by necrotic adipose cells, the increase in the flux of nonesterified fatty acids (increased rates of lipolysis), the reduction in the oxygen tension (hypoxia) leading to activation of hypoxia-induced factor (HIF)-1, which controls the expression of proinflammatory proteins, and the production of chemokines by adipose cells have been suggested to play a role in the initiation of inflammatory process [61]. Despite several advances in the field, the initial events involved in the beginning of inflammation in the AT and the complex interactions between them are not clearly understood, and new components are continuously described and added to the puzzle such as leukotrienes and the apoptosis inhibitor of macrophage (AIM) [62, 63]. This latter protein has been shown to stimulate lipolysis in adipocytes. The release of fatty acids, through interaction

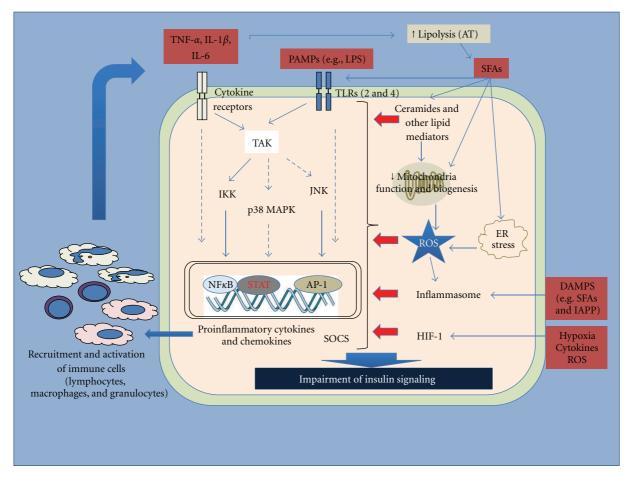


FIGURE 2: Inflammatory pathways activated during obesity and their cross talk with insulin signaling. Different signals act directly through membrane (e.g., toll-like receptors [TLRs] and cytokine receptors) and intracellular proteins (inflammasomes) or indirectly by their effect on cell organelles such as mitochondria and endosomal reticulum and generation of metabolites (e.g., ceramides and other lipid mediators) to activate inflammatory pathways. Transcription factor such as nuclear factor κB (NF κB), activator protein-1 (AP-1), and signal transducers and activators of transcription (STAT) are activated downstream to these pathways and lead to the expression of proteins that inhibit insulin signaling and induce a pro-inflammatory state by recruiting and activating immune cells. AT, adipose tissue; DAMPS, damage associated molecular patterns; ER, endoplasmic reticulum; HIF-1, hypoxia factor-1; IAPP, islet amyloid polypeptide; PAMPS, pathogen associated molecular patterns; ROS, reactive oxygen species; SFAs, saturated fatty acids; SOCS, suppressor of cytokine signaling; TAK, transforming growth factor β -activated kinase.

with TLR4, induces the expression of chemokines that lead to macrophage recruitment [63].

The obesity-induced inflammation is associated with activation of resident cells such as Kupffer cells in the liver [64] and leukocyte infiltration and shift in the polarized state of tissue/recruited leukocytes. These changes are better characterized in the adipose tissue, in which an increased infiltration by proinflammatory M1-type macrophages (classical macrophages), Th1, Th17 and CD8⁺T cells and a reduction in the content of less inflammatory cells such as M2-macrophage, T reg, and Th2 cells were demonstrated [65–68]. The overproduction of inflammatory mediators by the infiltrating cells together with changes in adipokines production and NEFA release by AT contribute to the tissue dysfunctions observed in obesity, as discussed below.

1.4. Relationship between Inflammation and Insulin Resistance. Several chronic diseases are characterized by increased inflammatory process and insulin resistance, such as obesity, type 2 diabetes mellitus, metabolic syndrome, cardiovascular diseases, and cancer [50]. The relationship between these two factors has been proposed by several authors. For example, various studies suggest the involvement of some inflammatory factors in the development of insulin resistance, including cytokines (TNF- α , IL-1, IL-6), ROS, and RNS (Figure 2). These factors lead to the activation of signaling pathways that ultimately impair insulin signaling (see below). In addition, other factors involved in inflammatory processes also can impair insulin sensitivity, particularly lipopolysaccharides (LPSs) and environmental stress (hypoxia, nutrients, and pH) (Figure 2).

TLRs comprehend a family of receptors involved in the recognition of microbes. It has been demonstrated that fatty acids, specially saturated fatty acids, are able to activate TLR-4 in skeletal muscle cells, resulting in increased activity of IKK and JNK. The first kinase degrades the inhibitor of κB $(I\kappa B\alpha)$, resulting in the nuclear factor- κB (NF κB) release and migration to the cell nucleus, where it induces the transcription of proinflammatory genes. The second kinase activates members of the signal transducers and activators of transcription (STAT) family, which are involved in several biological effects, such as expression of genes related to inflammation, apoptosis, differentiation, growth, morphogenesis, migration, and proliferation [69]. Both JNK and IKK β have been proposed to be the mediators of insulin resistance induced by saturated fatty acids (Figure 2). It has been shown that these kinases phosphorylate serine residues on IRS proteins, blocking IRS phosphorylation on tyrosine residues by the activated insulin receptor [70] and consequently inhibiting insulin effects [71]. Moreover, phosphorylation on serine/threonine residues also increases IRS protein degradation, contributing to the establishment of insulin resistance [71–73].

These effects are confirmed by several studies involving gene manipulation. Nonfunctional TLR-4 expression protects mice from insulin resistance and inflammation induced by high-fat diet [74], and TLR-4 gene silencing by small interference of RNA reduces inflammation in acute lung injury induced by lipopolysaccharide [75]. Obese and type 2 diabetic animals are prevented from insulin resistance and inflammation by specific inhibitors or gene mutation (knockout or nonfunctional gene) of IKK or JNK [76–78].

Possible mediators of inflammation and insulin resistance are the fatty acids. These metabolites have been linked to the establishment of inflammatory process, by modulating several signaling pathways related to inflammation. For example, fatty acids can directly activate toll-like receptors (TLRs), G-protein coupled receptors (GPCRs), and tumor necrosis factor- α (TNF- α) receptor as well as modulate inflammatory signaling pathways involved in the increase in cytokine secretion (TNF- α , IL-1 β , and IL-6) [60, 79, 80], oxidative and nitrative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and proinflammatory gene expression. Alterations in the expression of genes and proteins involved in the inflammatory process are clearly associated with insulin resistance and several metabolic abnormalities, including mitochondrial dysfunction, decreased fat oxidation, increased ectopic lipid storage, and impaired insulin signaling pathways (Figure 2) [81].

Proinflammatory cytokines were also involved in the reduction of mitochondrial function [79, 82]. Palmitate-stimulated IL-1 β production in macrophages occurs via NLRP3-ASC inflammasome and participates in the mitochondrial dysfunction induced by this fatty acid. This dysfunction is also observed when the cells are exposed to other cytokines, such as TNF- α or IL-6 [83, 84].

Therapies aimed at neutralizing proinflammatory cytokines such as TNF- α and IL-1 β , such as the monoclonal antibody infliximab and canakinumab, respectively, have been under investigation in the treatment of type 2 diabetic

patients. Considering anti-TNF antibodies, the results are disappointing as many clinical trials in type 2 diabetic patients have failed to demonstrate an effect of TNF neutralization on insulin sensitivity [85–89]. On the other hand, in patients with high grade inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis, anti-TNF therapy has been successfully associated with reduction in insulin resistance and metabolic syndrome components [90–95]. The molecular mechanisms of TNF- α blockade on insulin signaling were related to reduction in IRS-1 serine phosphorylation and increase in AKT phosphorylation in peripheral mononuclear cells from rheumatoid arthritis patients [95].

Potential effects of IL-1 β blockade on insulin sensitivity are current under investigation in humans. The long-term effects of anti-IL-1 β therapy are now examined in the large phase III clinical trial CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) [96]. The study included and treated 17,200 patients with various doses of anti-IL-1 β antibody every 3 months and followed up over 4 years. The primary endpoint of the CANTOS study will be cardiovascular events, and secondary endpoints include new onset type 2 diabetes and diabetes-specific markers. Such large and long-term trials could provide a novel cytokine-based therapy for the secondary prevention of new-onset diabetes as well as confirm the autoinflammatory nature of metabolic disorders.

Another potential molecular target for treatment of inflammatory diseases is JNK. This kinase regulates both the development of insulin resistance and inflammation. However, identification of pharmacologically potent and selective small molecule JNK inhibitors has been limited. Compound A, a reversible ATP-competitive aminopyridine inhibitor of JNK, was developed by Pfizer. Testing this compound in obese mice decreased their body weight as well as blood glucose and triglyceride concentrations and increased insulin sensitivity to levels comparable to those in lean control mice [97]. A substrate competitive inhibitor of JNK, BI-78D3, has also been shown to restore insulin sensitivity in a murine model of type 2 diabetes after a single dose [98]. A more recent drug discovery is the compound 19, a potent and selective dual substrate and ATP-competitive JNK bidentate inhibitor [99]. Glucose intolerant NONcNZO10/LtJ mice were injected intraperitoneally daily for four days with 25 mg/kg 19, and this compound was remarkably effective in restoring normoglycemia without inducing hypoglycemia compared to the vehicle control. These studies demonstrate that inhibition of INK is an effective strategy to ameliorate insulin resistance. However clinical trials are needed to test these compounds in humans and show their efficacy and long-term toxicity.

2. Identification of New Molecular Targets for Reducing Inflammation in Insulin Resistance Models

2.1. GPCRs. G-protein coupled receptors (GPCRs) constitute a family of membrane proteins characterized by

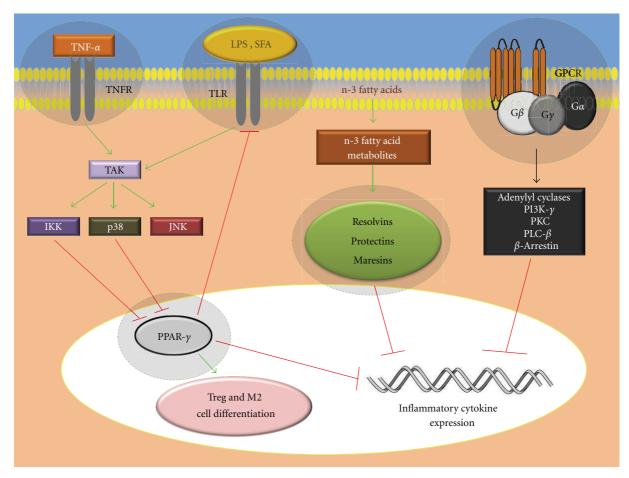


FIGURE 3: Potential molecular targets for reducing inflammation in insulin resistance conditions. Circulated proteins and lipid mediators are included as potential targets. Resolvins, protectins, and maresins are lipid mediators generated from n-3 fatty acid metabolism that have potent anti-inflammatory and immunoregulatory actions, promoting decreased inflammatory cytokine expression. Toll-like receptors (TLRs) are transmembrane receptors that are activated by saturated fatty acids (SFAs) and lipopolysaccharides (LPSs) inducing inflammatory responses. TLRs activate intracellular pathways that inhibit the peroxisome proliferator-activated receptor- γ (PPAR- γ activity). This transcriptional factor is involved with decreased inflammatory cytokine expression and increased Treg cell differentiation. Other cytokines, including tumor necrosis factor- α (TNF- α), also promote PPAR- γ inhibition. G-protein coupled receptor (GPCR) activation may attenuate the production of TNF- α , interleucin-6 (IL-6) and macrophage chemoattractant protein-1. GPR120 is a GPCR activated by n-3 fatty acids in insulin resistance models. COX: cyclooxygenase; IKK: IκB kinase; JNK: c-Jun NH(2)-terminal kinase; PI3 K- γ : phosphatidylinositol 3-kinase- γ ; PKC: protein kinase C; PLC- β : phospholipase C- β .

a common motif, the seven transmembrane domains. Ligand binding to these receptors leads to conformational changes in the receptor and activation of intracellular guanine nucleotide-binding proteins (G-proteins). Several enzymes including adenylyl and guanylyl cyclases, phospholipases A2 and C, phosphodiesterases and phosphatidylinositol 3-kinases (PI3Ks) and other intracellular transduction cascades are triggered downstream to these receptors. These pathways affect both the insulin signaling and the inflammatory proteins expression (Figure 3).

In the recent years, new GPCRs were described and deorphanized. Several of these receptors have been shown to regulate insulin secretion and tissue sensibility to this hormone, becoming potential targets for intervention in conditions of insulin resistance/deficiency. In this paper, we will briefly discuss a class of GPCRs that share in common the fact of being activated by fatty acids (FAs-GPCRs). Other

GPCRs implicated in the glucose metabolism and regulation of inflammation such as GLP-1, glucose-dependent insulinotropic polypeptide (GIP), the bile acid (TGR5), cholecystokinin, the cannabinoid receptors (CBs), and muscarinic receptors are beyond the focus of the present paper and are discussed elsewhere. Proteins that regulate GPCRs signaling such as GPCR kinases and arrestins, which are implicated in the control of food intake, regulation of insulin action, inflammation, adipogenesis, and other processes that are associated with weight gain and development of insulin resistance, are the focus of recent reviews [100, 101], so they are not discussed here.

The FAs-GPCRs receptors, which include the GPRs 40, 41, 43, 84, 119, and 120, present distinct ligand specificity and tissue distribution [102]. These receptors play a relevant role in physio- and pathological conditions [102]. Regarding their participation in the glucose metabolism, it has been

demonstrated that their activation (at least, GPR40 and GPR119) directly stimulates insulin secretion by β -cells and protects these cells from gluco- and lipotoxicity (GPR40) [103, 104]. Activation of FAs-GPCRs induces also the release of gut-derived hormones including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [105–109]. These latter two-gut-derived hormones not only modulate the gastrointestinal functions such as motility, but also the insulin secretion and food intake. In addition to these effects, FAs-GPCRs, mainly, GPR43, 84, and 120, present relevant effects in the inflammatory cell activation [102]. In this sense, activation of GPR120 and β -arrestin 2 by n-3 fatty acids (docosahexaenoic and α -linolenic acids) attenuates the production of TNF- α , IL-6, and macrophage chemoattractant protein-1 (MCP-1, also known as CCL2). This anti-inflammatory effect seems to be important for the beneficial action of these fatty acids in the model of obesity induced by high-fat diet, preventing development of glucose intolerance, insulin resistance, and obesity [110].

Altogether the findings herein discussed highlight the importance of these receptors for glucose homeostasis, control of inflammatory cells activation, and food intake, processes that are linked through complexes interaction, which are not completely understood.

2.2. Histone Deacetylases. Histone deacetylase (HDAC) is a family of enzymes that together with the histone acetyltransferases (HATs) controls the degree of protein acetylation. Inhibition of HDAC activity by different compounds (e.g., short chain fatty acids such as butyrate, valproic acid, trichostatin A, and other compounds) increases the acetylation of histone and nonhistone proteins including NF κ B, MyoD, p53, and N-FAT [111] and, consequently, affects gene expression and proteins activities leading to changes in different aspects of cell biology including cell motility, proliferation, differentiation, and apoptosis.

In addition to their well-known anti-inflammatory effects [112], other recent evidence has been obtained, which together strongly indicates HDAC as a target for novel therapies in insulin resistance and diabetes.

- (i) Histone hyperacetylation has been associated with an increase in insulin expression and protection of β -cells against cytokine-induced apoptosis, as reviewed by Christensen et al. [113].
- (ii) The isoforms 4, 5, and 9 of HDAC are associated with the development of β and δ cells of the pancreas [48].
- (iii) Oral administration of HDACi (ITF2357) reduced β-cells toxicity associated with streptozotocin administration. Additionally, the authors also showed that this HDACi protected islets from cytokine-induced toxicity and reduced production of NO and chemokines in islets [114];
- (iv) Administration of sodium butyrate (diet supplementation or oral tributyrin (a prodrug of butyrate)) to high-fat fed mice attenuated body weight gain, improved lipid and glucose metabolism parameters, and inhibited the development of obesity-associated

- changes including activation of inflammatory pathways and hepatic steatosis [115, 116]. Recently, it has been suggested that this effect of butyrate involves inhibition of HDAC 3 activity, an effect that leads to activation of PPAR- α and expression of FGF21, which stimulates lipid oxidation, triglyceride clearance, and energy expenditure [117];
- (v) HDAC 6 knockout mice are protected from hyperglycemia, glucose intolerance, and insulin resistance secondary to chronic corticoid administration [118];
- (vi) HDACi increases the number of T regulatory cells and their suppressive function, an effect that may be important in the context of adipose tissue inflammation [119, 120].
- 2.3. Peroxisome Proliferator-Activated Receptor-y (PPAR-y). PPAR-y receptor activation has been shown to have significant effects on increasing insulin sensitivity in fat and muscle cells. It improves glucose metabolism and reduces inflammation (Figure 3) [121] and has a crucial role in adipocyte differentiation. PPAR-y is a nuclear receptor that acts as a transcription factor upon activation, by regulating the transcription and expression of specific genes such as adipokines. There are two isoforms of PPAR-y: PPAR-y1 and PPAR-y2. PPAR-y1 is expressed ubiquitously and PPAR-y2 is mainly expressed in adipocytes.

Several studies have demonstrated the anti-inflammatory activities of PPAR- γ . Inhibition of PPAR- γ function by inflammatory cytokines may contribute to pathogenesis of many diseases such as insulin resistance, atherosclerosis, inflammation, and cancer cachexia [122–124]. Its inhibition by TNF- α is involved in inflammation pathogenesis characteristic of insulin resistance. Activation of serine kinases including IKK, ERK, JNK, and p38 may be involved in the TNF regulation of PPAR- γ (Figure 3) (reviewed in [125]). IKK acts through at least two mechanisms: inhibition of PPAR- γ expression [126] and activation of PPAR- γ corepressor [127].

In macrophages, where PPAR gamma is also expressed, it inhibits TLR and IFN- γ mediated inflammatory responses. In obesity, macrophages invade adipose tissue promoting the inflammation characteristic of insulin resistance [128]. Therefore, macrophage PPAR- γ function gained considerable pharmacological interest [129]. Diet-induced obesity influences the state of adipose tissue macrophages from an M2-polarized state (that protects adipocytes from inflammation) to an M1 proinflammatory state. Studies have demonstrated that this obesity-induced phenotypic alteration of macrophage polarization is orchestrated by PPAR- γ [130]. These researchers demonstrated that PPAR- γ is required for the maturation of alternatively activated macrophages (M2 macrophages) by using mice with specific macrophage deletion of PPAR- γ .

Prolonged nutrient excess promotes the accumulation and activation of leukocytes in visceral adipose tissue and other tissues, leading to metabolic abnormalities such as insulin resistance. Cipolletta et al. [131] showed that PPAR- γ is involved with adipose tissue-specific lymphocyte

accumulation and activation, leading to cell differentiation. T regulatory cells (Tregs) are a small subset of T lymphocytes, normally constituting only 5-20% of the CD4⁺ compartment. These cells are thought to be one of the most critical defenses against excessive immune responses, avoiding autoimmunity, allergy, inflammation, infection, and tumorigenesis [132, 133]. Typically, Tregs control other T cell populations, but can also influence the activities of innate immune system cells. Treg cells are characterized by highlevel expression of the forkhead/winged-helix transcription factor, Foxp3. Cipolletta et al. [131] have demonstrated that PPAR-γ collaborates with Foxp3 to impose on naive CD4⁺ T cells the characteristic of visceral adipose tissue Treg cells. In fact, Feuerer et al. [134] demonstrated that Treg cells with a unique phenotype were highly enriched in the abdominal fat of normal mice, but were specifically reduced at this tissue in an insulin-resistant model of obesity. Studies have suggested that adipokines may control T cell responses leading to Treg differentiation [135]. A recent study highlighted the negative effect of leptin on the proliferative capacity of Treg [136]. In fact, it is well demonstrated that obese subjects present low Treg cell number and adiponectin production and high leptin secretion [134]. Probably, PPAR-y is involved with adipokines regulation of lymphocyte differentiation in adipose tissue.

2.4. Toll-Like Receptors (TLRs). TLRs are transmembrane receptors that play a critical role in the detection of microbial infection and in the induction of inflammatory and immune responses against conserved microbial structures, called pathogen-associated molecular patterns [137]. Each member of TLR family recognizes a specific pathogen component which, upon activation, triggers a signaling cascade leading to cytokine production and adaptive immune response. Among the TLRs, TLR2 and TLR4 play a critical role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions [138, 139].

C3H/HeJ mice with a mutation in TLR4 are protected against the development of high fat diet-induced obesity. In addition, these mice demonstrate decreased adiposity, increased oxygen consumption, a decreased respiratory exchange ratio, improved insulin sensitivity, and enhanced insulin-signaling capacity in adipose tissue, muscle, and liver. Moreover, in all these tissues, control mice fed a high-fat diet showed an increase in IkappaB kinase complex and c-Jun NH(2)-terminal kinase activity, which is prevented in C3H/HeJ mice.

Studies in mice demonstrate that TLR-2 and TLR-4 activation and cytokine production stimulated by these receptors lead to the development of diabetes (Figure 3) [140, 141]. More recently, TLR4 has been indicated as a molecular link between free fatty acids, inflammation, and the innate immune system. Dasu et al. [139] studied TLR2 and TLR4 mRNA and protein expression, their ligands, and intracellular signaling in monocytes of recently diagnosed type 2 diabetic patients and observed that there is significant elevation of TLR2 and TLR4 protein, mRNA, endogenous

ligands, and cofactors which, together with hyperglycemia, contribute to the proinflammatory state of type 2 diabetes.

Koopet et al. [142] observed that TLR activation promotes upregulation of IL-6 and MCP-1 release in isolated human adipocytes via specific activation of Erk. TLR-4 deficient mice had also markedly lower circulating concentrations of MCP-1 and much less NF-kB protein in nuclear extracts prepared from adipose tissue. In contrast, TLR-4 deficiency did not attenuate the induction of tumor necrosis factor-alpha (TNF- α) or interleukin-6 (IL-6) expression in adipose tissue promoted by diet with high saturated fatty acids [143]. Nowadays, based on several studies it is clear that TLR4 inhibition is a pharmacologic tool to avoid inflammation in insulin resistance patients.

2.5. N-3 Fatty Acids-Derived Lipid Mediators. Arachidonic acid (n-6 fatty acid) serves as precursor of immune-active lipid mediators known as eicosanoids. Classes of eicosanoids include lipoxins, leukotrienes, and PGs, and their effects on the immune system have been extensively explored and reviewed [144].

First identified by Serhan et al. [145], resolvins are new mediators generated from n-3 fatty acids docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic (EPA, C20: 5n-3) identified first in resolving inflammatory exudates and in tissues enriched with DHA. The names resolvin (resolution phase interaction products) and docosatrienes were given because these bioactive compounds demonstrate potent anti-inflammatory and immunoregulatory actions (Figure 3). These mediators prevented neutrophil entry to inflammation sites and cytokine production and reduced exudates in rats with experimental peritonitis [146]. The compounds derived from EPA carrying potent biological actions are named the E series and are denoted as resolvins of the E series. Those synthesized from DHA are resolvins of the 17S-D series that have immunoregulatory [145] and neuroprotective actions [147]. Protectin D1 (formerly known as neuroprotectin D1) is also generated from DHA [148]. These compounds are produced after acetylation of COX-2 by aspirin. In addition, aspirin treatment triggers the formation of 15-epimeric lipoxins, termed aspirin-triggered lipoxins, that also play a role in resolution of inflammation through inhibition of neutrophil tissue infiltration [146] and stimulation of macrophage phagocytosis of apoptotic neutrophils [149].

Studies demonstrate that n-3 fatty acid feeding promotes endogenous production of resolving D1 and 17-hydroxy-DHA, a marker of resolvin biosynthesis, in adipose tissue of obese-diabetic mice [150]. Transgenic overexpression of fat-1, which encodes a desaturase enzyme that is able to convert ω -6 to ω -3 fatty acids, partially protects against obesity-induced insulin resistance in mice and is associated with an increase in the resolvin biosynthetic pathway marker 17-hydroxy-DHA [151]. These studies demonstrated that high-fat feeding results in a deficient endogenous resolvin and protectin biosynthesis and that these compounds are restored in fat-1 transgenic mice.

Horrillo et al. [152] provided evidence that adipose tissue expresses all the enzymes necessary for the formation of

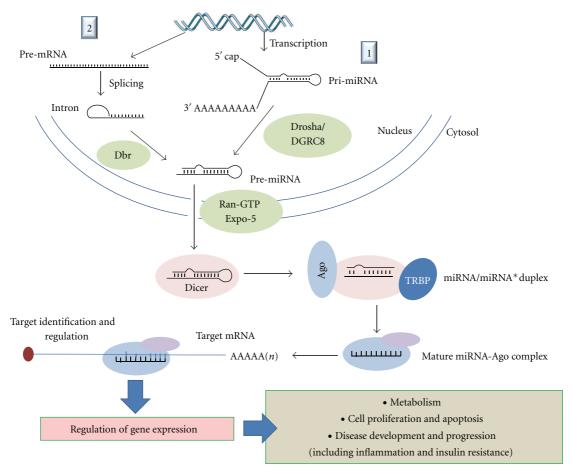


FIGURE 4: MicroRNA (miRNA) biogenesis and gene expression control in human cells. (1) Canonical pathway produces pre-miRNA by Drosha/DGCR8 cleavage of pri-miRNA. (2) Noncanonical pathway mirtrons are produced by spliced introns debranched by debranching enzyme (Dbr), after which they fold into pre-miRNA hairpins. Pre-miRNA hairpins are exported from the nucleus to cytosol by exportin-5 (Expo-5) and cleaved by Dicer to produce 22 nucleotides RNA duplexes. One strand of the duplex is transferred to Argonaute complex (Ago) and guided to base-pair with its target mRNA throughout its seed sequence. TRBP: tar-RNA binding protein.

bioactive lipid mediators derived from both omega-6 and omega-3-PUFAs. In ob/ob mice DHA significantly increased adipose tissue levels of adiponectin, which alleviated hepatic steatosis and insulin resistance [150]. Recent findings indicate also that DHA (at micromolar concentrations) and resolvin D1 (at nanomolar concentrations) consistently decrease M1 macrophage activation in adipose tissue and increase M2 cells. These effects are related to stimulation of arginase 1 expression and attenuation of IFNy/LPS-induced Th1 cytokine secretion [153].

Hellmann et al. [154] suggested that stimulation of the inflammation resolution with the endogenous proresolving mediator resolving D1 provides a novel therapeutic strategy for treating obesity-induced diabetes. The authors observed that in leptin-receptor deficient mouse resolvin D1 prevents the accumulation of macrophages in adipose tissue and restores systemic insulin sensitivity. Notably, these inflammation-resolving factors are important tools to decrease adipose tissue inflammation that is common in insulin resistance.

2.6. MicroRNAs. MicroRNAs (miRNAs or miRs) are short noncoding RNAs that have been demonstrated to be master regulators of the cellular transcriptome and proteome [155–157]. Regulatory miRNAs bind to the complementary segments within 3'-untranslated region (3'UTR) of target transcripts through Watson-Crick base pairing, causing translational inhibition or mRNA cleavage and suppression of gene expression. Over 1000 miRNAs have been identified in the human genome, which are estimated to regulate thousands of protein-coding genes [158, 159]. There is also increasing experimental evidence that miRNAs are involved in the control of several critical biological processes such as metabolism, cell proliferation, apoptosis, and disease development and progression [160–163].

Single-stranded mature miRNAs with 20–24 nucleotides in length are derived from genomically encoded sequences through transcription and complex mRNA processing. Change in gene expression or processing in dysfunctional or abnormal cells or tissues leads to an altered miRNA expression level. Figure 4 summarizes biogenesis and gene

expression control by miRNAs. Profile of these molecules may be used as biomarkers for classifying human diseases and disease status [162, 164, 165].

Recent studies have observed an association between specific miRNAs and insulin resistance [166, 167], supporting the fact that miRNAs may play a role in the pathological development of type 2 diabetes mellitus and leading also to the hypothesis that miRNAs may represent a new class of glucose metabolism regulators with therapeutic potential for improving insulin sensitivity in peripheral tissues. Supporting this idea, Frost and Olson [168] have demonstrated that global and pancreas-specific overexpression of the miRNA Let-7 in mice results in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion. Pharmacological inhibition of the miRNA Let-7 family with specific anti-miR was sufficient to prevent and treat impaired glucose tolerance in diet-induced obesity mice, at least in part, by improving insulin sensitivity in liver and muscle. In addition, miRNA Let-7 was able to block glucoseinduced insulin secretion from the pancreas, suggesting that knockdown of this miRNA also might improve pancreatic β cell function.

A role of the heart in systemic metabolic control and the involvement of the heart-specific microRNA, miR-208a, as potential therapeutic target for metabolic disorder have been proposed. Impaired metabolism of energy-providing substrates and myocardial lipid accumulation are early abnormalities in obese and insulin-resistant individuals [169]. Grueter et al. [170] have shown that MED13, a subunit of the Mediator complex, controls transcription by thyroid hormone and other functions of nuclear hormone receptors in heart, controlling metabolic homeostasis and energy expenditure in mice. They have also shown that miR-208a, a heart-specific miRNA encoded by an intron of the cardiac-specific α -myosin heavy-chain (MHC) gene, negatively regulates MED13 expression. Elevated cardiac expression of MED13 or pharmacologic inhibition of miR-208a in mice led to resistance to high-fat diet-induced obesity and improved systemic insulin sensitivity and glucose tolerance in mice. Conversely, genetic deletion of MED13 specifically in cardiomyocytes enhanced obesity in response to high-fat diet and exacerbated metabolic syndrome [170].

As discussed previously, inflammation and oxidative stress participate in the propagation and development of obesity and associated metabolic disorders associated to insulin resistance. Maladaptive production of various adipokines (e.g., adiponectin, resistin, visfatin, and leptin), migration of monocytes, and subsequent transformation into macrophages within affected tissues are key factors in the self-perpetuating inflammation associated with metabolic disorders [171, 172]. In particular, plasma levels of adiponectin are significantly lower in obese individuals and have been associated with inflammation, insulin resistance, and cardiovascular disease. The involvement of miRs in the protective effects of adiponectin has been recently evaluated. Hulsmans et al. [173] identified miR-146b-5p as a downregulated miR in monocytes of obese subjects with targets in the IRAK/NFkB-related gene cluster. They identified obesityassociated low levels of globular adiponectin as cause of the decrease in miR-146b-5p. The role of miR-146b-5p in the protection against inflammation was further supported by the finding that, after sequestration of miR-146b-5p, the cells lost their potency to raise their anti-inflammatory action in response to high levels of adiponectin.

A possible role of miR-107 in regulating the inflammatory process that might lead to type 2 diabetes mellitus has been proposed by Foley and O'Neill [174]. Because the TLR4 (LPS receptor) has been shown to downregulate miR-107 in activated macrophages located in adipose tissue [175], and miR-107 has been demonstrated to be dysregulated in murine and rodent models of obesity and insulin resistance, respectively [176-178], it has been suggested that miR-107 may be the link between obesity, inflammation, and insulin resistance. The authors hypothesize that decreased miR-107 by TLR4 is required to limit proinflammatory signaling pathways, since this effect will stabilize Caveolin-1, blocking the TLR4 pathway by displacement of MYD88. Decreased miR-107 is an attempt to increase insulin sensitivity in the resolution phase of inflammation. A defect in this process, particularly in the ability of TLR4 to decrease miR-107, could therefore promote inflammation and type 2 diabetes mellitus. Further investigations are warranted to assess this hypothesis. An interesting model would be transgenic mice, in which we could manipulate miR-107 in vivo and pharmacologically inhibit miR-107.

3. Concluding Remarks

The discovery and identification of new biomarkers involved in the pathogenesis of chronic inflammation and insulin resistance have been fundamental to understanding how these processes work and to direct further studies for the prevention or treatment of related disorders. Particularly, these discoveries have aided to identify new target genes, lipids, proteins, and other metabolites involved in the development or severity of chronic diseases, for example, obesity, type 2 diabetes mellitus, metabolic syndrome, cardiovascular diseases, cancer, dyslipidemia, and cancer. Probably, with the help of new tools and advanced methodologies, we will have a better characterization and understanding of the mechanisms and mediators involved in the different inflammatory and insulin resistance conditions, addressing further biotechnological studies for the development of new potential clinical therapies and interventions.

Acknowledgments

This study was supported by grants from São Paulo Research Foundation (FAPESP), Coordination for the Improvement of Higher Level Personnel (CAPES), National Council for Scientific and Technological Development (CNPq)/National Institute of Science and Technology in Obesity and Diabetes (INOD), Center of Lipid Research and Education (CLEaR), and Dean's Office for Research/University of São Paulo. A. Rodrigues is a recipient of fellowship from FAPESP (2011/05876-6).

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 249129, 11 pages doi:10.1155/2012/249129

Research Article

The Immunologic Injury Composite with Balloon Injury Leads to Dyslipidemia: A Robust Rabbit Model of Human Atherosclerosis and Vulnerable Plaque

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Received 21 March 2012; Revised 25 July 2012; Accepted 30 July 2012

Academic Editor: Renata Gorjão

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Atherosclerosis is a condition in which a lipid deposition, thrombus formation, immune cell infiltration, and a chronic inflammatory response, but its systemic study has been hampered by the lack of suitable animal models, especially in herbalism fields. We have tried to perform a perfect animal model that completely replicates the stages of human atherosclerosis. This is the first combined study about the immunologic injury and balloon injury based on the cholesterol diet. In this study, we developed a modified protocol of the white rabbit model that could represent a novel approach to studying human atherosclerosis and vulnerable plaque.

1. Introduction

Atherosclerosis is the most common pathological process that leads to cardiovascular diseases (CVDs), a disease of large- and medium-sized arteries, that is characterized by a formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, inflamed smooth muscle cells (SMCs), endothelial cells (ECs), leukocytes, and foam cells [1, 2]. Several features of atherosclerotic plaques illustrate that atherosclerosis is a complex disease, and many components of the vascular, metabolic, and immune systems are involved in this process [3]. Although dyslipidemia like higher low-density lipoprotein (LDL) or higher triglyceride (TC) remains the most important risk factor for atherosclerosis, immune and inflammatory mechanisms of atherosclerosis have gained tremendous interest in the past 20 years [4, 5].

The area of atherosclerotic plaques research is day by day expanding and the animal models play a very crucial role in this onward journey. An animal model is a nonhuman animal that has a disease or injury that is similar to a human condition [6]. They are due to focal accumulation of cells within the intima of the artery [7], both intra- and extracellular lipids [8], fibrous tissues [9], complex proteoglycans, and mineral blood and blood products [10]. Even though there is no one perfect animal model that completely replicates the stages of human atherosclerosis, cholesterol feeding and mechanical endothelial injury are two common features shared by most models of atherosclerosis [11, 12]. Several characteristics of the rabbit make it an excellent model for the study of atherosclerosis. Previous experimental models for studying this vessel disease consist of rabbits and rats undergoing cholesterol feeding and mechanical endothelial injury [13]. We have built a model based on rabbit that including cholesterol feeding, immunologic injury, and mechanical endothelial injury. Studies in these models have primarily focused on the rules of changes in blood lipids and the morphology and inflammation of the arterial walls [14].

The aim of this experimental study was to evaluate the applicability of a modified white rabbit model that

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were injured with immunologic and balloon base on the cholesterol diet. We evaluated the model from cholesterol metabolism, immune and inflammatory mechanisms and impaired level of reactive oxygen species. The model that we characterize here would be useful for studying the entire process of human atherosclerosis from dysfunction of cholesterol metabolism to the formation of atherosclerotic plaques.

2. Materials and Methods

2.1. Animals and Experimental Design. All animal experiments were performed with the approval of the Animal Care Committee of the Tianjin University of Traditional Chinese Medicine and complied with the Animal Management Rule of the Ministry of Public Health, People's Republic of China (Documentation 55, 2001). Twenty-four, adult (3 months old, 2.0 ± 0.2 kg), male, Japanese white rabbits were purchased from Vital River Lab Animal Technology Co., Ltd. (Beijing, China) and were housed in an animal room maintained at 22 ± 2 °C with 40% to 60% RH and a light period from 8:00 to 20:00 in the Laboratory Animal Center of Tianjin university of TCM. The rabbits were divided randomly into two groups, normal-diet group (normal, n =8) was fed the rabbit standard diet (100 g per rabbit per day) and experimental model group (CIB, n = 16) were fed an atherogenic diet (1% cholesterol, 5% yolk, 5% lard, and 89% standard diet). All the animals had free access to water. A scheme of the design of the atherosclerotic model study is shown in Figure 1.

2.2. Immunologic Injury and Balloon Injury. Initially, all experiment rabbits were fed with cholesterol diet (Yingbo, Tianjin, China) 2 weeks prior to injecting solcoseryl albumin (Sangon, Shanghai, China, 250 mg/kg) from the marginal ear vein and 4 weeks after balloon injury of the abdominal aorta, followed by 6 weeks of cholesterol chow diet constantly. All rabbits underwent balloon-induced endothelial injury in the abdominal aorta after being anesthetized with 3% pentobarbital sodium salt (Sigma-Aldrich P3761, US, 30 mg/kg body weight, iv), and balloon injury of the abdominal aortic wall was performed using a 4F Fogarty catheter (Shangyikangge, Shanghai, China) introduced through a right femoral artery cutdown. After the catheter was advanced to the diaphragm, the balloon was inflated and the catheter was gently retracted toward the iliofemoral artery. This was repeated three times. The method was demonstrated in previous reports [15, 16].

2.3. Biochemical Parameters. Animals were bled from the marginal ear vein at baseline at the end of weeks 3, 6, and 10 (3 mL each time). Blood samples were centrifuged at 2 000 g for 20 minutes to obtain serum and plasma. The serum was used for biomarkers assay.

Collected serum was analyzed in an automatic blood chemical analyzer (Humalyzer 2000, Nairobi, Kenya), and the serum concentrations of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were

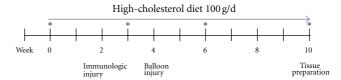


FIGURE 1: Schematic diagram of study timeline. Immunologic injury is followed by 2 weeks of high cholesterol feeding and balloon injury on week 4. *Means the times of serum collection and animal body weight.

obtained (enzyme-colorimetric method). At the same time, serum levels of superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO) were estimated by enzyme colorimetric method with kits from, Jiancheng Bioengineering Institute (Nanjing, China), and serum levels of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor- α (TNF- α), and oxidized LDL cholesterol (ox-LDL) were measured by use of enzyme-linked immunosorbent assay (ELISA) kits (Rapid Bio, California, US).

2.4. Tissue Preparation. The experiment was continued till 10 weeks. After euthanasia of the animals by intravenous injection of pentobarbital (Sigma-Aldrich P3761, US, 50 mg/kg body weight, iv) at the end of weeks 10, the blood sample was taken via the marginal ear vein. The aorta from the aortic valve to the femoral bifurcation tissue of animals was removed. The abdominal aortic tissue was collected for histological study and remainders stored at -80° C for other studies.

The abdominal aortas from the rabbits were immediately excised and cut into 10 serial 2.5 mm sections, with alternative sections embedded in paraffin and catalogued. The tissue sections (6 μ m thick) were cut from paraffinembedded blocks on a microtome and mounted from warm water (40°C) onto adhesive microscope slides. Sections were allowed to dry overnight at room temperature and for later general histological staining, as described previously [17, 18].

2.5. Histological and Morphometric Evaluation

2.5.1. Identification of Atherosclerotic Plaques. The fatty streak lesions of the thoracic aorta in each group were easily identified by staining with Sudan III (Sangon, Shanghai, China), as described above [19]. After formalin fixation of the arteries, paraffin blocks were prepared by the following routine histological procedures. The tissue sections were obtained from the prepared paraffin blocks and were stained with hematoxylin and eosin (H&E). All slides were examined by Olympus BX 40 Light Microscope (Olympus Corporation, Tokyo, Japan), and Champion HMIAS-2000 Image Analysis System (Wuhan Champion Image Technology Co., Ltd, Hubei, China) was used for image processing. The luminal intimal and medial cross-sectional areas of the arteries were measured and the index defined as intimal/medial area ratio was calculated from these measurements. The fibrous cap thickness was measured by drawing lines perpendicular to the lumen at three different locations of the fibrous cap

TABLE 1: Body weight in the two groups (kg)*.

Groups	Number of rabbits	0 wk (baseline)	3 wk	6 wk	10 wk
Normal	6	2.11 ± 0.20	2.250 ± 0.175	2.400 ± 0.167	2.73 ± 0.31
CIB	10	2.12 ± 0.14	2.257 ± 0.183	2.242 ± 0.156	2.57 ± 0.16

^{*}Data are given as the mean ± SD. No significant difference was found between the normal group and the CIB group.

Table 2: Lipid profile of serum in the two groups (mmol/L).

	Groups	n	0 wk	3 wk	6 wk	10 wk
TC	Normal	6	1.04 ± 0.13	1.43 ± 0.40	1.31 ± 0.17	1.35 ± 0.34
	CIB	10	1.08 ± 0.40	$10.38 \pm 4.99**$	$10.99 \pm 3.89**$	$24.04 \pm 4.73**$
TG	Normal	6	1.14 ± 0.39	0.69 ± 0.07	0.96 ± 0.34	0.77 ± 0.20
	CIB	10	1.19 ± 0.54	$1.25 \pm 0.57^*$	0.95 ± 0.47	$1.42 \pm 0.51^*$
LDL-C	Normal	6	0.53 ± 0.17	0.39 ± 0.13	0.35 ± 0.16	0.67 ± 0.21
	CIB	10	0.51 ± 0.12	$3.50 \pm 1.74**$	$2.39 \pm 1.17**$	$11.73 \pm 3.09**$
HDL-C	Normal	6	0.66 ± 0.18	0.68 ± 0.09	0.53 ± 0.08	0.37 ± 0.09
	CIB	10	0.60 ± 0.15	$1.54 \pm 0.59**$	$1.59 \pm 0.38**$	$4.00 \pm 0.90**$

TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol. Data are expressed as mean \pm SD, *P < 0.05; **P < 0.01 compared with normal.

on sections stained with the measurement as well, and the mean value was calculated. The other segment was used for cryosections and cut into $6\,\mu$ m thick sections for Masson's trichromatic and Oil Red O staining, and the methods used here were demonstrated in previous reports [20, 21].

2.5.2. Immunohistochemistry in Abdominal Aortas Lesions. Immunohistochemical staining involved several standard techniques as described previously [22]. In brief, all tissue sections were rinsed with PBS at pH 7.4; in order to block endogenous peroxidase activity; the sections were incubated in 0.3% H₂O₂ for 30 min, and then washed with PBS. Followed by incubating in secondary antibody for 30 minutes at 37°C. The secondary antibody and all subsequent reagents were diluted in PBS containing 0.1% bovine serum albumin; 200 µL of the diluted solution was added to each slide and incubated in a moisture chamber. Immunohisto-chemical staining was visualized by use of a diaminobenzidine kit (TBD Science, Tianjin, China) according to the manufacturer's instructions. The primary antibodies included α-smooth muscle cell actin (Thermo Fisher Scientific, US), anti-CD68 polyclonal antibody, anti-CD31 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) matrix metalloproteinase-9 antibody (Abcam, Cambridge, UK), vascular endothelial growth factor antibody (Santa Cruz Biotechnology, CA, USA), and NF-kB P65 subunit antibody (Thermo Fisher Scientific, USA).

3. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) 11.0 was used for the analysis. Results are given as mean \pm SD. Data were analyzed statistically using one-way ANOVA test followed by LSD posttest, and pairwise multiple comparisons were performed using LSD posttest. In all instances, P value less than 0.05 or 0.01 was considered significant.

4. Results

4.1. Design of the Animal Experiments. During the experiment, six rabbits in the CIB group and two rabbits in the normal group died of anesthesia overdose or diarrhea. Data was available for analysis for 6 rabbits in the normal group and 10 for CIB group. The animal body weight was not statistically significant in the CIB group compared with the normal group in week 0, 3, 6, 10, as shown in Table 1. We found that the animal body weight of CIB group was slightly decreased at the end of the sixth weekend it was probably due more to diet reduction after surgery.

4.2. Cholesterol Levels. Table 2 shows lipid profile of serum in two groups; the levels of serum TC, TG, LDL-C, and HDL-C were elevated in general. Serum TC levels in the CIB group were higher than those in the control group at 3rd, 6th, and 10th weekend, respectively (P < 0.01). Compared with the normal group, serum TG levels were increased in the CIB group at the 3rd weekend (P < 0.05), and the levels significantly increased at the 10th weekend (P < 0.05). However, which levels in CIB group not increased like TC than in the control group at the 6th weekend. Under a high cholesterol diet, the levels of the serum LDL-C of the rabbits were elevated as well. The serum LDL-C levels in the CIB group were higher than those in the normal group at the 3rd, 6th weeks, respectively, and showed a significant difference at the 10th weekend. Serum HDL-C levels kept the same trend in both groups.

4.3. Histological and Morphometric Evaluation

4.3.1. Identification of Atherosclerotic Plaques. The fatty streak lesions of the aorta in two groups were easily identified by staining with Sudan III or by macroscopic observation. Broad and fused fatty streak lesions were easily found in CIB

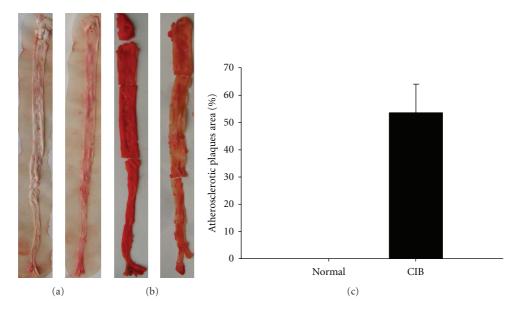


FIGURE 2: Macroscopic observation of atherosclerotic plaques area and staining with Sudan III. (a) Macroscopic observation of the whole aorta. (b) Representative en face Sudan staining of the whole aorta. (c) Percentage of the atherosclerotic plaques area in both groups. The Ratio of atherosclerotic plaques area to the whole aorta was $54 \pm 11\%$ in CIB group, but no atherosclerotic plaques area has been detected in the normal group.

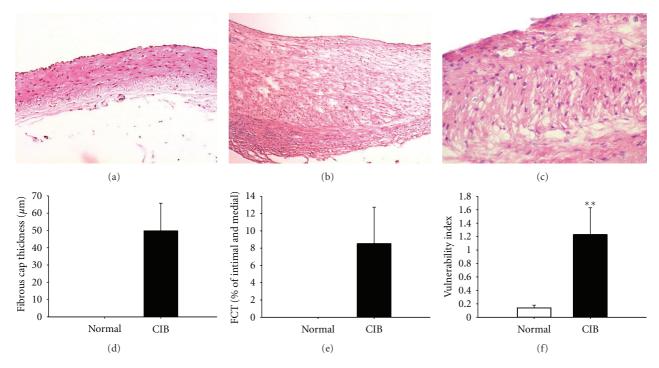


FIGURE 3: Histological examples of lipid core and the fibrous cap associated with rabbit atherosclerotic plaques. (a) A cross section of a normal rabbit aorta. (b) A cross section of a CIB rabbit atherosclerotic plaques. (c) Higher magnification of the atherosclerotic plaques site shows a thin fibrous cap. The fibrous cap thickness, (d) ratio of fibrous cap to intima media thickness (e), and vulnerability index (f) were calculated. Data are expressed as mean \pm SD, **P < 0.01 compared with normal.

group that was stained most seriously, almost the whole aorta was red, whereas no atherosclerotic plaques were observed in the normal group (Figures 2(a) and 2(b)). The percent area of the atherosclerotic plaques was measured in both groups as shown in Figure 2(c).

The fibrous cap thickness was measured by drawing lines perpendicular to the lumen at three different locations of the fibrous cap on sections. When compared with that of the normal group, fibrous cap thickness was significantly increased in CIB group ($49.80 \pm 16.96 \,\mu\text{m}$, Figure 3(a)), and

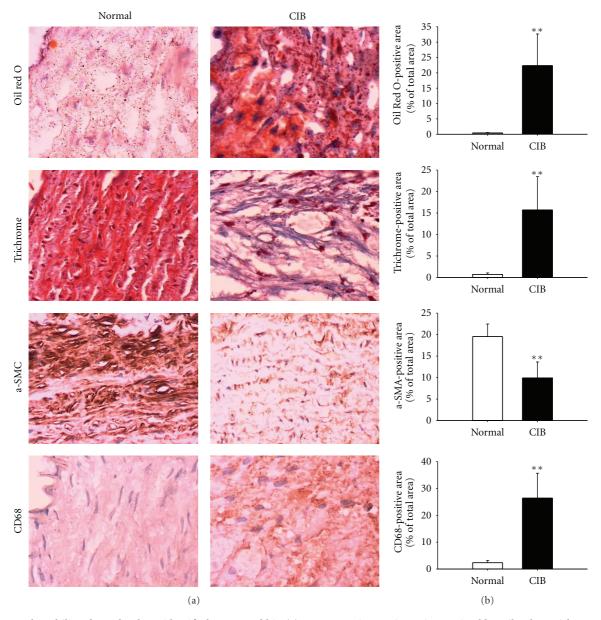


FIGURE 4: Vulnerability plaque has been identified on CIB rabbit. (a) Representative aortic sections stained by Oil red O, trichrome, CD68 antibody and a SMC-specific antibody (magnification $\times 40$). (b) Quantification revealed a significantly larger lipid core, collagen, SMA-positive area, and the density of macrophages (identified by CD 68 immunostaining) in CIB rabbits. Data are expressed as mean \pm SD, **P < 0.01 compared with normal.

the ratios of fibrous cap and intima media thickness were calculated (8.52 \pm 4.22, Figure 3(b)).

The atherosclerotic plaque content of SMCs was lower in the normal group while the content of lipid core area was lower in the CIB group. But CD68 and collagen content of the plaques were higher in the CIB group; as a result, the vulnerability index in the normal group was lower than that in the CIB group (Figure 4(a)). The ratio of lipid core area and plaque area, and ratio of plaque area and intima area were calculated as well (Figure 4(b)).

There was no detectable change in the size of the medial thickness of aortic arch thoracic aorta and abdominal aorta, but the intima thickness of the aorta and the ratio of intima and media in CIB group was the highest. The index of endometrial hyperplasia was remarkably different between the two groups (Figure 5).

4.4. Immunohistochemical Inflammatory Markers and Others. Immunologic injury and high cholesterol control induced a significant increase in systemic inflammatory response in CIB group. NF- κ B is a key factor that regulates the expression of many genes involved in the pathophysiology of tissue inflammation. In CIB group, NF- κ B was increased significantly compared to the normal diet group on the basis of atherosclerotic arteries sections, and strong MMP-9

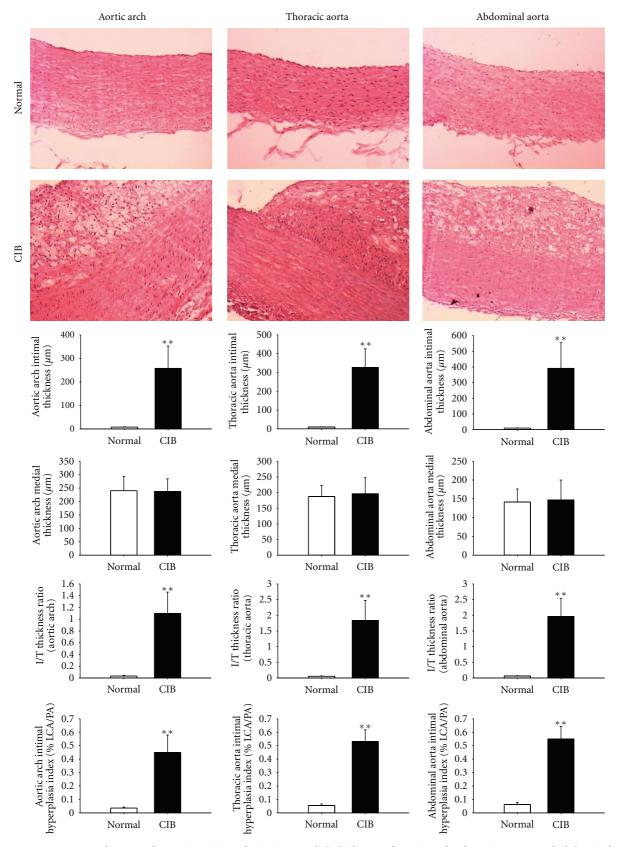


FIGURE 5: Representative hematoxylin-eosin staining for intima/medial thickness of aortic arch, thoracic aorta, and abdominal aorta (magnification $\times 40$). The pathology of the abdominal aortic was most serious, followed by the thoracic aorta and aortic arch. Data are expressed as mean \pm SD, *P < 0.01, **P < 0.01 compared with normal group.

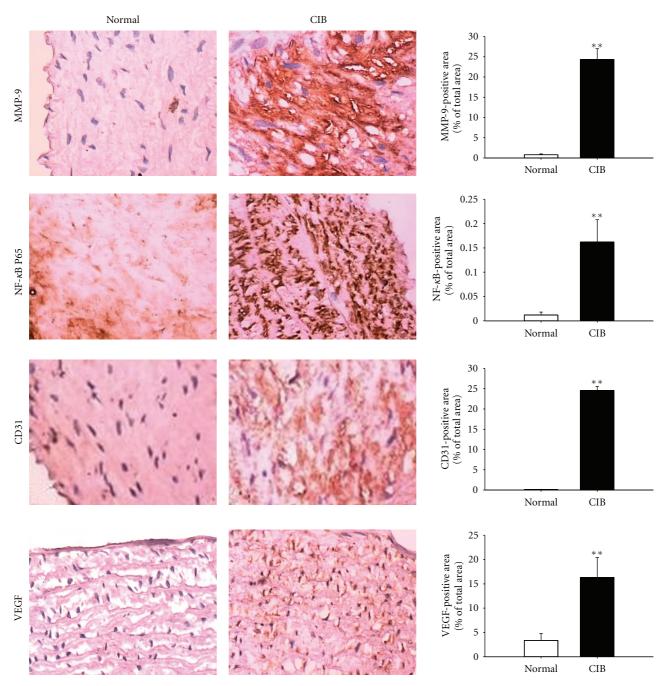


FIGURE 6: Representative immunohistochemical staining for MMP-9, NF- κ B P65 subunit, CD31 and VEGF. Strong positive staining was observed in CIB group and weak staining on the sections of arteries from normal group. MMP-9, matrix metalloproteinase-9; NF- κ B P65 subunit, nuclear factor- κ B P65 subunit; CD31, cluster of differentiation 31; VEGF, vascular endothelial growth factor. Data are expressed as mean \pm SD, **P < 0.01 compared with normal.

positive staining was also observed And CD31 and VEGF have the same trend with MMP-9 (Figure 6).

4.5. Oxidative Factors. Serum levels of ox-LDL were elevated at week 3 and 6 in the CIB group, but compared to the week 6, it has a mild reduction. The levels of NO decreased after the immunologic injury, however, at week 10, which was significantly higher in CIB group than in the control group. Serum level of SOD was reduced by the immunologic

injury due to the cholesterol diet, but it was elevated at week 6 maybe because the intima was destroyed by balloon injury. Compared to the control group, MDA was increased uniformly at week 3, 6, and 10. In CIB group, MCP-1 was increased significantly compared to the normal diet group at the end of the third week, whereas there was no obvious differences in week 6. The levels of IL-1 have slightly decreased at week 6 compared to week 3; TNF- α kept a same trend with MCP-1 (Figure 7).

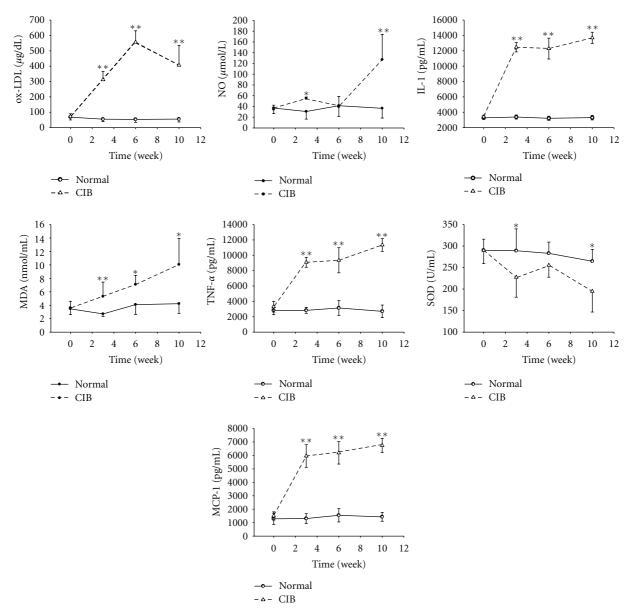


FIGURE 7: Analysis of biochemical measurements in two groups of rabbits. Serum level of IL-1, ox-LDL, NO, SOD, MDA, MCP-1, and TNF- α by using of enzyme-linked immunosorbent assay on week 0, 3, 6, 10. IL-1, interleukin-1; ox-LDL, oxidized LDL cholesterol; NO, nitric oxide; SOD, superoxide dismutase; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α . Data are expressed as mean \pm SD, *P < 0.05, **P < 0.01 compared with normal group.

5. Discussion

Atherosclerosis is characterized by lipid accumulation in the vessel wall, inflammation, oxidative stress, and immunologic injury [23]. There is no perfect animal model that completely replicates all stages of human atherosclerosis, yet the model may be a promising entity in exploring the aetiopathogenesis and regression of atherosclerosis [24, 25]. In this study, we developed a modified protocol of the JW rabbit that reproduced features of atherothrombosis observed in humans. All the experimental rabbits have been injected with foreign protein, remodeled mechanical endothelial injury, and undergone cholesterol feeding. Histological features of the aorta and the levels of cholesterol, inflammation,

and oxidative stress were measured and evaluated to the robust animal models. The key finding of our study is that the rabbit and human atherosclerotic plaques have remarkable similarities. In addition, several novel ideas which enhance our understanding of the mechanisms of human atherosclerotic stages were also introduced by this study.

Cholesterol feeding and mechanical endothelial injury are two common features shared by most models of atherosclerosis [26], but the occurrence of coronary or aortic atherosclerosis in immunopathology factors probably play a major role [27]. We retained the procedure of cholesterol diet for longer durations, which leads to lipid toxicity. And the

balloon injury could accelerate cholesterol deposition on the arterial wall and injury of the foreign protein maybe make inflammation and oxidative stress of atherosclerosis more serious [28, 29].

At the beginning of the experiment, blood lipid distributions of all animals were evaluated and there was no significant difference between the two groups. The highcholesterol diet containing egg yolk and lard oil is very similar to the characteristics of human diet. We chose to perform immunologic injury at two weeks after initiating the high-cholesterol diet, for the immunopathology factors may increase the severity of the local per oxidative damage and inflammatory response [30]. The experimental animals have been injured with balloon at four weeks after initiating the high-cholesterol diet so that endothelial damage occurred in a setting of increased disorders of lipid metabolism, which could further accelerate plaque formation [31]. What makes me surprised was that the serum cholesterol levels were significantly higher in CIB rabbits than in normal diet rabbits at the end of week 3, but not significantly increased after balloon injury comparing to the week 3. It is becoming increasingly evidential that immunologic injury to the system is probably a primary causative factor in atherosclerosis.

Plaque vulnerability is characterized by a large lipidrich atherosclerotic core, a thin fibrous cap, and infiltration by inflammatory cells, such as macrophages [32, 33]. Compared with intact caps, the ruptured ones usually are thinner and contain less collagen, have fewer SMCs, and are heavily infiltrated by macrophage foam cells. Atherosclerosis plaques with its constituents, including lipid core structure, proliferating SMCs, and collagen fibers, was observed in the experiment described above. The observations of plaque constituents suggested that there were a higher intima-tomedia thickness ratio and intimal hyperplasia index and were confirmed by previous reports of high macrophage density in areas of plaque rupture [34, 35]. This correlation proves that the plaque which is characterized by a large lipid-rich athermanous core and a thin fibrous cap is more inflammatory and vulnerable. The experiment also showed that the pathology of the abdominal aortic was most serious, followed by the thoracic aorta and aortic arch. The intima thickness was significantly higher in CIB rabbits than in normal diet rabbits, but not significant different in media thickness between two groups.

Inflammation plays a pivotal role in all stages of thermogenesis, from foam cell to plaque formation to rupture and ultimately to thrombosis, so the inflammatory response in animal blood was evaluated [36, 37]. In this regard, the data suggested that the presence of immunologic injury in high-cholesterol diet rabbits results in a more vulnerable vessel wall at the site of the endothelial lesion. IL-6, MCP-1, and TNF- α are three critical mediators of the systemic effects in inflammatory endothelial lesion, and they are also associated with the precipitation of atherosclerotic events. As expected, serum levels of IL-6, MCP-1, and TNF- α increased in the CIB group at week 3, 6, and 10 compared with the control group. The previous studies have demonstrated that MCP-1

expression occurs in the arterial wall in response to hypercholesterolemia in rabbits. Oxidized LDL also induces local vascular cells to produce MCP-1, which causes monocyte recruitment and promotes the release of lipids and liposomal enzymes into the extracellular space, thereby enhancing the progression of the atherosclerotic lesion [38].

It is now recognized that atherosclerotic plaques are characterized not only by the presence of inflammatory reactions but also by oxidative stress. Oxidative stress induces inflammatory responses causing damage to the vasculature and may play an important role in the development of many diseases including atherosclerosis. It also causes direct damage to proteins or leads to chemical modification of amino acids in proteins. This increases the protein content in carbonyls, which then serve as biomarkers of general oxidative stress. The level of per oxidative damage has also been evaluated in this experiment. Oxidized LDL is highly cytotoxic and may promote the development of atherosclerosis by several mechanisms. MDA is a main byproduct of lipid peroxidation; lipid peroxidation is evaluated by levels of circulating MDA in our research. MDA production increases in CIB group early at week 3, confirming previous reports that an increased formation of lipid peroxidation products occurs. NO is a powerful regulator of vascular function, and it appears that abnormalities in the production or actions of NO lead to endothelial dysfunction and abnormal vascular remodeling. In our experiment, the serum NO level in the CIB group was higher than those in the normal group at the 3rd, but there was a slightly decreased after treating with balloon angioplasty in the 4th week, respectively, and it showed a significant difference at the 10th weekend, which may be that the function of blood vessel endothelium of rabbit was recovered after injuring with balloon angioplasty in the 4th week.

In conclusion, CIB rabbit, a novel robust model of atherosclerotic plaques, not only has the disorders of lipid metabolism but also carries the metabolic syndrome, which partially caused by immunologic derangement. The pathophysiology of the CIB rabbit coincides well with the current concept of metabolic syndrome, which contends that lipid toxicity accumulation is the fundamental disorder and that inflammation, oxidative stress, and lipid metabolism disorders are intimately related to the evolution of atherosclerosis. As the metabolic pattern for rabbits resembles that in humans, CIB rabbits have superior clinical usefulness compared with other animal models, especially in herbalism fields.

Funding

This work was supported by the State Key Program of the National Natural Science Foundation of China (no. 30973731; 30901905).

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 945131, 9 pages doi:10.1155/2012/945131

Research Article

Sunflower Oil Supplementation Has Proinflammatory Effects and Does Not Reverse Insulin Resistance in Obesity Induced by High-Fat Diet in C57BL/6 Mice

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Received 15 June 2012; Accepted 5 August 2012

Academic Editor: Ji An Pan

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High consumption of polyunsaturated fatty acids, such as sunflower oil has been associated to beneficial effects in plasma lipid profile, but its role on inflammation and insulin resistance is not fully elucidated yet. We evaluated the effect of sunflower oil supplementation on inflammatory state and insulin resistance condition in HFD-induced obese mice. C57BL/6 male mice (8 weeks) were divided in four groups: (a) control diet (CD), (b) HFD, (c) CD supplemented with n-6 (CD + n-6), and (d) HFD supplemented with n-6 (HFD + n-6). CD + n-6 and HFD + n-6 were supplemented with sunflower oil by oral gavage at 2 g/Kg of body weight, three times per week. CD and HFD were supplemented with water instead at the same dose. HFD induced whole and muscle-specific insulin resistance associated with increased inflammatory markers in insulin-sensitive tissues and macrophage cells. Sunflower oil supplementation was not efficient in preventing or reducing these parameters. In addition, the supplementation increased pro-inflammatory cytokine production by macrophages and tissues. Lipid profile, on the other hand, was improved with the sunflower oil supplementation in animals fed HFD. In conclusion, sunflower oil supplementation improves lipid profile, but it does not prevent or attenuate insulin resistance and inflammation induced by HFD in C57BL/6 mice.

1. Introduction

Occidental diet is characterized by high caloric intake, mainly saturated fatty acids and glucose consumption, contributing to the development of obesity and insulin resistance. In the past 15 years, obesity has been associated to chronic inflammation in several tissues and cells, such as liver, adipose tissue, skeletal muscle, and immune cells.

In fact, chronic and subclinical "low-grade" inflammatory state is a hallmark of obesity, and this condition has been proposed to play a central role in the development of insulin resistance, type 2 *diabetes mellitus*, and steatosis [1]. During the past decades, the prevalence of obesity has

enormously increased worldwide [2]. A possible interaction factor between metabolic disorders and inflammation is the disruption in lipid metabolism caused by high levels of saturated fatty acids from high-fat and caloric diet [3, 4].

C57BL/6 mice develop obesity, insulin resistance (IR), diabetes mellitus, advanced fatty liver, and fatty pancreatic diseases when submitted to a high-fat diet (HFD), mainly enriched with saturated fatty acids [5]. The composition of fatty acids on diet can be an important modulator of lipid metabolism and inflammation. Some studies have showed that an increase in circulating levels of mono- or polyunsaturated fatty acids improves insulin sensitivity and ameliorates hepatic steatosis [6, 7]. Sunflower oil is rich in

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mono- (MUFA) and polyunsaturated fatty acids (PUFA), mainly linoleic acid, a member of the n-6 family.

Diet enriched with sunflower oil decreases plasma triacylglycerol and has beneficial effects on plasma lipid profile [8]. Moreover, acute administration of sunflower oil in rats potentially prevents the gastric damages generated by indomethacin administration through increased anti-inflammatory response [9]. However, mice fed with sunflower oil-enriched diet present increased IL-6 (a proinflammatory cytokine) levels in white adipose tissue[8]. Thus, further studies are required to adequately determine the pro- or anti-inflammatory effects of n-6 fatty acids.

Adipose tissue, skeletal muscle, liver, and immune cells have been identified as critical targets for the disruption in metabolic and inflammatory signaling in obesity. The imbalance between metabolic alterations and inflammatory signaling pathways leads to insulin resistance, hepatic steatosis, and related diseases [4, 10].

In this study, we evaluated the effect of sunflower oil supplementation on inflammation and insulin sensitivity in mice fed a balanced diet and a HFD (rich in saturated fatty acids). The protecting or potentiating effect of sunflower oil supplementation on insulin resistance and inflammation was investigated. Whole body (Insulin Tolerance Test—ITT and Glucose Tolerance Test—GTT) and skeletal muscle (glucose uptake and metabolism) insulin sensitivity was examined. Inflammatory markers were investigated in liver, adipose tissue, skeletal muscle, and peritoneal macrophages. The strategy used was to investigate the effect of sunflower oil supplementation in mice fed a balanced diet and a HFD. Under this last condition, a clear inflammatory state is established and so a possible anti-inflammatory effect could be pronounced.

2. Materials and Methods

2.1. Animals. All animal studies were performed according to protocols approved by the Animal Care and Use Committee from the Institute of Biomedical Sciences, University of São Paulo. C57BL/6 male mice (8 weeks-old) were used for the study. Animals were housed in a room with light-dark cycle of 12-12 h and temperature of 23 \pm 2°C. Animals were divided in four groups: (a) control diet (CD), (b) high-fat diet (HFD), (c) control diet supplemented with n-6 (CD + n-6), and (d) high-fat diet supplemented with n-6 (HFD + n-6). During the first 4 weeks preceding the induction of obesity by HFD, all the four experimental groups were fed ad libitum with a control diet (76% carbohydrates, 9% fat, 15% proteins). CD + n-6 and HFD + n-6 were supplemented with sunflower oil (n-6 PUFA source) by oral gavage at 2 g per Kg of body weight, three times per week, during 12 weeks. This dosage of oil was chosen based on previous studies using different oils from our group and others [11-13]. CD and HFD received water at the same dose.

2.2. HFD-Induced Obesity and Insulin Resistance. After the first 4 weeks, animals from the HFD and HFD + n-6 groups received high-fat diet (26% carbohydrates, 59% fat, 15%

proteins) during the next 8 weeks. CD and CD + n-6 groups remained on the control diet. Supplementation with n-6 (sunflower oil, 2 g/Kg b.w.) was kept until the end of the 12 weeks.

2.3. Glucose and Insulin Tolerance Tests. Tolerances to glucose (GTT) and to insulin (ITT) were evaluated after 6 h fasting. For GTT, mice were intraperitoneally injected (i.p.) with glucose (2 g/Kg body weight). Blood glucose measurements were performed at 0, 15, 30, 45, 60, and 90 min after glucose injection. Glucose concentration versus time was plotted and the area under the curve (AUC) was calculated for each animal. For ITT, animals were i.p. injected with insulin (Humulin R, Lilly, 0.75 UI/kg b.w.) and glucose measurements were performed at 0, 10, 20, 30, 40, 50, and 60 min after injection. Glucose concentration versus time was plotted and the glucose lowering rate was calculated.

In both tests, blood samples were collected from the tail vein. For GTT, serum glucose was measured by colorimetric assay commercially available (PAP Liquiform Glucose, Labtest) and for ITT, glucose was measured by using glucometer (One Touch Ultra, Johnson & Johnson).

2.4. Serum Parameters Analysis. After 6 hours fasting, animals were anesthetized and blood was collected by puncturing the orbital plexus. Serum glucose, triacylglycerol, total cholesterol, LDL-cholesterol, and HDL-cholesterol were determined by colorimetric assays (Labtest Diagnostics, Lagoa Santa, MG, Brazil).

2.5. Responsiveness to Insulin in Isolated Soleus Muscle. Animals were euthanized by cervical dislocation and soleus muscles rapidly and carefully isolated, weighed (8–10 mg), attached to stainless steel clips to maintain resting tension, and preincubated for 30 min, at 37°C, in Krebs-Ringer bicarbonate buffer (KRBB) containing 5.6 mM glucose and 1% bovine serum albumin (BSA), pH 7.4, pregassed for 30 min with 95% O₂/5% CO₂, with agitation at 100 oscillations per min. After this period, muscles were transferred to other vials containing the same buffer, but added of 0.3 µCi/mL D-[U- 14 C]-glucose and 0.2 μ Ci/mL 2-deoxy-D-[2,6- 3 H]-glucose. Phenylethylamine (0.2 mL), diluted 1:1 v/v in methanol, was added into a separate compartment for ¹⁴CO₂ adsorption. Incubation was then performed for 1 h under similar conditions, in the absence or presence of 7 nM insulin. Gasification with 95% O₂/5% CO₂ was maintained during the preincubation and the first 15 min of the incubation period and then stopped.

After the incubation period, muscles were briefly washed in cold KRBB at 4°C, dried on filter paper and frozen in liquid N₂. Samples were processed for measurements of uptake of 2-deoxy-D-[2,6-³H]-glucose, D-[¹⁴C]-glucose incorporation, [¹⁴C]-glycogen synthesis, and decarboxylation of D-[¹⁴C]-glucose, according to methods described by Challiss et al. [14], Espinal et al. [15] and Leighton et al. [16], respectively, and routinely have been used by our group [11, 17–22].

Table 1: Obesity characteristics from mice fed with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids).

	CD	HFD	CD + n6	HFD + n6
Body weight gain (g)	2.90 ± 0.42	$11.30 \pm 0.91^*$	3.99 ± 0.65	$10.33 \pm 0.50^{\#}$
Food ingestion (g/day/animal)	3.61 ± 0.091	$2.44 \pm 0.164^*$	$4.53 \pm 0.158^*$	$2.45 \pm 0.033^{\#}$
Food efficiency (body weight gain (g)/food ingestion (g))	0.014 ± 0.06	$0.083 \pm 0.022^*$	0.016 ± 0.008	$0.075 \pm 0.011^{\#}$
Epididymal adipose tissue (mg)	737.9 ± 89.4	$1777.0 \pm 234.3^*$	555.1 ± 45.5	$1450.0 \pm 176.8^{\#}$

Oral supplementation with sunflower oil rich in n-6 (2 g/Kg body weight, three times a week, oral gavage) or water, started four weeks before feeding or not with HFD, maintained until the end of experimental protocol. Animals were feed with HFD or CD for additional eight weeks. Mean \pm SEM (n = 8-10). Data were analyzed by two-way ANOVA and Bonferroni post-test. *P < 0.05 versus CD; *P < 0.05 versus CD + n6.

2.6. Analysis of Inflammatory Parameters

2.6.1. Tissue Cytokine and Adipokine Content Measurements. Mice were euthanized on CO₂ chamber and visceral adipose tissue (epididymal, retroperitoneal, and mesenteric), liver, gastrocnemius muscle, and peritoneal macrophages rapidly collected. About 100 mg of liver, gastrocnemius muscle and retroperitoneal adipose tissue were used for the determination of TNF- α , IL-6, and IL-10 content. Tissues were homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediamine tetra acetic acid at pH 7.4), containing 10 g/mL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 12,000 g for 10 min at 4°C, supernatant was collected, and protein concentration determined using Bradford assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a protein reference. Likewise, about 150 mg of epididymal adipose tissue were cultured in DMEM sterile medium (Gibco), containing 10% FBS, 2 mM glutamine and antibiotics for 24 h, at 37°C, 5% CO₂ humidified air environment. Thereafter, medium culture was collected and used for the determination of leptin, adiponectin, and resistin contents. For all determinations, we used colorimetric immunoassays ELISA (DuoSet kits, R&D System).

2.6.2. Peritoneal Macrophage Isolation and Culture. Cytokine and nitric oxide (NO) production was evaluated in macrophages obtained by washing the peritoneal cavity with 6 mL RPMI culture medium (Gibco), containing 10% FBS and 4 mM glutamine. Macrophage-rich cultures (more than 90% of the cells were F4/80⁺) were obtained by incubating peritoneal cells in 24-well polystyrene culture plates for 2 h at 37°C in a 5% CO₂, humidified air environment. Nonadherent cells were removed by washing with RPMI. Adherent cells were then incubated with 2.5 μg/mL of LPS ($E.\ coli$, serotype 0111:B4, Sigma Chemical Company, USA) for 24 h [23]. Medium was collected for determination of IL-6, IL-10, and TNF-α by ELISA, and nitrite content by Griess method [24].

2.7. Statistical Analysis. Data are presented as mean \pm SEM. All groups were compared by two-way ANOVA following Bonferroni posttests. P < 0.05 was considered to be significant.

3. Results

3.1. Exposure to HFD Induces Obesity Associated with Glucose and Insulin Intolerance. Animals fed with HFD for eight weeks showed increased (by 3.8 fold) body weight gain when compared to those fed with CD. Despite reduced food ingestion, the food efficiency of HFD was 6 fold higher than CD. Moreover, epididymal adipose tissues were increased by HFD. Sunflower oil did not change body weight gain, food efficiency, or adipose tissue increased (Table 1).

HFD increased fasted glucose in 18% (Table 2). An increase of 63% in area under the curve in glucose tolerance test (GTT) was also observed in animals treated with HDF, demonstrating glucose intolerance, which was associated with insulin intolerance, as observed by the reduction in glucose clearance during insulin tolerance test (ITT). Supplementation with sunflower oil maintained fasted glucose levels similar to CD. However, the supplementation did not prevent the glucose and insulin intolerance induced by HFD. Furthermore, sunflower oil supplementation induced insulin intolerance itself (CD + n-6 group), reducing the clearance of glucose similar to HFD without supplementation. Total cholesterol and LDL cholesterol levels were increased by HFD and sunflower oil supplementation in association with HFD showed protective effect in the prevention of the increased LDL cholesterol and cholesterol total levels found in HFD.

3.2. HFD Impaired Glucose Uptake and Metabolism in Soleus Muscle. Soleus muscles from animals fed with HFD did not respond to insulin stimulus in relation to glucose uptake and metabolism (Figure 1), characterizing muscle insulin resistance state. This effect was not prevented or reduced by sunflower oil supplementation. Interestingly, the supplementation itself (CD + n-6 group) increased basal glucose uptake and glucose oxidation, but it failed to respond to the insulin stimulus when compared to the control group (CD group).

3.3. HFD and Sunflower Oil Supplementation Induced Inflammation. Peritoneal macrophages stimulated with LPS showed increased production of nitrite, TNF α , IL6, and IL10 when compared with unstimulated cells (Figure 2). Macrophages from animals with obesity induced by HFD for 8 weeks showed a inflammatory profile, with increased production of nitrite, TNF- α , and IL-6, associated with decreased content of IL-10 when stimulated with LPS.

	CD	HFD	CD + n6	HFD + n6
Fasted glucose (mg/dL)	186.1 ± 5.66	221.0 ± 8.38*	173.8 ± 4.75	196.8 ± 10.66
GTT test (AUC)	7987 ± 779.3	$13090 \pm 643.9^*$	7568 ± 1359	$14790 \pm 1731^{\#}$
ITT test (% glucose/min)	2.97 ± 0.19	$2.13 \pm 0.21^*$	$1.94 \pm 0.30^*$	1.98 ± 0.22
Total cholesterol (mg/dL)	148.2 ± 6.72	$192.7 \pm 11.96^*$	164.3 ± 14.00	166.3 ± 12.33
LDL cholesterol (mg/dL)	127.4 ± 5.15	$165.1 \pm 14.49^*$	128.2 ± 13.77	136.7 ± 13.22
HDL cholesterol (mg/dL)	14.73 ± 0.83	15.52 ± 1.86	19.33 ± 2.21	15.00 ± 1.15
Triglycerides (mg/dL)	68.61 ± 5.56	63.00 ± 4.40	84.70 ± 8.53	72.78 ± 8.71

Table 2: Metabolic parameters from mice fed with control diet (CD) or high-fat diet (HFD), supplemented, or not with sunflower oil (rich in n-6 fatty acids).

Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water have started four weeks before HFD and continued until the end of experimental period. Animals were fed HFD or CD for eight weeks. GTT: glucose tolerance test; ITT: insulin tolerance test. Mean \pm SEM (n = 6–15). ANOVA two-way with Bonferroni post tests. *P < 0.05 when compared with CD; $^{\#}P < 0.05$ when compared with CD + n6.

Table 3: Adipokine production in adipose tissue from mice fed with control diet (CD) or high-fat diet (HFD), supplemented, or not with sunflower oil (rich in n-6 fatty acids).

	CD	HFD	CD + n6	HFD + n6
Epididymal adipose tissue ^a				
Leptin	42.51 ± 8.75	$81.38 \pm 9.90^*$	62.42 ± 8.80	88.03 ± 10.96
Adiponectin	659.9 ± 39.94	697.6 ± 43.85	730.3 ± 76.68	649.8 ± 45.38
Resistin	625.1 ± 100.8	528.6 ± 71.65	913.0 ± 181.0	$327.0 \pm 42.15^{\#}$

Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water started four weeks before HFD and continued until the end of experiment. Animals were feed with HFD or CD for eight weeks. Approximately 150 mg of tissue were incubated in DMEM medium for 24h and the supernatant were used for measures. Mean \pm SEM (n = 7-11). ANOVA two-way with Bonferroni post tests. *P < 0.05 when compared with CD; *P < 0.05 when compared with CD + n6. a Values are expressed as pg/mL/mg tissue.

Interestingly, supplementation with sunflower oil induced similar inflammatory response in macrophages. For TNF- α only, the association of HFD and n-6 supplementation (HFD + n-6 group) showed beneficial response, decreasing this cytokine production.

In general, HFD induced and increase in the proinflammatory cytokine IL6 and decrease in the anti-inflammatory cytokine IL10 contents in the insulin target tissues (liver, skeletal muscle, and adipose tissue; Figure 3). Interesting, sunflower oil supplementation itself (CD + n-6 group) for 12 weeks increased the content of the proinflammatory cytokine IL6 in all tissues. However, when the sunflower oil was associated with HFD (HFD + n-6 group), cytokine concentrations were similar to that found in the HFD group, in liver and adipose tissue.

Adipokines production by epididymal adipose tissue was determined after 24 h culture. Adipose tissues from HFD animals showed increased production of leptin (Table 3). Resistin was reduced only when HFD was associated to sunflower oil supplementation (HFD + n-6 group) in comparison to CD + n6 group.

4. Discussion

This is the first study to investigate the effects of sunflower oil supplementation on inflammation and insulin sensitivity in mice fed a balanced diet and a HFD. We showed that HFD induces decreased glucose tolerance (as demonstrated by GTT) and insulin sensitivity (as observed in ITT) in the whole body. Surprisingly, this last effect was also verified in the CD + n6 group. Moreover, proinflammatory response was increased in the HFD group without prevention or attenuation by sunflower oil supplementation. This effect was also observed in CD + n6 group. Lipid profile was impaired by HFD. Animals from HFD group showed an increase in total and LDL cholesterol plasma levels. These alterations were ameliorated by sunflower oil supplementation (HFD + n6 group).

HFD has been associated with insulin resistance and inflammatory condition, contributing to the development of several related diseases, such as obesity, diabetes mellitus type 2, metabolic syndrome, cancer, and cardiovascular diseases [25-27]. Various strategies have been investigated in order to decreasing insulin resistance and inflammation, including natural and modified compounds, cyclooxygenase inhibitors, and physical exercise [28-35]. Since skeletal muscle tissue represents 50-60% of the body weight and it is the main insulin-responsive tissue in association to liver and adipose tissue, it has been proposed that skeletal muscle dysfunctions (reduced oxidative capacity and impaired insulin sensitivity) play a central role in the abnormalities of chronic diseases [21, 36–40]. In this work, we investigated the effect of sunflower oil supplementation on insulin sensitivity (in whole animal in vivo and in soleus muscle in vivo) and inflammatory condition (liver, skeletal muscle, adipose tissue) in mice fed a balanced diet and a HFD. In recent study, Bjermo et al. [41] showed that isocaloric diet with n-6

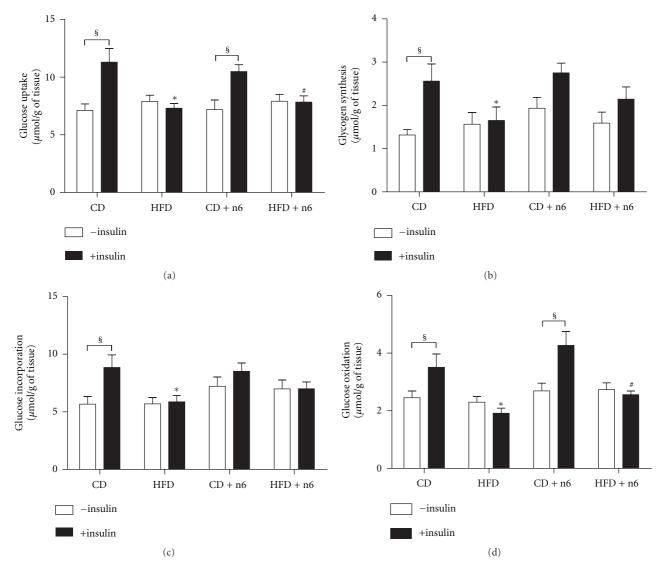


FIGURE 1: Effect of sunflower oil supplementation (rich in n-6 fatty acids) on glucose uptake and metabolism in isolated soleus muscle from C57BL/6 mice. The muscles were incubated in Krebs Ringer buffer, containing 5.6 mM glucose, $0.3 \,\mu\text{Ci/mL}$ of $[\text{U-}^{14}\text{C}]\text{-D-glucose}$ and $0.2 \,\mu\text{Ci/mL}$ of 2-deoxy- $[2,6^{-3}\text{H}]\text{-D-glucose}$, in the absence (white bars) or presence (black bars) of 7 nM insulin, for one hour. At the end of the incubation period, muscles were proceeded to analyze glucose uptake and metabolism as described in Materials and Methods. Results are presented as mean \pm SEM (n = 10–16). Two-way ANOVA and Bonferroni posttest were used for statistical analysis. $^{\$}P < 0.05$ versus the insulin stimulated (+insulin) of the same group; $^*P < 0.05$ versus CD with insulin; $^{\#}P < 0.05$ versus CD + n6 with insulin.

PUFA for 10 weeks reduce liver fat and modestly improves metabolic conditions, without weight loss in humans. In addition, it has been proposed that n-6 PUFA reduce risks of cardiovascular diseases, by improving lipid profile [42]. This improvement in lipid profile was found in our study, whereas HFD + n6 group showed decreased plasma LDL and total cholesterol levels.

Some studies have shown that n-6 PUFA intake positively correlate with obesity and insulin resistance [43, 44]. Treatment with subcutaneous injection of sunflower oil by 7 days in *Wistar* rats impaired insulin sensitivity by decreasing glucose clearance during ITT and GLUT-4 expression and translocation in white adipose tissue (WAT), with no effect in skeletal muscle [7]. In the CD + n6 group, KiTT was

reduced, but glucose uptake and metabolism in isolated soleus muscles were not altered after insulin stimulus. These results suggest that sunflower oil supplementation *per se* is not able to impair insulin response in skeletal muscle, but it possibly reduces total insulin sensitivity by decreasing the response to the hormone in other tissues, such as liver and adipose tissue. This proposition has to be investigated in further studies. In addition, both groups fed with HDF (HFD and HFD + n6 groups) showed skeletal muscle resistance to insulin, showing no beneficial effects of n-6 PUFA on peripheral insulin sensitivity.

Obesity is accompanied by a chronic low grade subclinical inflammation promoted by an increase in fuel availability, leading to infiltration of macrophages into adipose tissue.

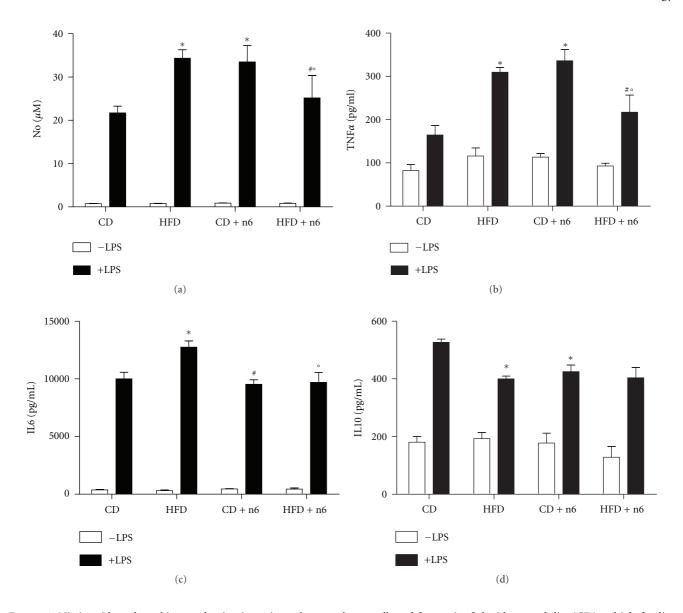


FIGURE 2: Nitric oxide and cytokine production in peritoneal macrophages collected from mice fed with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids, 2 g/Kg b.w., three times a week, by oral gavage). Animals were fed with HFD or CD for eight weeks. Peritoneal macrophages were collected and cultured for 24 h in the absence (white bars) or presence (black bars) of 2.5 μ g/mL LPS. Nitric oxide (a), TNF- α (b), IL-6 (c), and IL-10 (d) were measured as described in Material and Methods. Total number of cells in peritoneal exudates was not different among the groups (data not shown). Data are presented as mean \pm SEM (n = 5–10). The CD + n6 group had just 5 samples up to the detection limit in the TNF- α assay. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. All LPS stimulated conditions were different from unstimulated; *P < 0.05 versus CD with LPS; *P < 0.05 versus CD + n6 with LPS; *P < 0.05 versus HFD with LPS.

This condition results in enhanced inflammatory response, which stimulates the production of cytokines by adipose tissue [45]. Moreover, in obesity, increased lipotoxicity caused by high nonesterified fatty acids (NEFA) available, and triacylglycerol content leads to an increase in inflammatory markers and cytokine production in multiple organs [46, 47]. NEFA increases inflammatory response by activating TLR-4 [48]. HFD increased inflammatory response in macrophages, showing high content of IL-6, TNF- α , and NO, and low content of IL-10 (anti-inflammatory cytokine)

when compared to the control group. In peripheral tissues, IL-6 content was increased in the liver and gastrocnemius muscle whereas IL-10 content was decreased in liver and retroperitoneal adipose tissue. Adipose tissue showed increased production of leptin in the HFD and HFD + n6 groups, compared with mice fed with control diet, and resistin was reduced in the HFD + n6 group compared with CD + n6, in epididymal adipose tissue. Whereas in retroperitoneal adipose tissue, IL-6 was elevated in the HFD and CD + n6 groups compared with CD, IL-10 was reduced

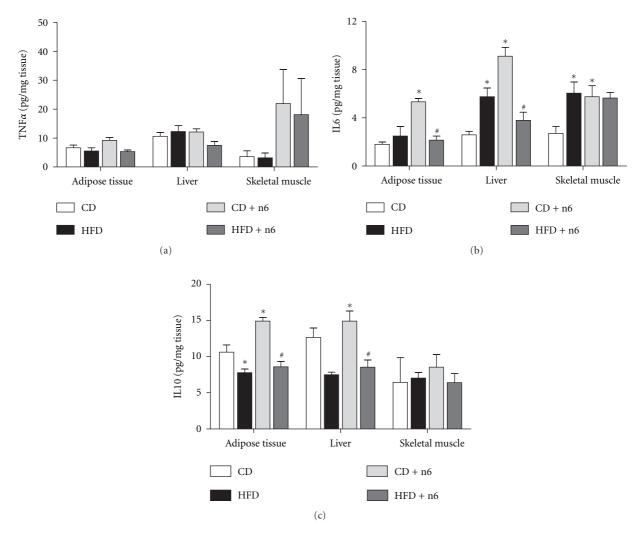


FIGURE 3: Tissue cytokine concentrations of TNF α (a), IL6 (b), and IL10 (c) in adipose tissue (retroperitoneal), liver, and gastrocnemius skeletal muscle from mice treated with control diet (CD) or high-fat diet (HFD), supplemented or not with sunflower oil (rich in n-6 fatty acids). Oral supplementation with n-6 (2 g/Kg body weight, three times a week, gavage) or water started four weeks before HFD and continued until the end of experiment. Animals were feed with HFD or CD for eight weeks. Cytokines were measured in tissue homogenate. Values are expressed as pg/mg protein. Mean \pm SEM (n=4–6). The statistical analysis was performed using two-way ANOVA with Bonferroni post tests. *P < 0.05 when compared with CD; *P < 0.05 when compared with CD + n6.

in the HFD and CD + n6, and adiponectin was reduced only in CD + n6. These results showed the inflammatory characteristics the sunflower oil supplementation and the high fatty diet fed.

Our results showed that sunflower oil supplementation causes a clear pro-inflammatory response in mice fed on control diet and it does not have additive or prophylactic effects in mice fed on HFD. Fatty acid effects on immune system have been studied since 1970's decade [49]. Linoleic acid is an essential fatty acid that leads to inflammatory response by increasing arachidonic acid production, a fatty acid related to elevated pro-inflammatory mediator generation [50]. High intake of linoleic acid has been associated to the development of chronic diseases in several studies [51, 52]. However, few studies have been carried out on the direct effect of this fatty acid on inflammatory markers [53].

In conclusion, our results showed that sunflower oil supplementation has pro-inflammatory effects in macrophages and insulin-sensitive peripheral tissues and induced insulin resistance *in vivo* and *in vitro*. These effects were demonstrated in mice fed a balanced diet and pronounced in mice fed a HFD. Interestingly, sunflower oil supplementation partially prevented the dislipidemia found in mice submitted to HFD, but it did not improve or even worsened inflammatory state and insulin resistance induced by this diet.

Acknowledgments

The authors declare no conflict of interests. This study was supported by Grants from São Paulo Research Foundation (FAPESP), Coordination for the Improvement of Higher Level Personnel (CAPES), National Council for Scientific

and Technological Development (CNPq)/National Institute of Science and Technology in Obesity and Diabetes (INOD), Center of Lipid Research and Education (CLEaR), and Dean's Office for Research/University of São Paulo.

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 634195, 11 pages doi:10.1155/2012/634195

Review Article

Role of Vitamin D in Insulin Resistance

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Received 15 June 2012; Revised 17 August 2012; Accepted 27 August 2012

Academic Editor: Hilton Kenji Takahashi

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Vitamin D is characterized as a regulator of homeostasis of bone and mineral metabolism, but it can also provide nonskeletal actions because vitamin D receptors have been found in various tissues including the brain, prostate, breast, colon, pancreas, and immune cells. Bone metabolism, modulation of the immune response, and regulation of cell proliferation and differentiation are all biological functions of vitamin D. Vitamin D may play an important role in modifying the risk of cardiometabolic outcomes, including diabetes mellitus (DM), hypertension, and cardiovascular disease. The incidence of type 2 DM is increasing worldwide and results from a lack of insulin or inadequate insulin secretion following increases in insulin resistance. Therefore, it has been proposed that vitamin D deficiency plays an important role in insulin resistance resulting in diabetes. The potential role of vitamin D deficiency in insulin resistance has been proposed to be associated with inherited gene polymorphisms including vitamin D-binding protein, vitamin D receptor, and vitamin D 1alpha-hydroxylase gene. Other roles have been proposed to involve immunoregulatory function by activating innate and adaptive immunity and cytokine release, activating inflammation by upregulation of nuclear factor κB and inducing tumor necrosis factor α , and other molecular actions to maintain glucose homeostasis and mediate insulin sensitivity by a low calcium status, obesity, or by elevating serum levels of parathyroid hormone. These effects of vitamin D deficiency, either acting in concert or alone, all serve to increase insulin resistance. Although there is evidence to support a relationship between vitamin D status and insulin resistance, the underlying mechanism requires further exploration. The purpose of this paper was to review the current information available concerning the role of vitamin D in insulin resistance.

1. Introduction

The incidence of type 2 diabetes mellitus (type 2 DM) is increasing at an alarming rate both nationally and worldwide. Defects in pancreatic β -cell function, insulin sensitivity, and systemic inflammation all contribute to the development of type 2 DM. Since insulin resistance is a risk factor for diabetes, understanding the role of various nutritional and other modifiable risk factors that may contribute to the pathogenesis of diabetes is important. Obesity and other lifestyle factors such as exercise, alcohol consumption, smoking, and certain dietary habits can also play an important role. Recently, a novel association between insulin resistance and vitamin D deficiency has been proposed. Vitamin D has in vitro and in vivo effects on pancreatic β -cells and insulin sensitivity. In this study, we

place specific emphasis on the epidemiological evidence and possible mechanisms of these effects. In addition, we also review the therapeutic strategies involving vitamin D in the treatment of insulin resistance.

2. Synthesis and Metabolism of Vitamin D

2.1. Synthesis of 1,25-Hydroxyvitamin D. Vitamin D is obtained from exposure to sunlight, diet (fortified foods), and dietary supplements. When the skin is exposed to solar ultraviolet B radiation (wavelength, 290 to 315 nm), 7-dehydrocholesterol is converted to previtamin D₃, which is rapidly converted to vitamin D₃ (cholecalciferol) (Figure 1). Vitamin D from the skin and diet is transported in the blood by circulating vitamin D-binding protein

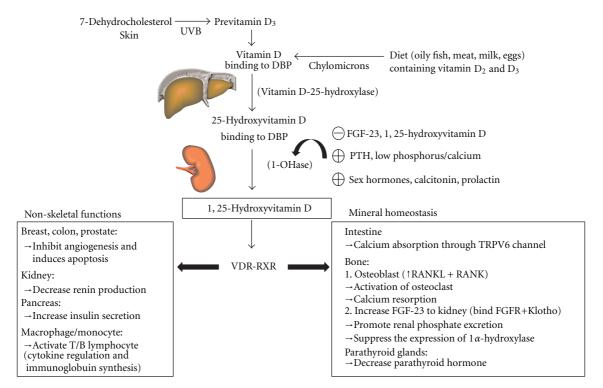


FIGURE 1: The synthesis and metabolism of vitamin D in the regulation of mineral homeostasis and nonskeletal functions. When under exposed to solar UVB (ultraviolet B), 7-dehydrocholesterol in the skin is converted to previtamin D_3 , which is immediately converted to vitamin D can also be obtained from dietary vitamin D_2 and D_3 incorporated into chylomicrons. Vitamin D in the circulation is bound to DBP (vitamin D-binding protein), which transports it to the liver where it is converted to 25-hydroxyvitamin D by vitamin D-25-hydroxyvitamin D₃ 1 α -hydroxyvitamin D must be converted in the kidneys to active 1,25-hydroxyvitamin D by 1-OHase (25-hydroxyvitamin D₃ 1 α -hydroxylase). Serum PTH (parathyroid hormone), low phosphorus/calcium, sex hormones, calcitonin, and prolactin can increase (\oplus) the renal production of 1,25-hydroxyvitamin D. However, FGF-23 (fibroblast growth factor 23) and 1,25-hydroxyvitamin D have feedback functions to inhibit (\ominus) 1-OHase. Finally, the active 1,25-hydroxyvitamin D can bind to VDR-RXR (vitamin D receptor-retinoic acid x-receptor complex) in the intestine, bone, and parathyroid glands and then exert the classical function of mineral homeostasis. In addition, it also has nonskeletal functions when bound to VDR-RXR in other organs (breast, colon, prostate, kidney, pancreas) or immune cells (macrophages/monocytes). FGFR: FGF-23 receptor; TRPV6: transient receptor potential cation channel, subfamily V, member 6; RANKL: receptor activator of nuclear factor- κ B ligand; RANK: the receptor for RANKL on preosteoclasts.

(DBP, a specific binding protein for vitamin D and its metabolites in serum) to the liver. In the liver, vitamin D is metabolized by P 450 vitamin D-25-hydroxylase to 25hydroxyvitamin D, which is the major circulating metabolite and used to determine a patient's vitamin D status [1–5]. Almost all 25-hydroxyvitamin D is bound to circulating DBP and is filtered by the kidneys and reabsorbed by the proximal convoluted tubules. In the kidney, megalin and cubilin, members of the LDL receptor superfamily, play essential roles in endocytic internalization of 25hydroxyvitamin D [6, 7]. In the proximal renal tubules, 25-hydroxyvitamin D is hydroxylased at the position of carbon 1 of the A-ring by the enzyme 25-hydroxyvitamin D_3 1 α -hydroxylase (CYP27B1) to its active form, 1,25hydroxyvitamin D. This enzyme is also found in extrarenal sites including the placenta, monocytes and macrophages [8-11].

2.2. Regulation of 1,25-Hydroxyvitamin D. The production of 1,25-hydroxyvitamin D is regulated by serum calcium and phosphorus levels, plasma parathyroid hormone (PTH)

levels, and fibroblast growth factor 23 (FGF-23). Low serum calcium and phosphate levels result in enhanced activity of 1α -hydroxylase. PTH stimulates the transcription of 1α hydroxylase and nuclear receptor 4A2 (NR4A2) is a key factor involved in the induction of 1α -hydroxylase transcription by PTH. 1,25-hydroxyvitamin D in turn suppresses PTH production at the level of transcription [12]. FGF-23 is a phosphaturic factor that promotes renal phosphate excretion by inactivating the sodium-phosphate cotransporter in the proximal tubule. 1,25-hydroxyvitamin D stimulates the production of FGF 23 in the bone, and an increased level of FGF-23 suppresses the expression of 1α -hydroxylase in the kidneys. FGF-23 requires a klotho (a multifunctional protein involved in phosphate and calcium homeostasis) as a cofactor for FGF signaling, and 1,25-hydroxyvitamin D upregulates klotho gene expression in the kidneys [12, 13].

2.3. Vitamin D Binding Protein (DBP) and Vitamin D Receptor (VDR). Vitamin D signaling may occur by binding of circulating 1,25-hydroxyvitamin D to VDR in β -cells

or by local production from the main circulating form, 25-hydroxyvitamin D. DBP (calbindin-D_{28 K}), encoded by the Gc (group-specific component) gene, functions as a specific transporter of circulating vitamin D metabolites [14] and is essential for vitamin D endocytosis and metabolism [15]. DBP is a highly polymorphic single-chain serum glycoprotein synthesized and secreted by the liver that forms a complex with vitamin D ensuring that circulating vitamin D is delivered to target tissues [16]. Vitamin D exerts its actions in a variety of cell types through binding to the cytosolic/nuclear vitamin D receptor (VDR), which is a member of the steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes [17-19]. VDR is widely distributed in more than 38 types of tissue, where it clearly controls vital genes related to bone metabolism, oxidative damage, chronic diseases, and inflammation [20]. The VDR gene, located on chromosome 12q13.1, consists of 14 exons and has an extensive promoter region capable of generating multiple tissue-specific transcripts [21, 22]. Upon ligand binding, this nuclear hormone receptor in conjunction with its heterodimeric partner, the retinoid receptor (RXR), regulates gene transcription through vitamin D responsive elements (VDRE) in the promoter regions of vitamin D target genes, thereby modifying their expression [23]. In addition to cytosolic/nuclear VDR mediating transcriptional regulation, the possible existence of a vitamin D receptor localized to the plasma membrane VDR (mVDR) has been postulated recently [24]. Pancreatic β -cells express both the specific cytosolic/nuclear VDR and putative mVDR.

2.4. Extrarenal 1,25-Hydroxyvitamin D Production (Nonskeletal Functions). Vitamin D is characterized as a regulator of homeostasis of bone and mineral metabolisms. In addition to its classical actions on mineral homeostasis, 1,25-dihydroxyvitamin D also has nonskeletal actions (Figure 1). It has been reported that the brain, prostate, breast, colon, and pancreas, as well as immune cells, have vitamin D receptors (VDR-RXR) and respond to this active form of vitamin D [4, 25-27]. More than 200 genes are controlled by 1,25-hydroxyvitamin D directly or indirectly to regulate cellular proliferation, differentiation, apoptosis, and angiogenesis [28]. For example, breast, colon, prostate, and other tissues express 25-hydroxyvitamin D₃ 1α-hydroxylase and produce 1,25-dihydroxyvitamin D which binds to VDR-RXR and then regulates a variety of genes that control proliferation, including p21 and p27, as well as genes that inhibit angiogenesis and induce differentiation and apoptosis. 1,25-dihydroxyvitamin D, an immunomodulator favoring the induction of regulatory T cells, can render dendritic cells tolerogenic with increased selective expression of immunoglobulin-like transcript 3 (ILT3) [29]. Meanwhile, 1,25-dihydroxyvitamin D inhibits rennin synthesis, and increases insulin production, myocardial contractility, the reproductive system, and hair growth [30-34]. Therefore, vitamin D may play an important role in modifying the risk of cardiometabolic outcomes, including type 2 DM, hypertension, and cardiovascular diseases.

3. Vitamin D Deficiency and Insulin Resistance

3.1. Vitamin D Deficiency. Vitamin D deficiency has been linked to a wide field of health problems including several types of cancer and autoimmune and metabolic diseases such as type 1 DM and type 2 DM. More than 30-50% of all children and adults are at risk of vitamin D deficiency, defined as a serum 25-hydroxyvitamin D level below 50 nmol/L [35]. However, this cutoff value is significantly higher than the 25 nmol/L (10 ng/mL) [36]. The association of vitamin D status and cardiometabolic disorders (cardiovascular disease, diabetes, and metabolic syndrome) was reviewed recently in a meta-analysis of 28 independently published studies [37]. The findings showed a significant 55% reduction in the risk of diabetes (9 studies), a 33% reduction in the risk of cardiovascular diseases (16 studies), and a 51% reduction in metabolic syndrome (8 studies) associated with a high serum 25-dihydroxyvitamin D concentration [37].

3.2. Evidence Linking Vitamin D to Insulin Resistance and Diabetes. Several studies have indicated a relationship between vitamin D status and the risk of diabetes or glucose intolerance. Vitamin D has been proposed to play an important role and to be a risk factor in the development of insulin resistance and the pathogenesis of type 2 DM by affecting either insulin sensitivity or β -cell function, or both [31, 38, 39]. Type 1 DM has been also reported to be associated with vitamin D deficiency based on animal and human observational studies [19, 23, 40]. The prevalence of hypovitaminosis D was found to be higher in diabetic patients (24%; P < 0.001) than in controls (16%) in one study [41]. Increasing evidence shows that vitamin D levels are also lower in patients with type 1 DM, especially at the onset [42].

3.3. Association between Vitamin D and Insulin Resistance. 1,25-dihydroxyvitamin D plays an important role in glucose homeostasis via different mechanisms. It not only improves insulin sensitivity of the target cells (liver, skeletal muscle, and adipose tissue) but also enhances and improves β -cell function. In addition, 1,25-dihydroxyvitamin D protects β -cells from detrimental immune attacks, directly by its action on β -cells, but also indirectly by acting on different immune cells, including inflammatory macrophages, dendritic cells, and a variety of T cells. Macrophages, dendritic cells, T lymphocytes, and B lymphocytes can synthesize 1,25-dihydroxyvitamin D, all contributing to the regulation of local immune responses [43]. The potential role of vitamin D deficiency in insulin resistance is shown in Table 1.

4. Role of Vitamin D Deficiency in Insulin Resistance

4.1. Vitamin D Associated Gene Polymorphisms and Insulin Resistance. Gene polymorphisms of the DBP, VDR, or vitamin D lalpha-hydroxylase (CYPlalpha) genes may affect insulin release and result in insulin resistant. In addition, these gene polymorphisms may disturb vitamin D production, transport, and action.

TABLE 1: Role of vitamin D deficiency in insulin resistance.

Role	References
Inherited gene polymorphisms	
(1) Including DBP, VDR, and CYP1alpha gene polymorphisms	[44–68]
(2) Disturbance of vitamin D transport, action, and production	
Immunoregulatory function	
(1) Activating innate and adaptive immunity	
(2) Enhancing dendritic cell maturation and macrophage differentiation, and cytokine release	[69–83]
(3) Enhancing T-cell proliferation	
(4) Releases of IL-12, IL-2, INF- γ , and TNF α (destruction of the β -cell)	
Inflammation	
(1) Upregulation of NF- κ B and inducing TNF α proinflammatory actions	[84–90]
(2) Downregulates $I\kappa B-\alpha$ by decreasing mRNA stability and increasing $I\kappa B-\alpha$ phosphorylation.	[04-70]
(3) Enhancing the expression of TLR2 and TLR4 protein and mRNA in human monocytes, reducing the release of cytokines	
Other molecular actions of vitamin D to alter glucose homeostasis	
(1) Low calcium status: hypocalcemia can lower glucose-stimulated insulin secretion in β -cell	[90–95]
(2) PTH level: elevating PTH reduces glucose uptake by liver, muscle and adipose cell	[70-93]
(3) Obesity: vitamin D deficiency can increase adiposity, and increasing sequestration of vitamin D in adipose tissue	

DBP: vitamin D binding protein; VDR: vitamin D receptor; CYP1alpha: vitamin D 1alpha-hydroxylase; II.-12: interleukin-12; INF- γ : interferon- γ ; TNF α : tumor necrosis factor α ; NF- κ B: nuclear factor κ B; $I\kappa$ B- α : the inhibitor of NF- κ B; TLR: Toll-like receptors; PTH: parathyroid hormone.

4.1.1. Gene Polymorphisms of the DBP Gene. Electrophoretic variants of DBP have been associated not only with diabetes, but also with prediabetic traits. Two frequent missense polymorphisms at codons 416 GAT → GAG (Asp → Glu) and 420 ACG → AAG (Thr → Lys) in exon 11 of the DBP gene are the genetic basis for the three common electrophoretic variants of DBP (Gc1F, Gc1S, and Gc2) and the resulting circulating phenotypes (Gc1F/Gc1F, Gc1F/Gc1S, Gc1S/Gc1S, Gc1F/Gc2, Gc1S/Gc2, and Gc2/Gc2) [44]. These variants of DBP are the serum carriers of vitamin D metabolites and have been associated with a higher risk of type 2 DM or prediabetic phenotypes in several studies [45–49]. However, some studies have shown that the genetic variants of the DBP gene are not associated with diabetes [50, 51].

4.1.2. Gene Polymorphisms of the VDR Gene. VDR functions as a transcription factor when bound to 1,25dihydroxyvitamin D. VDRs are present in pancreatic β cells and vitamin D is essential for normal insulin secretion [52]. Several VDR polymorphisms have been found since the early 1990s, including Apa1 [53], EcoRV, Bsm1 [54], Taq1 [55], Tru91 [56], Fok1 [57], and Cdx2 [58]. To date, three adjacent restriction fragment length polymorphisms for Bsm1, Apa1, and Taq1at the 3' end of the VDR gene have been the most frequently studied [59]. VDR polymorphisms have been reported to be related to type 1 DM [60-62]. The Bsm1 polymorphism has been shown to be associated with type 1 DM in Indians living in the south of the country [60], and combinations of Bsm1/Apa1/Taq1 have been shown to influence susceptibility to type 1 DM in Germans [61]. In a Taiwanese population, the AA genotype of the Apa1 polymorphism was found to be associated

with type 1 DM [62]. In type 1 DM, four well-known polymorphisms (Fok1, Apa1, Bsm1, and Taq1) in the VDR gene have been implicated in the susceptibility to type 1 DM, however the results to date have been inconclusive. A meta-analysis (57 case-control studies in 26 published studies) indicated that the Bsm1 polymorphism is associated with an increased risk of type 1 DM (BB + Bb versus bb: OR = 1.30, 95% CI = 1.03 – 1.63), while the Fok1, Apa1, and Taq1 polymorphisms were not, especially in Asians [63]. The VDR genotype may affect insulin resistance, both with regards to insulin secretion (the Apa1 VDR polymorphism) and insulin resistance (the Bsm1 VDR polymorphism) [64].

In type 2 DM, the VDR gene polymorphism aa genotype was found to be associated with defective insulin secretion in Bangladeshi Asians, a population at increased risk of type 2 DM [65]. The associations of the Fok1, Apal, Bsm1 and Taq1 polymorphisms of the VDR gene with type 2 DM were also explored in a case-control study (308 type 2 DM patients and 240 control cases). In this study, no associations were found between the four polymorphisms examined and type 2 DM [66]. In another study, the distributions of alleles and genotypes of the four single-nucleotide polymorphisms in intron 8 (Bsm1, Tru91, Apal) and exon 9 (Taq1) of the VDR gene were similar in patients with type 2 DM (n = 309) and controls (n = 143) [67]. Therefore, the evidence supporting an association of VDR genotypes with the risk of diabetes is conflicting.

4.1.3. Gene Polymorphisms of the CYP1alpha Gene. Polymorphisms of the CYP1alpha gene involved in the metabolism of vitamin D may influence the susceptibility to type 2 DM. A study on the association of two markers, one in intron 6 and the other located upstream from the 5' end of the CYP1alpha

gene, with type 2 DM in a Polish population found no differences in the distributions of genotypes, haplotypes, and haplotype combinations between the groups. However, the T-C/T-T heterozygous haplotype combination was more prevalent in the subgroup of obese type 2 DM patients (BMI \geq 30) than in the controls (41.5% versus 28.6%, P=0.01), suggesting an association with the risk factors for diabetes and obesity [68].

4.2. Effects of Vitamin D on the Immune System and Insulin Resistance

4.2.1. Immunoregulatory Function of Vitamin D. Basic science and epidemiological studies indicate that vitamin D has importance not only for cardiovascular health, but also for the immune response. Vitamin D has been shown to have a role in the development and function of the immune system. In fact, inadequate vitamin D and other nutrients during the development of the immune system may play a critical role in the development of autoimmune diseases. Evidence from animal models and prospective studies of rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and type 1 DM suggests that vitamin D has an important role as a modifiable environmental factor in autoimmune diseases [69–71].

4.2.2. Immunoregulatory Function of Vitamin D on Insulin Resistance. The immune system plays a central role in the destruction of β -cells [72]. The detection of VDR in almost all cells of the immune system, especially antigenpresenting cells (macrophages and dendritic cells) and activated T cells [73-75], led to the investigation of a potential role for vitamin D as an immunomodulator. In addition, activation of nuclear VDR is also known to modify transcription via several intracellular pathways and influence proliferation and differentiation of immune cells [76, 77]. The importance of vitamin D in immune regulation is highlighted by the facts that VDR is expressed in activated inflammatory cells, that T-cell proliferation is inhibited by 1,25-dihydroxyvitamin D, and that activated macrophages produce 1,25-dihydroxyvitamin D [74, 78]. Vitamin D signaling pathways regulate both innate and adaptive immunity, maintaining the associated inflammatory response within physiological limits.

The innate immune response involves the activation of Toll-like receptors (TLRs) on polymorphonuclear cells, monocytes, macrophages, and a number of epithelial cells [79]. 1,25-dihydroxyvitamin D primarily influences dendritic cell maturation and macrophage differentiation, and also reduces the release of cytokines [80]. The adaptive immune response is initiated by cells specializing in antigen presentation, including dendritic cells and macrophages, which are responsible for presenting antigens for specific recognition by T lymphocytes and B lymphocytes [81]. 1,25-dihydroxyvitamin D exerts an inhibitory effect on the adaptive immune system by modifying the capacity of antigen-presenting cells (APCs) to induce T lymphocyte activation, proliferation and cytokine secretion [82]. 1,25-dihydroxyvitamin D decreases the maturation of dendritic

cells and also inhibits the release of interleukin-12 (IL-12) (stimulating T-helper 1 cell development), IL-2, interferon- γ (INF- γ), and tumor necrosis factor α (TNF α) (stimulators of inflammation), which involves the destruction of β -cells resulting in insulin resistance. Overall, 1,25-dihydroxyvitamin D directly modulates T-cell proliferation and cytokine production, decreases the development of T helper 1 (T_H1) cells, inhibits T_H17 cell development, and increases the production of Thelper 2 (T_H2) cells and T regulatory cells [83]. These immunomodulatory effects of 1,25-dihydroxyvitamin D can lead to the protection of target tissues, such as β -cells.

4.3. Inflammation, Vitamin D, and Insulin Resistance. Chronic inflammation is involved in the development of insulin resistance, which increases the risk of type 2 DM. VDR is known to be expressed by macrophages and dendritic cells, suggesting that vitamin D plays an important role in the modulation of inflammatory responses [84]. Both cell types express the enzymes vitamin D-25-hydroxylase and 1α-hydroxylase and can produce 1,25-dihydroxyvitamin D [85]. Several studies have supported the role of vitamin D and 1,25-dihydroxyvitamin D as an anti-inflammatory agent. Macrophages are cells with a large capacity for cytokine production, in particular TNF α , which is one of the most important products released from these cells [78]. The transcriptional activation of the TNF α gene in macrophages is largely dependent on nuclear factor κB (NF- κB) dependent transcriptional activation [86]. In lipopolysaccharide-(LPS-) stimulated murine macrophages, 1,25-dihydroxyvitamin D upregulates $I\kappa B-\alpha$ (the inhibitor of NF- κ B) by increasing mRNA stability and decreasing I κ B- α phosphorylation. Furthermore, increased IkB- α levels can reduce the nuclear translocation of NF- κ B [87]. In addition, 1,25-dihydroxyvitamin D suppresses the expressions of TLR2 and TLR4 proteins and mRNA in human monocytes in a time- and dose-dependent fashion [88]. Recently, it has also been suggested that inflammation and activation of the innate immune system could be downregulated by hydroxyvitamin D by increased levels of inflammatory markers (TNFα, IL-6, IL-1, IL-8, cyclooxygenase-2, intercellular adhesion molecule-1, and B7-1) in monocytes from type 2 DM compared with monocytes from healthy controls [89]. In summary, 1,25-dihydroxyvitamin D inhibits the release of the pro-inflammatory cytokine TNF α and regulates the activity of NF- κ B, [90] and suppresses the expressions of TLR2 and TLR4 proteins and mRNA in human monocytes, reducing the release of cytokines. Therefore, vitamin D may also function to reduce insulin resistance and the risk of diabetes by decreasing inflammatory responses.

4.4. Other Molecular Actions of Vitamin D to Alter Glucose Homeostasis. Several mechanisms have been proposed to explain the impact of vitamin D on insulin resistance including gene polymorphisms and the immunoregulatory function of vitamin D and inflammation as mentioned previously. The regulation of serum calcium via PTH and 1,25-dihydroxyvitamin D following changes in dietary

Study	Intervention	Subjects	Effect	
Pittas et al. [26]	vitamin D (700 IU/d for 3 years) and calcium intake	314	Decreased insulin resistance	
Inomata et al. [99]	1α(OH) vitamin D (400 IU/d for 3 wks)	14 noninsulin-dependent diabetes	(1) Increased insulin secretion(2) A reduction in serum free fatty acid levels	
Gedik and Akalin [100]	$1,25 \text{ (OH)}_2 \text{ vitamin D}$ (2000 IU/d for 6 months)	4 patients with vitamin D deficiency	Increased insulin secretion	
Borissova et al. [101]	$1,25 \text{ (OH)}_2 \text{ vitamin D}$ (1332 IU/d for 1 month)	10 females with type 2 diabetes	A decrease of 21.4% in insulin resistance	
Orwoll et al. [102]	1,25 (OH) ₂ vitamin D (200 IU/d for 4 d)	20 patient with type 2 diabetes	No effect on insulin, glucose, c-peptide	
Taylor and Wise [103]	$1,25 \text{ (OH)}_2 \text{ vitamin D}$ (300000 IU intramuscular)	3 Asians with type 2 diabetes	Increased insulin resistance	
Ljunghall et al. [104]	1α(OH) vitamin D (30 IU/d for 3 months)	65 Caucasian men with impaired glucose tolerance	No difference in insulin resistance	
Kumar et al. [105]	1,25 (OH) ₂ vitamin D (2000 IU/d for 1 month)	1 vitamin D deficient hypocalcemic woman	Increased glucose tolerance and β -cell function	

Table 2: Effects of vitamin D supplementation in insulin resistance.

calcium and obesity has been proposed to mediate the effects of vitamin D on insulin resistance.

4.4.1. Stimulation of Insulin Secretion by Vitamin D and Calcium. There is evidence that vitamin D may stimulate pancreatic insulin secretion directly. Vitamin D exerts its effects through nuclear vitamin D receptors [91]. The stimulatory effects of vitamin D on insulin secretion may only manifest when calcium levels are adequate. Insulin secretion is a calcium-dependent process, and therefore alterations in calcium flux can have adverse effects on β -cell secretary function. Glucose-stimulated insulin secretion has also been found to be lower in vitamin D-deficient rats when concurrent hypocalcemia has not been corrected [92].

4.4.2. Parathyroid Hormone (PTH). PTH regulates the activity of renal 1α-hydroxylase to convert 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. However, extra-renal 1α hydroxylase, which may lead to the local production of 1,25-dihydroxyvitamin D under conditions of high vitamin D status [93], has also been identified in a variety of tissues including muscles and adipocytes [94]. PTH may mediate insulin resistance by reducing glucose uptake by liver, muscle and adipose cells. PTH treatment (16h) was found to decrease insulin-stimulated glucose transport [90, 95] in an osteoblast-like cell type. Another study indicated that PTH decreased insulin-stimulated glucose uptake in rat adipocytes [96]. These studies suggest that PTH may elicit insulin resistance by reducing the number of glucose transporters (both GLUT1 and GLUT4) available in cell membranes to promote glucose uptake [90]. PTH has also been shown to suppress insulin release [97] and to promote insulin resistance in adipocytes [90]. Therefore, PTH may negatively affect insulin sensitivity through altering body composition and inhibiting insulin signaling.

4.4.3. Muscle and Obesity. Vitamin D and PTH have also been associated with a variety of other actions beyond their classical functions, including cell growth, differentiation and apoptosis. Both hormones have been shown to increase levels of intracellular calcium and other rapid signaling pathways in a variety of tissues including adipocytes and muscle cells. Vitamin D may reduce adiposity, thereby improving insulin sensitivity indirectly through improving muscle mass and the reduction in vitamin D status with increased adiposity [90]. In addition, obesity, increasing sequestration of vitamin D in adipose tissue, is also known to be associated with reduced vitamin D status [98].

5. Therapeutic Interventions on Insulin Resistance with Vitamin D

5.1. Effect of Vitamin D on Insulin Resistance. Vitamin D may have a beneficial effect on improving pancreatic β -cell function, decreasing insulin resistance, and improving systemic inflammation [26].

5.1.1. Pancreatic β -cell Function. Several studies support a role of vitamin D in pancreatic β -cell function through direct and indirect effects. The direct effect is where vitamin D binds directly to the β -cell vitamin D receptor. The indirect effect may be via its important and well-recognized role in regulating extracellular calcium and calcium flux through β -cells [26].

5.1.2. Insulin Resistance. Vitamin D may have a beneficial effect on insulin action either directly, by stimulating the expression of insulin receptors and thereby enhancing insulin responsiveness for glucose transport [106], or indirectly via its role in regulating extracellular calcium and ensuring normal calcium influx through cell membranes and an adequate intracellular cytosolic calcium pool because calcium is essential for insulin-mediated intracellular processes

in insulin-responsive tissues such as skeletal muscles and adipose tissues [107].

5.1.3. Inflammation. Systemic inflammation has been linked primarily to insulin resistance, but elevated cytokines may also play a role in β -cell dysfunction by triggering β -cell apoptosis. Vitamin D may improve insulin sensitivity and promote β -cell survival by directly modulating the generation and effects of cytokines. Vitamin D interacts with vitamin D response elements in the promoter region of cytokine genes to interfere with nuclear transcription factors implicated in cytokine generation, and its action and can downregulate activation of NF κ B [108–110].

5.2. Evidence of Intervention with Vitamin D Supplementation. The mainstay management for vitamin D deficiency is vitamin D supplementation to prevent or ameliorate the disease. Several studies support that vitamin D supplementation may affect glucose homeostasis or improve insulin resistance [99–101, 105] (Table 2). Restoration of vitamin D levels was shown to ameliorate glucose tolerance in a study on one hypocalcemic woman with vitamin D deficiency [105]. A significant increase in serum calcium levels and a reduction in serum free fatty acid levels have been found after taking vitamin D supplementations [99]. Recently, a New Zealand study found that south Asian women with insulin resistance improved markedly after taking vitamin D supplements [111]. The optimal vitamin D concentrations for reducing insulin resistance have been shown to be 80 to 119 nmol/L, providing further evidence for an increase in the recommended adequate levels [43].

Nevertheless, some studies have shown conflicting results of vitamin D supplementation for insulin resistance or improvement of type 2 DM [102–104] (Table 2). One report found that Asian patients with type 2 DM with vitamin D deficiency even had a worsening of glycemic control and an increase in insulin resistance [104]. These contrasting results suggest that the dose and method of supplementation, and the genetic background and baseline vitamin D status of individuals seem to be more important for the efficacy of vitamin D supplementations in insulin resistance.

6. Conclusion

Vitamin D is not only a regulator of bone and mineral metabolism, but also a potent immunomodulator linked to many major human diseases including glucose homeostasis and insulin resistance. Vitamin D deficiency has been shown to affect insulin secretion in both humans and animal models. Accumulating evidence suggests the role of vitamin D in the pathogenesis of insulin resistance including several vitamin-D-related gene polymorphisms and vitamin-D-related metabolic and immune pathways. Supplementations of vitamin D may provide for suitable management and act to ameliorate insulin resistance. Additionally, there is a need for randomized trials to evaluate the significant effects of vitamin D supplementations in insulin resistance.

Authors' Contribution

C.-C. Sung and M.-T. Liao contributed equally to this work.

Conflict of Interests

There is no conflict of interests.

Acknowledgments

This work was supported by the National Science Council, Taiwan (NSC 100-2314-B-016-027), and Ministry of National Defense, Taiwan (I-4 and MAB101-60), to C.-C. Wu.

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 805418, 7 pages doi:10.1155/2012/805418

Research Article

Maternal Moderate Physical Training during Pregnancy Attenuates the Effects of a Low-Protein Diet on the Impaired Secretion of Insulin in Rats: Potential Role for Compensation of Insulin Resistance and Preventing Gestational Diabetes Mellitus

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Received 14 June 2012; Revised 16 July 2012; Accepted 18 July 2012

Academic Editor: Renata Gorjão

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The effects of pregestational and gestational low-to-moderate physical training on insulin secretion in undernourished mothers were evaluated. Virgin female Wistar rats were divided into four groups as follows: control (C, n = 5); trained (T, n = 5); low-protein diet (T, n = 5); trained with a low-protein diet (T, n = 5). Trained rats ran on a treadmill over a period of 4 weeks before mate (5 days week⁻¹ and 60 min day⁻¹, at 65% of VO_{2max}). At pregnancy, the intensity and duration of the exercise were reduced. Low-protein groups were provided with an 8% casein diet, and controls were provided with a 17% casein diet. At third day after delivery, mothers and pups were killed and islets were isolated by collagenase digestion of pancreas and incubated for a further 1 h with medium containing 5.6 or 16.7 mM glucose. T mothers showed increased insulin secretion by isolated islets incubated with 16.7 mM glucose, whereas LP group showed reduced secretion of insulin by isolated islets when compared with both C and C and C are C are C are C and C are C and C are C are C are C are C and C are C are C and C are C are C are C are C and C are C and C are C are C are C and C are C are C are C are C and C are C and C are C and C are C are C and C are C are C and C are C are C are C and C are C are C are C and C are C and C are C are C are C are C and C are C are C and C are C are C are C and C are C and C are C and C are C are C are C are C are C are C and C are C are C are C and C are C are C and C are C are C a

1. Introduction

Pregnancy requires an increase in insulin secretion-induced glucose metabolism, in order to compensate for the insulin resistance at the end of gestation [1]. In pregnant rats, the threshold of glucose-stimulated insulin secretion begins to diverge from controls by day 10 and islet cell proliferation and insulin secretory profiles are inhibited by day 20 to avoid gestational diabetes [2]. Return to normal values of insulin secretion and inhibition of cell division is particularly important when the adequate nutrient intake is not provided

during pregnancy. For example, maternal low-protein diet (6% casein) causes a loss of glucose sensitivity and secretory capacity in pancreatic islets, which is probably the result of alterations to the coupling of stimuli with insulin secretion [3]. In fact, a maternal low-protein diet decreases insulin secretion and impairs glucose homeostasis, leading to gestational *diabetes mellitus* and increased risk of chronic diseases in later life [1, 4].

Recently, it has been recognized that an active maternal lifestyle, including regular to moderate physical activity, improves aerobic fitness and the maternal-fetal physiological

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reserve by enhancing nutrient and oxygen availability to the fetus [5–7]. Maternal physical activity is associated with normalised body weight gain, enhanced cardiorespiratory, and improved oxidative capacity and insulin sensitivity of skeletal muscle [8]. These physiological benefits mainly occur according to the type and volume of the exercise, physical fitness of the mother, the time point in the pregnancy when the exercise is carried out and also the duration and intensity of the exercise [9, 10]. Physical exercise training has been associated with reduced risk of metabolic disease and enhances both cardiorespiratory and metabolic functions [11–14]. These effects are mainly due to improved oxidative capacity and insulin sensitivity of skeletal muscle, since reduced muscle dysfunction and insulin resistance have been associated to the development of several chronic diseases [15–20]. Exercise is considered moderate intensity when oxygen consumption is 50-70% of the maximal oxygen consumption ($VO_{2 \text{ max}}$). In our previous study, we demonstrated in rats that a controlled moderate to low-intensity exercise before and during gestation (5 days week⁻¹, 60 min day⁻¹, 40%-70% of VO_{2 max}) increased the mothers' resting oxygen consumption [21]. In humans, the rate of placental bed blood flow increases, and more glucose and oxygen delivery to the placental site are observed in women submitted to a physical training [22].

The effects of maternal moderate physical training can be expected modifying glucose metabolism in pregnancy once regular physical activity in nonpregnant individual is known to improve peripheral insulin sensitivity, glucose tolerance, and may aid in preserving β -cell function [11]. Regular practice of moderate exercise has been associated with improved fitness, reduction of body weight, and increased muscle mitochondrial biogenesis and antioxidant defence [5, 23]. Our previous experimental data supports the idea that maternal physical training may protect mothers against perinatal undernutrition environment [21, 24]. Recently, we showed in rats that physical training was able to normalise the effects of a low-protein diet on fasting serum glucose at the second and third weeks of gestation [25]. This differential effect observed in previously active compared with undernourished dams may be related to reduced risk of gestational diabetes mellitus, since an exercise program may improve insulin sensitivity and fasting plasma glucose concentrations of women at risk for gestational diabetes mellitus [26].

Thus, the main goal of this study was to evaluate the impact of a moderate to low intensity protocol of physical training during gestation on the secretion of insulin whose mothers were undernourished. Our hypothesis is that maternal physical training-induced physiological adaptations during gestation attenuate the impact of a perinatal low-protein diet on the secretion of insulin. This is a topic of particular interest as a maternal lifestyle can be considered a therapeutic means of countering the effects of either maternal undernutrition or overnutrition.

2. Methods

Experimental protocol was approved by the Ethical Committee of the Biological Sciences Center, Federal University of

Table 1: Composition of the diets (control 17% and low protein 8%).

Ingredients	Amount for 1 Kg of diet			
ingredients	Low protein	Control		
Casein	79.3 g	179.3 g		
Vitamin mix*	10 g	10 g		
Mineral mixture#	35 g	35 g		
Cellulose	50 g	50 g		
Bitartarato of choline	2.5 g	2.5 g		
DL-methionine	3.0 g	3.0 g		
Soya oil	70 mL	70 mL		
Corn starch	750.2 g	650.2 g		

*Mineral mixture contained the following (mg/kg of diet): CaHPO₄, 17200; KCI, 4000; NaCl, 4000; MgO, 420; MgSO₄, 2000; Fe₂O₂, 120; FeSO₄·7H₂O, 200; trace elements, 400 (MnSO₄·H₂O, 98; CuSO₄·5H₂O, 20; ZnSO₄·7H₂O, 80; CoSO₄·7H₂O, 0.16; KI, 0.32; sufficient starch to bring to 40 g [per kg of diet]). *Vitamin mixture contained the following (mg/kg of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicottinic acid, 200; choline, 2720; folic acid, 10; paminobenzoic acid, 100; biotin, 0.6.

Pernambuco, Brazil and followed the Guidelines for the Care and Use of Laboratory Animals [27].

2.1. Animals. Virgin female albino Wistar rats (Rattus novegicus) aged 60 days and weighting 180 ± 11 g (mean ± S.E.M.) were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Brazil. Female rats were maintained at a room temperature of 22 ± 1°C and controlled light-dark cycle (dark 6:00 a.m.-6:00 p.m.). The standard laboratory chow for animals (52% carbohydrate, 21% protein, 4% lipids-Nuvilab CR1-Nuvital) and water were given ad libitum. Animals were randomly divided into two groups: untrained rats (NT, n = 10) and trained rats (T, n = 10). Trained rats were submitted to a training program of moderate running over a period of 4 weeks (5 days per week and 60 minutes per day) on a treadmill (Millennium Inbramed, Brazil) at a controlled intensity based on their VO_{2 max}. After a 4-week training period, the rats were mated (2 females for 1 male). The day on which spermatozoa were present in a vaginal smear was designated as the day of conception (day 0 of pregnancy). Pregnant rats were then transferred to individual cages. Half of rats of each group received either 17% casein diet or 8% casein (low-protein group, LP) isocaloric diet ad libitum (Table 1). Thus, two more groups were formed: untrained (C, n = 5), trained (T, n = 5), untrained with lowprotein diet (LP, n = 5), and trained with low-protein diet (T + LP, n = 5). Mother's body weight was weekly determined throughout the experiment. On the time of delivery, the litter size and pup's birth weight were recorded. The litters of six pups represent the sample that was evaluated: control (C, n =5); trained (T, n = 5); low-protein diet (LP, n = 5); trained with low-protein diet (T + LP, n = 5). The evaluation of body weight and gain of body weight of pups was recorded until the 3rd d of life with a Marte scale with 100 mg precision. Afterwards, mothers and pups were killed by decapitation.

2.2. Protocol of Physical Training. Considering that maternal physical exercise-induced changes depends on intensity, duration, and frequency of effort, our group standardized an experimental protocol of maternal physical training (based on blood lactate concentration and oxygen consumption) [21]. Briefly, rats ran on a treadmill during the four weeks prior to pregnancy (5 days week⁻¹ and 60 min day⁻¹, at 65%VO_{2 max}). The protocol was divided into four progressive stages in each session: (i) warm-up (5 minutes); (ii) intermediary (20 minutes); (iii) training (30 minutes); (iiv) cool-down (5 minutes) periods. The percentage of VO_{2 max} during the sessions of training was kept around 55–65% [21]. During pregnancy, rats ran at a progressively lower intensity of effort (40%VO_{2 max}, 5 days week⁻¹, and 20 min day⁻¹) until the 19th day of gestation [21].

2.3. Mother's Body Weight and Food Intake. Mother's body weight was daily recorded. Daily food consumption was determined by the difference between the amount of food provided at the onset of the dark cycle (06.00 hours) and the amount of food remaining 24 h later [28]. Body and food weights were recorded with a Marte Scale (AS-1000) with a 0.01-g accuracy.

2.4. Islet Isolation and Insulin Secretion. Islets were isolated from five fasting mothers in each group by collagenase digestion of pancreas followed by separation from pancreatic debris by centrifugation on Ficoll gradients as previously described [29]. Groups of five islets were initially incubated for 45 min at 37°C in Krebs-bicarbonate buffer containing 5.6 mM glucose and equilibrated with 95% O₂/5% CO₂, pH 7.4. The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were incubated for a further 1 h with medium containing 5.6 or 16.7 mM glucose. The incubation medium contained (in mM): NaCl 115, KCl 5, NaHCO₃ 24, CaCl₂ 2.56, MgCl₂ 1, and BSA 0.3% (w/v). The cumulative insulin release during 1 h was quantified by radioimmunoassay using rat insulin as standard.

2.5. Statistical Analyses. Values are presented as means \pm S.E.M. For statistical analysis, data were analyzed by two-way repeated measures ANOVA, with mothers' diet and physical training as factors. Bonferroni's post-hoc test was used. Each litter of six pups was considered one sample, and statistical analyses were performed by using the mean values of each litter. Significance was set at P < 0.05. Data analysis was performed using the statistical program Graphpad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

Body weight was lower in trained rats during the third and fourth weeks pregestation (Figure 1(a)). LP mothers showed a reduction in the body weight when compared to C mothers during gestation (P < 0.01), whereas physical training had no effect in the body weight (Figure 1(a)).

The number of pups (litter size) born from mothers was similar among groups. Litter weight, birth weight, and body

weight in the first three days of life of pups from mothers submitted to a low-protein diet during gestation, trained or not, were lower when compared to control (Table 2).

Trained mothers showed an increase of insulin secretion by isolated islets incubated with stimulatory (16.7 mM) concentration of glucose (Figure 2). However, low-protein diet during gestation induced a reduction in the secretion of insulin by isolated islets when compared with both control and LP + T groups (Figure 2).

4. Discussion

Maternal exercise guidelines preconize that 30 min, at least, of moderate-intensity exercise a day on most, if not all, days of the week is satisfactory for health [8]. Following these recommendations, a controlled prospective studies have demonstrated that moderate pregestational exercise (approximately 50% to 70% of VO_{2 max}) is useful to increase metabolic rate (reduction of body weight) and improve cardiorespiratory fitness and maternal-fetal physiological reserve [30]. In addition, previous study has demonstrated an improved cardiovascular function, limited gain of body weight, and reduced risk of gestational diabetes mellitus and hypertension [31]. In the present study, trained mothers demonstrated reduced body weight in the second and third weeks pregestation, but this normalized throughout gestation. Our results are in agreement with previous studies [21, 24]. It is well established that regular physical exercise increases lean body mass and induces a higher utilisation of fatty acids as fuel by skeletal muscles once the intensity of the exercise is maintained at 65–70% of $VO_{2 \text{max}}$ [32].

Maternal physical training and low-protein diet did not alter the amount of food intake as seen in previous studies [25, 33]. However, dams submitted to a low-protein diet showed a reduction in body weight in the last week of gestation and the first days postdelivery. Maternal protein restriction is associated with lower stores of maternal nutrients, and it has been found previously that an undernutrition-induced reduction of maternal gain of body weight is positively correlated with a low birth weight, an impaired offspring growth rate, and a loss of lean mass during development [34].

During pregnancy, there is an increase in basal hepatic glucose production, a progressive decrease in insulin sensitivity, and an associated 3.0-3.5-fold increase in insulin response in the last third of gestation [2]. Environmental stimulus as undernutrition can impair the normal hormonal/metabolic response during gestation. Herein we showed that maternal low-protein diet induced a reduction in the secretion of insulin. Our results are in accordance with previous studies where maternal low-protein (6% casein) diet induced impairment in the metabolism of glucose and secretion of insulin [1, 35, 36]. It has been suggested that the poor secretory response to glucose observed in islets from low-protein diet rats may be related to a defect in the ability of glucose to increase Ca2+ uptake and/or to reduce Ca²⁺ efflux from β -cells [36] and a reduced islet mass and/or insulin biosynthesis [3].

Given that previous studies have described an inverse relationship between maternal insulin and maternal insulin

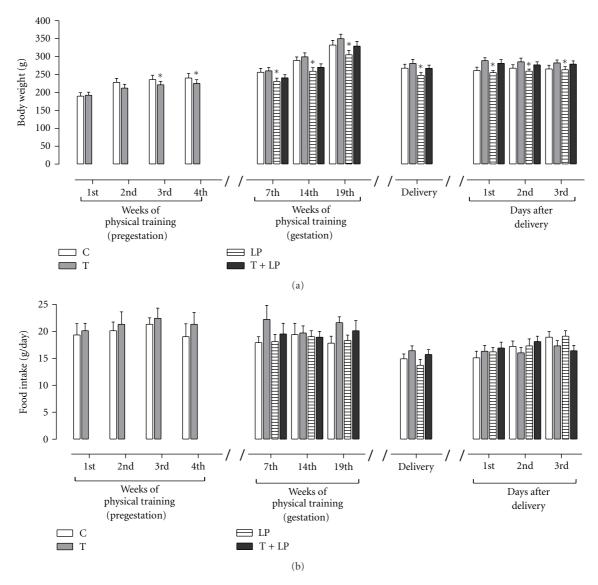


FIGURE 1: Body weight (a) and food intake (b) during pregestation and gestation by control (C, n = 5), trained (T, n = 5), low-protein diet (T, n = 5), and trained + low protein dams (T + T, n = 5). The protocol of physical training was initiated four weeks before pregnancy (5 days per week, and 60 minutes per day). During gestation, duration and intensity of each session of physical exercise were progressively reduced and each group received either 17% casein diet (T or 8% casein (low-protein group, T and T isocaloric diet *ad libitum*. The values are presented as means T S.E.M. *T < 0.05 *versus* T group using two-way ANOVA and Bonferroni's *post-hoc* test.

sensitivity with fetal growth restriction, it is expected that the processes governing fetal growth will be also affected. Indeed, we and previous investigators have showed that pups from mothers submitted to a low-protein diet present a reduction in body weight at postnatal period [24, 37–39]. These changes may result from a reduction in nutrient delivery to the fetus, then it would result in lower fetal insulin concentrations and a decrease in fetal IGF-I and IGF-II that would downregulate fetus-placental growth [40].

Little is known about the effects of maternal physical activity on the secretion of insulin. Here, we demonstrated for the first time that a controlled protocol of physical training before and during gestation increased pancreatic islets glucose-stimulated insulin secretion. This effect was

particularly important once maternal protein-restriction has been shown to impair secretion of insulin leading to gestational diabetes as seen in previous study [1]. The underlying mechanism to the effects of maternal physical training on glucose-stimulated insulin secretion remains unknown but can be associated to increased pancreatic β -cell mass by upregulation of growth and survival pathways (AKT, ERK pathways) in pancreatic islets [41]. In addition, it can be resulted from the high peripheral glucose uptake by skeletal muscle in response to moderate physical training. Indeed, our previous study demonstrated that dams submitted to a low-protein diet (8% casein) during gestation present a higher fasting glycaemia than control (17% casein) while trained mothers remained with normal glycaemia [25]. Our

	Groups							
	Control		Trained		Low protein diet		Trained + low protein diet	
	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
Litter size	11.0	0.3	10	0.2	10	0.3	10	0.2
Litter weight (g)	66.9	0.9	65.2	0.9	55.7*	1.1	55.8*	0.9
Birth weight (g)	7.1	0.09	6.9	0.1	5.7*	0.1	5.9*	0.1
Body weight (g)								
1st d	7.9	0.1	7.1	0.08	5.9*	0.1	6.1*	0.2
2nd d	8.9	0.2	8.7	0.2	5.9*	0.03	5.9*	0.1
3rd d	9.4	0.1	9.7	0.1	6.1*	0.1	6.5*	0.2

Table 2: Indicators of litter size, litter weight, and body weight at birth to 3rd d of life.

During gestation, the dams were submitted to physical training (5 days per week, with a progressively reduction of duration and intensity) and fed a low-protein diet. The pups into each litter were evaluated at birth to 3rd d of life. The values are presented as mean and S.E.M. $*P < 0.05 \ versus \ C$ using two-way ANOVA and Bonferroni's post-hoc test.

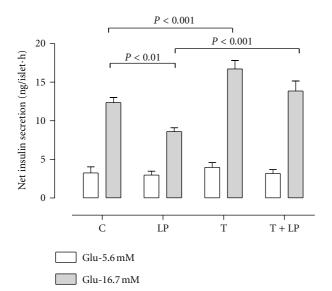


FIGURE 2: Glucose-induced insulin secretion in islets from rats submitted to a protocol of physical training four weeks before gestation and during gestation. During gestation, duration and intensity of each session of physical exercise were progressively reduced and each group received either 17% casein diet (C and T) or 8% casein (low-protein group, LP and LP + T) isocaloric diet *ad libitum*. Groups of five islets were initially incubated for 45 min at 37°C in Krebs-bicarbonate buffer containing 5.6 mM glucose. The solution was then replaced with fresh Krebs-bicarbonate buffer and islets were incubated for a further 1 h with medium containing 5.6 or 16.7 mM glucose. The values are presented as means \pm S.E.M. $^*P < 0.05\ versus$ C group using two-way ANOVA and Bonferroni's post-hoc test.

data reinforce the impaired effects of a maternal low-protein diet on the pancreatic islets glucose-stimulated insulin secretion that is preventable by moderate physical training.

The present study demonstrated that a controlled protocol of physical training during gestation resulted in increased insulin secretion, and mothers fed a low-protein diet during gestation showed similar results. This differential effect observed in the present study refers to dams which were previously trained when were contrasted with pregnancy and low-protein diet. These observations indicate that maternal physical exercise initiated in early pregnancy induces maternal adaptations and can be considered as a therapeutic mean of countering the effects of maternal undernutrition, which may provide a useful strategy since an exercise program may improve insulin sensitivity and fasting plasma glucose concentrations of women at risk for gestational diabetes [26].

Similarly to our results, previous study demonstrated that light-to-moderate physical activity during pregnancy is associated with reduced risk of abnormal glucose tolerance and hyperglycemia during pregnancy [42]. Rats submitted to 10 degrees slope treadmill for 5 days/week at 20 m/min, starting with a 20 min run and with a progressive daily increase of 5 min, throughout gestation showed a lower rise in blood glucose after an oral glucose load (2 g/kg body weight) than untrained pregnant rats [33]. In human, previous study has found that associations between the physical activity and insulin sensitivity and β -cell response do not appear to differ in pregnant versus nonpregnant women [43]. Further experiments are necessary to understand the mechanism underlying to those effects.

5. Conclusions

The present study showed that low-protein diet during gestation affects the secretion of insulin. In contrast, pregestational and gestational moderate physical training acts as an environmental stimulus by increasing insulin secretion and attenuating the effects of perinatal undernutrition in rats, suggesting a potential strategy for compensation of insulin resistance and preventing gestational *diabetes mellitus* during late pregnancy.

Acknowledgments

The authors declare no conflict of interest. This research was supported by National Council for Scientific and Technological Development (CNPq), Foundation to Support

Science and Research from Pernambuco State (FACEPE), São Paulo Research Foundation (FAPESP), Coordination for the Improvement of Higher Level Personnel (CAPES), and Dean's Office for Research/University of São Paulo. Authors acknowledge the National Institute of Science and Technology in Obesity and Diabetes (INOD) and the Center of Lipid Research and Education (CLEAR). All authors were responsible for critical revisions to the paper and approval of the final version. The authors are indebted to José Roberto Mendonça, Érica Portiolli, and Geraldina de Souza for technical assistance.

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 936486, 14 pages doi:10.1155/2012/936486

Review Article

Reactive Oxygen Species in Health and Disease

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Received 16 June 2012; Revised 17 July 2012; Accepted 18 July 2012

Academic Editor: Sandro Massao Hirabara

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During the past decades, it became obvious that reactive oxygen species (ROS) exert a multitude of biological effects covering a wide spectrum that ranges from physiological regulatory functions to damaging alterations participating in the pathogenesis of increasing number of diseases. This review summarizes the key roles played by the ROS in both health and disease. ROS are metabolic products arising from various cells; two cellular organelles are intimately involved in their production and metabolism, namely, the endoplasmic reticulum and the mitochondria. Updates on research that tremendously aided in confirming the fundamental roles of both organelles in redox regulation will be discussed as well. Although not comprehensive, this review will provide brief perspective on some of the current research conducted in this area for better understanding of the ROS actions in various conditions of health and disease.

1. Introduction

The first published article in Pubmed on reactive oxygen species (ROS) dated since 1945; this was retrieved using the keyword "reactive oxygen species." Since then, and at the time this review article was written, the use of ROS as keyword in Pubmed search resulted in more than 117,000 English-written articles, almost 12,000 of which are review articles. Most studies have linked ROS to disease states such as cancer, insulin resistance, diabetes mellitus, cardiovascular diseases, atherosclerosis, and aging, just to list examples. However, numerous articles have also linked ROS to various physiological processes and essential protective mechanisms that the living organisms use for their survival; obvious examples would be their role in immune defense, antibacterial action, vascular tone, and signal transduction. Soon it became clear that in order to maintain a state of homeostasis, living organisms are striving to keep those highly reactive molecules under tight control with the help of an intricate system of antioxidants.

The tremendous amount of research published in this field makes it almost unfeasible for any review article to be comprehensive. Thereby, the main objective of this review is to present recent studies that examine the roles played by ROS in various states of health and disease, with special emphasis on the involvement of two subcellular organelles, the endoplasmic reticulum and mitochondria, in the metabolism of ROS as it relates to these states. Metabolic disorders such as insulin resistance, diabetes mellitus, obesity, and chronic inflammation are focused on. A discussion of several molecules with antioxidant properties is also presented as these might prove to be promising in preventing and/or treating ROS-related diseases. As such, it was not within the scope of this review to deal with all the details.

2. Redox Stress or Redox Regulation?

ROS are highly reactive molecules that originate mainly from the mitochondrial electron transport chain (ETC). Almost all cells and tissues continuously convert a small proportion of molecular oxygen into superoxide anion by the univalent reduction of molecular oxygen in the ETC. The ROS are produced by other pathways as well, including the respiratory burst taking place in activated phagocytes,

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ionizing radiation's damaging effect on components of cell membranes, and as byproducts of several cellular enzymes including NADPH oxidases (Nox), xanthine oxidase (XO), and uncoupled endothelial nitric oxide synthase (eNOS) [1].

Due to the potential beneficial role of ROS demonstrated by several lines of research, ranging from their role as signaling molecules [2] to the more unexpected role in improvement of certain cancers [3], the term "redox regulation" might prove to be more accurate than "redox stress"; there have been even some situations where antioxidants are described to be "bad" [4]. However, the term "redox stress" is more commonly used.

3. Examples of ROS Role in Normal Physiological Processes

3.1. Role of ROS in Normal Vascular Diameter Regulation. Mitochondrial ROS, specifically superoxide anion (O2 --) and hydrogen peroxide (H₂O₂), were demonstrated to play a role in normal vascular physiology in response to such factors as shear-stress [2]. In the vascular system, ROS were demonstrated to originate mainly from the mitochondria in a study performed on human coronary resistance arteries. The mitochondrial origin of ROS was confirmed using electrobiophysical methods that assessed the ROS generation and the response of vessel diameter to the presence of inhibitors of mitochondrial complexes and antioxidants [2]. Go and colleagues have studied in more detail the mitochondrial role in the signaling response to oxidized milieu that might be encountered in the vascular system. Their results provide a model whereby more oxidized environment in the plasma will lead to oxidation of cellular plasma membrane and cytoskeletal proteins. Oxidized proteins will then stimulate mitochondrial production of ROS that will initiate signaling pathways upregulating the cellular inflammatory response [5]. The model described by Go et al. also provides explanation for the protective antioxidant role of small molecular weight-mitochondrial proteins such as thioredoxin 2 (Trx2). Earlier works have previously demonstrated Trx2 "regulatory" redox signaling pathway against mitochondrial ROS [6-8]. The described model also provides partial explanation for the paradox that whereas moderate ROS levels contribute to regulation of vascular cell function [2], their excessive production is linked to pathological situations where redox damage and inflammation prevail in several chronic diseases. This kind of studies linking the cellular responses to alterations in redox potential on one hand to the intracellular signaling pathways on the other hand is promising; since it can be translated into designing novel therapeutic agents that target relevant signaling pathways as an alternative to the use of nonspecific antioxidant agents in clinical trials, or perhaps as a complementary tool to these agents.

3.2. Role of ROS in Oxygen Sensing. Oxygen sensing is so critical to cellular health as it allows cells to initiate adaptive responses that will increase the likelihood of survival in anticipation for limited oxygen availability. Guzy and

Schumacker have proposed that the ETC acts as an O2 sensor by releasing ROS in response to hypoxia. The hypoxiainduced released ROS act as signaling molecules that trigger diverse functional responses, among which is the increased production and stabilization of the hypoxia-inducible factor-1 (HIF-1). This has been demonstrated at least in normal (nontransformed) cells. As a matter of fact, a mutual regulation was reported for both HIF-1 and ROS. Under acute hypoxic conditions, the mitochondrial ETC produces excess ROS. This is required for the induction of HIF-1 expression [9], which in turn mediates adaptive metabolic responses culminating in a normalization of ROS levels and maintenance of redox homeostasis. Likewise, hypoxic induction of HIF-1 activity will end in normalization of the tissue O₂ levels by stimulating angiogenesis, which augments oxygen delivery to tissues and solves the problem of tissue hypoxia. However, in some cancer cells the picture is not the same, as cells transformation is expected to result in alterations in the above-described normal adaptations, hence even though angiogenesis might occur, it is less effective in maintaining oxygen homeostasis; this was extensively reviewed by Semenza [10].

3.3. Role of ROS in the Immune System. Essentially, ROS are deeply involved in both arms of the immunological defense system, the innate and the acquired responses. Upon exposure to environmental pathogens, exaggerated ROS production as a part of the oxidative burst in activated phagocytes present in the local inflammatory milieu represents one of the first lines of defense mounted against the invading pathogens. Although rapid, this innate immunity is usually only partially effective, since certain fraction of pathogens might escape and proliferate, thereby producing a larger number of pathogens. Acquired immunity will be initiated when pathogen-derived antigenic peptides that are the result of phagocytosis and digestion by activated phagocytes are presented to the T lymphocytes. As a result, the latter will proliferate and differentiate producing a large progeny of immunological effector cells that are capable of mounting an efficient and antigen-specific immune response. ROS are involved in the acquired immune response because excess ROS continue to be locally produced by the activated phagocytes and consequently enhance the intracellular signal transduction cascades within the T lymphocytes and thereby decrease their activation threshold [1]. The role exerted by ROS in immune responses will be revisited when the inflammasome assembly as a part of chronic inflammation response will be discussed later in this review.

3.4. Role of ROS in Skeletal Muscle Physiology. The skeletal muscle is a target organ for oxidative regulation and/or oxidative stress since it requires a large supply of energy to ensure efficient contraction, and consequently it is liable to be exposed to excess mitochondrial ROS. The skeletal muscle production of ROS is promoted by multiple stimuli including muscle contraction, insulin, and hypoxia. Although under normal physiological conditions, antioxidant systems control the level of ROS in skeletal muscle, oxidative stress can take place if ROS levels exceed the muscle antioxidant

capabilities, and this can have damaging functional effects [11]. Recent research has suggested that ROS can act as signaling intermediates in the regulation of skeletal muscle glucose uptake during contraction. However, results of such research have to be interpreted with caution as they have been somewhat inconsistent depending on the model studied and the experimental design [11-13]. Interestingly, muscle activity has been recently reported to affect the antioxidant defenses as well. Berzosa and colleague have reported an augmented effect of acute exercise in healthy untrained male subjects on the circulating total antioxidant status and antioxidant enzymes activities after both maximal and submaximal exercise periods [14]. Others [15] have shown that the elevated levels of antioxidant enzymes activity was also detected in various body organs. Thus, it is thought that exercise, whether acute or chronic, helps in maintaining redox homeostasis since it increases the antioxidant defense mechanisms, and due to the fact that long-term heavy exercise renders both animals and humans more resistant to oxidative damage [14]. Not only muscle contraction has drawn the attention of scientists interested in the field of muscle physiology, but also muscle immobilization, where ROS production was reported to increase in skeletal muscle tissue after immobilization: a finding that warrant further studies specially if we consider that immobilized subjects manifest great loss of their muscle mass [1].

3.5. Role of ROS in Genomic Stability, Regulation of Transcription, and Signal Transduction. Cellular redox status is considered an emerging regulatory factor for genomic stability and transcription. In a recent review article by Rajendran and colleagues, the posttranslational enzymatic covalent modification of histone and nonhistone proteins in the form of acetylation/deacetylation for finely regulating transcription was discussed in relation to the cellular redox status. Various physiological processes such as cell cycle regulation, response to DNA damage, regulation of intermediary metabolism, programmed cell death, and autophagy, listing only few, are known to be regulated at the level of transcription of relevant genes. The authors have reviewed in detail various factors regulating transcription via modulation of chromatin dynamics. They have indicated that oxidative stress and cellular energy consumption are among the key transcription regulating factors since the deacetylase activity of sirtuins, members of class III histone deacetylases, depends on the cellular redox status and NAD⁺ availability, respectively. In fact, the gene expression level of sirtuins has been shown to be under the control of the oxidative stress- and DNA damage-responsive transcription factor, E2F1, which regulates cell cycle and directly binds to the promoter of sirtuin 1, the most studied member of the sirtuins family. Moreover, exposure of cells to excess ROS such as H₂O₂ results in posttranslation modification of Sirt 1 in the form of desumoylation and hence inactivation of Sirt 1 deacetylation function, and consequently to acetylation and hence activation of pro-apoptotic Sirt 1 substrates such as p53, and eventually cell death will take place. Under oxidative stress, the role played by ROS in transcription regulation is of critical importance and is able to affect vital processes such

as glucose homeostasis, inflammation, cellular lifespan, and multiple aging-related diseases including cancer [16].

Cells have an elaborate system to respond to redox status. This has been well studied in bacteria where the existence of a number of different ROS and redox statusresponsive signaling pathways is well established [17], as well as in the yeast Saccharomyces cerevisiae [18]. In mammalian cells, similar yet incompletely understood protective redoxresponsive signal cascades have been described. These cascades are critical for the survival of cells which happen to be in the midst of highly oxidizing environment such as sites of infection and inflammation. While activated phagocytes utilize their capability of creating "oxidative burst" to kill invading pathogens, and this implies the overproduction of ROS, recruited lymphocytes on the other hand need to possess an armament of oxidative stress-induced signal transduction cascades to protect themselves against this same oxidative burst. The oxidizing milieu modulates lymphocytes signal transduction cascades and increases the activities of redox-responsive transcription factors such as activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B). The latter will bind and activate the promoters of various genes. One of those genes is the gene for the protective protein thioredoxin (Trx). Trx is an oxidoreductase that works together with the glutathione system for establishing and maintaining a reducing intracellular redox state. Other set of genes whose products are protective antioxidants are peroxiredoxin I (i.e., a Trx peroxidase), heme oxygenase-1, the cystine transporter xc2, and manganese SOD (MnSOD) [1, 19].

3.6. "Potential Beneficial" Role of ROS in Cancer. Recently an interesting hypothesis arises that examines the following question: "Can antioxidants promote disease situations?" or as Perera and Bardeesy stated it: "When antioxidants are bad?" [4]. This is a hot area of research and is finding increasing implications in cancer-related studies. Classically, ROS were demonstrated to promote various types of cancers. This was explained by different facts: the ROS ability to induce DNA damage and thus to enhance the rate of tumor-causing mutations and genetic instability, their pro-inflammatory effect, and their stabilizing influence on HIF essential for energy regulation. Accordingly, antioxidants were able to decrease tumorigenesis by neutralizing the deleterious effects of ROS [20–23].

Recently, a different face of the ROS coin has been revealed based on studying the effect of mutations activating the transcription nuclear factor, nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2 is a redox stress-sensitive transcription factor that induces several antioxidant and detoxification genes. In the absence of redox stress states, Nrf2 is kept inactive by binding to another protein, Kelchlike ECH-associated protein 1 or KEAP1, ensuring effective Nrf2 repression. Somatic mutations in either Nrf2 or KEAP1 that prevent their binding will result in constitutive Nrf2 activation and transcription of Nrf2 target genes. Such mutations have been isolated from patients with lung cancer suggesting a protumorigenic role of Nrf2. Furthermore, drug resistance in some antitumor therapy may take place as a result of such somatic mutations; this was reviewed by Hayes

and McMahon [24]. More recently, DeNicola and colleagues have demonstrated that in mice several endogenous oncogenes such as Kras, Braf, and Myc actively induce Nrf2 expression, promoting an ROS detoxification program and hence creating a more "reduced" intracellular environment, a program that the authors suggest to be required for tumor initiation [3]. As Hansson and Libby elegantly described the immune response in atherosclerosis as "double edged sword" [25], the description seems to perfectly fit the ROS. Therefore, the big picture reflecting the contributions of various mediators plus local environmental factors seems to be the actual determinant for ROS-induced consequences in both physiology and pathology, and hence it is essential to unravel the not-yet-well understood parts of this intricate picture for better understanding of the ROS induced alterations.

Key Messages from Section 3. Although ROS have been classically known for their damaging effects, increasing evidence of their use in regulating and maintaining normal processes in living organisms has been accumulating. Therefore, the term redox regulation seems to better describe the redox status and its consequences. Both ROS and the protective antioxidant systems have to work in coordination to reach a state of redox homeostasis. Evidence of the roles played by ROS in several physiologic processes has been presented such as maintaining vascular diameter and normal vascular cell function, participating with HIF in sensing the oxygen availability and initiating responses appropriate for cell survival, mounting effective immune response, acting as possible signaling molecules in regulating skeletal muscle glucose uptake, and regulating gene stability and transcription via affecting chromatin stability. Antioxidants are equally essential, and their genes expression is regulated by the ROS. In addition, muscle exercise is beneficial in rendering us more resistant to oxidative damage. Recent evidence points out to a potential link between the "reduced" cellular environment and tumor initiation.

4. ROS at the Cellular Organelles Level: The Roles of the Endoplasmic Reticulum and Mitochondria in Oxidative Stress/Regulation

Both the endoplasmic reticulum (ER) and the mitochondrion have proven to be fascinating intracellular organelles that have stimulated a tremendous amount of research due to their unique characters. Their well-established roles in proper protein folding, posttranslational modifications, cellular trafficking, ions storage, energy production, cellular thermogenesis, and intermediary metabolism are just some examples. Both organelles have strong and interrelated ties to the redox cellular homeostasis, disturbance of which is implicated in many diseases. Increasing evidence accumulates that ROS contribute to endothelial cell dysfunction, atherosclerosis, aging, diabetes mellitus (DM) and diabetic complications, and CVD, to name only few [26–30].

4.1. Endoplasmic Reticulum and Endoplasmic Reticulum Stress. Impaired biological processes within the cell, collectively defined as cellular stress, together with chronic

inflammation have been causally associated to various metabolic diseases, such as DM, obesity and CVD [25-31]. The ER, ubiquitously present in eukaryotic cells, plays a key role in protein folding and modification as well as in dynamic storage of calcium. It is through its role in maintaining protein folding that the ER is intricately involved in the overall ROS production as will be discussed shortly. Although protein folding is a multistep process that is not yet fully understood, two factors are known to be essentially required for the formation of intra- and intermolecular disulphide bonds that are fundamental to the folding process; these are the availability of energy and an ER oxidizing environment. In addition, two ER enzymes, the protein disulphide isomerase (PDI) and ER oxidoreductin 1 (ERO1), are critical for the oxidative formation of disulphide bonds [32]. The reactions they catalyze involve transfer of electrons and oxidation of cysteine residues in nascent proteins and utilize flavin adenine dinucleotide (FAD) and molecular oxygen. Electron transfer to molecular oxygen as a terminal electron receiver produces H₂O₂; hence excess load of protein folding can result in accumulated ROS. The latter will trigger cellular inflammatory response.

The ER is thought to sense signals of altered cellular states triggered by a variety of stimuli such as certain growth factors and hormones, limited availability of energy or nutrients, and the cellular redox state. The ER then acts accordingly aiming at restoring the normal cellular homeostasis. The ER itself might experience a state of ER stress, in which its capacity to correctly fold and modify proteins is overwhelmed by an excessive demand for protein folding or by conditions accompanied by excessive unfolded or misfolded proteins. This will increase the amount of proteins of abnormal structure in the ER, triggering a defensive set of reactions collectively known as "unfolded protein response" or UPR, during which the cellular transcriptional and translational machineries are altered in order to restore the normal protein folding process. However, if the stress is extreme or prolonged, cellular homeostasis cannot be established and, alternatively, cellular pathways culminating in apoptosis will be activated [33, 34]. A less well-understood UPR system was recently described in the mitochondria (UPR mt) and its involvement in protecting cellular and specifically mitochondrial components against damaging consequences of metabolic stressors is increasingly acknowledged [35]. At the molecular level, the relation between ER stress and oxidative stress can be explained by various routes. As mentioned earlier, during electron transfer to molecular oxygen as the terminal electron recipient in the ER protein folding process, some ROS will be generated. Furthermore, under ER stress conditions, manifested by excess accumulation of unfolded or misfolded proteins, the cell consumes extra reduced glutathione (GSH) to correctly fold these aberrantly folded proteins, adding more to the cellular stress. Consequently, the ER stress can result in oxidative stress which as mentioned earlier might trigger an inflammatory state. Thus, it seems that the ER is placed in a vicious cycle where ER stress can be caused by oxidative stress, and will also augment the perturbed oxidative redox state. Therefore, protective mechanisms essentially exist in the ER to limit the consequences of this damaging cycle. These include the protein kinase R-like ER Kinase (PERK) pathway-induced activation of an antioxidant program that utilizes the transcription factors: activating transcription factor-4 (ATF4) and Nrf2 [36–38]. As previously mentioned, activated Nrf2 will be translocated to the nucleus to increase the rate of expression of a group of antioxidant and oxidant-detoxifying genes [39, 40].

4.2. Role of Mitochondria in ROS Production. The mitochondrial ETC represents the major source for cellular ROS production; therefore, it is mentioned in various sections of this review. The superoxide anion is nonenzymatically formed by the ETC semiubiquinone compound and then enzymatically converted into hydrogen peroxide by superoxide dismutase (SOD). Superoxide anion can also be nonenzymatically converted into hydrogen peroxide and singlet oxygen. Hydrogen peroxide can be converted into the highly reactive hydroxyl radical in the presence of reduced transition metals. Alternatively, hydrogen peroxide may be enzymatically converted into water by the enzymes catalase or glutathione peroxidase [1].

Mitochondria possess several unique characters among which are the presence of mitochondrial DNA (mtDNA), their mode of inheritance, the dynamic nature of their structure, their indispensable roles in fuel metabolism and energy production, and the established links to various metabolic abnormalities. Therefore, it is expected that a defected mitochondrion is the underlying mechanism for a myriad of pathological conditions. The strong association between mitochondrial dysfunction, whether genetically determined or acquired, and chronic metabolic diseases such as type 2 DM (T2DM) and obesity was observed in many studies; yet a cause-effect relationship remained tentative for some time, till further studies demonstrated that impaired mitochondrial capacity and function are potential causes for insulin resistance and/or DM progression; this will be discussed below in more detail [41].

The central regulatory role played by the mitochondria in whole body metabolism, energetics, and homeostasis necessitates that it will be under tight control. Its ultimate functional capacity in certain tissue and under certain physiological conditions is the result of a network of interfering parameters. These include the mitochondrial DNA copy number, the mitochondrial density, and levels and activity of specific mitochondrial proteins [41]. Both transcriptional and posttranscriptional mechanisms exist to ensure tight control of the mitochondrial functional outcome. The nuclear DNA is deeply involved as well in implementing this control, and a strong link between nuclear and mitochondrial gene expression was demonstrated more than 15 years ago [42].

As mentioned before, mitochondrial ETC is a potent source of ROS, and for obvious reasons such as the physical proximity to mtDNA, mitochondrial ROS generation is under tight control by various mechanisms, among which are the uncoupling proteins 1, 2, and 3 (UCP1, 2 and 3). UCPs are inner mitochondrial membrane proteins that are considered as natural regulators of mitochondrial ROS, responding

to and controlling ROS production by diminishing the formation of a large proton gradient [41]. It is thought that UCP1, which is present in the brown adipose tissue, evolved a thermogenic role in mammals as a side pathway of the original, more general function of protecting cells against the cold-induced production of ROS. On the other hand, UCP2 (ubiquitously expressed at low levels) and UCP3 (preferentially expressed in skeletal muscle) maintain their original function of decreasing ROS production through uncoupling and hence buffering ROS levels and do not appear to play a thermogenic role [43-45]. Other emerging roles of UCP have been suggested, for example, UCP2 is thought to exert a negative regulatory effect on pancreatic insulin secretion, as well as an ROS buffering effect on hypothalamic neurons controlling eating behavior; this will be detailed later [46, 47].

Several years ago, the dynamic nature of the mitochondrial structure was elucidated and was demonstrated to be attained by complex molecular machinery, several components of which have been well characterized [48]. Abnormality in this machinery is linked to mitochondria—associated metabolic diseases. As an example, reduced expression of mitofusin 2 (Mfn2), one of the mitochondrial proteins responsible for its dynamic morphology, was demonstrated to be partly responsible for decreased glucose oxidation and cell respiration in obesity [49].

Key Messages from Section 4. Both the ER and the mitochondria participate in maintaining normal cellular homeostasis. It is through the ER role in maintaining proper protein folding that this organelle is intricately involved in the overall ROS regulation. The ER senses signals of altered cellular redox states and then acts accordingly in order to restore and maintain normal homeostasis. During the UPR of the ER, ROS will be accumulated either due to actual production of ROS or due to consumption of the antioxidants such as GSH. Because the ER can be a part of a vicious cycle, where oxidative stress leads to ER stress, and the latter will further worsen the redox status, there are several protective mechanisms to limit the anticipated damage. A strong association and a potential cause-effect relationship exist between defective mitochondria and metabolic diseases. As in the ER case, several protective mechanisms exist to protect the mitochondria from oxidative damage. The antioxidants, as superoxide dismutase, catalase, and glutathione peroxidase/reductase system, are not in the scope of this review. UCPs are natural regulators for mitochondrial ROS, responding to and controlling the ROS production by diminishing the mitochondrial large proton gradient. Recently, UCP2 has been linked to other functions as well.

5. Role of ROS in Metabolic Diseases and Chronic Inflammation

5.1. Macromolecular "Toxicity". In DM and obesity, the prevalent metabolic state is the one described by the term "glucolipotoxicity," in which excess extracellular glucose and

fatty acids (FAs) exert various damaging effects. Excess glucose increases oxidative stress through several biochemical mechanisms, including glyceraldehydes autoxidation, protein kinase C activation, glycation, methyl glyoxal and sorbitol production, hexosamine pathway, and oxidative phosphorylation [50]. Likewise, excess FA leads to peripheral insulin resistance and accumulation of lipid in nonadipose tissue locations as the liver, heart, and pancreas, potentially resulting in failure of these organs. At the cellular organelles level, lipotoxicity has been recently linked to both oxidative and ER stress [51].

The link between excess glucose and lipid "that is, macromolecules" cell stressors and inflammation was recently demonstrated in adipocyte where excess glucose and saturated FA, through ROS generation and activation of the nuclear transcription factor NF-κB, induced inflammation as manifested by upregulation of active inflammatory mediators involved in monocyte adhesion and chemotaxis. The Toll-like receptor 4 (TLR4) was implicated in mediating the effect of excess saturated FA-but not excess glucoseon the expression of these inflammatory mediators [52]. Moreover, and in contrast to excess saturated FAs, polyunsaturated FA were reported to exert anti-inflammatory effect on adipocytes that were linked to the nuclear receptor PPARy [52]. These observations were supported by in vivo studies on experimental animals [53-55], but not yet in human.

5.2. Role of ROS in Insulin Resistance. Insulin resistance (IR) is not only a key feature of T2DM, but is also a characteristic of a wide range of clinical conditions such as obesity, metabolic syndrome, pregnancy, and sepsis [56]. IR can also occur, both *in vivo* and *in vitro*, as a consequence of certain experimental treatments with inflammatory cytokines such as tumor-necrosis factor- α (TNF- α) or with glucocorticoids such as dexamethasone, both treatments have obvious clinical implications. As a matter of fact, it is well established that elevated levels of TNF- α and/or glucocorticoids are detected in patients with the above-mentioned IR-associated clinical states [57–60].

Several factors have been demonstrated to play a role in IR. ROS hold a unique position among these factors, based on studies conducted on cell lines or in vivo. When the murine adipocyte cell line 3T3-L1 was treated with the ROS H₂O₂ or with ROS inducers, it clearly developed resistance to insulin [61, 62]. Moreover, markers of oxidative stress have been significantly associated with obesity, IR, DM, and sepsis [63, 64]. Similarly, conditions that increase ROS levels, for example, diseases with primary defects affecting ROS balance such as familial amyotrophic lateral sclerosis, were found to be associated with IR [65]. Albeit strong, the association between ROS and IR in various pathologic settings did not initially imply a cause-effect relationship; it just elucidated a strong association state. Nevertheless, such a causal effect was demonstrated few years ago by Houstis and colleagues. Using two approaches, cell lines (3T3-L1) and animal model of genetic obesity (ob/ob mice), the authors have undoubtedly demonstrated that increased levels of ROS were indeed the cause for TNF-α- or dexamethasone-induced IR determined

by the lowered glucose uptake rate. Experimental intervening by either pharmacological agents or in transgenic animals designed to decrease ROS levels was shown to substantially prevent the IR status [56]. c-Jun NH₂-terminal kinase (JNK) activation, which was detected upon stimulating the cell line with TNF- α or dexamethasone, was suggested to mediate the ROS-induced IR and was demonstrated to be linked to differential translocation of two important transcription factors; the pancreatic and duodenal homeobox-1 (PDX-1; which will be translocated from the nucleus to the cytosol thereby suppressing insulin biosynthesis) and the Forkhead transcription factor Foxo1, which will be translocated in the opposite direction, from the cytosol to the nucleus, thereby contributing to insulin resistance by enhancing gluconeogenesis [66]. Because the sphingolipid ceramide was reported to be increased in TNF- α - and dexamethasoneinduced IR in 3T3-L1 cell line and in diabetic muscle, it was suggested as a potential ROS source in insulin resistance [67–

5.3. Role of ROS in Mitochondrial Dysfunction and in Diabetes Mellitus. Normally, the β -cells of the pancreas adapt their insulin secretion to the fluctuations in blood glucose concentration sensed by their glucose sensor, glucokinase. During hyperglycemia, the rate of insulin-dependent glucose utilization by glycolysis in the β -cells will increase. Compared to other cell types, the β -cells manifest an unusually high proportion of glucose-derived carbon skeleton entering the mitochondria in the form of pyruvate that will then enter the tricarboxylic acid (TCA) cycle. Mitochondrial ETC promotes ATP generation, which will then be exported to the cytosol. Under high ATP: ADP ratio, the β -cells plasma membrane will be depolarized, and the potassium-ATP channels (K_{ATP}) will be closed allowing the opening of voltage-sensitive Ca²⁺ channels. Increased intracellular Ca2+ is the key trigger for exocytosis and insulin release from the secretory granules [70, 71]. This is referred to as stimulus-secretion coupling in the β -cells or glucose-stimulated insulin secretion (GSIS), as it was initiated by glucose utilization.

The pivotal role of normal mitochondrial ETC in the pancreatic β -cells glucose homeostasis has been established by a number of elegant studies over the past 30 years. Exposing the mitochondria to poisons or to restricted oxygen supply has established the finding that blockade of the mitochondrial ETC inhibits GSIS from β -cells [72]. This was later confirmed in experiments using rho⁰ β -cells, where the mtDNA-encoded subunits of the ETC enzymes are suppressed, while insulin biosynthesis and cell viability are preserved. The mitochondrial dysfunction in these cells and the consequent loss of mitochondrial ATP production have resulted in loss of GSIS [73–75]. The lost response was restored by introducing normal mitochondria into the rho⁰ β-cells, confirming the mitochondrial origin of the defect [73]. The stimulus-secretion coupling in the β -cells was further studied in transgenic animals with β -cellstargeted deletion of the nuclear encoded mitochondrial transcription factor (TFAM), which is the major transcription factor controlling the mtDNA genes expression. The β cells of this animal model manifest diabetic phenotype with both the ATP production and GSIS greatly diminished [76]. These animals represent a model for human mitochondrial diabetes, a rare form of DM, that is maternally inherited, caused by mutations in the mtDNA, and usually associated with other pathological findings as bilateral sensory-neural deafness [77].

In patients with T2DM, the form of disease that affects almost 90% of all diabetic patients, some reports have demonstrated a decrease in the copy number of mtDNA in skeletal muscles and in peripheral blood cells [78, 79].

As previously mentioned, accumulation of ROS in the mitochondria (due to excessive production and/or defective defense mechanisms) is accompanied by mitochondrial dysfunction; this was found to be an age-related process [80]. Apparently, with advanced age the β -cells will be particularly susceptible to ROS damage, based on their low expression of the antioxidant protective enzymes, which will allow for the buildup of damaging effect of ROS [81, 82].

The mitochondrial uncoupling protein UCP2 is considered as a negative regulator of insulin secretion. Overexpression of UCP2 in β -cells diminishes ATP production and GSIS [46]. Likewise, deletion of UCP2 in mice enhances pancreatic islet ATP generation and GSIS. Furthermore, increased UCP2 in obesity was suggested to be one of the links between obesity and β -cell dysfunction in obesity-induced T2DM [47]. However, the role of UCPs is not fully understood, and specifically their response to the state of glucolipotoxicity that is highly manifested in uncontrolled DM and obesity requires further studies.

It is increasingly acknowledged that diabetic complications are also strongly linked to a state of oxidative stress. Diabetic retinopathy, being a major cause of blindness among adults worldwide, has been the focus of intensive research, which demonstrated that oxidative stress plays a vital role in its pathogenesis. In a recent review article, Zhu and Zou have presented data published from research studying the pigment-epithelium-derived factor (PEDF), which is a small secreted glycoprotein that was shown to exert protective effect on the retina based on its antioxidant properties in addition to other functions as the neurotrophic, antiangiogenic, antivasopermeability, anti-inflammatory, and antifibrosis properties. Therefore, PEDF or its peptide derivatives might represent a potential therapeutic approach in the prevention and/or treatment of diabetic retinopathy, an area that still needs further assessment [83].

5.4. Role of ROS in Obesity and Obesity-Associated Comorbidities. Obesity—defined as a body mass index of 30 Kg/m² or higher—is a chronic disease with serious adverse consequences and is currently a leading cause of preventable deaths worldwide. It is an established independent risk factor for CVD [84]. Obesity is also associated with a state of chronic inflammation in the adipose tissues as well in other organs, where tissue-infiltrating monocytes/macrophages increase in number and in activity. Several active mediators, chemotactic molecules, cytokines, and adipokines, augment the chronic inflammatory state and result in the excessive production of ROS causing systemic oxidative stress. This is considered a

potential mechanism linking obesity, vascular abnormalities, and the elevated risk of atherosclerosis and CVD. One of the main sources of ROS in those situations is believed to be the NADPH oxidase (Nox), a multiprotein complex that is expressed both in phagocytes and endothelial cells. Feeding mice high-fat diet for 22 weeks to cause dietinduced obesity was associated with activation of Nox. The latter is believed to elevate the expression of TLR in the vascular tissues, and probably in adipocytes as well. TLR4, which is the receptor for endotoxin and lipid, and its intracellular signaling consequences induce overexpression of proinflammatory cytokines, as TNF- α and IL6, and of transcription factors such as NF-κB. Therefore, Nox-induced elevated TLR4 expression and signaling might be involved in the obesity-induced inflammation and insulin resistance. Such findings propose the components of Nox system as potential novel therapeutic targets for obesity-associated comorbidities [84].

In recent years, novel roles have been assigned to the ROS as they relate to the central nervous system control over our body weight. The site of these new roles for ROS is the hypothalamus, where there are neurons controlling our satiety and others controlling our hunger behavior. Such roles have been implicated as contributing factors underlying diverse findings such as the age-related decrease ability to lose weight and the caloric restriction-induced longevity. Interesting findings demonstrated that different hypothalamic neurons have distinct preference to fuel utilization, so that glucose is the preferred fuel for proopiomelanocortin (POMC) neurons that are responsible for satiety, while FAs are preferred fuel to neuropeptide Y/Agouti-related protein (NPY/AgRP) neurons responsible for feeding. Although ROS are produced in both types of neurons as a result of oxidation of glucose and FA, yet it was demonstrated that the ROS produced in the POMC neurons will be accumulating and hence impairing the POMC neurons over time and this is thought to be responsible, at least in part, for our inability to lose weight as we get older. On the other hand, the ROS produced in the NPY/AgRP neurons that are active during negative energy balance will be buffered by UCP2 and this is thought to play a role in the mechanism of longevity induced by caloric restriction. This delicate neuronal system, although not completely well-understood, emphasizes the real need to be extra cautious with the use of any antiobesity pharmacological approach attempting to promote satiety or suppress hunger at the hypothalamic level [85, 86].

5.5. Role of ROS in Inflammation and Infection

5.5.1. Role of ROS in Inflammation. Recently, ROS were demonstrated to induce the assembly and activation of inflammasomes, which are multiprotein cytoplasmic complexes involved in mediating cellular inflammation in response to various damaging agents [87–91]. One of the major inflammasomes studied in depth is the NOD-like-receptor- (NLR-) related protein 3 nucleotide-binding domain, leucine-rich repeat containing receptors-related protein 3 (NLRP3) inflammasome, which have been strongly linked to aging and age-related diseases. The mitochondria

are believed to be the main source of inflammasomeactivating ROS, although other sources may exist. Excess ROS not only will result in the assembly and activation of the NLRP3 inflammasome but will also inhibit the process of mitophagy, which is a specialized type of autophagy responsible for removal of malfunctioning mitochondria. Therefore, the damaged mitochondria will persist, producing more ROS, and continuing the activation of inflammasome. Alternatively, the cells containing these damaged mitochondria might undergo apoptosis; which is, surprisingly, dependent on ROS as well. Likewise, the voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane are also involved in both inflammation and apoptosis. Although it is so far uncertain what will direct the cell to either chronic inflammation or apoptosis, it is expected that this type of decision is under tight control [92].

In line with the strong association between ROS and chronic inflammation, it was reported that ROS generation correlates with toxicity and pathogenicity of different types of pollutant, such as asbestos and silica particles. In a recent study, Dostert et al. have demonstrated the key role of ROS in mediating the injurious effects of these pollutants that may end in chronic inflammation or even tumor formation. Their findings indicate that upon particles phagocytosis by the immune cells, Nox will be assembled and activated, which will produce ROS in an iron-dependent process [93]. Again, Nox might not be the only source of ROS; other ROS producers may be involved. In any case, the ROS will then activate the inflammasome complex formed of the protein NLRP3, the adaptor ASC, and the substrate procaspase-1. The described stress-related response will end in caspase-1 formation and processing followed by secretion of proinflammatory mediators, including IL-1 β and IL-18 [92].

5.5.2. Role of ROS in Infection. ROS production has been used by human cells to fight infection, both bacterial and viral. Although the bactericidal effect of ROS is known since the 50s of the last century [94], active research in this area is still ongoing, especially with the aim to discover novel agents targeting bacterial strains with multiple antibiotic resistance, a serious clinical problem that is increasingly encountered. Recent experimental methodologies have been applied to this area; for instance, a genome-wide transcriptional profiling of the response of Staphylococcus aureus (S. aureus) to cryptotanshinone, a medicinal plant-isolated chemical agent exhibiting antimicrobial activity against a broad range of bacteria [95]. Cryptotanshinone (CT) demonstrated effective in vitro antibacterial activity against all S. aureus strains tested. Affymetrix GeneChips were used to determine the global transcriptional response of S. aureus to treatment with subinhibitory concentrations of CT. Both antibacterial and active oxygen radical generation functions of CT were positively correlated. Moreover, the S. aureus was found to undergo a defensive oxygen-limiting state upon exposure to the drug. Hence, the authors suggested that both actions of the drug, the antibacterial and the oxygen radical generation, may be responsible for its pharmacologic efficiency. This type of studies is promising since it sets the platform for developing and characterizing novel antibacterial agents with optimum activity against antibiotic resistant bacterial strains.

The ROS involvement in viral infection has also been studied since quite a long time (late 1980s and early 1990s) [96]; more research is still being conducted and producing interesting results, especially in the field of human immunodeficiency virus-1 (HIV-1) infection and treatment. HIV-1 infection is known to be associated with a state of oxidative stress. Interestingly, HIV-1 treatment using highly active antiretroviral therapy (HAART) seems to worsen the oxidative stress status. This was recently published by Mandas and colleagues who have compared the HIV-1-infected patients treated with HAART with untreated patients and with normal control. Moreover, optimal adherence to the HIV-1 therapy further worsened the oxidative stress status as compared to poor adherence [97]. More recently, higher oxidative stress status was demonstrated in patients coinfected with HIV-1 and HCV as manifested by higher oxidized glutathione level and more severe mitochondrial DNA damage as compared to patients who are monoinfected with HIV-1 [98].

Key Messages from Section 5. Glucolipotoxicity is associated with both oxidative and ER stress. This is linked to activation of the transcription factor NF- κ B and consequently of the proinflammatory gene expression. In vitro polyunsaturated FA anti-inflammatory effect is partly mediated by the nuclear receptor PPAR- γ .

TNF- α or glucocorticoids-induced insulin resistance is mediated by excess ROS production, a potential source of which is the sphingolipid ceramide. Excess ROS in turn is thought to work through activation of the JNK signaling pathway that results in differential translocation of two transcription factors; PDX-1, and Foxo1. The biochemical consequences that will take place include suppression of insulin biosynthesis and activation of gluconeogenesis, both will enhance the progression of IR into diabetes.

In normal β -cells, there is a glucose-stimulated insulin secretion that is dependent on the level of ATP production and is diminished by the mitochondrial UCP2. The age-dependent mitochondrial dysfunction is particularly important in the β -cells due to their relative deficiency of antioxidant protective enzymes.

The redox state will not only affect the incidence of DM, but it is also involved in the incidence of diabetic complications. PEDF is believed to be protective against the occurrence of diabetic retinopathy and hence is suggested to be of therapeutic potential and must be further investigated.

Activation of Nox enzyme is believed to elevate the expression of TLR4 in vascular tissues and is involved in the obesity-induced inflammation and associated vascular abnormalities.

ROS exert different effects on the hypothalamic neurons involved in satiety or hunger behaviors; therefore, caution should be exerted with attempting to design anti-obesity approach working at the hypothalamic level.

ROS induce the assembly and activation of inflammasomes and inhibit mitochondrial autophagy, both processes are related to aging and age-related diseases.

Certain pollutants-induced chronic inflammation or tumor formation is induced by Nox-released ROS-induced activation of inflammasome complex.

Both bacterial and viral infections have been related to ROS generation. Novel approaches are utilized to develop antibacterial agents with optimum activity.

6. Antioxidant Therapeutics

Several natural antioxidants have been investigated *in vitro* or in animal models to assess their potential therapeutic effect in conditions linked to oxidative stress. Interestingly not all antioxidants are identical, results from recent studies emphasize that point, and some will be briefly summarized in the following section.

In order to determine the protective role of vitamin E and/or dithiothreitol (DTT), Tsai and colleagues have studied rat hepatocytes that have been exposed to oxidative stress by treating them with Tert-butyl hydroperoxide and have assessed the cellular calcium homeostasis in these cells. Their results indicated that vitamin E not only blocks the elevation of intracellular ionic calcium ions but also prevents the loss of protein thiols from the cellular membranes, leading the authors to suggest that vitamin E conserves the integrity of cell membranes and this might be important for the maintenance of intracellular calcium homeostasis [99].

Another natural antioxidant, rottlerin, was studied by Maioli et al., in human breast cancer and human colon cancer cell lines, MCF-7 and HT-29, respectively [100]. Rottlerin is a pigment that exerts a pleiotropic inhibitory effect on specific intracellular kinases and hence is thought to interfere with the NF- κ B activation process. Similar polyphenolic phytochemical compounds as curcumin, resveratrol, and mangiferin were also reported to exert antioxidant activity that is mediated by NF- κ B inhibition [101]. Because not all the antioxidant phytopolyphenols are identical in their mechanism of action, resveratrol and rottlerin, both are antioxidants that act as protein kinase C δ (PKC δ) inhibitors, inhibit NF- κ B via different mechanisms. In addition, rottlerin exerts a free radical scavenging effect [100].

Similar to rottlerin, curcumin, which is commonly used as food additive in many parts of the world, exerts anti-inflammatory and antioxidant effect by scavenging free radicals and inhibiting NF- κ B. Curcumin also inhibits lipid peroxidation as manifested by decreasing the hepatic malondialdehyde (MDA) level in a rat model of alcoholic liver disease. Samuhasaneeto and colleagues have induced liver injury in rats by feeding them ethanol and then assessed the protective effect of orally administered curcumin. On the other hand, and at least in this studied model, curcumin did not affect the SOD activity nor did it affect the PPAR γ protein expression level. Curcumin seems to inhibit the early stages of alcohol liver disease in rats. As a matter of fact, early stages of the disease are mainly linked to oxidative stress that is induced by excessive accumulation of ROS. To

a lesser extent, curcumin was found to decrease hepatocytes apoptosis that is caused by mitochondrial dysfunction and cytochrome C release [101].

While curcumin did not affect the level of SOD activity, another natural antioxidant and anti-inflammatory compound, the purple sweet potato color (PSPC), was recently reported to increase the activity of Cu²⁺/Zn²⁺ SOD, as well as of catalase. This was reported in the brain tissue of a D-Gal-induced mouse model for aging, where Shan et al. first evaluated the animal spontaneous behavior and its cognitive performance and then thoroughly evaluated the biochemical changes taking place in the animal brain. In this model, oral administration of PSPC resulted in improvement in the mice behavior and cognitive performance in the intact animal. At the level of the animal isolated brain tissues and in addition to the increased activity of Cu²⁺/Zn²⁺ SOD and catalase; the demonstrated low expression levels of induced NOS (iNOS) and of cyclooxygenase 2 (Cox2), the decreased nuclear translocation of NF- κ B, and the lowered content of MDA have all led the authors to suggest that PSPC, through its antioxidant and anti-inflammatory capacity, ameliorates the cognition deficits and attenuates oxidative damage and inflammation in aging mouse brain [102].

Ginsenoside Rb1, a natural plant steroid belonging to the family of glycosides and triterpene saponins, was recently reported by Xia and colleagues to attenuate the myocardial oxidative stress and tissue histological damage in a model of streptozotocin-induced diabetes and myocardial ischemia/reperfusion injury. Since this protective effect was abolished by the eNOS inhibitor, L-NAME, it was suggested that ginsenoside Rb1 exerts its protective effect by enhancing the expression of eNOS and hence increasing the NO content, in addition to its antioxidant effect [103].

Interestingly, not all anti-inflammatory agents are antioxidants as well; diclofenac, a nonsteroidal anti-inflammatory drug (NSAID) that is usually prescribed to treat pain, fever, and inflammation is a clear example. It was recently reported that diclofenac resulted in apoptosis of neuroblastoma cell line. Diclofenac-induced apoptosis was related to its ability to cause mitochondrial dysfunction in the form of lowering the mitochondrial membrane potential and consequently releasing cytochrome C, and eventually causing cellular apoptosis. The diclofenac-induced mitochondrial dysfunction was related to its prooxidant activity since it was found to decrease the protein level and activity of mitochondrial SOD, though not its mRNA level. Furthermore, exogenous administration of the antioxidant Trx lowered the diclofenac-induced apoptosis and improved the mitochondrial SOD protein level. Such research has the potential to be of clinical significance as it can be applied in determining the optimum dosage and avoiding side effects and drug interactions caused by diclofenac [104].

Epoetin δ is an erythropoietin that is prescribed to patients who are at increased risk of developing anemia. It is unique because, unlike other erythropoiesis-stimulating agents, epoetin δ is produced by gene-activation technology in a human cell line, and hence it has a human-type glycosylation profile. The antioxidant capacity of epoetin δ was recently assessed in primary human renal tubular cells.

Oxidative stress was first induced by treating the cells with glucose oxidase enzyme. The protective antioxidant capability of epoetin δ was then assessed using a commercial oxidative status indicator (2′, 7′-dichlorodihydrofluorescein diacetate; H₂DCFDA). The authors have demonstrated that epoetin δ antioxidant capacity has protected the renal tissue through upregulation of several renoprotective genes, some of them, as carboxypeptidase M, dipeptide peptidase IV, and cytoglobin, were reported for the first time to be involved in the antioxidant renoprotection process [105].

7. Potential Novel ROS Targeted Therapeutics

Taken together, the results of recently conducted research studying the molecular, subcellular organelles, and cellular mechanisms involved in mediating the ROS actions offer promising venues as they propose novel potential therapeutic agents for the ROS-linked diseases. Few examples were presented in this review that should be further studied. The complexity and multifaceted nature of the process of redox regulation make it essential to better understand the key players in the process and then to design a targeted means of controlling these players. An obvious example is the JNK signaling pathway, which is activated by various cell stressors including ROS, glucolipotoxicity, and ER stress [106–108]. In the case of chronic ER stress, such as that seen in obesity [85], the ER stress-induced metabolic disturbance would result in insulin resistance and, ultimately, T2DM. Could inhibitors for JNK signaling pathway be designed to specifically ameliorate the ER-stress associated activation of this pathway? Results published by Özcan and colleagues have suggested that interventions that regulate the ER stress response offer new opportunities for preventing and treating T2DM [107].

In addition, the serine/threonine kinase, IkappaB kinase β (IKK) pathway is also activated by such stressors and is strongly involved in the development of β cell dysfunction, insulin resistance, and T2DM [108-111]. Therefore, it is possible that such pathways could be targeted as an approach that is complementary to the classical antioxidants in the prevention and/or treatment of ROS-associated chronic diseases. However, this approach is usually neither predictable nor straightforward; therefore in vitro, as well as experimental animal models studies have to be conducted first, and based on their results, carefully designed human intervention studies could be proposed. Even with such design, the hypothesis of targeting a specific signaling pathway with the objective of ameliorating the redox stress-associated diseases remains subject to either approval or refutation. The recent published work by Meijer and colleagues is a clear case for the inherent complexity of metabolic disorders. Based on results from animal studies implicating that the transcription factor activator protein-1 (AP-1) proinflammatory pathway is a promising target in the treatment of vascular diseases as atherosclerosis, this group has evaluated the profile of AP-1 activation in human aortic wall samples and tested the potential benefit of AP-1 inhibition in a clinical trial involving patients with symptomatic peripheral arterial disease.

Using doxycycline (an AP-1 inhibitor) or placebo in those patients did not affect any of the markers of inflammation and vascular dysfunction, except for the C-reactive protein which only revealed a borderline reduction in the group treated with doxycycline. This has led the authors to conclude that their findings did not corroborate the animal studies results and that AP-1 proved not to be a therapeutic target for progressive human vascular diseases [112].

This review summarizes the key roles played by ROS, which are considered major redox species, although not the only ones; the thiol/disulfide redox system plays key roles as well in redox signaling and oxidative stress. In fact, the limited benefit of the classical antioxidant therapeutic agents used so far in several clinical trials might be the result of the untargeted approach of these agents as mentioned above and importantly due to the fact that they are not affecting the cysteine-based redox regulators. Further research is indeed required for better clarifying the big picture of redox regulation both by ROS and non-ROS mediators.

Key Messages from Sections 6 and 7. In the field of antioxidant therapeutics, ongoing research is being conducted to better understand the mechanism of action of known antioxidant agents and to design and test novel therapeutic agents. As the case in any novel medication testing, systematic approach has to be undertaken utilizing *in vitro* and *in vivo* animal models and human trials; nevertheless, results might not be predictable.

8. Concluding Remarks

In conclusion, oxidative regulation is a term that better describes the actions of ROS, as some of these actions are considered physiological and others, especially if uncontrolled, are deeply involved in so many pathological situations

There is growing evidence that redox regulators, related active mediators, cellular organelles functions, and surrounding environments are all tied together in intricate networks affecting the whole body energetic, metabolism, state of health and disease and even lifespan.

Although at present the use of antioxidants seems disappointing in preventing the progression of the ROS-associated diseases, current research findings have proposed novel targets that might prove to be more appropriate antioxidants. Further research is needed to investigate the possible preventive and/or therapeutic values of these molecules.

Abbreviation

ROS: Reactive oxygen species

ETC: Electron transport chain

Nox: NADPH oxidases

H₂O₂: Hydrogen peroxide

Trx2: Thioredoxin 2

HIF-1: Hypoxia-inducible factor-1

Nrf2: Nuclear factor-erythroid 2-related factor 2

CVD: Cardiovascular diseases

DM: Diabetes mellitus

ER: Endoplasmic reticulum
UPR: Unfolded protein response
mtDNA: Mitochondrial DNA
T2DM: Type 2 diabetes mellitus
UCP: Uncoupling protein

FA: Fatty acid

NF- κ B: Nuclear transcription factor κ B

TLR: Toll-like receptor IR: Insulin resistance TNF- α : Tumor-necrosis factor- α JNK: c-Jun NH₂-terminal kinase

GSIS: Glucose-stimulated insulin secretion

POMC: Pro-opiomelanocortin

NPY/AgRP: Neuropeptide Y/Agouti-related protein NLRP3: NOD-like-receptor- (NLR-) related protein

3 nucleotide-binding domain leucine-rich repeat containing receptors-related protein 3

inflammasome

Acknowledgment

This work was supported by the Strategic Technologies Program of the National Plan for Sciences and Technology and Innovation in the Kingdom of Saudi Arabia (Project no. 08-MED513-02).

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 691369, 12 pages doi:10.1155/2012/691369

Review Article

Insulin Resistance in Patients with Chronic Kidney Disease

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Received 8 June 2012; Accepted 15 July 2012

Academic Editor: Sandro Massao Hirabara

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Metabolic syndrome and its components are associated with chronic kidney disease (CKD) development. Insulin resistance (IR) plays a central role in the metabolic syndrome and is associated with increased risk for CKD in nondiabetic patients. IR is common in patients with mild-to-moderate stage CKD, even when the glomerular filtration rate is within the normal range. IR, along with oxidative stress and inflammation, also promotes kidney disease. In patients with end stage renal disease, IR is an independent predictor of cardiovascular disease and is linked to protein energy wasting and malnutrition. Systemic inflammation, oxidative stress, elevated serum adipokines and fetuin-A, metabolic acidosis, vitamin D deficiency, depressed serum erythropoietin, endoplasmic reticulum stress, and suppressors of cytokine signaling all cause IR by suppressing insulin receptor-PI3K-Akt pathways in CKD. In addition to adequate renal replacement therapy and correction of uremia-associated factors, thiazolidinedione, ghrelin, protein restriction, and keto-acid supplementation are therapeutic options. Weight control, reduced daily prednisolone dosage, and the use of cyclosporin decrease the risk of developing new-onset diabetes after kidney transplantation. Improved understanding of the pathogenic mechanisms underlying IR in CKD may lead to more effective therapeutic strategies to reduce uremia-associated morbidity and mortality.

1. Introduction

Despite recent advances in the treatment of chronic kidney disease (CKD), it remains an important public health challenge [1]. CKD is a major risk factor for end stage renal disease (ESRD) and cardiovascular disease [2], and cardiovascular disease is the leading cause of mortality in uremic patients. Cardiac mortality in dialysis patients is more than 10-fold greater than that of the general population [2]. The excess cardiovascular mortality in patients with CKD is not explained by traditional risk factors such as hypertension and hypercholesterolemia. Identifying and treating risk factors for early CKD may be the best approach to prevent and delay adverse outcome [3].

The metabolic syndrome (MetS), characterized by abdominal obesity, hypertriglyceridemia, depressed serum high-density lipoprotein cholesterol (HDL-C), high blood

pressure, and high fasting glucose level [4], is commonly seen in patients with CKD. Insulin resistance (IR) plays a central role in the MetS and is associated with increased risk for CKD in nondiabetic patients [5, 6]. The MetS and its components are associated with the development of CKD, microalbuminuria, and overt proteinuria [7, 8]. There is a strong association between HDL-C, inflammatory surrogates, and IR in this nondiabetic, nonobese hemodialysis (HD) patient group. In addition, serum HDL-C level is a good screening parameter in high-risk patients [9].

Rodents are widely used to mimic human diseases to improve understanding of the causes and progression of disease symptoms and to test possible therapeutic interventions. For instance, Diabetic Zucker fatty rats (ZDFs), a model of early onset obesity, have a mutation in the leptin receptor gene. Also, Goto-Kakizaki (GK) rodents, which are nonobese to begin with and genetically engineered to have

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malfunctioned β -cells, can mimic noninsulin-dependent disorder [10]. These models can utilize urine concentration of energy metabolites to evaluate the metabolic syndrome in prediabetic insulin-resistant and hyperglycemia stage. Chronic consumption of a high-carbohydrate, high-fat diet by normal rodents may provide an adequate rodent model to mimic the human metabolic syndrome and for testing potential therapeutic interventions [10, 11].

IR is common in patients with mild-to-moderate CKD [12]. IR is also an independent predictor of cardiovascular mortality in ESRD [13] and is increasing recognized as a nontraditional risk factor [14, 15]. IR is common in patients with ESRD [16] and is linked to protein energy wasting and malnutrition [17, 18]. Nutritional, metabolic, and cardiovascular complications of renal disease may be a consequence of abnormal insulin action [19]. Therefore, IR may be an important therapeutic target for reduction of cardiovascular mortality in patients with CKD.

2. Measurement of IR

There are a number of methods to assess insulin sensitivity in the dynamic versus static state. Estimates of IR based on fasting insulin concentration may not be adequate in patients with CKD as it largely reflects hepatic defects, whereas CKD impairs insulin catabolism [20]. Hyperinsulinemic euglycemic clamp is the gold standard for IR determination because it provides a direct measure of wholebody sensitivity to insulin, primarily skeletal muscle. At lower doses of insulin infusion, the addition of labeled glucose can allow for specific assessment of the ability of insulin to suppress endogenous glucose production. This method can differentiate between peripheral IR and hepatic IR and provides a direct and precise IR measurement [20, 21]. The oral glucose tolerance test (OGTT) primarily measures glucose tolerance, which reflects both IR and beta-cell function; IR can be calculated from OGTT using validated formulae. On the other hand, the hyperglycemic clamp is the gold standard for the measurement of beta-cell function. Due to the complexity of this methodology, more practical methods such as homeostatic model assessment (HOMA) and the quantitative insulin sensitivity check index (QUICKI) are widely used in clinical studies. The HOMA test estimates IR from fasting glucose and insulin concentrations, but primarily reflects hepatic IR rather than peripheral IR. There is an inverse linear correlation between IR by euglycemic hyperinsulinemic clamp and log-transformed IR by HOMA [22]. Therefore, IR by HOMA can be used as an alternative method to assess IR in patients with renal failure.

3. IR in CKD

IR is a well-known complication of ESRD based on hyperinsulinemic euglycemic clamp testing. IR is also present in early stage CKD, even when the glomerular filtration rate (GFR) is within the normal range. Glucose disposal rate (GDR) correlates negatively with serum creatinine level and positively with creatinine clearance [12]. A large prospective study using data from the Atherosclerosis Risk in Communities (ARICs) cohort confirmed a step-wise increase in risk for CKD development with the number of MetS criteria met in nondiabetic adults, ever after controlling for the development of diabetes mellitus (DM) and hypertension [5]. A prospective cohort study found that MetS predicts the risk of prevalent and incident CKD. This same study found IR to be associated with prevalent CKD and rapid decline in renal function among elderly individuals in an older Asian nondiabetic cohort [23]. However, there is little evidence that preventing or treating symptoms of the MetS protects patients with renal impairment. IR, along with oxidative stress and inflammation, is suggested to play a role in the development of albuminuria and declining kidney function [23, 24]. Hyperglycemia may not only cause poor renal function but also lower expression of Klotho protein in diabetic kidney, and these defects could be reversed by correction of blood glucose levels using insulin treatment in diabetic rats [25].

3.1. IR Accentuates Kidney Injury. IR promotes kidney disease by worsening renal hemodynamics through mechanisms such as activation of the sympathetic nervous system [26], sodium retention, decreased Na+, K+-ATPase activity, and increased GFR [24]. Endoplasmic reticulum (ER) stress seems to be the factor-linking inflammation and IR at the molecular level. The suppression of insulin signaling via phosphorylation of the insulin receptor substrate (IRS-1) due to activation of c-Jun N-terminal kinase (JNK) plays an important role [27]. Renal ER stress is associated with proteinuria-induced podocyte damage and alteration of nephrin N-glycosylation in podocytes, which is the underlying factor in the pathogenesis of proteinuria, and is involved in the pathophysiology of chronic kidney injury with tubulointerstitial damage [28]. According to Sowers [29], IR and inflammatory cytokine release may be partly responsible for glomerular mesangial expansion, basement membrane thickening, podocytopathy, and the loss of slit pore diaphragm integrity ultimately leading to glomerulosclerosis and tubulointerstitial injury. Megalin, an endocytic receptor, mediates the conservation of nutrients and carrier bound vitamins in glomerular filtrates via interaction with various molecules in renal proximal tubular epithelial cells (PTECs) [30]. In metabolic syndrome or dyslipidemia, free fatty acids are delivered to PTECs with the carrier proteins such as albumin or liver-type fatty acid-binding protein [31]. Metabolically overloaded PTECs are activated to express proinflammatory cytokines, such as MCP1 and TNF α , and lead to epithelial-mesenchymal transition [32].

IR may drive the overproduction of very low-density lipoprotein cholesterol and contribute to hypertriglyceridemia [36]. Triglyceride-rich apolipoprotein B-containing lipoproteins clearly promote the progression of renal insufficiency [37]. High triglyceride levels are a risk factor for proteinuria development [38].

3.2. IR in CKD Patients with Dialysis Therapy. Adequate HD has a positive effect on IR (Table 1), but there is

Patients	n	GDR (mg	HOMA index	
Controls [33]	10	9.93	1.85–1.95	
CKD [12]	29 ^a	6.91	2.24 ± 1.06	
Before Tx	After Tx	Before KT	After KT	
With PD [34]	9a	6.35 ± 1.65	8.18 ± 1.76	
With HD [34]	10 ^a	6.53 ± 1.84	9.74 ± 2.88	
1 M after KT [35]	34	3.05 ± 2.72	1.67 ± 1.25	
6 M after KT [35]	34	3.05 ± 2.72	2.32 ± 2.83	

Table 1: Clinical studies assessing IR in healthy controls, CKD patients, and patients receiving PD, HD, or kidney transplantation (KT).

CKD: chronic kidney disease; GDR: glucose disposal rate; HOMA: homeostasis model assessment for insulin resistance; ESRD: end stage renal disease; CAPD: continuous ambulatory PD; Tx: dialysis treatment; KT: kidney transplantation.

aNondiabetic.

little clinical data regarding the effect of peritoneal dialysis (PD) on insulin sensitivity. After comparing the effect of continuous cycling PD (CCPD) to that of HD on IR in younger adolescent uremic patients [39], the percentage increase and the final insulin sensitivity were significantly higher in the CCPD group than the HD group. This may be because PD provided better clearance of middle-molecule-weight uremic toxins than HD. In contrast, in 19 nondiabetic patients with ESRD, CAPD therapy for 5.4 weeks normalized IR similar to HD. There is close correlation between changes in blood urea nitrogen (BUN) or HCO₃⁻ and IR [34]. The variations in effect on IR with PD may be due to the different modalities used.

3.3. IR in CKD Patients with Kidney Transplantation. After renal transplantation, serum leptin level dramatically decreased immediately and correlated significantly with serum insulin level and HOMA index. Serum leptin levels at six months after transplant were significantly higher than that of controls and correlation between serum leptin levels and HOMA index persisted [35]. Five years after transplant, serum leptin, IR, body fat percentage, and serum lipids had a profile similar to those in the pretransplant period. A positive correlation between leptin and IR in the late postrenal transplantation period persisted [40]. Distribution of obesity and prednisolone treatment are predominant determinants of IR in the long term after transplantation [41]. These findings suggest that IR might play a role in increased leptin concentration in renal transplant recipients together with increased body fat mass. IR after transplantation could be managed favorably by weight control in overweight patients and prednisolone dose reduction. Pretransplant glycemia and high posttransplant BUN were associated with a greater risk of posttransplant hyperglycemia [42].

4. Factors Affecting IR in CKD

IR in CKD exists mainly at periphery. As adipose tissue accounts for the disposal of less than 2% of the glucose load, muscle tissue must represent the primary site of IR in patients with ESRD. Hepatic glucose production is not increased and is suppressed normally in response to

insulin in patients with ESRD. IR may not lead to a compensatory increase in insulin secretion, as compared to patients without advanced kidney disease [43]. In contrast to animal models with high-fat intake-induced IR, IR is first manifested in the liver and then in white adipose tissue, while skeletal muscle remains insulin sensitive [44]. A postreceptor defect is recognized as the primary defect in CKD. One study demonstrated that IRS-1-associated phospho-inositol (PI) 3-kinase (K) activity is suppressed. The potential mechanism for the reduction in IRS-1-associated PI3-K activity is induction of the p85 PI3-K regulatory subunit, but another mechanism may also be involved [45]. Downstream function of the IRS-PI3 K-Akt pathways was associated with chronic inflammation, metabolic acidosis, vitamin D and parathyroid hormone status, anemia, uremic toxins, and adipokines.

4.1. IR Associated with Inflammation in CKD. A complex network of nutritional and metabolic alterations underlies CKD, including chronic inflammation, oxidative stress, IR, and protein energy wasting. Like other chronic diseases, patient with CKD demonstrates low-grade systemic inflammation marked by elevated levels of proinflammatory cytokines such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF- α), inerleukin-6 (IL-6), and interleukin-1 beta (IL-1 β) [46]. Inflammation and oxidative stress are evident in the early stage of CKD [47] and are also known to induce IR, primarily via increased production of proinflammatory cytokines.

In the presence of insulin, the insulin receptor phosphorylates IRS, which is linked to the activation of two main signaling pathways: the phosphatidylinositol 3-kinase (PI3 K)-AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic action such as glucose transport, and the Ras-mitogen-activated protein kinase (MAPK) pathway, which regulates gene expression and cooperates with the PI3 K pathway to control cell growth and differentiation [48]. In animal models of CKD, PI3 K activity is reduced and both apoptosis [49], and ATP-ubiquitin-proteasome pathways are activated [50]. This mechanism could increase muscle atrophy in conditions with impaired insulin action.

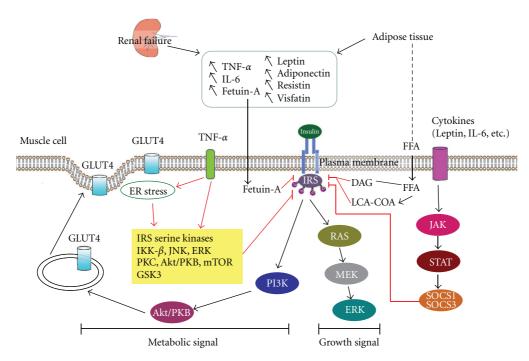


FIGURE 1: Insulin resistance in chronic kidney disease. Renal failure and adipose tissue lead to systemic inflammation and increased plasma levels of adipokines. TNF- α activates adipose tissue lipolysis, which generates free-fatty acids (FFAs). On muscle cells, FFAs activate transcription factors, such as peroxisome proliferator-activated receptor (PPAR), and generate messengers including diacylglycerol (DAG) and long-chain acyl-CoA (LCA-CoA), which will lead to protein kinase C (PKC) activation and dephosphorylation of insulin receptor substrate (IRS)-1/2. On muscle cells, TNF- α activates a series of kinases including IKK- β , c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), protein kinase C (PKC), Akt (PKB), mammalian target of rapamycin (mTOR), and glycogen synthase kinase 3 (GSK3) responsible for phosphorylation of insulin receptor (InsR) and IRS-1 on serine/threonine residues. Inhibition of IRS-1 function will block Akt leading to cytosol glucose transporter 4 (GLUT4) sequestration. Fetuin-A could also inhibit IRS and induce low-grade inflammation. IL-6 is responsible for the induction of different suppressors of cytokine signaling (SOCS) proteins through the Janus kinase/signal transducer and activator of transcription (Jak/STAT)-signaling pathway. SOCS will inhibit IRS-1/2 and protein kinase A. Endoplasmic reticulum stress seems to be another factor linking inflammation and insulin resistance at the molecular level. Red line illustrates the mechanism of CKD-related factors in IR.

TNF- α induces IR through direct or indirect mechanisms (Figure 1). TNF- α can induce IRS-1 serine phosphorylation through activation of serine kinases including IKK- β [51], c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), protein kinase C (PKC), Akt (PKB), mammalian target of rapamycin (mTOR), and glycogen synthase kinase 3 (GSK3) [49]. After infusion of TNF- α in healthy humans, IR develops in skeletal muscle that is associated with impaired phosphorylation of Akt substrate 160, leading to dysfunction of glucose transporter 4 (GLUT4) translocation and glucose uptake [52]. Excessive concentrations of TNF- α negatively regulate insulin signaling and whole-body glucose uptake in humans. Additionally, TNF- α stimulates lipolysis and release of free-fatty acids [53]. Free-fatty acids induce intracellular accumulation of diacylglycerol and long-chain acyl CoA, with subsequent protein kinase C activation and dephosphorylation of IRS-1/2 in human skeletal muscle cell. Other studies demonstrate that IL-6 can also inhibit the insulin-signaling pathway at the level of the insulin receptor and IRS-1 interaction [54]. Administration of an IL-1 receptor antagonist in patients with type II DM not only decreases serum CRP and IL-6

levels but also improves insulin sensitivity [55]. Defects in insulin signaling through the protein kinase Akt underlie the development of IR. Akt acts as a nodal point in the control of both the metabolic and pleiotropic effects of insulin. Imbalance among these effects leads to cardiac hypertrophy, fibrosis, and apoptosis, reduced angiogenesis, metabolic remodeling, and altered calcium cycling, all key features of uremic cardiomyopathy [56].

Fetuin-A, also called a2-Heremans-Schmid glycoprotein (Ahsg), is a powerful circulating inhibitor of vascular calcification [57]. CKD patients have significantly lower levels of fetuin-A than healthy controls. Short-term treatment with sevelamer increases serum fetuin-A concentration in stage 4 CKD patients [58]. In dialysis patients, calcimimetic therapy for secondary hyperparathyroidism significantly decreases fetuin-A levels [59]. However, serum fetuin-A levels increased obviously following parathyroidectomy in patients with refractory renal hyperparathyroidism [60]. Fetuin-A inhibits insulin receptor tyrosine kinase activity by blocking autophosphorylation of tyrosine kinase and IRS-1 and induces lower-grade inflammation [61, 62], resulting in IR [63, 64]. Recently, epidemiological studies showed that

serum fetuin-A was associated with IR [65] and its comorbidities, such as MetS [66] and type 2 DM [67, 68]. Higher fetuin-A concentrations were associated with type 2 DM and IR in middle-aged and elderly Chinese patients [69]. However, the possible effects of fetuin-A on IR in CKD deserve further elucidation.

Suppressors of cytokine signaling (SOCS) are a family of intracellular proteins, several of which have emerged as key physiological regulators of cytokine-mediated homeostasis, including innate and adaptive immunity. In CKD or ESRD patients, increased expression of SOCS in monocytes and lymphocytes, accompanied by increased plasma levels of the inflammatory cytokines IL-6, CRP, and TNF α , was reported [70, 71]. Recent observations also suggest that SOCS expression levels are also profoundly altered in kidney disease [72].

SOCS proteins act as a classical feedback loop to ensure fine regulation of cytokine-signaling pathways, and the classical target of the SOCS proteins is inhibition of the JAK-STAT-signaling pathway. SOCS proteins can also inhibit insulin signaling; SOCS-1 knock-out mice show hypoglycemia and increased insulin signaling. Recent studies showed that SOCS proteins not only exacerbate IR but also act as an important link between elevated cytokine levels and IR [73]. SOCS-3 can bind phosphorylated Tyr960 on the insulin receptor, which is important for IRS-1 binding [74]. Inhibition of SOCS-1 is likely mediated through binding to the kinase domain of the insulin receptor, preventing further phosphorylation [75]. Targeting of IRS-1 and -2 for ubiquitin-dependent degradation via the proteasomal machinery may be another inhibitory mechanism [76].

Elevated IL-6 induced SOCS-3 expression and consequently inhibited insulin signaling in human differentiated myotubes grown *in vitro* [77]. SOCS-3 expression was not elevated in muscle from nondiabetic obese and type 1 DM patient. Increased SOCS-3 expression in patients with type 2 DM may be explained by the combination of high glucose and IL-6 levels in the blood. High levels of inflammatory cytokines lead to increased SOCS-1 and -3 expression in insulin sensitive tissues, which induce IR via inhibition of the insulin-signaling pathway.

4.2. Role of Adipokines. Adipokines are markedly elevated in the plasma of uremic patients, mainly due to reduced renal excretion. Obesity superimposed on uremia may further aggravate hyperadipokinemia. Abnormal adipocytokine levels, including leptin and adiponectin, may further promote IR and a proinflammatory state in CKD [78]. The exact pathophysiological role of adipokines [79, 80] in the pathogenesis of IR in CKD is still unclear. Leptin may be considered as a uremia toxin and is associated with reduced energy intake and protein-energy wasting in uremic patients [81]. Leptin signaling in the central nervous system was recently shown to be an important cause of anorexia [82]. Leptin also significantly interferes with neutrophil chemotaxis [83] and produces vascular damage through proatherogenic and proinflammatory effects [84]. Adiponectin could mediate insulin-sensitizing [85,

86], antiatherogenic, and anti-inflammatory actions [87]. However, whether the removal of leptin by dialysis may translate into prospective improvements in appetite, nutrition status, neutrophil chemotaxis, and other clinical benefits in uremic patients still needs to be clarified [88]. Experiments conducted on animal models and in vitro on cell culture demonstrated that adiponectin signaling via MAPK regulates oxidative stress, segmental fusion of podocyte foot processes, and albuminuria [89, 90]. Adiponectin treatment decreased albuminuria, glomerular hypertrophy, and tubulointerstitial fibrosis due to the restoration of VCAM-1, monocyte chemoattractant protein-1, TNF- α , TGF- β 1, collagen type I/III, and Nox to the levels found in wildtype mice [91]. In patients on HD, plasma adiponectin levels were inversely related to body mass index values, insulin levels, and HOMA index [92]. Plasma adiponectin is an inverse predictor of cardiovascular outcome. However, the paradoxical unfavorable effect of high adiponectin could be a consequence of a concurrent process of wasting and salt and volume overload.

In humans, resistin is expressed by macrophages residing in adipose tissue. Resistin appears to disturb the immune response and contribute to increased atherosclerotic risk by modulating the activity of endothelial [93]. Circulating resistin levels are strongly associated with both GFR and inflammatory biomarkers in CKD, but not with IR [94]. Visfatin is mainly synthesized in visceral fat and is linked to impaired endothelial function and angiogenesis [95]. In PD patients, serum visfatin might be a sensitive marker for cardiac performance [96]. Chemerin is another novel adipokine linked to obesity and is also increased in uremic patients. In incident dialysis patients, elevated chemerin is associated with a survival advantage despite its significant positive correlation with markers of inflammation and dyslipidemia [97].

Previous study showed that adipose-derived proinflammatory cytokines exert direct inhibitory effects on muscle insulin signaling, accounting for the close link between IR and systemic inflammation [98]. Obesity or abdominal fat accumulation is associated with oxidative stress and impaired insulin action in patients with CKD. There is a plausible relationship between inflammatory biomarkers and truncal fat mass in patients with ESRD. Weight loss may decrease the inflammatory and oxidative burden in CKD and attenuate cardiovascular risk in this population [99].

4.3. Role of Vitamin D and Secondary Hyperparathyroidism. There are evidences that the vitamin D and or parathyroid hormone (PTH) axis is important in the pathogenesis of glucose intolerance and IR in patients with CKD. IR is present in the early stages of CKD and has an inverse association with 25-hydroxyvitamin D levels [100]. Vitamin D metabolism is profoundly disturbed in CKD; abnormalities begin during early CKD stages, that is, stage 3 or earlier, and progress as renal function declines [101]. Vitamin D is now recognized as having pleiotropic roles beyond bone and mineral homeostasis, with vitamin D receptor and metabolizing machinery identified in multiple tissues.

Circulating 25-hydroxyvitamin D concentration correlates with glucose tolerance, beta-cell function, and insulin sensitivity, measured using oral glucose tolerance tests and hyperglycemia clamps [102, 103]. Intravenous vitamin D3 corrects glucose intolerance and stimulates insulin secretion in response to a glucose challenge in 1,25-dihydroxyvitamin D3 (1,25(OH)₂D₃)-deficient animals [104]. After four weeks of intravenous $1,25(OH)_2D_3$ therapy, glucose intolerance, IR, hypoinsulinemia, and hypertriglyceridemia were corrected in patients on HD, in the absence of PTH suppression [105]. A single dose of $1,25(OH)_2D_3$ also improved insulin sensitivity [106].

The mechanisms through which excess PTH blunts insulin sensitivity are uncertain. Medical treatment of hyperparathyroidism in patients with CKD led to correction of glucose intolerance and increased insulin secretion [107]. In a CKD animal model, PTH did not affect tissue resistance to insulin, and the normalization of glucose metabolism in the absence of PTH was due to increased insulin secretion [108]. One study indicates that PTH could stimulate calcium influx into cells and that increased cellular calcium concentration enhances insulin secretion [109]. Contrary to some published data, peripheral blood mononuclear cells from patients with CKD had higher cytosolic calcium concentration and intracellular calcium stores, and long-term vitamin D3 supplement can only normalize the cytosolic calcium concentration without any effect on intracellular calcium store in the absence of PTH [110]. Calcitriol treatment of patients on HD with secondary hyperparathyroidism is associated with increased insulin secretion; this is linked to decreased intracellular free calcium [111]. It is possible that the effect of altered calcium content in beta cells on insulin secretion depends on the magnitude and duration of the change. Recent evidence indicates that the ER mediates cytosolic calcium concentration, and ER stress is a central feature of IR. Cytosolic calcium levels or calcium modulation of the signal transduction of the insulin receptor might play a central role in the mechanisms of IR [112].

4.4. Other Clinical Conditions in CKD. Accumulation of uremic toxins may contribute to IR in ESRD. The middle-molecular range peptides in uremic serum induce IR in adipose cells [113]. Pseudouridine [65], which accumulates in the circulation, and asymmetric dimethyl arginine (ADMA) [114] are recognized as an important uremic toxins associated with CKD and IR. Urea, which was previously considered to have negligible toxicity, induced reactive oxygen species generation and caused IR in an animal model [115].

Metabolic acidosis may also play a significant role in IR in CKD. It is linked to protein-energy malnutrition and inflammation. Evidence suggests that the catabolic effects of metabolic acidosis may be due to increased activity of the adenosine triphosphate (ATP)-dependent ubiquitin-proteasome and branched-chain ketoacid dehydrogenase [116]. Treatment of metabolic acidosis with oral bicarbonate increased both insulin sensitivity and insulin secretion in patients with uremia measured by hyperinsulinemic euglycemic clamp [117].

Patients with ESRD generally have low-exercise tolerance, and this may contribute to IR and metabolic abnormalities [118]. Moderate endurance training programs improved some metabolic abnormalities and insulin sensitivity. Correction of anemia by erythropoietin (EPO) therapy in HD reportedly improves exercise tolerance. Significant improvements in glucose tolerance and insulin sensitivity have been found in CKD patients after six [65] or nine months [119] of EPO therapy for anemia. Improvement of exercise tolerance following EPO treatment may be the important factor in the improvement in glucose metabolism in patients with CKD.

5. Treatment of IR in CKD

IR is highly prevalent in CKD patients and is associated with a complex network of nutritional and metabolic alterations including chronic systemic inflammation, oxidative stress, and protein-energy wasting. Whether IR is antecedent to CKD or a consequence of impaired kidney function has been the subject of debate. The goal of IR treatment is traditionally aimed at etiologies including uremic toxins, protein catabolism, vitamin D deficiency, metabolic acidosis, anemia, poor physical fitness, and cachexia.

Thiazolidinediones (TZDs) are a class of oral diabetic medications that function via binding peroxisome proliferator-activated receptor γ , thereby increasing insulin sensitivity in peripheral tissue [120]. Evidence demonstrates that TZD therapy can alter cardiovascular risk factors leading to increased HDL-C and circulating adiponectin, decreased triglycerides, visceral adiposity, circulating inflammatory mediators, albuminuria, improved flow-mediated vasodilation, and blunted carotid intima-media thickening. Observations suggest that improvement in glycemic control is not the dominant operative mechanism for the effects of TZD. In patients with noninsulin-dependent DM on HD, TZD treatment was associated with significantly lower allcause mortality among insulin-free but not insulin-requiring patients [121]. Although a meta-analysis suggested that TZD was associated with increased cardiovascular mortality [122], no conclusions can be drawn regarding the association between TZD and nonfatal cardiovascular events in dialysis patients.

Vitamin D supplementation for the general population may lead to a small but significant improvement in mortality but did not appear to prevent the development of DM in the largest clinical trial to date [123]. Data from the HD population consistently suggest that calcitriol improves short-term insulin secretion and insulin sensitivity in ESRD [105, 111]. This may be due to the fact that patients with CKD have profound vitamin D deficiency at baseline, and vitamin D therapy may be most effective when intrinsic 1-a hydroxylase activity is most impaired. Further long-term clinical trials in the setting of CKD are warranted to test this hypothesis.

IR was at least partially correctable by maintenance HD after EPO therapy. Treatment of malnutrition by intravenous nutrition [124] or low-protein diet supplement with amino acid-keto acid supplements improves IR. Recent studies show

that dairy proteins and meat stimulate insulin/insulin-like growth factor-1 signaling and provide high amounts of leucine, a primary and independent stimulator for mTORC1 activation. The downstream target of mTORC1, kinase S6 K1, induces IR by phosphorylation of IRS-1. Attenuation of leucine-mediated mTORC1 signaling by defining appropriate upper limits of the daily intake of leucine-rich proteins were advocated for the prevention of type II DM and obesity [125]. However, this cannot apply to the advanced CKD patients as leucine, and essential amino acids have a critically important role in muscle protein turnover. Although there is some resistance to leucine in CKD, leucine-rich supplements in the management of uremic muscle wasting were suggested [126]. IR in children with CKD improved during treatment with dietary protein restriction and essential amino acid and keto-acid supplement. These metabolic changes were not related to hyperparathyroidism and were probably due to a reduction in nitrogen toxicity [127].

Early detection of MetS or high triglyceride levels might be beneficial if accompanied by early intervention such as fibrate therapy to lower triglyceride levels and suppress pathways for renal injury [128]. However, studies showed a controversial role of hypertriglyceridemia in CKD. Patients with hypertriglyceridemia had a relative risk of 2.01 for nephropathy, and higher risk of CKD was found in patients with triglyceride levels greater than 200 mg/dl [129]. The Helsinki Heart Study, a randomized clinical trial of gemfibrozil for the prevention of coronary heart disease, found no association between triglyceride levels and CKD [130].

Sympathetic hyperactivity, activation of the reninangiotensin-aldosterone system (RAAS), and the related development of hypertension are implicated in CKD; this may be further aggravated by excess dietary salt intake and effects of IR and hyperinsulinemia on sodium retention [78]. IR in patients on maintenance HD was ameliorated after 12 weeks of treatment with angiotensin II receptor blockers [131]. However, insulin per se may have a modest anti-inflammatory effect as evidenced by a reduction in serum CRP that appears to have a persistent effect over the 24 h after dialysis [132].

Ghrelin is a gastric hormone [133] discovered in its acylated form and extensively studies for its appetite-stimulating effect. Evidence shows that total circulating ghrelin is positively associated with insulin sensitivity in general [134] and in patients with CKD. Nonobese, nondiabetic HD patients may present with higher circulating total ghrelin [135]. In a nephrectomy animal model, two weeks of subcutaneous ghrelin administration reduced loss of muscle mass, associated with enhanced muscle transcription of PPAR- γ coactivator 1- α and anti-inflammatory effects [136]. Ghrelin may be considered a novel potential therapeutic agent to effectively counteract CKD-related loss of lean body mass.

Lowering daily prednisolone toward 5 mg/day has beneficial effects on insulin action after renal transplantation, but withdrawal of 5 mg prednisolone may not influence IS significantly [137]. New onset DM after renal transplantation increases the risk of morbidity and mortality. The use of cyclosporine rather than tacrolimus decreases the risk of

developing new-onset DM after transplantation, while the potential long-term benefits of this strategy require further study [138].

6. Conclusion

IR is prevalent in CKD patients and plays a role in declining renal function. The etiology of IR is multifactorial and associated with a complex network including chronic inflammation, oxidative stress, vitamin D deficiency, anemia, and malnutrition. These factors are associated with elevated inflammatory cytokines, adipokines, ER stress, and SOCS, leading to an acquired defect of the insulin receptor-signaling pathway. TZD treatment is associated with significantly reduced all-cause mortality and higher serum albumin among an insulin-free population but is used sparingly due to possible side effects. Proper nutritional supplementation and prevention of uremia-associated complications including dyslipidemia, vitamin D and EPO deficiency, and anemia may also improve insulin sensitivity among the CKD population. Ghrelin may be a novel intervention to enhance appetite in malnourished CKD patients. Further studies with larger patient populations are necessary to clarify longterm effects of these treatments. More novel approaches to improving IR in the CKD population might lead to potential strategies for preventing excess mortality.

Authors' Contributions

M.-T. Liao and C.-C. Sung contributed equally to this work.

Acknowledgments

The Research Fund from Cardinal-Tien Hospital (CTH-99-1-2A06) and Teh-Tzer Study Group for Human Medical Research Foundation (B-99-10-53) supported this study.

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 902351, 7 pages doi:10.1155/2012/902351

Research Article

Treatment with Aqueous Extract from *Croton cajucara* **Benth Reduces Hepatic Oxidative Stress in Streptozotocin-Diabetic Rats**

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Received 12 March 2012; Revised 1 May 2012; Accepted 7 May 2012

Academic Editor: Sandro Massao Hirabara

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Croton cajucara Benth is a plant found in Amazonia, Brazil and the bark and leaf infusion of this plant have been popularly used to treat diabetes and hepatic disorders. The present study was designed to evaluate the oxidative stress as well as the therapeutic effect of Croton cajucara Benth (1.5 mL of the C. cajucara extract i.g.) in rats with streptozotocin-induced diabetes. Croton cajucara Benth was tested as an aqueous extract for its phytochemical composition, and its antioxidant activity in vitro was also evaluated. Lipid peroxidation and superoxide dismutase, catalase, and glutathione reductase activities were measured in the hepatic tissue, as well as the presence activation of p65 (NF- κ B), through western blot. Phytochemical screening of Croton cajucara Benth detected the presence of flavonoids, coumarins and alkaloids. The extract exhibited a significant antioxidant activity in the DPPH-scavenging and the hypoxanthine/xanthine oxidase assays. Liver lipid peroxidation increased in diabetic animals followed by a reduction in the Croton-cajucara-Benth-treated group. There was activation of p65 nuclear expression in the diabetic animals, which was attenuated in the animals receiving the Croton cajucara Benth aqueous extract. The liver tissue in diabetic rats showed oxidative alterations related to the streptozotocin treatment. In conclusion the Croton cajucara Benth aqueous extract treatment effectively reduced the oxidative stress and contributed to tissue recovery.

1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels [1]. There are convincing experimental and clinical pieces of evidences that the generation of reactive oxygen species (ROS) is increased

in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [2]. Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins [3].

Oxidative stress, the prevalence of oxidant factors over antioxidant mechanisms, plays a central role in the pathogenesis and progression of diabetes and its complications [4]. Enhanced formation of oxygen free radicals occurs in tissues during hyperglycemia [5]. The hyperglycemia can also activate transcription factors, such as nuclear factor NF- κ B. This factor regulates the expression of a large number of genes including those who have deeper connection to the complications of diabetes [6]. Hyperglycemia also favors, through the activation of NF- κ B, an increased expression of inducible nitric oxide synthase (iNOS), which is accompanied by increased generation of nitric oxide [7].

Traditional medicines and extracts from medicinal plants have been extensively used as alternative medicine for better control and management of DM [8]. *Croton cajucara* Benth (*C. cajucara*), commonly known as Sacaca, is a shrubby plant found in Amazonia, Brazil [9]. The bark and leaf infusions of this plant have been popularly used to treat diabetes, diarrhea, malaria, fever, gastrointestinal, renal, and hepatic disorders, as well as in the control of high levels of cholesterol [10, 11]. Antioxidant effects of *C. cajucara* leaf extracts was investigated both *in vitro* and *in vivo* models. Leaf extracts showed radical inhibitory scavenging activity against the stable radical DPPH and reduced oxidative stress in animals treated with paraquat [12].

The liver is the main organ of oxidative and detoxifying processes. In many diseases, biomarkers stress oxidative are elevated in the liver at an early stage [13]. Thus, the present investigation was carried out in order to study the possible antioxidant effect and the expression of NF- κ B of *C. cajucara* aqueous extract in streptozotocin-diabetic rats.

2. Materials and Methods

2.1. Plant Material and Preparation of C. cajucara Bark Aqueous Extract. Bark fragments of C. cajucara were collected in Amazônia-Santarém, Brazil. A voucher specimen (number 247) identified by Nelson A. Rosa was deposited in the IAN Herbarium (Belém, PA, Brazil).

The bark (5 g) was ground and mixed with boiling water (100 mL) to provide a 5% aqueous extract. After 10 minutes, the mixture was filtered with filter paper and the extract was administered to the rats. After cooling and filtering, the extracts were frozen and concentrated by lyophilization for five days overnight.

- 2.2. Phytochemical Screening. The phytochemical analysis (flavonoids, tannins, anthraquinones, alkaloids, saponins, coumarins, and cardiac glycosides) of *C. cajucara* bark was carried out according to the methods described by Harbone [14]. The thin layer chromatography analyses were performed following systems and developers indicated by Wagner and Bladt [15].
- 2.3. In Vitro Test for Antioxidant Activity of Bark Aqueous Extract
- 2.3.1. Hypoxanthine/Xanthine Oxidase Assay. The method employed to assay the hydroxyl radical (OH•) scavenging ability of C. cajucara aqueous extract or infusions was based on the method developed by Owen et al. [16]. Briefly, C. cajucara aqueous extract or infusions were dissolved in the assay buffer (hypoxanthine, Fe (III), EDTA, and salicylic

acid) at a concentration of 2.0 mg/mL and diluted appropriately (in triplicate) in assay buffer to a final volume of 1.0 mL, giving a range of 0.1-2.0 mg/mL for extract and volumes of 10-200 µL of aqueous infusions of C. cajucara bark. A 5 μ L aliquot of xanthine oxidase dissolved in 3.2 M (NH₄)₂SO₄ was added to initiate the reaction. The sample tubes were incubated for 3 h at 37°C, and the reaction stopped afterwards by adding 10 µL of HCl. A 30 µL aliquot of the reaction mixture was analyzed by HPLC under chromatographic conditions as described by Owen et al. [17, 18] Chromatographic analysis was done using a gradient based on methanol/water/acetic acid with a µBondaPak C18 reverse phase column (Waters) and detection at 325 nm. The HPLC equipment had a 2695 separation module (Waters) and a UV detector 2487 (Waters). Hydroxylation of salicylic acid and hypoxanthine was monitored at A = 325 and A = 278 nm, respectively. The amount of dihydroxyphenols (2,5-dihydroxybenzoic acid and 2,3-dihydroxibenzoic acid) (2,5-DHBA and 2,3-DHBA) produced by (OH[•]) attack on salicylic acid was determined from standard curves prepared with the respective pure dihydroxyphenols.

2.3.2. DPPH Scavenging Assay. Scavenging of the DPPH free radical was measured using a modified Yamaguchi et al. [19] method in which C. cajucara aqueous extract or C. cajucara bark infusions were added to Tris-HCl (100 mM) buffer, pH 7.0, containing 250 mM DPPH dissolved in methanol. At least six different dilutions of C. cajucara aqueous extract or C. cajucara bark infusions were tested and allowed to stand for 20 min in the dark, before absorbance was measured at 517 nm using a Shimadzu spectrophotometer model UV-1602PC (Kyoto, Japan). The experiment was conducted in triplicate. Antioxidant activity was expressed as IC_{50} (inhibitory concentration in $\mu g/mL$ of samples or positive controls necessary to reduce the absorbance of DPPH by 50%, as compared to the negative control). The lower the IC_{50} , the higher is the antioxidant activity.

2.3.3. Animals and Experimental Protocol. The experimental protocol used complied with the norms established by the Ethical and Health Research Committee of the Group of Research and Postgraduate Studies of the Hospital de Clínicas of Porto Alegre as well as with the Principles for Research Involving Animals. Only male Wistar rats were used, obtained from the breeding colony of the Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul (UFRGS). The mean weight of animals at the start of the study was 200-300 grams. They were kept under a 12:12 hours light/dark cycle (light from 7 am to 7 pm) in a temperature-controlled environment (22 \pm 4°C). The rats were randomly divided into 6 groups. In 3 groups, diabetes was induced by a single intraperitoneal (i.p) injection of streptozotocin (70 mg/kg body weight; Sigma Chemical) in freshly prepared 10 mmol/L sodium citrate, pH 4,5. Five days after the STZ injection, plasma glucose concentration was measured using retro orbital blood samples obtained from rats after overnight food deprivation. A plasma glucose level > 250 mg/dL was considered indicative of diabetes. The experimental groups comprised the following: (i) normal control group (CO: n=10) that received 1,5 mL of distilled water administered intragastrically (i.g.); (ii) group treated with C. cajucara for 5 days (CO5D: n=10) 1,5 mL of the C. cajucara extract i.g. during the last 5 days before being killed; (iii) group treated with C. cajucara for 20 days (CO20D: n=10) 1,5 mL of the C. cajucara extract i.g. for 20 days before being killed; (iv) diabetic group (DM: n=10) 1,5 mL of distilled water i.g.; (v) diabetic group treated with C. cajucara for 5 days (DM5D: n=10), 1,5 mL of the C. cajucara extract i.g. during the last 5 days before killed; (vi) diabetic group treated with C. cajucara for 20 days (DM20D: C0 days before being killed.

2.3.4. Biochemical Analyses of Oxidative Stress and Antioxidant Assay. The livers were homogenized with 9 mL of phosphate buffer (KCL 140 mM, phosphate 20 mM, pH 7.4) per gram of tissue. The protein concentration in these liver homogenates was determined using a standard solution of bovine albumin according to Lowry et al. [20]. Hepatics lipoperoxidation was determined by the method of thiobarbituric acid reactive substances (TBA-RS) [21]. Superoxide dismutase (SOD) activity in the liver tissue was determined using a technique based on the inhibition of adrenochrome formation in epinephrine autoxidation [22]. Catalase (CAT) activity in the liver tissue was determined as described elsewhere [23]. For Glutathione reductase (GSH) measurement, it was made according to adapted method by Kolberg et al. [24].

2.3.5. Western Blot for p65 NF-κB Subunit. 75 μg of nuclear extracts, prepared as described by Gutiérrez et al. [25], was loaded on an SDS/PAGE gel (10%), electroblotted, and p65 protein was detected using specific polyclonal antibodies (65 kDa) (NF-κB p65 (C22B4) Rabbit mAb-cell signaling). Bound primary antibody was detected, HRP—with Antirabbit IgG antibody Cell Signaling and blots were developed using an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified with an imaging densitometer Software (Scion Image, Maryland, MA).

2.4. Statistical Analysis. The results were expressed as mean values \pm SEM. The data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Student Newman Keuls test. The level of significance used was P < .05.

3. Results

- 3.1. Phytochemical Analyses. Phytochemical analyses of *C. cajucara* indicated the presence of flavonoids, coumarins, and alkaloids. Other secondary metabolites such as saponins, anthraquinones, cardiac glycosides, fenolic acids, and tannins were not detected.
- 3.2. Hypoxanthine/Xanthine Oxidase In Vitro Assay. The in vitro antioxidant activity of the extract was determined

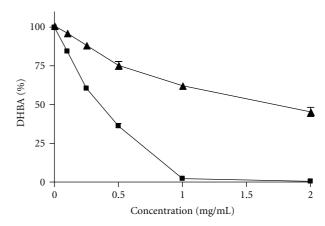


FIGURE 1: Dose-dependent inhibition of reactive oxygen species attack on salicylic acid by the *C. cajucara* aqueous extract (**A**) and of Trolox (**II**), as positive control, in the HPLC-based hypoxanthine/xanthine oxidase assay (mean of 3 assays). DHBA: dihydroxybenzoic acid.

by monitoring the production of hydroxyl benzoic acids (DHBA) as a product of the hydroxyl radical attack on salicylic acid in the hipoxanthine-xanthine oxidase assay. The reduction of total oxidation products as a function of the concentration of C. cajucara added to the assay is shown in Figure 1. The aqueous extract ($IC_{50} = 1.61 \text{ mg/mL}$) displayed a pronounced in vitro antioxidant capacity in a dose-dependent manner, reducing the formation of both DHBA species to 45.49% in the highest concentration used (2 mg/mL), whereas Trolox ($IC_{50} = 0.34 \text{ mg/mL}$), as positive control, could reduce both compounds totally at the same concentration.

3.3. DPPH Scavenging Assay. The free radical scavenging effect of extracts, as well as ascorbic acid and rutin, both acting as positive controls, was tested using the DPPH free radical scavenging assay. The free radical scavenging effect of *C. cajucara* showed a concentration-dependent activity, based on a 91.38% inhibition at the concentration of $1000 \,\mu\text{g/mL}$ and of 57.32% at $100 \,\mu\text{g/mL}$ (Table 1). The respective IC50 value for the extract was 63.34 $\mu\text{g/mL}$. In comparison, the results of the free radical scavenging effect of ascorbic acid (IC50 = $4.03 \,\mu\text{g/mL}$) and trolox (IC50 = $15.52 \,\mu\text{g/mL}$) were respectively, 99.62% and 99.03% inhibition at the concentration of $1000 \,\mu\text{g/mL}$ and 99.40% and 91.27% at $100 \,\mu\text{g/mL}$.

3.4. Biochemical Analysis and Oxidative Stress. C. cajucara administration failed to reduce glycemia in diabetic rats. The treatment with C. cajucara during 5 (DM5D) and 20 (DM20D) days significantly reduced the lipoperoxidation. Normal animals treated with the aqueous extract during 20 days (CO20D) had the lipoperoxidation increased, although significantly less than the diabetic animals. SOD activity was higher in diabetic animals if compared to the controls. This effect was partially abolished by administration of the bark extract. Animals that received only bark extract for 20 days

Table 1: Inhibition of DPPH, IC₅₀ values for the DPPH assay of the *C. cajucara* aqueous extract, Trolox, rutin, and ascorbic acid, as well as the AEAC**.

Sample	Inhibition (%)			IC ₅₀ (μg/mL)	
concentration	$1 \mu \mathrm{g/mL}$	$10\mu\mathrm{g/mL}$	$100 \mu\mathrm{g/mL}$	$1000\mu\mathrm{g/mL}$	1050 (118)
Ascorbic acid	9.70	98.11	99.40	99.62	4.03 ± 0.16
Trolox	8.94	45.82	91.27	99.03	15.52 ± 2.64
Rutin	5.22	31.83	82.86	97.89	18.64 ± 0.94
C. cajucara	2.61	13.74	57.32	91.38	63.34 ± 29.28

Mean \pm standard deviation of three individual determinations. Results were based on the values measured at 20 min. Ascorbic acid, vitamin E, and rutin were used as positive controls.

Table 2: Biochemical analyses of oxidative stress in liver tissue.

Group	TBARS (nmoles/mg of protein)	SOD (U/mg protein)	CAT (pmoles/mg protein)	GSH (nmoles/mg of protein)
CO	0.31 ± 0.04	2.38 ± 0.29	0.34 ± 0.04	4344.01 ± 433.64
CO5D	0.38 ± 0.06	3.39 ± 0.19	0.42 ± 0.07	5188.72 ± 233.98
CO20D	$0.54 \pm 0.05^*$	$5.57 \pm 0.67^*$	0.5 ± 0.07	4654.82 ± 480.51
DM	$0.92 \pm 0.05**$	$8.34 \pm 1.05**$	0.57 ± 0.05	$2851.94 \pm 378.76**$
DM5D	$0.55 \pm 0.06***$	$4.14 \pm 0.48***$	0.49 ± 0.07	4094.46 ± 293.00***
DM20D	$0.48 \pm 0.08***$	$3.93 \pm 0.44***$	0.48 ± 0.08	3020.94 ± 151.43

CO: control (n = 10); CO5D: control treated with C. cajucara for 5 days (n = 10); CO20D: control treated with C. cajucara for 20 days (n = 10); DM: diabetes mellitus (n = 10); DM5D: diabetes mellitus treated with C. cajucara for 5 days (n = 10); DM20D: Diabetes Mellitus treated with C. cajucara for 20 days (n = 10). C. cajucara 5% aqueous extract.

Data appear as mean \pm SEM.

(CO20D) also had SOD activity increased. However, activity of antioxidant enzyme CAT did not show any differences between the groups. GSH activity was significantly higher in liver. The treatment with *C. cajucara* during 5 days decreased significantly GSH activity (Table 2).

3.5. Western Blot for p65 NF- κ B Subunit. To study the effects on p65 NF- κ B subunit expression, liver nuclear extracts were studied by western blot. As shown in Figure 2 *C. cajucara* affects NF- κ B binding activity in CO20D. Experimental diabetes markedly induced NF- κ B, an effect that was abolished by *C. cajucara* treatment.

4. Discussion

The potential mechanism underlying the antioxidant property of *C. cajucara* extract was investigated using the *in vitro* assay hypoxanthine/xanthine oxidase, which revealed an expressive reduction in the concentration of DHBAs, the byproducts of the reaction between the hydroxyl radical and salicylic acid, as shown in Figure 1. Therefore, it can be suggested that the compounds present in *C. cajucara* extract may act as scavengers of the hydroxyl radical generated by the Haber-Weiss/Fenton reaction, acting as O2 • and/or OH• scavenger, and that the protection provided by extract is probably due to its ability to quench free radicals. Several plant extracts and secondary metabolites derived from plants show *in vitro* antioxidant activity by quenching hydroxyl radicals, evaluated using this assay [26]. In addition, the

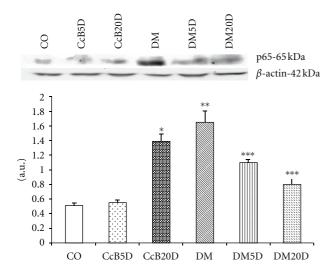
results of the antioxidant evaluation using DPPH reduction confirm the dose-response manner pattern of the antioxidant effect of this extract (Table 1). When the DPPH solution is mixed with some hydrogen donor compound, it is reduced to diphenylpicrylhydrazine and loses its violet color [27]. The degree of coloration loss bears a direct correlation with the activity of elimination of free radicals in the compound evaluated [28]. Several series of chemical compounds have been studied and presented a close correlation between DPPH sequestering activity and antioxidant activity in biological and nonbiological models [29, 30]. The differences of the C. cajucara extract IC₅₀ values for both antioxidant assays might be explained due to different antioxidant compounds responsible for radical-scavenging in both tests. Through of the phytochemical screening were detected alkaloids, flavonoids, and coumarins. Based on the antioxidant activity reported in the literature for alkaloids, flavonoids, and coumarins we can link the presence of these secondary metabolites to the antioxidant activity detected [31, 32].

Oxidative stress is produced under diabetic condition and it is likely to be involved in progression of pancreatic β -cell dysfunction [33]. High levels of free radicals, due to insufficiency of the antioxidant defense system, may lead to disruption of cellular function, oxidative damages to membranes, and enhance their susceptibility to lipoperoxidation [34]. In recent years, dietary plants with antioxidative property have been the center of focus. It is believed that these plants can prevent or protect tissues by antiobesity and hypoglycaemic effects [35]. The protection of hepatocyte cells from the effects of oxidative stress by treatment with

^{*}P < .05 CO20D, versus CO, CO5D.

^{**}P < .05 DM versus CO, CO5D, CO20D, DM5D, DM20D.

^{***}P < .05 DM versus CO, DM.



Data appear as mean ± SEM

- *P < .05 CO20D, versus CO, CO5D
- **P <.05 DM versus CO, CO5D, CO20D, DM5D, DM20D
- ***P <.05 DM versus CO, DM

FIGURE 2: Effect of streptozotocin-induced diabetes and *C. cajucara* aqueous extract on liver in p65 NF- κ B protein by Western blot analysis.

vitamin E was evidenced in a recent study [36]. In addition, it has been shown that dietary supplementation with natural antioxidants such as vitamins C and E, melatonin, and flavonoids attenuates the oxidative stress and diabetic state induced by STZ [37, 38].

Increased levels of TBARS, an end-product of lipoperoxidation, were found previously in the liver of streptozotocin-induced diabetic rats [39–41]. In this study, the TBARS increase confirms this finding, which indicates an overall oxidative stress increase in diabetic rats. Treatment with *C. cajucara* bark extract suggests an amelioration of oxidative stress. *trans-dehydrocrotonin* (t-DCTN), the main component of the bark extract, showed hypoglycemic, anti-inflammatory, analgesic, antiulcerogenic, and antilipidemic effects [42].

Oxidative stress is the result of a redox imbalance between the generation of ROS and the compensatory response from the endogenous antioxidant network. Although some studies which measured activities of SOD, CAT, and glutathione peroxidase in diabetes mellitus showed reduction in the levels of theses enzymes [43], other authors reported an increase in SOD and CAT activities in streptozotocin-induced diabetic rats [44]. These apparently contradictory results could be due to tissue specificity, variation in severity and duration of the disease, or other experimental conditions. Hyperglycemia results in increased enzymatic conversion of glucose to the polyalcohol sorbitol with concomitant decreases in NADPH and glutathione [43, 45]. The resulting loss of antioxidant reducing equivalents results in enhanced sensitivity to oxidative stress associated with intracellular

ROS. In the present study, SOD activity increased in diabetic rats. This effect may thus be an adaptive response for increased oxidative stress in the liver tissue; C. cajucara bark extract by scavenging ROS prevents the elevation of this antioxidant enzyme activities in diabetic rat liver. This suggests the possibility of a radical superoxide scavenger activity. In our study, CAT activity remained unchanged. One the consequences of hyperglycemia is increase the metabolism of glucose by sorbitol pathway. Besides this, other pathways, such as fatty acid and cholesterol biosynthesis, also compete for NADPH with GSH. We also observed the decreased in GSH in the liver of streptozotocin-induced diabetic rats. Under in vivo condition, GSH acts as an antioxidant and its decrease is reported in diabetes mellitus studies in [46]. showed that GSH level was significantly lower in diabetic rats than normal rats. In the present study we found that treatment with C. cajucara bark extract significantly increased the glutathione when compared to diabetic control rats where the levels were significantly decrease. This increased GSH in rats treated with C. cajucara bark for 5 days extract may be one of the factors responsible for the inhibition of lipoperoxidation.

The NF- κ B has been proposed to form a critical bridge between oxidant stress and the cellular response [47, 48]. One mechanism by which hyperglycaemia-induced oxidative stress might alter cellular functions is activation of the transcription factor NF-κB [39]. Rodrigues et al. [49] showed than glucose concentration in the blood plasma of streptozotocin-treated rats was significantly higher than in the normal control group and in the animals treated with *C*. cajucara. In our study, to evaluate the liver tissue of diabetic animals found significant increase in the activation of NF- κ B. In contrast, the treatment with *C. cajucara* prevented NF- κ B activation [49]. However, a significant increase in oxidative stress and in the expression of p65 NF- κ B was found in the group that underwent treatment for 20 days. Although the previous study did not demonstrate genotoxic effects using the same period, other studies show reports of fulminant hepatitis related to the indiscriminate use of the plant [50].

In summary, the results show that in situations where there is no oxidative stress the extended use of *C. cajucara* behaves acts as a prooxidant and in situations where there is oxidative stress the treatment with *C. cajucara* may be an antioxidant scavenger of free radicals. Furthermore, the results seem to support the hypothesis that the oxidative stress in DM stimulates NF-κB expression and that *C. cajucara* aqueus extract administration reduces this expression.

Acknowledgments

This study was supported by the CAPES and FIPE (Financiamento de pesquisas do Hospital de Clínicas de Porto Alegre).

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Hindawi Publishing Corporation Journal of Biomedicine and Biotechnology Volume 2012, Article ID 205752, 7 pages doi:10.1155/2012/205752

Research Article

Association of Polymorphisms in Mitofusin-2 Gene with Type 2 Diabetes in Han Chinese

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Received 6 March 2012; Revised 13 April 2012; Accepted 20 April 2012

Academic Editor: Sandro Massao Hirabara

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MFN2 and ESRRA are candidate genes involved in the pathogenesis of T2D. Five tag-SNPs in MFN2 gene and three in ESRRA gene were selected and genotyped with TaqMan or PCR-RFLP method in stage 1 populations (555 patients with T2D and 649 control subjects) and stage 2 populations (546 patients with T2D versus 419 control subjects) in Han Chinese. And combining our published data, we estimated the interactions between genetic variants in the MFN2, ESRRA, and PGC-1α genes on the T2D risk using MDR. rs873458 (G > A) and rs2878677 (C > T) in MFN2 gene were significantly associated with T2D (P = 0.005 and 0.01) in stage 1 populations, and the association of other SNPs with T2D was not found. In stage 2 populations, we further confirmed the association between rs2878677 and T2D (P = 0.01). Combining the two stage populations, the data supported more significant effect of rs873458 and rs2878677 on T2D risk (P = 0.003 and 0.0001). A-C-G-T-C and G-T-C-T-C in MFN2 had significant association with T2D (P = 0.007 and 0.009). The present study also provided the evidence that MFN2 had interactions with PGC-1α (P < 0.0001) or ESRRA (P < 0.0001). This study suggested a role of MFN2 polymorphism in the risk of T2D; however, further studies are needed.

1. Introduction

Type 2 diabetes (T2D) is a chronic disease characterized by high blood glucose which is induced by both insulin resistance and relative insulin deficiency [1], and the complications of T2D, such as cardiovascular disease, renal failure and blindness, always bring heavy burden to society. The latest studies from the International Diabetes Federation (IDF) reported that currently there were 366 million people with diabetes in the world by 2030 this number is expected to rise to 552 million with diabetes if no action is taken [2]. Some 90% of patients with diabetes have T2D, which is always as a result of a combination of lifestyle and genetic factors [3].

Peroxisome proliferator-activated receptor γ coactivator 1α ($PGC-1\alpha$) gene is originally identified as a coactivator of PPAR γ . It is a multifunctional regulatory factor involved in mitochondrial biosynthesis, β -oxidation of fatty acids, beta-cell insulin secretion, hepatic gluconeogenesis, glucose

transport in muscle [4–6]. Our and other genetic association studies showed that the associations of $PGC-1\alpha$ loci with T2D were found in different populations [7–10].

Recently, it was reported that estrogen-related receptor- α (ESRRA) protein binding PGC-1 α protein could regulate the expression of *Mitofusin-2* (*MFN2*) gene by stimulating the activity of the *MFN2* promoter [11]. *ESRRA* gene is located on chromosome 11q13. *ESRRA* gene is an orphan nuclear receptor (NR) belonging to the NR superfamily, group III, and it is a vital regulator involved in a wide variety of cell functions such as mitochondrial biogenesis, mitochondrial oxidative metabolism, carbohydrate metabolism, adaptive energy metabolism, and lipid metabolism [12–14]. Therefore, we hypothesize that *ESRRA* gene may be a candidate gene involved in the pathogenesis of T2D.

MFN2 gene maps on chromosome 1p36.22. MFN2 serve as a mitochondrial fusion protein regulating their morphology and distribution [15, 16]. Tissues with high energetic requirement, such as skeletal muscle and heart, show high

expression of MFN2 gene [17]. Some evidences demonstrated that it also played an important role in the oxidative metabolism [17] and ER-mitochondria juxtaposition [18], and repression of *MFN2* gene reduced glucose oxidation, mitochondrial membrane potential, cell respiration, and mitochondrial proton leak [17]. In addition, the *MFN2*-dependent mechanism of mitochondrial control is disturbed in skeletal muscle in animal or human obesity and in patients with T2D [17, 19], but no study has confirmed that *MFN2* gene was associated with T2D.

In the present study, we evaluated the associations between the *MFN2* and *ESRRA* genetic polymorphisms and T2D, and then combining our published data of $PGC-1\alpha$ [7], we estimated the interactions between genetic variants in the *MFN2*, *ESRRA*, and $PGC-1\alpha$ on the T2D risk in Han Chinese.

2. Materials and Methods

2.1. Subjects. The stage 1 populations are summarized in Table 1. The total of stage 1 populations comprised 1204 unrelated subjects, including 555 patients with T2D and 649 nondiabetic control subjects, ascertained from Han Chinese. All subjects were randomly enrolled from three top-grade hospitals in Beijing in 2010. Total case subjects were diagnosed according to the World Health Organization criteria (WHO 1998). As total control subjects, the age was between 35 and 70 years old, and they had normal fasting plasma glucose levels $(5.0 \pm 0.5 \, \text{mmol/L})$ without diabetic record or T2D family history. The genomic DNA was extracted from peripheral blood using QIAGEN kit. The clinical characteristics of stage 2 populations are also summarized in Table 1; it included 546 unrelated patients with T2D and 419 nodiabetic control subjects and of Han Chinese origin, as described previously [20]. All subjects provided written informed consent under protocols approved by the local ethics committee.

2.2. Single-Nucleotide Polymorphism (SNP) Selection and Genotyping. International HapMap Project SNP database (http://www.hapmap.org/) was implemented to search for SNPs in MFN2 and ESRRA gene. Using tagging with an $r^2 > 0.8$ and minor allele frequency (MAF) of >5% and sequencing data in 20 nondiabetic control subjects, we selected 5 tag SNPs (rs873458, rs2878677, rs2236058, rs3766742, rs3766741) in MFN2 gene and 3 tag SNPs (rs731703, rs650008, rs11600990) in ESRRA gene; they were all located in intron region and could capture 100% of common variations across MFN2 and ESRRA gene region (containing 21 HapMap SNPs). 7 of 8 SNPs were successfully genotyped using the TaqMan method on a Bio-Rad iQ5 system. Primers and probes of rs873458C_8861262_10, rs3766741C_25606040_10 and rs650008 AHWR23M were purchased from Applied Biosystems, the others from Shanghai GeneCore BioTechnologies (primers showed in Table 2). Only one SNP (rs3766742) in the MFN2 was genotyped with PCR-restriction fragment-length polymorphism (PCR-RFLP) method, using the following (5'-AGCAGGACATGATAGGTTAG-3' and CACAGCTTGTCACAGTTTAG-3') and PCR Tm was 57°C,

PCR product length is about 900 bp, and restriction enzyme (Hha I) provided by MBI Fermentas. A $10\,\mu\text{L}$ aliquot of PCR product was digested overnight at 37 in a $20\,\mu\text{L}$ reaction containing 5 units of Hha I. After overnight digestion, the products were separated on a 3% agarose gel stained with ethidium bromide. TT genotypes were represented by a DNA band with a size of 900 bp, TC genotypes were represented by DNA bands with sizes of 900, 700, and 200 bp, and CC genotypes were represented by DNA bands with sizes of 700 and 200 bp. Call rates for all SNPs were >99%. 20 samples randomly selected from the whole sample bank were sequenced for 8 tag SNPs and the results showed excellent correspondence between sequencing and TaqMan genotyping or PCR-RFLP technique.

2.3. Statistical Methods. Genotype distributions for all studied SNPs were tested for Hardy-Weinberg equilibrium (HWE) by chi-square tests and no significant deviation was found in control subjects. Allele frequencies and genotype distributions, linkage disequilibrium (LD), and haplotypes were tested using the online software SHEsis (http://analysis .bio-x.cn/myAnalysis.php). Logistic regression analysis with additive model was used to adjust for sex, age, and body mass index (BMI), and the analysis was performed by SPSS (version 16.0). Tests for association between genotypes and quantitative traits were performed in control subjects using Kruskal-Wallis analysis of ranks for traits with nonnormal distribution or, alternatively, ANOVA for normally distribution in SPSS. We examined the analyses of genegene interactions on the risk of T2D using multifactor dimensionality reduction (MDR) version 2.0 beta 6 (http://www.epistasis.org/). P value <0.05 is nominally significant.

3. Results

In stage 1 populations, we found that the A allele and C allele frequencies of rs873458 and rs2878677 of MFN2 gene in the diabetic group were lower than in the control group (P=0.005, OR=0.79, 95% CI=0.67-0.93; P=0.01, OR=0.81, 95% CI=0.68-0.95) (Table 3); it showed A allele and C allele of rs873458 and rs2878677 of MFN2 gene confered protection agasist T2D. After adjusting for age, sex, and body mass index by using analysis of logistic regression with additive model, the results showed that the genotype distributions of rs873458 and rs2878677 of MFN2 gene also were significantly different in case-control subjects (Pc=0.01, OR=0.80, 95% CI=0.67-0.95; Pc=0.02, OR=0.83, 95% CI=0.71-0.97) (Table 3).

Replicating the two associated SNPs in stage 2 populations, data suggested that the C allele of rs2878677 of MFN2 gene in the diabetic group was lower than in the control group (P=0.01, OR = 0.79, 95% CI = 0.65–0.94) (Table 3). And it revealed that there was a borderline association between genotype distributions of rs873458 of MFN2 gene and type 2 diabetes (adjusted Pc=0.08, OR = 0.84, 95% CI = 0.69–1.02) (Table 3). Additionally, analyses in the combined populations showed that the allele frequencies and genotype distributions of rs873458 and rs2878677 of MFN2 gene had

Stage 1 (n = 1204) Stage 2 (n = 965)Characteristic T₂D Control T₂D Control Total (male %) 555 (55.9) 649 (46.4)* 546 (50.7) 419 (48.7) Age (years) 50.5 (8.5)* 63.2 (8.2)* 53.6 (8.2) 58.8 (11.8) BMI (kg/m^2) 25.8 (3.4) 25.4 (3.3)* 24.9 (3.5) 24.6 (3.4) DBP (mmHg) 82.7 (11.4) 79.7 (11.8)* 80.3 (11.0) 75.6 (10.1)* SBP (mmHg) 129.6 (18.5) 128.3 (16.9) 131.4 (19.2) 127.7 (17.2) FPG (mmol/L) 9.0(2.9)5.0(0.5)*9.2 (3.2) $5.0(0.5)^*$ TG (mmol/L) 2.1(2.3) $1.6(1.1)^*$ IFCC-HbA1c (mmol/mol) 63 (19.0) 41 (3.0)* DCCT-HbA1c (%) 7.9 (1.7) 5.9 (0.3)*

TABLE 1: Characteristics of the study populations.

Data are means (SD), or male (%), unless otherwise indicated. *P < 0.05. BMI: body mass index; DBP: diastolic blood pressure; SBP: systolic blood pressure; FPG: fasting plasma glucose; TG: triglycerides; HbA1c: glycated haemoglobin A_{1c}.

SNPs ID	Primer (5'-3')	Probe (5'-3')
rs2878677	F: GTGGAATAAAAGTTTTATGAATGGAG	P1: FAM-ATTCCACTTACGTGAGACaTCTAGAATAGTC-BHQ
1820/00//	R: TAGCCATTAAACAATAACTCCCTG	P2: HEX-TCCACTTACGTGAGACgTCTAGAATAGTC-BHQ
rs2236058	F: GGTGAGGAGGGCGCT	P1: FAM-CTTCATCACCCCACCTGGTCTGTG-BHQ
182230036	R: TGTGTTCAGGCTCTGAGAATGG	P2: HEX-CTTCATCACCCGACCTGGTCTGTBHQ
rs731703	F: AGCCAGAGTCCCTGTTCGG	P1: FAM-TTGCTGTCCTGGCCAGTGTTCC-BHQ
18/31/03	R: TGCTTCAGCCCCAGCTATG	P2: HEX-TTTGCTGTCCTGACCAGTGTTCCAG-BHQ
rs11600990	F: AAGTCATTGTTCTTCCCTAAGCCTC	P1: FAM-TGCTACCGTCACTGAG-MGB
	R: GAGGTGTCTCCGTAAGGTCTTCA	P2: HEX-TGCTACCATCACTGAGT-MGB

TABLE 2: Primers and probes of all SNPs.

more significant effects on type 2 diabetes susceptibility (P = 0.002, OR = 0.83, 95% CI = 0.73–0.93; P = 0.0001, OR = 0.79, 95% CI = 0.69–0.89 and adjusted Pc = 0.002, OR = 0.82, 95% CI = 0.72–0.93; Pc = 0.0004, OR = 0.81, 95% CI = 0.72–0.91) (Table 3). No significant association of other SNPs in *MFN2* or *ESRRA* gene with type 2 diabetes was found (See Supplementary Table 1 in Supplementary material available online at doi:10.1155/2012/205752).

Analyses of association between haplotypes and phenotypes were performed in nondiabetic control subjects, for most patients with T2D included in the present study had medical treatments, which may affect the real parameters. We found that the genotype distributions of SNPs in MFN2 and ESRRA gene in control subjects displayed no significant association with quantitative traits of type 2 diabetes including body mass index, diastolic blood pressure, systolic blood pressure, fasting plasma glucose, triglycerides, and glycated haemoglobin A1c (data not shown).

We further examined the degree of linkage disequilibrium of SNPs in MFN2 and ESRRA gene (Supplementary Tables 2 and 3). To test whether haplotypes represent the causal variants better than single SNPs, we estimated the frequencies of haplotypes between the case-control subjects in stage 1 populations (all those frequencies <0.03 were ignored). It was showed that A-C-G-T-C and G-T-C-T-C in MFN2 gene were significantly associated with T2D (P = 0.007, OR = 0.79, 95% CI = 0.66–0.94; P = 0.009, OR = 1.26, 95% CI = 1.06–1.49) (Table 4), while the frequencies

of haplotypes in *ESRRA* gene did not significantly differ in case-control subjects (data not shown).

The results of the exhaustive MDR analysis are summarized in Table 5. The model was considered to be statistically significant with a testing accuracy >50% and P < 0.05 via permutation test of 1000 iterations. The rs2878677/rs3766741/rs731703/rs11600990 model (P < 0.0001) in MFN2-ESRRA from stage 1 populations, the rs2878677/rs3774923/rs7656250 model (P < 0.0001) and the rs2878677/rs3774923/rs7656250/rs13131226 model (P < 0.0001) in MFN2-PGC- 1α gene from stage 2 populations (shown in Table 5), data of PGC- 1α gene from our previously study [7], suggest that there are interactions between genetic variants in the MFN2, ESRRA, and PGC- 1α gene.

4. Discussion

In the present study, we examined eight common variants in *MFN2* and *ESRRA* genes and confirmed that G allele of rs873458 and T allele of rs2878677in *MFN2* gene are associated with higher T2D risk compared with A and C allele, respectively. Consistently, in haplotypes analyses of *MFN2* gene, results show that individuals carrying G-T-C-T-C present increased T2D risk compared with those carrying A-C-G-T-C.

Because rs873458 and rs2878677 map within intron 2 and 3, it is difficult for us to shed light on their downstream consequences, given our rudimentary knowledge of the

Table 3: Allele frequencies and genotype distributions of rs873458 and rs2878677 in MFN2 gene among stage 1 and stage 2 populations.

	SNP		Genotype		Allele						
	SINF		T2D (%)	Control (%)	P/Pc	OR (95%CI)		T2D (%)	Control (%)	P	OR (95% CI)
	rs873458		555	638							
		A/A	50 (9.0)	105 (16.5)	0.0007/0.01	0.80 (0.67–0.95)	A	382 (34.4)	510 (40.0)	0.005	0.79 (0.67–0.93)
Stage 1		A/G	282 (50.8)	300 (47.0)			G	728 (65.6)	766 (60.0)		
Stage 1		G/G	223 (40.2)	233 (36.5)							
	rs2878677		549	643							
		T/T	124 (22.6)	108 (16.8)	0.03/0.02	0.83 (0.71–0.97)	T	487 (44.4)	503 (39.1)	0.01	0.81 (0.68–0.95)
		C/T	239 (43.5)	287 (44.6)			С	611 (55.6)	783 (60.9)		
		C/C	186 (33.9)	248 (38.6)							
	rs873458		539	412							
		A/A	72 (13.4)	58 (14.1)	0.21/0.08	0.84 (0.69–1.02)	A	386 (35.8)	321 (39.0)	0.16	0.87 (0.72–1.05)
C4 2		A/G	242 (44.9)	205 (49.8)			G	692 (64.2)	503 (61.0)		
Stage 2		G/G	225 (41.7)	149 (36.2)							
	rs2878677		545	417							
		T/T	135 (24.8)	78 (18.7)	0.05/0.02	0.80 (0.67–0.97)	T	516 (47.8)	348 (41.8)	0.01	0.79 (0.65–0.94)
		C/T	248 (45.5)	192 (46.0)			С	564 (52.2)	484 (58.2)		
		C/C	162 (29.7)	147 (35.3)							
	rs873458		1094	1050							
		A/A	122 (11.2)	163 (15.5)	0.005/0.002	0.82 (0.72–0.93)	A	768 (35.1)	831 (39.6)	0.002	0.83 (0.73–0.93)
6 1: 1.1		A/G	524 (47.9)	505 (48.1)			G	1420 (64.9)	1269 (60.4)		
Combinded		G/G	448 (41.0)	382 (36.4)							
	rs2878677		1089	1059							
		T/T	259 (23.8)	186 (17.6)	0.0006/0.0004	0.81 (0.72–0.91)	T	1003 (46.1)	851 (40.2)	0.0001	0.79 (0.69–0.89)
		C/T	485 (44.5)	479 (45.2)			С	1183 (53.9)	1267 (59.8)		
		C/C	345 (31.7)	394 (37.2)							

P had no adjustment, whereas Pc were adjusted for age, sex, and BMI.

Table 4: Common haplotypes in the MFN2 gene.

Haplotype	T2D (%)	Control (%)	P	OR (95% CI)
A-C-G-T-C	347.95 (31.9)	474.88 (37.7)	0.007	0.79 (0.66–0.94)
G-C-C-C	66.82 (6.1)	100.87 (8.0)	0.10	0.76 (0.55-1.05)
G-C-C-T-C	35.18 (3.2)	40.09 (3.2)	0.90	1.03 (0.65–1.63)
G-C-G-T-G	114.87 (10.5)	111.74 (8.9)	0.14	1.23 (0.94-1.62)
G-T-C-T-C	443.08 (40.6)	454.77 (36.2)	0.009	1.26 (1.06-1.49)
others	84.1 (7.7)	75.65 (6.0)		
Total	1092 (100%)	1258 (100%)		

Haplotype analysis was conducted with rs873458, rs2878677, rs2236058, rs3766742, rs3766741. P had no adjustment.

Table 5: Models of multi-loci interaction on the risk of T2D by MDR.

Gene-gene	Combination of multiloci	*P value	CV consistency	Testing accuracy (%)
	rs873458/rs3766742	0.0007	7/10	56.1
MFN2-ESRRA	rs873458/rs2878677/rs3766741	< 0.0001	4/10	56.2
	rs2878677/rs3766741/rs731703/rs11600990	< 0.0001	3/10	57.7
	rs7656250/rs13131226	0.0002	7/10	56.4
MFN2-PGC-1α	rs2878677/rs3774923/rs7656250	< 0.0001	10/10	59.9
	rs2878677/rs3774923/rs7656250/rs13131226	< 0.0001	9/10	63.9

Rs873458, rs2878677, rs3766742, rs3766741 located in MFN2, rs731703 and rs11600990 located in ESRRArs7656250, rs13131226, and rs3774923 located in PGC -1 α . P value based on 1,000 permutations.

mechanics of gene regulation. In multifactorial disease, most susceptibility variants, mapping outside the coding regions of genes, are assumed to influence transcript regulation rather than gene function [21]. Locations of rs873458 and rs2878677 are surrounded by the regions coding the GTPase domain of MFN2 which drive GTP hydrolysis to provide energy for the mitochondrial fusion activity induced by MFN2 [16, 22]. In addition, MFN2 gene is the first structural tether identified gene in the molecular basis of ER-mitochondria juxtaposition, it has a role in the intercommunication during Ca²⁺ signaling [18]. A study revealed that ablation or silencing of MFN2 gene in mouse embryonic fibroblasts or HeLa cell created confusion in ER morphology and increased distance between the ER and mitochondria, hence retarding the mitochondrial Ca²⁺ uptake [18], which may lead to insulin secretion disorder and insulin resistance. Besides, positive correlation was observed between the expression of MFN2 gene and insulin sensitivity [23]. In addition, the MFN2-dependent mechanism of mitochondrial control is disturbed in skeletal muscle in animal or human obesity, and in patients with T2D [17, 19]. It is possible that the associated SNPs may be in strong linkage disequilibrium with unidentified causal variant(s) that may regulate the expression or function of MFN2

Although many genomewide association studies (GWASs) of T2D are now emerging, MFN2 gene is not involved. Indeed, the effect sizes of the known, common variants influencing the risk of type 2 diabetes are modest, and the proportion of overall predisposition explained is approximately 5 to 10% for T2D [24]. It has been found that T2D loci identified by linkage analyses and GWAS lack the correlation of T2D [25]. Additionally, most of them were conducted in Caucasians. Differences in genetic background, risk-factor profile, environment, and study design may lead to ethnic differences in susceptibility loci [26]. Different allele frequencies of rs873458 and rs2878677 among the HapMap populations indicate that differences in genetic architecture may play a role (Supplementary Table 4). Therefore, further studies including GWAS are needed to confirm our findings.

ESRRA is an orphan nuclear receptor, it could regulate a number of downstream genes to control energy balance in animals. ESRRA gene KO mice showed altered expression of several target genes implicated in the regulation of adipogenesis and energy metabolism [27]. However, in the present study, no association between the phenotypes of T2D, such as BMI, was found. Our data also showed that there were three common variants (rs731703, rs650008, rs11600990) in ESRRA in Han Chinese populations, while only one common variant rs11600990 was found in Danish populations [28]. The analysis of rs11600990 in the two populations is consistent. Three tag SNPs could capture 100% of common variations (MAF > 5%) across ESRRA gene region, but we cannot exclude rare causal genetic polymorphisms in ESRRA gene, for the rare variations may be responsible for disease [29-31].

 $PGC-1\alpha$ is an important transcriptional coactivator involved in the regulation of genes related to energy metabolism [32]. Mice lacking $PGC-1\alpha$ developed fasting hepatic steatosis which may lead to lower rates of fatty acid oxidation that might play a causative role to develop insulin resistance [33, 34]. In addition, $PGC-1\alpha$ maintained higher number of active mitochondria and OXPHOS proteins that were reduced in T2D subjects. As it relates to the human genetics, several SNPs of $PGC-1\alpha$ gene in certain populations were associated with T2D in certain populations [7–10]. The analysis of association between $PGC-1\alpha$ gene and T2D has been published in our previous paper [7].

In addition, it was found that the interactions between genetic variants of the MFN2-ESRRA and MFN2-PGC-1α in the pathogenesis of type 2 diabetes. Moreover, a positive result of association analyses regarding single SNP rs2878677 in MFN2 gene indicates MFN2 gene may be the main factor during the interaction and has an important role in the pathogenesis of T2D. It is consistent with the study that it proved the interactions between MFN2, ESRRA, and PGC- 1α proteins, which revealed the stimulatory effect of PGC- 1α on the activity of MFN2 gene needed to integrate the ESRRA, ESRRA, and PGC-1 α caused a synergic effect on activity of the MFN2 gene expression, and the stimulatory effect of $PGC-1\alpha$ gene on mitochondrial membrane potential can be weakened by MFN2 loss of function [11]. Therefore, further studies are needed to explore the functions and interactions of the MFN2, ESRRA, and PGC-1 α genes.

In most patients, T2D is due to alterations of many genes, each of which has a partial and additive effect [35]. Consequently, we hypothesize that these three genes may serve as partial or additive factors leading to T2D, while the intrinsic mechanism is unknown. Functional studies, multiplex-gene analyses, and computational biology are needed to further characterization of their interactions in the pathogenesis of T2D.

However, some limitations should be noted in the present study. First, the effect of gene-environment interactions was not evaluated; environmental risk factors may influence the effect estimates. Second, given the study samples were relatively small, we need to replicate our finding in additional samples in future studies. Third, clinical characteristics were not defined strictly.

In conclusion, the present analysis shows that *MFN2* gene has significant associations with T2D in Han Chinese. It may represent a potential explanation for T2D. But more studies with different ethnic populations are needed to verify this finding and the intrinsic mechanism is expected to be uncovered.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

The authors thank all the participants of this research. This study was supported by the Science Fund for Creative Research Groups (no. 30721063) and Genome-Wide

Association Studies of T2D and Pharmacogenomics Study (2009AA022704).

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