Experimental Diabetes Research

Pathophysiological Insights into Cardiovascular Health in Metabolic Syndrome

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Editorial

Pathophysiological Insights into Cardiovascular Health in Metabolic Syndrome

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Obesity, diabetes mellitus, dyslipidemia, and hypertension often cluster together as the most significant risk factors for cardiovascular diseases [1–3]. Even a modest increase in body weight may lead to a rather significant increase in the prevalence of cardiovascular morbidity and mortality. Ever since its appearance in the medical vernacular three decades ago, the term "metabolic syndrome" or "cardiorenal metabolic syndrome" has caught sufficient attention due to its unfavorable impact on the overall human health in particular cardiovascular diseases. In fact, metabolic syndrome is not a particular disease but rather a cluster of symptoms encompassing large waist circumference, hypertension, hyperglycemia, dyslipidemia, and insulin resistance, all commonly associated with the increased prevalence of obesity and type 2 diabetes mellitus [3]. Patients with metabolic syndrome display nonspecific symptoms, obesity, and a sedentary lifestyle. Ample of epidemiological, clinical, and experimental evidence has defined a unique role of insulin resistance and central obesity for the occurrence of metabolic syndrome and, later on, type 2 diabetes [3, 4]. However, confirmed metabolic syndrome provides little therapeutic value or guidance other than treating each single component individually, which may or may not reduce the overall cardiovascular risks. To better address the health care issues of metabolic syndrome, intensive effort has been geared towards elucidation of the contributing cardiovascular risk factors and how they contribute to metabolic syndrome. Several contributing factors have been identified for the onset and development of metabolic syndrome. In particular, food intake and lifestyle are perhaps the most devastating factors responsible for the rapid rise in the prevalence of obesity and metabolic syndrome [5]. Excessive caloric intake and inadequate physical activity are deemed the modern lifestyle traits responsible for overweight and obesity [6]. In addition, socioeconomic factors also play an important role in the ever-increasing prevalence of metabolic syndrome including physical exercise, technological advances, and higher workload. Therefore, there is a critical need for a better understanding of the mechanisms responsible for regulation of satiety, lipid metabolism, energy balance, thermogenesis, adiposity, and weight gain, as well as central and peripheral regulation of metabolic processes. Given that recent advances in biology and medicine have introduced new technologies to study the genetics and pathophysiology of metabolic syndrome. Knowledge and understanding of these conditions lead to the development of animal models, successful therapies, and novel concepts to characterize the cardiovascular complications in metabolic syndrome, which should help to greatly improve the efficacy of clinical therapies for management of metabolic syndrome.

This special issue examines some of the critical issues in our understanding of the pathophysiology of metabolic syndrome and its cardiovascular complications. The paper by J. Palios and colleagues discusses the association between human immunodeficiency virus (HIV) and metabolic syndrome. HIV and the highly active antiretroviral therapy (HAART) may contribute to the onset of metabolic syndrome. These authors suggest a role of adipokines such

as visfatin, apelin, and vaspin in the pathogenesis of the HIV/HAART-related metabolic syndrome, which should shed some lights for cardiovascular therapeutic strategy in HIV patients. The paper by N. Martinelli and coworkers addressed the rising role of low plasma concentrations of paraoxonase (PON1) in metabolic syndrome. PON1 activities are found lower in patients with metabolic syndrome, with a progressively decreasing trend by increasing the number of metabolic syndrome components. These findings suggest a role of low PON1 concentrations in predicting metabolic syndrome-associated cardiovascular diseases. The paper by I. Isordia-Salas and colleagues found a rather high prevalence of metabolic syndrome (59.7% and 68.7% based on definitions of American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) and the International Diabetes Federation (IDF), resp.) in an urban Mexican sample, alerting the necessity for early detection of metabolic syndrome and its components to prevent type 2 diabetes and atherothrombotic complications in these patients. In the paper by D. Bharadwaj and coworkers, the variant gene MTHFR-rs1801133 involved in homocysteine metabolism was found to be associated with type 2 diabetes, postload glucose, high-density lipoprotein cholesterol, and total cholesterol in Indians. the paper by, S.-C. Chen and colleagues assessed the determinants of left ventricular mass index (LVMI) and left ventricular ejection fraction (LVEF) in diabetic patients at various stages of chronic kidney disease, and their findings suggest increases in LVMI and decreases in LVEF coincide with advances in chronic kidney disease stages in diabetic patients. the paper by H., Upur and colleagues reported an overtly higher prevalence of diabetes in periodontitis patients than individuals without periodontitis, suggesting the role of moderate periodontitis as an independent risk factor for type 2 diabetes mellitus.

In the second part of this special series involving basic science reports, J. R. Sowers and colleagues discussed the role of chronic alcohol consumption in cardiac insulin resistance. Chronic alcohol consumption inhibits mTOR/S6K1 activation in cardiac tissue. These authors suggested the activation of mTOR/S6K1 regulated by the mTOR-AT2R loop and microRNA that target S6K1. The paper by L. Brown and colleagues tested the therapeutic potential pf soluble epoxide hydrolase inhibition in obesity and metabolic dysfunction using a rat model of diet-induced metabolic syndrome. These authors reported that chronic oral treatment with an epoxide hydrolase inhibitor alleviates signs of metabolic syndrome including glucose, insulin, and lipid abnormalities, changes in pancreatic structure, systolic blood pressure, cardiovascular structural and functional abnormalities, and structural and functional changes in the liver. Then, L. Li and colleagues evaluated the effect of dietary supplementation of short-chain fatty acid propionate on cardiac contractile dysfunction in an Akt2 knockout-induced insulin resistance model, revealing the beneficial role of propionate or short-chain fatty acids against Akt2 deficiency-associated cardiac anomalies. The last but not least, L. Cai and colleagues review the unfavorable impact of diabetes mellitus on ischemic-preconditioning- (IPC-) and ischemic-postconditioning- (Ipost-) mediated myocardial protection. Diabetes has been shown to inhibit IPC- and Ipost-mediated myocardial protection through inhibition of the PI3K/Akt-GSK-3β pathway. To this end, activation of PI3K/Akt-GSK-3β pathway may relief the diabetic inhibition of both IPC and Ipost-mediated myocardial protection during ischemia/reperfusion injury.

As with all areas of cutting-edge human and experimental research, the advances in the pathophysiology of cardiovascular complications in metabolic syndrome described in this special raise as many questions as they answer. First, we need to better understand how individual component of metabolic syndrome work in concert to trigger cardiovascular complications. In particular, the clinical value of these individual factors in the diagnosis and treatment of cardiovascular anomies needs further scrutiny. Second, effective intervention for achieving and maintaining a healthy weight remains a major challenge for health care. Third, experimental model reminiscent of human metabolic syndrome remains a critical issue to advance metabolic syndrome research. Despite the availability of animal models for individual metabolic syndrome component, special caution is needed to translate findings from experimental setting to the clinic.

In summary, the metabolic syndrome research agenda remains a lengthy one with a large number of important questions to be answered. We hope that this special issue will help clarify the research agenda and so provide a launching pad for future progress in the field.

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Review Article

The Pathophysiology of HIV-/HAART-Related Metabolic Syndrome Leading to Cardiovascular Disorders: The Emerging Role of Adipokines

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Individuals infected with human immunodeficiency virus (HIV) frequently demonstrate metabolic syndrome (MS) associated with increased incidence of cardiovascular disorders. Characteristics of HIV infection, such as immunodeficiency, viral load, and duration of the disease, in addition to the highly active antiretroviral therapy (HAART) have been suggested to induce MS in these patients. It is well documented that MS involves a number of traditional cardiovascular risk factors, like glucose, lipids, and arterial blood pressure abnormalities, leading to extensive atherogenic arterial wall changes. Nevertheless, the above traditional cardiovascular risk factors merely explain the exacerbated cardiovascular risk in MS. Nowadays, the adipose-tissue derivatives, known as adipokines, have been suggested to contribute to chronic inflammation and the MS-related cardiovascular disease. In view of a novel understanding on how adipokines affect the pathogenesis of HIV/HAART-related MS and cardiovascular complications, this paper focuses on the interaction of the metabolic pathways and the potential cardiovascular consequences. Based on the current literature, we suggest adipokines to have a role in the pathogenesis of the HIV/HAART-related MS. It is crucial to understand the pathophysiology of the HIV/HAART-related MS and apply therapeutic strategies in order to reduce cardiovascular risk in HIV patients.

1. Introduction

Treatment with highly active antiretroviral therapy (HAART) in patients infected with human immunodeficiency virus (HIV) has been documented to significantly increase life expectancy [1]. However, adverse metabolic effects like dyslipidemia, increased blood pressure, and in-sulin resistance have been attributed to HAART [2, 3]. Therefore, the use of HAART raises concerns regarding metabolic disorders and cardiovascular risk in HIV-infected patients who now present an extended life expectancy. An increase of approximately 26% of the risk for myocardial infarction has been reported in patients on HAART [4]. The detrimental effect of HAART on the arterial wall properties [5–8] has been proposed as an underlying mechanism, while it has been documented that HIV infection per se may promote atherosclerosis through immunodeficiency, chronic inflammation progress, viral load, and endothelial cell dysfunction, and either directly or indirectly via metabolic risk factors [9–13]. The exact pathophysiological events underlying the development of metabolic changes in HIV-infected patients are still under investigation. Several studies have identified specific defects in adipocyte function as main drivers in the pathogenesis of some of the metabolic changes in these patients. The list of adipocyte-secreted cytokines, known as adipokines, has been continuously expanded to include biomolecules, such as leptin, adiponectin, resistin, visfatin, apelin, acylation stimulating protein, omentin, and vaspin. In addition to this, TNF-α, adipose-derived interleukins, and acute-phase proteins have been also considered as adipokines by some researchers [14]. We should mention that the current terminology refers to a cytokine as an immunomodulating agent. Taking this into account, adiponectin, leptin, and resistin are not appropriately considered as adipokines, since they do not act on the immune system. Nevertheless, these peptides are still referred to as adipokines in the

Table 1: The main differences between the pathogenesis of MS in HIV-infected patients and other patients.

	HIV-infected patients	Non-HIV-infected patients
(A)	HAART-induced dyslipidemia, hypertriglyceridemia, HDL reduction, especially if PI used	Fat abnormal metabolism leading to hypertriglyceridemia and dyslipidemia
(B)	HAART-induced leptin deficiency and hypoadiponectinemia leading to insulin resistance	Hypoadiponectinemia leading to insulin resistance and abnormal glucose metabolism
(C)	HIV-associated "lipodystrophy" syndrome—body fat abnormalities—fat accumulation around the neck, dorsocervical region as "buffalo hump," abdomen, and trunk	Waist circumference enlargement due to abdominal fat accumulation

literature; however, they could be more accurately put into the larger, growing list of adipose-tissue-derived hormones. The role of those adipose-tissue-secreted hormones in the pathophysiology of HIV-/HAART-related metabolic syndrome (MS) in HIV-infected patients is still the subject of intense research. Therefore, we decided to review published data regarding the emerging role of adipokines in the increased cardiovascular risk in HIV-infected patients related to the HIV/HAART-associated MS. The main differences between the pathogenesis of MS in HIV-infected patients and other patients are summarized in Table 1.

2. Body Fat and Waist Circumference Abnormalities in HIV-Infected Patients

The prevalence of the HIV-associated "lipodystrophy" syndrome according to previous studies approaches 80% of patients receiving HAART [15], while other studies report only a prevalence of 17% [16]. Significant differences in "lipodystrophy" rates exist when comparing patients with or without HAART. In one of these studies the prevalence of any body change was 62% in protease inhibitor (PI)-experienced patients, 33% in PI-naive patients and 21% in antiretroviral-naive patients [17]. There seems to be a lower prevalence of morphological body shape and fat redistribution changes in HIV-infected children, while an increase in serum adipokine levels has been additionally described [18]. According to the European Paediatric Lipodystrophy Group, approximately a quarter of children and adolescents show signs of lipodystrophy, similar to those described in adults [19].

Lipoatrophy typically includes decreased subcutaneous fat in the upper or lower extremities with prominent veins, loss of buttock subcutaneous fat, and facial atrophy [20]. The fat wasting should be differentiated from other wasting conditions associated with HIV infection, including the AIDS-wasting syndrome, malnutrition, cachexia, adrenal insufficiency, and severe chronic infections.

Lipodystrophy is characterized by lipoatrophy/fat loss, lipohypertrophy/fat accumulation, or both [20]. Fat accumulation can be seen around the neck, the dorsocervical region as "buffalo hump," the abdomen, and the trunk or as subcutaneous fat deposits, that is, lipomas, particularly in the dorsocervical area. These findings can be either symmetric or asymmetric. Breast enlargement has also been observed.

3. Dyslipidemia and Insulin Resistance in HIV-Infected Patients

The association of dyslipidemia with many antiretroviral regimens and especially PIs has been well established [21]. The effect on total cholesterol levels appears to be regimen dependent as shown in the Swiss HIV-1 Cohort Study [22]. Potential mechanisms for PI-associated dyslipidemia include (a) inhibition of sterol regulatory element-binding protein-1 (SREBP-1) activation in the liver and/or adipocytes along with the protease-mediated breakdown of apolipoprotein-B [23], (b) direct enhancement of the formation of very-low-density lipoproteins (VLDLs) [24] and the reduction of lipoprotein lipase activity [25], and (c) changes in the mobilization of lipid stores [26]. Nevertheless, lipid disorders can also occur during therapies not including PIs [27, 28]. Insulin resistance is also a significant metabolic side effect associated with HAART. PIs affect insulin sensitivity through various mechanisms such as IRS-1 phosphorylation and subsequent glucose uptake from adipocytes [29]. Lipodystrophy may also result in B-cell dysfunction [30] and is associated with impaired feedback of insulin on B-cells [31]. On the other hand, HIV-1 infection itself may be independently linked to the attenuation of insulin sensitivity. The HIV-1 accessory protein Vpr induces transcription of glucocorticoid-responsive promoters, *in vitro*, thus increasing sensitivity to glucocorticoids [32]. It also attenuates peroxisome-proliferator-activated receptor-γ (PPAR-γ) activity [33] and interferes with the suppressive effects of insulin on forehead transcription factors [34]. Therefore, it contributes to the tissue-selective insulin resistance. The end result of all the described factors is the attenuation of insulin sensitivity.

4. Parameters of HIV-/HAART-Induced Metabolic Syndrome and Cardiovascular Disorders

Concerning arterial stiffness, expressed by pulse wave velocity (PWV), and markers of metabolic profile, we recently compared HIV-infected patients age- and sex-matched individuals with either with hypertension or without any chronic disease [35]. In that study, HIV-infected patients had higher PWV levels than healthy controls, but lower than hypertensive patients. Notably, patients on HAART had similar PWV to hypertensive patients. In multivariate analysis, the independent determinants of increased arterial

stiffness were HAART duration and MS parameters, like serum lipids and blood pressure.

In our previously published study, we performed a comparative evaluation of endothelial dysfunction between HIV-positive individuals and age- and sex-matched controls with similar risk factors and a group of patients with established coronary artery disease (CAD). HIV-infected patients presented endothelial dysfunction to a similar extent as patients with CAD. Moreover, HIV-infected patients taking PIs had higher blood pressure, cholesterol, and triglycerides than those not taking PIs. Importantly, endothelial dysfunction was associated with elevated serum triglycerides. Therefore, we concluded that HAART-induced hypertriglyceridemia might have been a plausible mechanism explaining endothelial dysfunction in HIV-infected individuals. In the same study, we found an increased carotid intima media thickness (IMT), an index of subclinical carotid atherosclerosis, in HIV-infected patients. Most importantly, carotid IMT levels were equivalent in HIV-infected and CAD groups. So, we suggested that subclinical carotid atherosclerosis was closely related to PI-related changes of metabolic parameters in HIV-infected patients.

Current recommendations by the National Cholesterol Education Program for HIV-infected persons focus on LDL-C levels, as the primary target of the lipid-lowering therapy. The LDL cholesterol goal has been set <160 mg/dL for persons with 0-1 cardiovascular risk factors, <130 mg/dL for persons with multiple (2+) risk factors, and <100 mg/dL for persons with established coronary heart disease (CHD) or CHD risk equivalents. After lifestyle modifications, statins should be used to lower LDL-C levels. Therapy with fibrates is recommended to lower triglycerides levels. However, omega-3 fatty acids can be effective means of triglycerides lowering as well, particularly in patients with markedly elevated triglycerides levels. The efficacy of statins in HIV-infected persons appears to be lower than expected, although adherence to statins therapy has not been well assessed. Statins combining high potency and minor interactions with antiretroviral therapy (pravastatin, fluvastatin, atorvastatin, and rosuvastatin) should be preferred as the initial therapy, though comparative studies in HIV-infected persons are scarce.

Adequate choice and dosing of lipid-lowering drugs, given as single agents or in combination therapy, and care for drug compliance in HIV-infected patients at moderate or high cardiovascular risk should help maximize their long-term health.

5. HIV-/HAART-Induced Metabolic Syndrome: The Role of Adipokines

Visceral adipose tissue (VAT) is the predominant adipose tissue compartment responsible for the production of adipokines. A growing body of evidence supports the emerging role of adipokines in metabolic homeostasis and atherosclerosis. In this paper, we have reviewed the recent progress regarding the role of adipokines in the HIV/HAART-induced MS and cardiovascular disease (CVD). A better understanding of the molecular mechanisms will lead to the discovery of new drugs and reduce the incidence of lipodystrophy and related metabolic complications in HIV-infected patients receiving HAART.

5.1. Leptin. Leptin, which was the first adipokine identified, influences food intake through direct effects on the hypothalamus [36]. This adipocyte-derived hormone has actions in the brain (e.g., hypothalamus, cortex, and limbic areas) and in a number of peripheral tissues as well as cells of the pancreas, liver, and immune system. The central actions of leptin include energy and glucose homeostasis, reproductive functions, and immunity [37, 38]. The relationship between adiposity and leptin levels appears similar to controls and HIV infected but untreated patients [39]. On the other hand, severe lipodystrophy syndromes are characterized by loss of subcutaneous adipose tissue and a relative deficiency of leptin [40]. The effect of HAART on leptin levels is subject of controversy. In few studies, HAART administration had been associated with lipodystrophy and hypoleptinemia [41], while numerous studies had predominantly demonstrated no effect of HAART on leptin concentrations [42]. The above discrepancy was mainly attributed to the differential effects of HAART on fat-mass distribution and not directly to leptin per se [43]. Indeed, HAART without fat-mass re-distribution did not influence leptin levels [44]. Moreover, there is a weak correlation of leptin with insulin sensitivity in HIV-infected population [45].

Accumulating data support the proinflammatory and proatherogenic properties of leptin in either noninfected or HIV-infected patients [46, 47]. Although there is no prospective study evaluating the association of leptin with long-term cardiovascular events in HIV infected patients, the high levels of leptin in that population apparently increases the inflammatory milieu and perhaps the total cardiovascular risk. Future studies will elucidate the role of leptin in CVD progression in HIV-infected patients.

5.2. Adiponectin. Adiponectin, a well-studied adipokine, is secreted by fatty cells and is widely regarded to exert a counterregulatory role in atherogenesis, by its antioxidant, anti-inflammatory, antithrombotic, and direct anti-atherosclerotic properties [48]. Adiponectin expression is suppressed in patients with obesity and type 2 diabetes, showing an inverse relationship with insulin resistance and visceral adiposity [49]. Treatment-naïve, HIV-1-positive patients appear with suppressed adiponectin levels [50]. In previous studies, circulating adiponectin levels were suppressed in patients with chronic HIV infection and fat redistribution, but the underlying mechanisms remain obscure [51, 52]. Moreover, patients treated with HAART, especially those with lipodystrophy, showed gradual downregulation in adiponectin serum levels [53]. Notably, in the latter subgroup of patients, the HAART-induced hypoadiponectinemia was associated with accelerated cardiovascular impairment [54]. Taken all together, the suppression of adiponectin levels in HIV-infected patients under HAART may deteriorate numerous metabolic parameters (e.g., insulin resistance, lipid profile, etc.) leading to detrimental cardiovascular events.

5.3. Resistin. Despite the quite promising data from rodent studies, human data did not consistently confirm the association of resistin with insulin resistance, diabetes, and obesity [55]. Contrary to the aforementioned findings, elevated resistin levels have been found in HIV-infected patients compared to uninfected individuals. That difference was ascribed to HAART-related metabolic changes [56, 57]. Perhaps, HIV/HAART-related MS alters the regulatory mechanisms of resistin, but this hypothesis requires further investigation. On the other hand, the predominant sources of human resistin are macrophages and mononuclear leukocytes, and to a lesser extent, adipocytes [58]. Conditions of low-grade systemic inflammation, such as diabetes and atherosclerosis, may induce macrophage expression of resistin and increase circulating levels, independently of metabolic changes. The latter notion is also supported by the previously reported contributory role of resistin to pathologic processes, like inflammation, endothelial dysfunction, thrombosis, and smooth muscle cell dysfunction, leading to CVD [59]. Unambiguously, future studies will shed more light on the interplay between resistin and HIV/HAART-related MS and CVD.

5.4. Visfatin. Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT), functions as a growth factor for early B cells within the immune system [60]. Although visfatin is expressed and regulated by the adipose tissue, its relationship with adiposity-related insulin resistance is controversial [61, 62]. Regarding the impact of HAART on visfatin, a single study demonstrated significantly increased serum visfatin levels after HAART initiation, along with insulin resistance augmentation, and without concomitant changes in fat mass [63]. Thus, the fluctuations of insulin resistance and glucose homeostasis in HIV-positive patients may explain the regulation of visfatin.

Importantly, patients with stable coronary and carotid disease appear with high circulating levels of visfatin [64, 65], while macrophages derived from human unstable carotid and coronary plaques increasingly express visfatin [66]. Future studies clarifying the involvement of proinflammatory visfatin in the HIV/HAART-associated MS may better define the cardiovascular risk.

5.5. Apelin. Apelin, an adipocyte-secreted factor, has been recently identified as a contributor to glucose homeostasis and insulin resistance [67]. Abundant expression of apelin and its receptor, APJ, has been detected in endothelial cells from large arteries and coronary blood vessels and in the heart [68]. Moreover, previous trials have suggested apelin as a potent regulator of cardiovascular function [69]. We and other investigators have recently documented the inverse relationship between circulating apelin levels and CHD [70, 71].

The interplay between apelin with HIV infection and initially reported by Zou et al. who described the inhibition of HIV-1 and HIV-2 entrance in CHO and NP-2 cells expressing CD4 and its receptor after preincubation with apelin [72]. Moreover, apelinreceptor has been shown *in vitro* to act as HIV-1 coreceptor [73]. Taken together, more functional studies are required to determine the precise role of apelin/APJ in cardiovascular regulation, insulin resistance, and the susceptibility to HIV infection. This information would help to evaluate its potential as a future drug target.

5.6. Vaspin. A novel adipokine, vaspin, has been recently designated as a mediator of obesity, insulin resistance, and type 2 diabetes [74]. Both animal and clinical studies suggest that elevated vaspin levels in serum and adipose tissue may be a compensatory response to elevated insulin resistance, secondary to metabolic complications [75]. Extremely limited data implicate the association of low serum vaspin levels with atherosclerosis development and progression [64, 76]. Although vaspin exerts insulin-sensitizing and atheroprotective actions, its relationship with cardiovascular complications in HIV/HAART-related MS has not been investigated.

6. Conclusions

Adipokines appear to have a leading role in the pathogenesis of the HIV/HAART-related MS [77]. Leptin deficiency and hypoadiponectinemia, for example, correlate with insulin resistance and body fat abnormalities. These disorders affect the cardiovascular health of HIV patients through the amelioration of atherosclerosis and endothelial dysfunction. Furthermore, novel adipokines, such as visfatin, apelin, and vaspin, have emerged as potential mediators of the interplay between MS and atherosclerosis in HIV-infected patients. It is of great interest to study the pathological mechanism of the HIV/HAART-related MS and its cardiovascular complications and try to apply therapeutic strategies in order to reduce cardiovascular risk in HIV patients.

Abbreviations

AIDS: Acquired immunodeficiency syndrome
CHD: Coronary heart disease
CHO: Chinese hamster Ovary
CVD: Cardiovascular disease
HIV: Human immunodeficiency virus
HAART: Highly active antiretroviral therapy
IMT: Intima media thickness
LDL: Low-density lipoprotein
MS: Metabolic syndrome
NAMPT: Nicotinamide phosphoribosyltransferase
NNRTI: Non-nucleoside reverse transcriptase inhibitors
NP: Neural progenitors
NRTI: Nucleoside reverse transcriptase inhibitors
PI: Protease inhibitors
PPAR-γ: Peroxisome-proliferator-activated receptor-γ
PWV: Pulse wave velocity
SREBP: Sterol regulatory element-binding protein
TNF: Tumor necrosis factor
VAT: Visceral adipose tissue
VLDL: Very-low-density lipoprotein.

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Research Article

Prevalence of Metabolic Syndrome Components in an Urban Mexican Sample: Comparison between Two Classifications

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Background. The aim of this study was to examine the prevalence of metabolic syndrome (MS) components in an urban Mexican sample. *Methods*. A total of 854 subjects were included. Anthropometric, blood pressure measurements, clinical data, and overnight fasting blood samples were obtained from all subjects. *Results*. In accordance with definitions by the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) and the International Diabetes Federation (IDF), the prevalence of MS among participants was 59.7 and 68.7%, respectively. The prevalence of MS was higher in women and in individuals older than 45 years of age. More than 40% of the subjects fulfilled four criterions of MS according to both definitions. *Conclusions*. There was a high prevalence of MS components in an urban Mexican sample. Therefore, strong strategies had to be developed for early detection of MS and its components to prevent DMT2 and atherothrombotic complications in these patients.

1. Introduction

he original description of the metabolic syndrome (MS) by Reaven [1] consisted of abdominal obesity, insulin resistance, high blood pressure, impaired glucose tolerance or diabetes, hyperinsulinemia and dyslipidemia characterized by elevated triglyceride, and low HDL concentration. All of the features described above are risk factors for atherosclerosis, and thus MS constituted a significant risk for atherothrombosis disease. The World Health Organization in 1998 [2] and the Adult Treatment Panel III (ATP III) in 2001 standardized the definitions of MS [3]. In 2005, The International Diabetes Federation (IDF) formulated a new definition of the MS in a global consensus statement [4]. The prevalence of MS in adults varies from one population to another worldwide. Several studies indicate that, in USA, one-third of adults [5] with an alarming proportion of adolescents and children [6, 7] have the MS. Also a high prevalence of MS in Europe has been demonstrated [8]. It was reported that non-diabetic subjects under 40 years of age had an MS prevalence of 14–41%, depending on the age range [8]. In Mexico, several studies had documented a high prevalence of MS [9, 10], with an increased tendency due to changes in lifestyle behavior (overweight and obesity, physical inactivity, high carbohydrate diets, alcohol, and tobacco consumption) and genetic predisposition. The prevalence of the components

of MS is increased in obesity [11] and is associated with atherothrombotic complications in micro- and macrovascular territories [12, 13]. The syndrome is also strongly associated with the increased risk of coronary heart disease and type 2 diabetes mellitus (T2DM) [14–17]. The abnormal metabolic state that accompanies diabetes causes arterial dysfunction. Relevant abnormalities include chronic hyperglycemia, dyslipidemia, and insulin resistance [18, 19]. Atherothrombotic disease is the leading cause of dead worldwide and is the result of genetic and environmental factors [20, 21].

We have previously identified genetic variants associated with myocardial infarction and stroke in Mexican young population, in whom components of MS are present [22, 23]. Therefore, the aim of this study was to examine the prevalence of components of syndrome (MS), among Mexican adult population, and to evaluate the genetic participation in this group of patients with high vulnerability to T2DM and atherothrombotic disease.

2. Materials and Methods

We conducted a study to identify the prevalence of MS components using two definitions: the IDF and AHA/NHLBI in an urban Mexican sample from Mexico City. Individuals >20 years of age were invited to participate in the study if they were interested to know the risk to develop cardiovascular disease or T2DM. The recruitment period lasted for 1 year (1 May–30 May 2011). All included subjects provided the informed written consent to participate in the study.

2.1. Methods. Subjects were interviewed privately by a physician using pretest questionnaires. The following, demographic and clinical data were collected at the time of the interview: sex, age, cigarette smoking previous diseases, familial history of diabetes, and atherothrombotic disease.

Waist circumference (WC) was measured at the midpoint between the last rib and the iliac crest with participants standing and wearing only undergarments. Body weight was measured by precision scale while subjects were minimally clothed without shoes. Height was measured in a standing position without shoes using tape meter while the shoulders were in a normal state. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Patients were defined as overweight with BMI of 25.0–29.9 kg/m^2, and obese with a BMI $\geq$30 kg/m^2. All measurements were taken by the same person. Blood pressure (at rest) was measured with the participant seated. Two readings were taken in 5-minute interval between these two separated measurements, and thereafter the mean of the two measurements was considered to be the participant's blood pressure. The subjects were considered smokers if they were currently smoking (regularly or occasionally, including also former smokers defined as people who stopped smoking at least one year before the examination). A familial history of atherothrombotic disease was defined as acute myocardial infarction (AMI), stroke, or sudden death in a first-degree male relative younger than 55 years of age or a female relative younger than 65 years of age.

2.2. Laboratory Methods. In the morning, after an overnight fast, venous blood was sampled for the measurements of the serum glucose, total and HDL cholesterol, triglycerides, hs-CRP, uric acid, and plasma concentration of fibrinogen. Plasma low-density lipoprotein (LDL) cholesterol was calculated with the equation of Friedewald et al. [24], except when triglycerides exceeded 400 mg/dL. Buffy coat was collected and frozen for genetic studies.

Plasma levels of LDL, fibrinogen, uric acid, hs-CRP, and HbA1c were considered high if they were above 160 mg/dL, 400 mg/dL, 6 mg/dL in women and 7 mg/dL in men, 3.0 mg/L, and 6.5%, respectively.

The study protocol was reviewed and approved by the Human Ethical Committee and Medical Research Council of Instituto Mexicano del Seguro Social and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Informed written consent was obtained from all subjects before enrollment.

2.3. Diagnostic Criteria of the Metabolic Syndrome. The MS was defined according to each of the IDF and AHA/NHLBI definitions as described in Table 1 [4, 25].

2.4. Statistical Analysis. Continuous data are expressed as the mean ± standard deviation (SD); categorical data are expressed with percentages. The significance of differences between continuous variables was determined by Student's t-test. Differences between categorical variables were determined with the chi-square test. A P value <0.05 was considered as statistically significant. All statistical analyses were performed using SPSS (statistical package for the social sciences) statistical software package (version 16: SPSS Inc, Chicago, Il, USA).

3. Results

A total of 854 subjects were included in this study (see Figure 1). To ascertain whether different definitions may yield different prevalence, MS was diagnosed on the bases of American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) and International Diabetes Federation (IDF) criterions (Table 1).

Table 2 shows demographic, clinical, and biochemical features of subjects with MS (MS+), and without MS (MS−). The metabolic syndrome was identified in 607 individuals (407/woman versus 200/men). The mean of age of the subjects MS+ and MS− was similar (53.4 ± 11.0 versus 49.3 ± 13.4, $P = 0.43$), respectively. The body mass index (BMI) was MS+ 29.9 ± 4.8 versus MS− 26.7 ± 4.2, $P < 0.001$. There was a statistical significance in terms of waist circumference (MS+ 97.3 ± 10.8 cm versus MS− 88.5 ± 11.5 cm) ($P = 0.001$). There was a higher triglycerides levels in the group of MS+ compared with MS− (233.8 ± 220.7 versus 129.0 ± 7.3) ($P < 0.001$). The high-density lipoprotein cholesterol (HDL-C) was lower in the group of patients with MS ($P < 0.001$). There was no differences in total cholesterol between both groups ($P = 0.58$). The concentration of fibrinogen, uric acid, and HbA1c was higher in the group of MS+, compared with MS−.

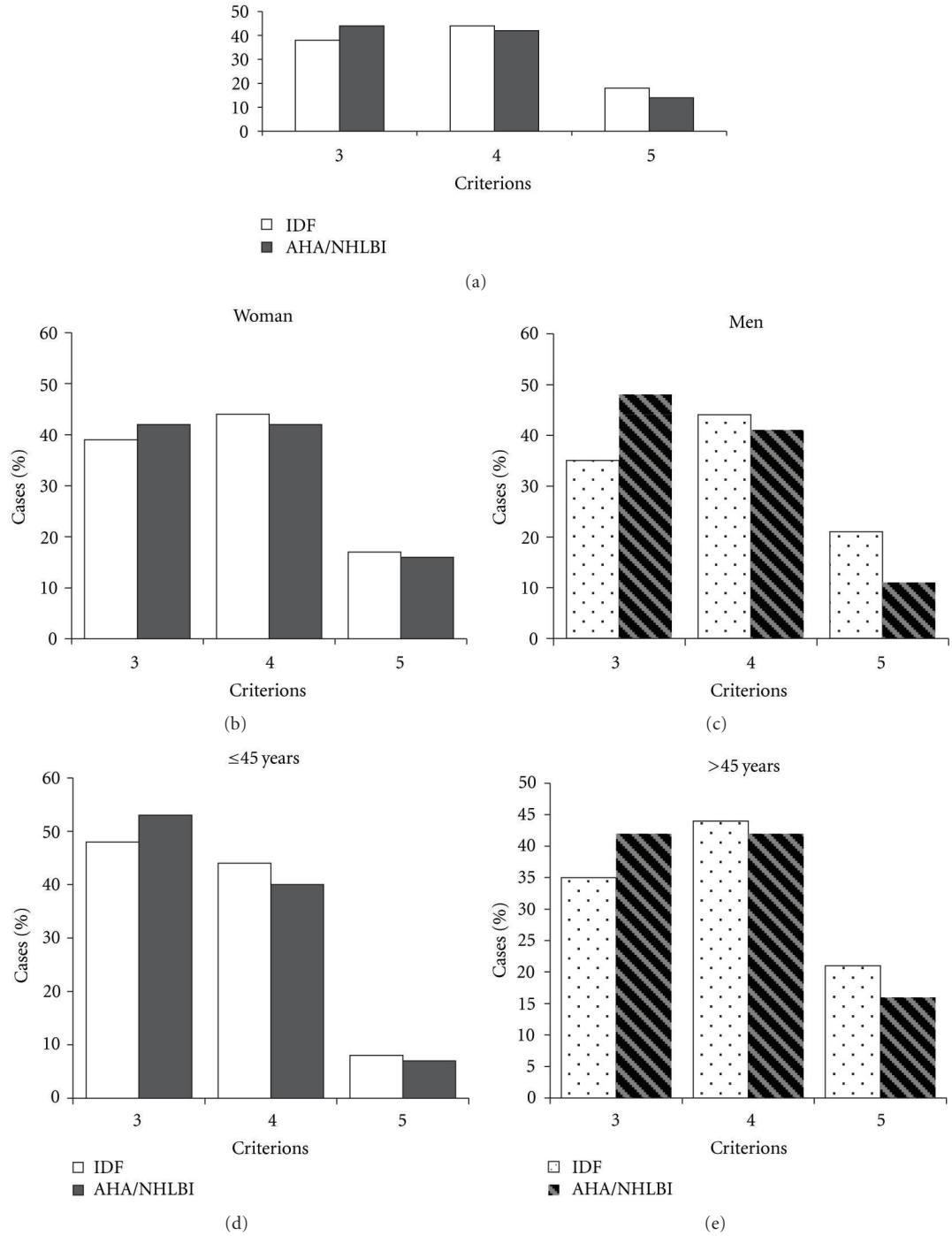

Figure 1: Percent of patients with number of criterions based on IDF y AHA/NHBLI definitions stratified by sex and age. Regardless of the severity of the metabolic syndrome, we found that 43.7% of the individuals fulfilled four criterions when MS was diagnosed by IDF, and 45.3% of the subjects had three components when was used the AHA/NHLBI definition. Similar results were found when we did the analysis stratified by gender. As we expect, in the analysis stratified by age-based MS by IDF and AHA/NHLBI definitions, we found that individuals ≤45 years old fulfilled 3 criterions, whereas four components were present in the older group (43.8%).

As it was expected there was a higher frequency of cardiovascular risk factors such as smoking, hypertension, and familial history of atherothrombotic disease in the group of individuals with MS. The prevalence of MS diagnosed based on IDF, AHA/NHLBI, or combination of both definitions was (68.7%, 59.7%, and 57.2%), respectively.

Table 3 shows the analysis of metabolic syndrome according to the IDF definitions. There was no difference in mean of age between men (53.1 ± 9.5 years) and women (53.7 ± 11.3 years). It was more frequent in women (67.7%) than in men (32.3%). Reduced HDL cholesterol was more frequent in woman (93.2%) compared with the group of

Table 1: Criteria for Clinical diagnosis of the metabolic syndrome by the IDF and AHA/NHLBI definitions.

The IDF definition	The AHA/NHLBI definition
Central obesity: defined as waist circumference ≥90 cm for Asian men and ≥80 cm for Asian woman plus any two of the following four criteria	Three or more of the following
	Central obesity: defined as waist circumference ≥102 cm for men and ≥88 for woman
Raised fasting plasma glucose (FPG): ≥100 mg/dL, or previously diagnosed type 2 diabetes	Raised fasting plasma glucose (FPG): ≥100 mg/dL or previously diagnosed type 2 diabetes
Raised triglycerides ≥150 mg/dL, or specific treatment for this lipid abnormality	Raised triglycerides ≥150 mg/dL or specific treatment for this lipid abnormality
Reduced HDL cholesterol: <40 mg/dL in males and <50 mg/dL in females	Reduced HDL cholesterol: <40 mg/dL in males and <50 mg/dL in females
Elevated blood pressure ≥130/≥85 mm Hg, or previous medical diagnosis of hypertension	Elevated blood pressure ≥130/≥85 mm Hg, or previous medical diagnosis of hypertension

Table 2: Demographic, clinical, and biochemical features of subjects with and without metabolic syndrome included in the study.

	MS+	MS−
Subjects *n* (%)	607 (70%)	247 (30%)
Gender *n* (%)		
Man	200 (33.0%)	74 (30.0%)
Women	407 (67.0%)	173 (70.0%)
Age (years)	53.4 ± 11.0	49.3 ± 13.4
Body mass index (kg/m^2)	29.9 ± 4.8	26.7 ± 4.2
Waist circumference (cm)	97.3 ± 10.8	88.5 ± 11.5
Triglycerides (mg/dL)	233.8 ± 220.7	129.0 ± 7.3
HDL-C (mg/dL)	36.02 ± 9.0	48.0 ± 13.7
Total cholesterol (mg/dL)	211.8 ± 61.5	205.8 ± 42.4
FPG (mg/dL)	158.3 ± 48.1	94.8 ± 16.9
Fibrinogen (mg/dL)	377.0 ± 89.2	3 69.1 ± 71.5
Uric Acid (mg/dL)	5.4 ± 2.8	3.9 ± 1.3
hs-CRP (mg/dL)	1.02 ± 0.5	0.78 ± 0.22
HbA1c (%)	5.0 ± 2.6	4.3 ± 2.1
Currents smoking *n* (%)	136 (22.4)	45 (18.2)
Blood pressure ≥130/≥85 mmHg or previous diagnosis of hypertension		
n (%)	301 (49.58)	
Previously diagnosed hypertension *n* = (%)	282 (46.45)	23 (30.2)
New cases of hypertension *n* = (%)	19 (3.13)	
Elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM		
n (%)	398 (65.56)	
Previously T2DM *n* = (%)	165 (27.18)	33 (30.2)
New cases of T2DM *n* = (%)	26 (4.28)	
No T2MD patients with FPG ≥100 mg/dL–125 mg/dL *n* = (%) 209 (34.43)		
FH of AT *n* (%)	112 (18.45)	30 (14.5)

HDL-C = high-density lipoprotein cholesterol, FG = fasting glucose, hsCRP = high sensitivity C protein reactive, HbA1c = glycosylate hemoglobin, T2DM = type 2 diabetes mellitus, IDF = International Diabetes Federation, AHA/NHLBI = American Heart Association/National Heart, Lung, and Blood Institute, FH of EAT = familial history of atherothrombosis.

men (90.0%). The prevalence of elevated triglycerides was similar in both groups. There was a similar percent of individuals with elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM women (64.8%) versus men (67.3%). In the group of women (64.8%) with the criteria before mentioned, 35.8% had glucose level between ≥100 mg/dL and <126/mg/dL, and 29.0% had been already diagnosed with T2DM whereas, in the group of men, 33.1% had ≥100 mg/dL

Table 3: Prevalence (%) of MS and its components to IDF definition, by gender.

Measure (any 3 of 5 constitute diagnosis of MS)	Men	Women
	Total = 587	
	n = 190 (32.3%)	n = 397 (67.7%)
Age (years)	53.1±	53.7±
Waist circumference n (%)	190 (100%)	397 (100%)
Men ≥90 cm		
Women ≥80 cm		
Reduced HDL-C n (%)	171 (90.0%)	370 (93.2%)
Men <40 mg/dL		
Women <50 mg/dL		
Triglycerides ≥150 mg/dL or medical treatment of elevated TG n (%)	142 (74.7%)	293 (73.8%)
Elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM	128 (67.3 %)	257 (64.8%)
Blood pressure ≥130/≥85 mmHg or previous diagnosis of hypertension n (%)	104 (54.7%)	183 (46.1%)

Table 4: Prevalence (%) of MS and its components to AHA/NHLBI definition, by gender.

Measure (any 3 of 5 constitute diagnosis of MS)	Men	Women
	Total = 510	
	n = 155 (30.4)	n = 355 (69.6%)
Age (years)	53.6±	53.9±
Reduced HDL-C n (%)	148 (95.5%)	332 (93.5%)
Men <40 mg/dL		
Women <50 mg/dL		
Triglycerides ≥150 mg/dL or medical treatment of elevated TG n (%)	124 (80.0%)	270 (76.0%)
Elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM	114 (73.5%)	250 (70.4%)
Blood pressure ≥130/≥85 mmHg or previous diagnosis of hypertension n (%)	101 (65.2%)	178 (50.2%)
Waist circumference n (%)	73 (47.0%)	295 (83.0%)
Men ≥102 cm		
Women ≥88 cm		

and <126/mg/dL, and 34.2% had been diagnosed with the disease. Hypertension was more common in men (54.7%) than in woman (46.1%).

Table 4 shows the MS diagnosed based on AHA/NHLBI criterions, and low HDL cholesterol was the most common with a similar frequency in both sexes (men 95.5%) versus (women 93.5%), followed by increased triglyceride levels with higher percentage in men (80.0%) and women (76.0%). There was a slightly high percent of individuals with elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM in the group of men (73.5%) versus women (70.4%). Hypertension was the least frequent component with 65.2% in the group of man versus 50.2% in woman. There was a very significant difference in waist circumference between both groups: men (47.0%) versus woman (83%).

Table 5 shows the MS diagnosed based on IDF definition and stratified by age (≤ y >45 years old). There was a higher percent of men in the group of younger individuals, whereas the women were the predominant gender in the older group. Followed by the waist circumference, the reduced HDL-C was the second most frequent criteria with 94.4% in individuals ≤45 years old versus >45 years (91.5%). We found a significant difference in elevated triglycerides levels between both groups of age, with higher levels in the young group (83.3%) versus the oldest one (71.6%). In terms of individuals with previous diagnosis of T2DM or elevated fasting glucose ≥100 mg/dL, there was a higher percent in the group of older individuals (68.3%) compared to the youngest one (55.6%). The percent of elevated blood pressure was higher in the old group against the youngest one (54.9% versus 27.0%).

Table 6 shows the results of the analysis of individuals with MS diagnosed based on the AHA/NHLBI stratified by age. The female group was predominant in both groups of age in the group of younger women (61.9%) and (69.4%) in the oldest one. The most common criterion was reduced HDL-C with 97.0% in the youngest group versus 93.4% in the old one. In contrast, as we expected we found elevated triglycerides levels in both groups, with higher percent in the group ≤45 years of age (86.1%) compared with the oldest group (75.0%). There was a slight difference in the percent of waist circumference between the groups (≤45 years, 74.3% versus >45 years, 71.6). Increased fasting glucose ≥100 mg/dL or previous diagnosis of T2DM was found in 67.3% of young individuals versus 72.4% <45 years of age. As it was expected, there was a higher percent of individuals with

Table 5: Prevalence (%) of MS and its components to IDF definition, by age.

Age (years)	≤45	Total = 587	>45
	$n = 126$		$n = 461$
Sex (%)			
Men	38.1		30.6
Women	61.9		69.4
Waist circumference *n* (%)	126 (100)		461(100)
Men ≥90 cm, women ≥80 cm			
Reduced HDL-C *n* (%)	119 (94.4)		422 (91.5)
Men <40 mg/dL			
Women <50 mg/dL			
Triglycerides ≥150 mg/dL or medical treatment of elevated TG *n* (%)	105 (83.3)		330 (71.6)
Elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM	70 (55.6)		315 (68.3)
Blood pressure ≥130/≥85 mmHg or previous diagnosis of hypertension *n* (%)	34 (27.0)		253 (54.9)

Table 6: Prevalence (%) of MS and its components to AHA/NHLBI definition, by age.

Age (years)	≤45	Total = 510	>45
	$n = 101$		$n = 409$
Sex	53.1±		53.7±
Men	38.1		30.6
Women	61.9		69.4
Waist circumference *n* (%)	75 (74.3)		293 (71.6)
Men ≥102 cm, women ≥88 cm			
Reduced HDL-C *n* (%)	98 (97.0)		382 (93.4)
Men <40 mg/dL			
Women <50 mg/dL			
Triglycerides ≥150 mg/dL or medical treatment of elevated TG *n* (%)	87 (86.1)		307 (75.0)
Blood pressure ≥130/≥85 mmHg or previous diagnosis of hypertension *n* (%)	29 (28.7)		250 (61.1)
Elevated fasting glucose ≥100 mg/dL or previous diagnosis of T2DM	68 (67.3)		296 (72.4)

high blood pressure or previous diagnosis of hypertension in the group of >45 years of age (61.1%) versus (28.7%) in the youngest group.

We want to point out that we identified an increased level of fasting overnight glucose ≥100 mg/dL and <126 mg/dL in the group of individuals ≤45 years of age when MS was diagnosed by IDF criterions (38.9%), versus >45 years of age (33.8%), and when diagnosed by AHA/NHLBI ≤45 years (46.5%) versus >45 years old (35.5%) (data not shown).

4. Discussion

Patients with MS die from complication of T2DM and atherothrombotic disease such as acute myocardial infarction and stroke. In previous studies, a high frequency of metabolic syndrome (MS) has been identified in our population. Therefore, the aim of this study was to identify phenotypic, specific and genotypic profile that may help to improve the prevention of T2DM, dyslipidemia, and atherothrombotic complication such as AMI and Stroke. Although the final results with respect to this principal aim are still awaited, the study clearly confirms that an early screening should be performed in individuals anticipated to be at risk of T2DM, dyslipidemia, and obesity by their primary care physician and should be treated in metabolic syndrome clinic.

The MS was present in 68.7% of the total sample, according to IDF definitions versus 59.7%, compared with AHA/NHLBI. In 57.2% of the individuals the MS was diagnosed base by either one definition IDF or AHA/NHLBI. We identify more individuals with MS by IDF classification compared to AHA/NHLBI. This result is probable due to the waist circumference lower cutoff applied by the IDF. In contrast, by AHA/NHLBI, we found an increased percentage of individuals with hypertension, dyslipidemia, and high levels of glucose, compare to IDF classification, because the waist circumference is not an absolute required criterion. This combination of criterions might represent a different severity of the MS between patients classified by one or another.

Those results are in agreement with those previously reported by Rojas et al. [26], who identified a less frequency of the syndrome by AHA/NHLBI compared with IDF in our population, but are in disagreement with those obtained by other investigators in American population >20 years [4].

Another relevant issue found in this study is the high prevalence of the numbers of criterions in each subject from this sample stratified by groups of age and gender by both classifications IDF and AHA/NHLBI. In the present study, we found a high percent of individuals who fulfilled four or more criterions. However, for the IDF classification, the waist circumference was the most frequent criterion, followed by lowered HDL-C and increased level of triglycerides, whereas for the AHA/NHLBI, the more frequent component was HDL-C, triglycerides, and waist circumference. The most important is that all three criterions are associated with an increased risk for cardiovascular disease, and they are frequently present in the same individual.

In approximately 42.2% of the individuals ≤45 years of age, who were diagnosed with MS by either classification, IDF, or AHA/NHLBI, registered glucose levels were between ≥100 mg/dL and 125 mg/dL. The most important thing is that they were not under lowering glucose therapy, because they did not have a recent blood glucose test.

On the other hand, as we expect there was a higher percent of individuals with T2DM >45 years old (35.7%) compared with the subjects ≤45 years old (18.7%) in both groups of patients diagnosed with either definition IDF or AHA/NHLBI. Those results corroborated that insulin resistance (IR) is more frequent in young individuals, whereas T2DM is predominant in older individuals. Therefore, an early detection and lifestyle changes have to be implemented in young individuals to avoid the development of chronic disease such as T2DM and atherothrombotic disease complications such acute myocardial infarction and stroke.

Also, similar results were obtained when we analyzed the triglycerides levels by age.

In 73.97% of individuals with MS diagnosed by either definitions, IDF or AHA/NHLBI were found to have an increased triglycerides levels (≥150 mg/dL), and HDL-C below normal ranges was diagnosed. Most of them had history of dyslipidemia, but only 19.6% were under lowering lipid therapy. In this particular case is necessary a rigorous and constant monitoring in patients with previous history of dyslipidemia.

Several new features have been added to the syndrome over time. These include elevated plasminogen activator inhibitor-1 (PAI-1) and C-reactive protein (CRP) concentrations. These features were added on the basis that they were frequently found in association with the metabolic syndrome. These features are probably related to both insulin resistance and obesity [27]. In the present study, we failed to identify, an association between high levels of hs-CRP and MS, and those results are in agreement with those previously published by Han et al. [28].

In contrast, we have previously reported that higher levels of plasma PAI-1 represent a risk factor for development of ST elevation acute myocardial infarction (STEAMI) [29].

Therefore, the following step will be determinate PAI-1 levels in the same group of patients with MS. Those results probabley allow us to include a new atherothrombotic marker in this type of patients in our population.

A previous study by Madrid-Miller [30] explores the clinical impact of MS in patients with acute coronary syndrome (ACS). The MS was more frequent in older patients with ACS and was associated with poorer in-hospital outcomes.

Since most patients with T2DM die from complications of atherosclerosis, they should receive intense preventive interventions proven to reduce their cardiovascular risk.

Using the AHA/NHLBI definitions, MS is present in 82.3 of self-reported coronary heart diseases (CHD), 87.5% of type 2 diabetes cases, 43.1% self-reported with high triglycerides, 47.6% of subjects with low HDL-cholesterol levels, and 70.5% of adults with hypertension [26].

That percentage provides a gross estimate of the contribution of MS to the outcomes mentioned above and justifies the screening of MS components in persons with those conditions. These results will be useful for updating local guidelines for the prevention and treatment of specific chronic disorders, which requires a multidisciplinary team approach that implements lifestyle changes and a combination of drugs (when appropriate). The primary goal of clinical management in individuals with MS is to reduce the risk of clinical atherosclerosis disease.

Therefore, our data describes the significant challenges that MS represents to our health system. The Mexican health system should develop specific management programs for all identified cases; failure to identify or treat cases of MS will result in a considerable increase in new forms of T2DM and/or CHD.

5. Conclusions

Our results identify that more than 40% of individuals with MS has four or more criterions, which represent the severity of the syndrome among urban Mexican sample. Therefore, strong strategies had to be developed for early detection of MS and its components in order to prevent DMT2 and atherothrombotic complications in these patients with myocardial infarction and stoke.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Pharmacological Inhibition of Soluble Epoxide Hydrolase Ameliorates Diet-Induced Metabolic Syndrome in Rats

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The signs of metabolic syndrome following chronic excessive macronutrient intake include body weight gain, excess visceral adipose deposition, hyperglycaemia, glucose and insulin intolerances, hypertension, dyslipidaemia, endothelial damage, cardiovascular hypertrophy, inflammation, ventricular contractile dysfunction, fibrosis, and fatty liver disease. Recent studies show increased activity of soluble epoxide hydrolase (sEH) during obesity and metabolic dysfunction. We have tested whether sEH inhibition has therapeutic potential in a rat model of diet-induced metabolic syndrome. In these high-carbohydrate, high-fat-fed rats, chronic oral treatment with *trans*-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB), a potent sEH inhibitor, alleviated the signs of metabolic syndrome *in vivo* including glucose, insulin, and lipid abnormalities, changes in pancreatic structure, increased systolic blood pressure, cardiovascular structural and functional abnormalities, and structural and functional changes in the liver. The present study describes the pharmacological responses to this selective sEH inhibitor in rats with the signs of diet-induced metabolic syndrome.

1. Introduction

Chronic metabolic dysfunction leading to obesity and diabetes represents a major challenge to health worldwide [1, 2]. Lifestyle changes such as an excessive caloric intake are indicated as important factors in initiating obesity and the associated metabolic and cardiovascular disorders [1–4]. This prevalent human condition is now collectively referred to as "metabolic syndrome" [3, 4]. Leading definitions of metabolic syndrome differ mainly in threshold values but in general emphasise the clustering of abdominal obesity, dyslipidaemia, hyperglycaemia, a prothrombotic state, and hypertension in increasing an individual's risk of developing type II diabetes mellitus, insulin resistance, and cardiovascular disease earlier in adult life [5–10]. This syndrome is associated with complications throughout the body such as excessive visceral fat deposition, hypertension, endothelial damage, cardiovascular hypertrophy, inflammation, atherosclerosis, ventricular contractile dysfunction, fibrosis and fatty liver disease [5, 8]. Identifying adequate therapeutic and preventive alternatives for this multifactorial syndrome has so far been challenging.

Arachidonic acid is the precursor of the eicosanoid family of signalling lipid mediators that modulate immune and inflammatory responses in the body [1, 11]. They are metabolised by cyclooxygenases, lipoxygenases, and cytochrome P450 monooxygenases to form biologically active products, including prostaglandins, leukotrienes, epoxyeicosatrienoic acids (EETs), and hydroxyeicosatetraenoic acids [1, 12, 13]. Pharmacological manipulation of the cyclooxygenase and lipoxygenase pathways improves adiposity and insulin sensitivity in humans and animal models [1, 14–16]. The cytochrome P450 enzymes generate EETs by catalysing the epoxidation of arachidonic acid [17]. EETs are endothelium-derived hyperpolarising factors that protect cells from ischaemic injury and possess anti-inflammatory responses

in canine and rodent disease models [17]. These endogenous lipid mediators are converted into inactive diols by soluble epoxide hydrolase (sEH), and so inhibiting this enzyme would be expected to enhance the stability and therapeutic actions of EETs [17]. Orally active and selective sEH inhibitors that are based on 1,3-disubstituted urea and to a lesser extent amides and carbamates have been developed for chronic *in vivo* studies; *in vivo* these derivatives have proven to be antihypertensive and anti-inflammatory and protect the brain, heart, and kidney from damage [17]. Further, these derivatives produced analgesic responses in pain models [18].

Although it is well established that sEH inhibition improves cardiovascular and renal diseases [19–21], the therapeutic potential of sEH inhibition in diet-induced metabolic syndrome is still largely unknown [17]. To test the hypothesis that EETs are important in metabolic control, this study has evaluated *trans*-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) (Figure 1), a potent sEH inhibitor [22] with greater *in vivo* metabolic stability than many other sEH inhibitors [22], in a rat model of diet-induced metabolic syndrome. We have investigated whether chronic oral treatment with *t*-AUCB improved adiposity, control of blood glucose, insulin, and lipid homeostasis and the associated structural and functional changes in metabolic, liver, and cardiovascular systems induced by high-carbohydrate, high-fat feeding in rats.

2. Materials and Methods

2.1. Diet-Induced Metabolic Syndrome in Rats. All experimental protocols were approved by the University of Queensland Animal Experimentation Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old) were obtained from The University of Queensland Biological Resources facility. The rats were randomly divided into four experimental groups and were fed with either corn starch diet (CS; $n = 24$) or high-carbohydrate high-fat diet (HCHF; $n = 24$) for 16 weeks. The CS diet contained 570 g corn starch, 155 g powdered rat food (Specialty feeds, Glen Forest, WA, Australia), 25 g Hubble, Mendel, and Wakeman (HMW) salt mixture, and 250 g water per kilogram of food. HCHF diet contained 175 g fructose, 395 g sweetened condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g HMW salt mixture, and 50 g water per kilogram of food [23]. In addition, the drinking water for the HCHF group was supplemented with 25% fructose [23]. 12 rats from each CS and HCHF group were randomised into *t*-AUCB treatment at 8 weeks. Rats were given *ad libitum* access to food and water and were individually housed in a temperature-controlled 12 hours light-dark conditions. *t*-AUCB was administered in the drinking water in both CS and HCHF groups with the final dose calculated from daily drinking water consumption. Final doses of *t*-AUCB were 1.4 mg/Kg/day in CS-fed and 0.95 mg/Kg/day in HCHF-fed rats. *t*-AUCB was dissolved in 10 mL of distilled water made slightly basic with sodium hydroxide and then diluted to 1 L with distilled water.

2.2. Physiological Parameters. Body weight and food and water intakes were measured daily. Oral glucose tolerance and clearance tests have been previously described [23, 24]. Plasma lipids and enzyme concentrations were measured by the Veterinary Pathology Service of The University of Queensland, QLD, Australia [23, 24]. Systolic blood pressure of rats was measured as previously described [23–26].

2.3. Body Composition Measurements. Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 16 weeks of feeding (2 days before rats were euthanised for pathophysiological assessments) as previously described [23].

2.4. Experimental Protocol. Eight rats from each group were used for isolated Langendorff's preparations and vascular reactivity studies, and four rats per group were taken exclusively for histopathological analysis. For terminal experiments, rats were euthanised with Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac, Peakhurst, NSW, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, NSW, Australia) was injected through the right femoral vein. The abdomen was then opened, and blood (~6 mL) was withdrawn from the abdominal aorta and collected into heparinised tubes and centrifuged at 5,000 ×g for 15 minutes to obtain plasma. Plasma was stored at −20°C for further characterisation. Hearts were removed from rats for isolated Langendorff's preparation, and thoracic aortic rings were used for vascular reactivity studies as described below [25]. Liver, kidneys, and fat pads were removed from these rats and weighed. After perfusion studies, right ventricles from the hearts were removed and weighed whereas the left ventricle was weighed with septum. Weights of these organs were normalised relative to the tibial length at the time of removal (expressed as tissue weight in mg/mm tibial length) [23]. Heart, liver, kidney, and pancreas from the rats used for histopathological analysis were removed and fixed in appropriate solutions [23].

2.5. Structural Changes in Liver, Pancreas, and Adipose Tissue. Liver and pancreas were fixed with 10% neutral buffered formalin for three days. These tissue samples were dehydrated and then embedded in paraffin wax. Thin sections (5 μm) of these tissues were cut and stained with Wright's staining for determination of inflammatory cell infiltration and for determining the fat vacuoles in liver. Liver sections were also stained with Milligan's stain to determine collagen deposition and to determine fat droplets. Routine histological stains used were Wright's for general histology and Milligan's for assessment of collagen deposition. Milligan's stained slides were visualised using a Nikon Eclipse 50*i* microscope (Kanagawa, Japan) fitted with a DSF*i*1 camera, and images were captured directly digitally (magnification ×200). Pancreatic sections were stained with aldehyde fuchsin staining following pretreatment with potassium permanganate (0.5%) and haematoxylin and eosin staining to determine infiltration of inflammatory cells. After staining, tissues were mounted, and pictures were taken with a Zeiss Microscope

(magnification ×40 for haematoxylin and eosin and ×20 for aldehyde fuchsin, oil red "O", and Milligan's stain). Islet area was quantified using NIH image J software (National Institute of Health, USA). Results are presented as mean ± SEM of the area of view. α and β cells in 12 islets were counted in each rat using NIH-image J software. Numbers of these cells are presented as mean ± SEM per islet [23]. Adipose tissue was fixed with 10% neutral buffered formalin for 1 day. Cryostat sections were prepared of this tissue in OCT as per normal histological procedures. Thick sections (40 μm) of these tissues were cut and stained with Wright's staining for determination of inflammatory cell infiltration.

2.6. Cardiovascular Structure and Function. Echocardiographic examination was performed as previously described in all rats after 16 weeks [23, 27]. The isolated Langendorff's heart preparation was used to assess left ventricular function of the rats in all the groups as in previous studies [23, 25]. End diastolic pressures were obtained from 0 mmHg up to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) as described in previous studies [23, 24, 26]. Thoracic aortic rings (4 mm in length) were suspended in an organ bath filled with Tyrode's physiological salt solution bubbled with 95% O_2–5% CO_2, maintained at 35°C, and allowed to stabilise at a resting tension of 10 mN. Cumulative concentration-response curves (contraction or relaxation) were obtained as in previous studies [25, 26]. Hearts were processed by two different procedures for histopathological studies as in previous studies [23, 25, 26].

2.7. Statistical Analysis. All data are presented as mean ± SEM. Differences between the groups were determined by one-way analysis of variance. Statistically significant variables were treated with the Neuman-Keuls *post hoc* test to compare all the groups of animals. For body weight data, Student's *t*-tests were performed. All statistical analyses were performed using Graph Pad Prism version 5 for Windows (San Diego, Calif, USA) with a P value of <0.05 considered as statistically significant.

3. Results

3.1. HCHF Diet Induces Signs of the Metabolic Syndrome. Young male Wistar rats fed HCHF diet showed progressive increases in body weight, abdominal fat deposition, and whole body fat mass along with impaired glucose tolerance, plasma lipid abnormalities, hyperinsulinaemia, and increased plasma leptin concentrations [23]. Cardiovascular changes included increased systolic blood pressure and endothelial dysfunction together with inflammation, fibrosis, hypertrophy, and increased stiffness of the left ventricle of the heart [23]. The liver showed increased wet weight, fat deposition, inflammation, and fibrosis with increased plasma activity of liver enzymes. The pancreas showed increased islet size. Treatment with *t*-AUCB from week 8 to 16 attenuated the metabolic, liver, and cardiovascular abnormalities, outlined below, but did not affect the increased body weight, abdominal fat deposition, and whole body fat mass (Table 1).

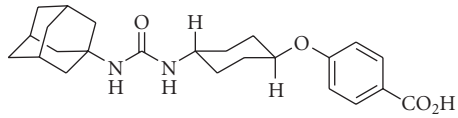

Figure 1: Chemical structure of the potent sEH inhibitor, *trans*-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB).

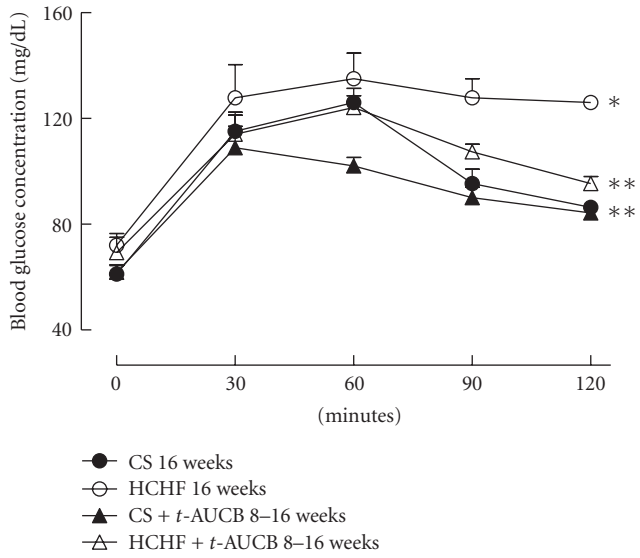

Figure 2: Plasma glucose concentrations following oral gavage of glucose (2 g/kg) recorded after 16 weeks for rats fed with corn starch (CS), high carbohydrate high fat (HCHF), corn starch with *t*-AUCB (CS + *t*-AUCB), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB). $^*P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

3.2. Treatment with t-AUCB Attenuated Metabolic Abnormalities. Overnight fasting blood glucose concentrations did not differ among the experimental groups. Following oral glucose loading, blood glucose concentrations in CS-fed rats increased after 30 mins then decreased rapidly and returned to fasting glucose concentrations at 120 mins after glucose loading (Figure 2). In HCHF-fed rats, blood glucose concentrations decreased much more slowly and were higher at 120 mins after glucose loading compared to CS-fed rats, suggesting glucose intolerance (Figure 2). Blood glucose concentrations at both 4-hour fasting and 75 mins after intraperitoneal administration of insulin were much higher in HCHF-fed rats compared to CS-fed rats suggesting insulin intolerance (Figure 3). Both of the above features were attenuated with *t*-AUCB treatment in HCHF-fed rats (Figures 2 and 3). Plasma concentrations of insulin were increased with HCHF diet after 16 weeks in comparison to CS diet (Table 1). Plasma leptin concentrations did not increase with the HCHF diet compared to CS diet at 16 weeks (Table 1). *t*-AUCB did not prevent the increased insulin concentrations in HCHF-fed rats but further increased insulin concentrations in CS-treated rats (Table 1). Further, plasma concentrations of NEFA, total cholesterol, and triglycerides were

Table 1: Physiological parameters of CS-, HCHF-, CS + *t*-AUCB-, HCHF + *t*-AUCB-treated rats. $^{*}P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

Parameter	CS (16 weeks)	HCHF (16 weeks)	CS + *t*-AUCB (16 weeks)	HCHF + *t*-AUCB (16 weeks)
Body weight @ week 16 (g)	422 ± 6 ($n = 6$)	515.8 ± 11.9 ($n = 6$)	415 ± 6 ($n = 6$)	515.7 ± 13 ($n = 6$)
Abdominal fat deposition (mg/mm tibial length)	401 ± 56 ($n = 6$)	746 ± 67* ($n = 6$)	270 ± 38 ($n = 6$)	674 ± 99* ($n = 6$)
Total fat mass (g)	56 ± 5 ($n = 6$)	139.4 ± 16* ($n = 6$)	46.7 ± 8 ($n = 6$)	154.3 ± 19* ($n = 6$)
Heart rate (bpm)	249 ± 9 ($n = 6$)	251 ± 21 ($n = 6$)	262 ± 13 ($n = 6$)	265 ± 8 ($n = 6$)
LVIDd (mm)	6.73 ± 0.2 ($n = 6$)	7.54 ± 0.18* ($n = 6$)	6.8 ± 0.2** ($n = 6$)	6.8 ± 0.2** ($n = 6$)
LVPWd (mm)	1.69 ± 0.1 ($n = 6$)	1.95 ± 0.1 ($n = 6$)	1.69 ± 0.08 ($n = 6$)	1.9 ± 0.1 ($n = 6$)
LVIDs (mm)	3.9 ± 0.3 ($n = 6$)	4.9 ± 0.2* ($n = 6$)	3.6 ± 0.2** ($n = 6$)	4.2 ± 0.2** ($n = 6$)
E/A ratio	1.6 ± 0.1 ($n = 6$)	1.2 ± 0.1* ($n = 6$)	2.0 ± 0.2** ($n = 6$)	1.5 ± 0.04** ($n = 6$)
Ejection fraction (%)	79 ± 4 ($n = 6$)	72 ± 2 ($n = 6$)	85 ± 2** ($n = 6$)	76 ± 3 ($n = 6$)
Cardiac output (mL/min)	63.4 ± 6.4 ($n = 6$)	83.8 ± 9 ($n = 6$)	81.8 ± 10 ($n = 6$)	72.2 ± 7 ($n = 6$)
Estimated LV mass (g)	0.74 ± 0.05 ($n = 6$)	1.0 ± 0.05* ($n = 6$)	0.77 ± 0.05** ($n = 6$)	0.84 ± 0.04** ($n = 6$)
Diastolic stiffness constant (κ)	20.5 ± 1.9 ($n = 6$)	28.9 ± 1* ($n = 6$)	22.8 ± 0.4** ($n = 6$)	20.1 ± 0.1** ($n = 6$)
Plasma insulin concentrations	2.2 ± 0.4 ($n = 5$)	4.2 ± 0.3* ($n = 5$)	4.7 ± 1.1* ($n = 5$)	5.2 ± 1* ($n = 5$)
Plasma leptin concentrations	6.8 ± 0.86 ($n = 5$)	8.6 ± 1.1 ($n = 5$)	6.9 ± 0.8 ($n = 5$)	9.7 ± 0.5 ($n = 5$)
Plasma ALT activity, U/L	37 ± 0.6 ($n = 5$)	59.6 ± 0.3* ($n = 5$)	52.3 ± 2* ($n = 5$)	49.4 ± 3.2* ($n = 5$)
Plasma AST activity, U/L	73.2 ± 5.6 ($n = 5$)	105.3 ± 9* ($n = 5$)	68.2 ± 3.2** ($n = 5$)	69.7 ± 5.7** ($n = 5$)
Plasma ALP activity, U/L	174 ± 19 ($n = 5$)	251 ± 21* ($n = 5$)	192 ± 11** ($n = 5$)	274 ± 29* ($n = 5$)
Plasma LDH activity, U/L	204 ± 23 ($n = 5$)	497 ± 14* ($n = 5$)	180 ± 56** ($n = 5$)	184 ± 26** ($n = 5$)
LV + septum (mg/mm tibial length)	18.9 ± 0.9 ($n = 6$)	21.8 ± 1.1 ($n = 6$)	20.3 ± 1.5 ($n = 6$)	20.5 ± 0.8 ($n = 6$)
Right ventricle (mg/mm tibial length)	4.14 ± 0.3 ($n = 6$)	4.48 ± 0.3 ($n = 6$)	3.7 ± 0.2 ($n = 6$)	4.4 ± 0.3 ($n = 6$)
Liver (mg/mm tibial length)	234 ± 14 ($n = 6$)	287 ± 12* ($n = 6$)	264 ± 15 ($n = 6$)	292 ± 19* ($n = 6$)
Islets as % area of pancreas	6.2 ± 1.4 ($n = 6$)	15.3 ± 1.5 ($n = 6$)	5.2 ± 0.1 ($n = 6$)	16.7 ± 1.3 ($n = 6$)
Number of α cells/islet	16.9 ± 0.5 ($n = 6$)	39.3 ± 1.2 ($n = 6$)	22.4 ± 1.9 ($n = 6$)	38.6 ± 1.4 ($n = 6$)
Number of β cells/islet	72.3 ± 2.8 ($n = 6$)	124.7 ± 7.2 ($n = 6$)	117.1 ± 4.1 ($n = 6$)	149.8 ± 6.3 ($n = 6$)

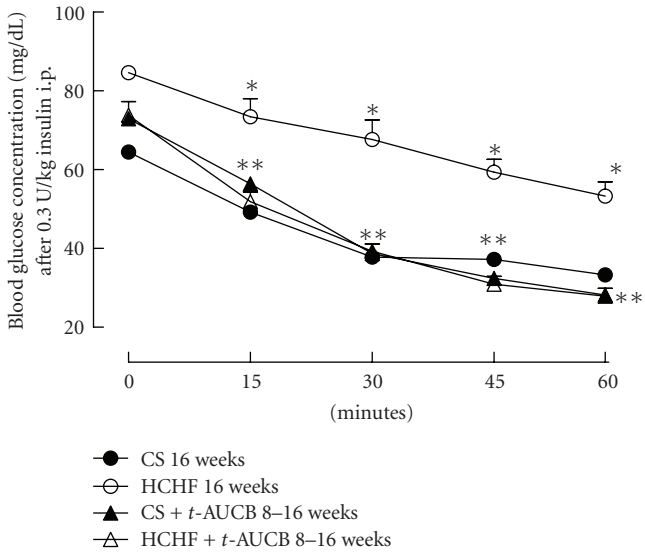

Figure 3: Plasma glucose concentrations following 0.3 U/Kg insulin i.p. dose after 16 weeks for rats fed with corn starch (CS), high carbohydrate high fat (HCHF), corn starch with *t*-AUCB (CS + *t*-AUCB), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB). $^{*}P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

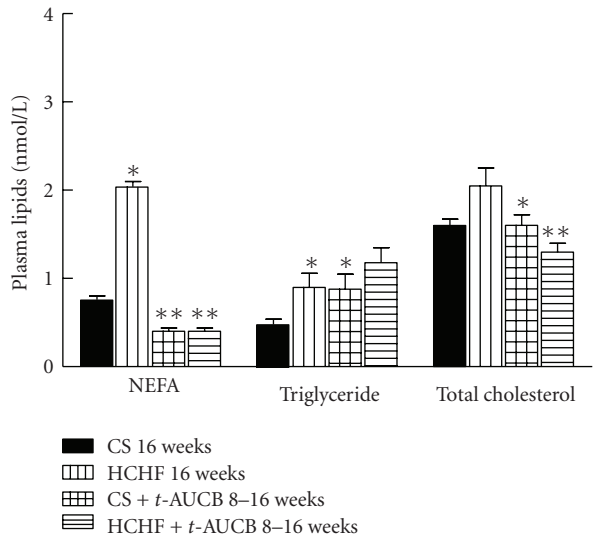

Figure 4: Plasma lipid concentrations after 16 weeks for rats fed with corn starch (CS), high carbohydrate high fat (HCHF), corn starch with *t*-AUCB (CS + *t*-AUCB), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB). $^{*}P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

elevated with HCHF feeding compared to CS-fed controls (Figure 4). Treatment with *t*-AUCB completely normalised these increased NEFA and total cholesterol concentrations in HCHF-fed rats (Figure 4). In CS-fed rats treated with *t*-AUCB, there was a further reduction in NEFA concentrations compared to control rats (Figure 4). There was no change in increased plasma triglycerides with *t*-AUCB treatment in HCHF-fed rats (Figure 4).

3.3. Structure and Function of Liver, Pancreas, and Adipose Tissue Are Moderated by t-AUCB. Postmortem liver weights (normalised to tibial length) from HCHF-fed rats were increased compared to CS-fed rats (Table 1). Treatment with *t*-AUCB did not prevent this organ's hypertrophy induced by HCHF feeding (Table 1). Histological analysis of liver samples showed HCHF diet-fed rats to have increased infiltration of inflammatory cells and increased deposition of collagen around the blood vessels compared to CS diet-fed rats (Figure 5). HCHF diet-fed rats showed deposition of fat droplets in liver, which were rarely observed in livers from CS diet-fed rats (Figure 5). Also, the size of fat vacuoles was larger in HCHF diet-fed rats (Figure 5). *t*-AUCB treatment attenuated the mild steatosis and increased hypertrophy and vacuole size seen in HCHF-fed rats without affecting the increased inflammatory cell infiltration (Figure 5). HCHF diet resulted in increased plasma activity of AST, ALT, ALP, and LDH compared to CS diet-fed rats indicating liver cell damage (Table 1). Treatment with *t*-AUCB attenuated the increased AST and LDH concentrations but not the ALT and ALP concentrations (Table 1).

The pancreas from HCHF diet-fed rats showed an increased number of inflammatory cells (Figure 5) with increased size of islets of Langerhans compared with CS diet-fed rats. Numbers of both α and β cells were also increased in HCHF diet-fed rats compared to CS diet-fed rats. Treatment with *t*-AUCB did not change the islets size in CS- or in HCHF-fed rats. *t*-AUCB treatment increased the number of both α and β cells in CS diet-fed rats but only increased β-cell numbers in HCHF-fed rats (Figure 6). Histological analysis of the adipose tissue samples showed increased infiltration of immune inflammatory cells with HCHF feeding at 16 weeks compared to CS-fed rats (Figure 7). Treatment with *t*-AUCB did not reduce this increased infiltration (Figure 7).

3.4. Treatment with t-AUCB Attenuated Changes in Cardiovascular Structure and Function. Heart rate did not vary among the experimental groups (Table 1). Systolic blood pressure in HCHF-fed rats increased sharply at week 4 and remained elevated until the end of the study protocol compared to CS-fed rats (Figure 8). Treatment with *t*-AUCB completely reversed the increased systolic blood pressure in HCHF-fed rats at 16 weeks (Figure 8). Echocardiographic assessment of HCHF-fed rats showed ventricular dilatation (increased left ventricular end diastolic dimensions), increased systolic volume, and increased estimated left ventricular mass (Table 1). Treatment with *t*-AUCB from week 8 to week 16 attenuated all these structural changes in HCHF-fed rats (Table 1).

Many inflammatory cells were observed in the left ventricle of HCHF-fed rats, whereas the number of inflammatory cells in the left ventricle of CS-fed rats was very low (Figure 9). Treatment with *t*-AUCB, in general, did not prevent this

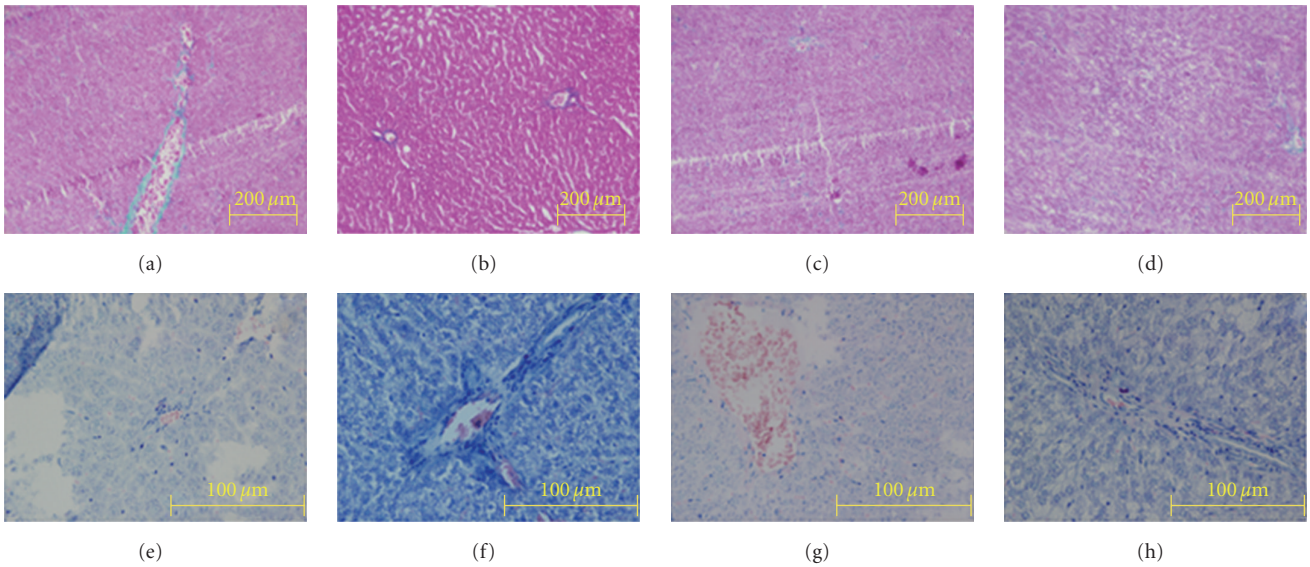

Figure 5: Structural changes and mild inflammation in the liver after 16 weeks for rats fed with corn starch (CS) (a) and (e), high carbohydrate high fat (HCHF) (b) and (f), corn starch with *t*-AUCB (CS + *t*-AUCB) (c) and (g), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB) (d) and (h).

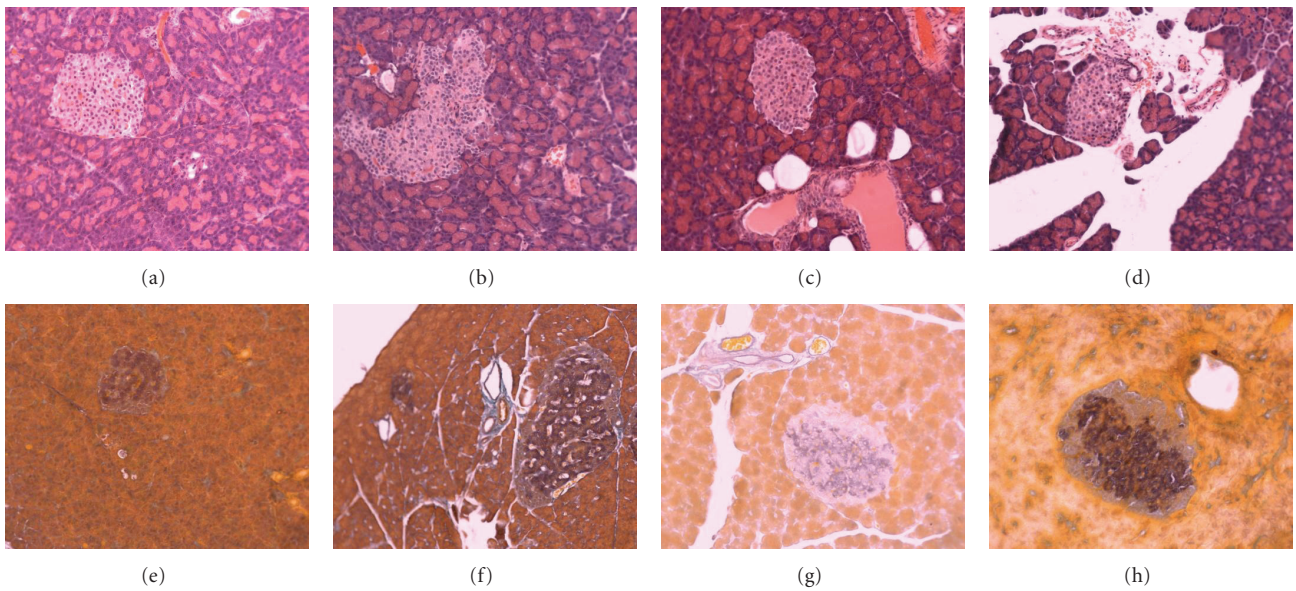

Figure 6: Structural changes in the pancreatic islets of Langerhans after 16 weeks for rats fed with corn starch (CS) (a) and (e), high carbohydrate high fat (HCHF) (b) and (f), corn starch with *t*-AUCB (CS + *t*-AUCB) (c) and (g), and high carbohydrate high fat with *t*-AUCB (HCHF) (d), (h), Magnification 20x.

increased infiltration of inflammatory cells but selectively prevented increased mast cells into the left ventricle in HCHF-fed rats. Interstitial collagen contents in left ventricle were increased in HCHF-fed rats compared with CS-fed rats (Figure 9). Treatment with *t*-AUCB attenuated this increase in interstitial collagen deposition in HCHF-fed rats (Figure 9). The isolated Langendorff's heart preparation showed increased diastolic stiffness in HCHF-fed rats at 16 weeks compared to CS-fed rats (Table 1). Treatment with *t*-AUCB from week 8 to week 16 completely reversed this increase in left ventricular diastolic stiffness in HCHF-fed rats (Table 1).

Lastly, organ bath studies showed no change in vascular responses to noradrenaline (constriction) and sodium nitroprusside (relaxation) among all treatment groups (Figure 10). In contrast, HCHF-fed rats showed pronounced endothelial dysfunction, seen as reduced vascular relaxation responses to acetylcholine compared to both CS-fed rats (Figure 10). Treatment with *t*-AUCB attenuated this decreased response to acetylcholine (Figure 10).

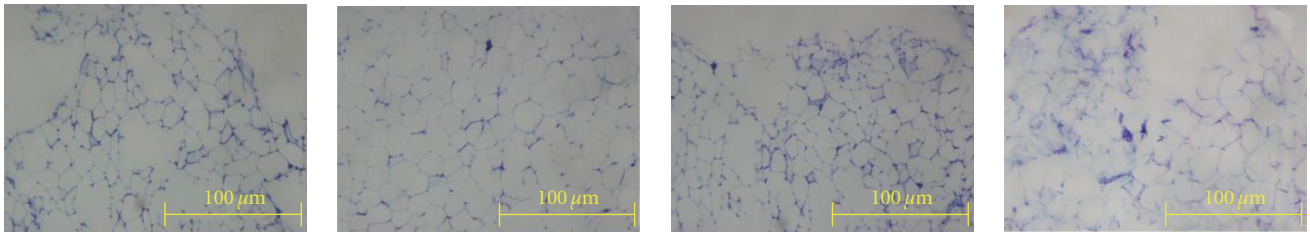

Figure 7: Structural changes and mild inflammation in the adipose tissue after 16 weeks for rats fed with corn starch (CS) (a), high carbohydrate high fat (HCHF) (b), corn starch with *t*-AUCB (CS + *t*-AUCB) (c), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB) (d).

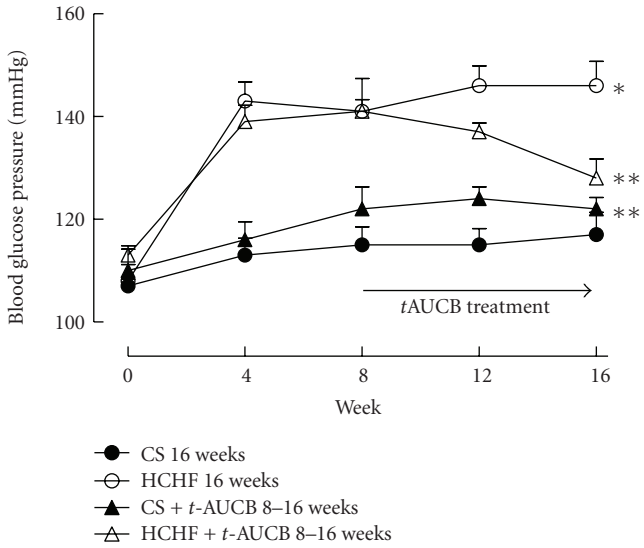

Figure 8: Systolic blood pressure measurements for rats fed with corn starch (CS), high carbohydrate high fat (HCHF), corn starch with *t*-AUCB (CS + *t*-AUCB), and high carbohydrate high fat with *t*-AUCB (HCHF + *t*-AUCB). $^{*}P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

4. Discussion

The signs of metabolic syndrome following chronic excessive macronutrient intake include body weight gain, excess visceral adipose deposition, hyperglycaemia, glucose and insulin intolerances, hypertension, dyslipidaemia, endothelial damage, cardiovascular hypertrophy, inflammation, ventricular contractile dysfunction, fibrosis, and fatty liver disease. Although it is well recognised that the inhibition of sEH lowers blood pressure in different rat models [19, 21, 28], the therapeutic potential of sEH inhibition in the control of diet-induced adiposity, metabolic and cardiovascular dysfunction are unknown. In this study, we show that sEH inhibition has therapeutic potential in the control of diet-induced prediabetes and metabolic syndrome. We report that chronic oral treatment with *t*-AUCB (~1 mg/kg/day), a potent sEH inhibitor [22], alleviated the symptoms of metabolic syndrome *in vivo* including glucose, insulin and lipid abnormalities, changes in pancreatic structure, increased systolic blood pressure, and cardiovascular and liver structural and functional abnormalities induced by chronic high-carbohydrate high-fat feeding in rats.

Clinical, animal and *in vitro* studies support links between obesity, insulin resistance, type II diabetes, metabolic dysfunction, and inflammation. Anti-inflammatory agents such as NSAIDs, salicylates, and aspirin reduce the severity of metabolic dysfunction [1, 2]. Local adipose tissue inflammation and inflammatory lipid mediators including EETs have been suggested to play important roles in regulating adipocyte function and metabolic homeostasis [1]. A recent study reported that sEH mRNA and protein concentrations in adipose tissue did not differ between normal- and fat-fed animals, but total adipose sEH activity was increased in obese mice, with a large increase during maturation of adipocytes [29]. Given the involvement of sEH in inflammation and also the increased activity of sEH during obesity and metabolic dysfunction, we hypothesised that increasing EET concentrations by sEH inhibition may be important in controlling obesity and the symptoms of the metabolic syndrome. Chronic oral treatment with *t*-AUCB improved metabolic and cardiovascular symptoms but did not alter body weight gain, excess visceral adipose deposition, or infiltration of immune inflammatory cells into the adipose tissue compared to untreated rats. This implies that *t*-AUCB acts to increase the anti-inflammatory EETs as metabolites of arachidonate produced by phospholipase A2 from inflammatory cells, rather than preventing infiltration of these immune inflammatory cells. It is well accepted that inhibition of sEH increases EET concentrations by decreasing their conversion into inactive diols (DHETs) [20]. Further, *t*-AUCB treatment reduces the production of DHETs and increases the ratios of EETs to DHETs in the plasma of lipopolysaccharide-treated mice [22]. Similarly, sEH knock-out mice have higher plasma ratios of EETs to DHETs than wild-type mice [30]. Thus, it is likely that sEH inhibition by *t*-AUCB may prevent adipocyte dysfunction downstream of immune inflammatory cell infiltration without affecting adiposity [1].

Further, changes in glucose, insulin, and lipid homeostasis are characteristic of insulin resistance, type II diabetes, and metabolic syndrome. There is growing incidental evidence to suggest the involvement of sEH in glucose, insulin, and lipid abnormalities. CYP2J protein, which generates EETs, is expressed in human and rat pancreatic tissues where

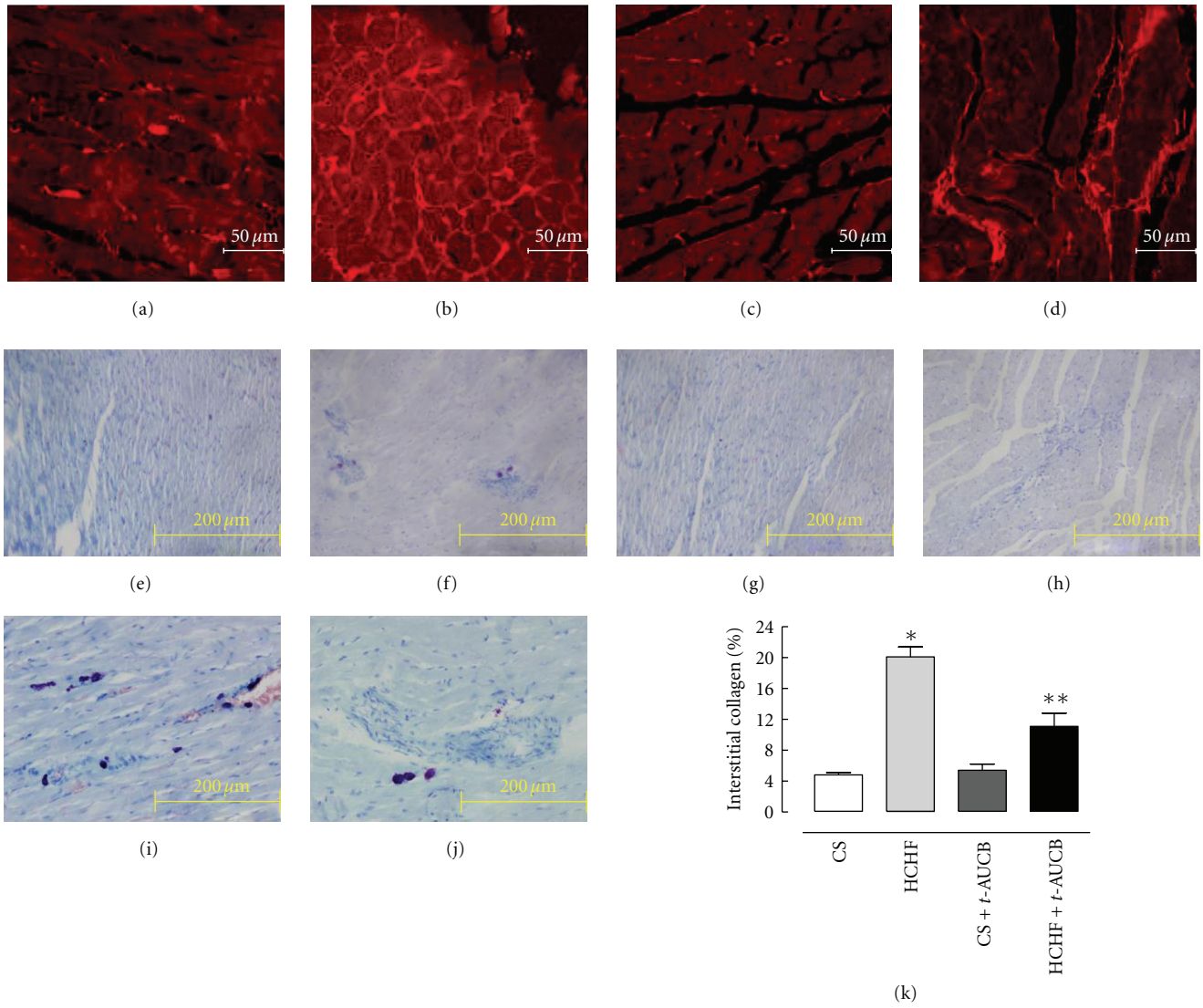

Figure 9: Inflammation and fibrosis in the heart. Representative images of left ventricular interstitial collagen deposition stained in picrosirius red staining (×40) after 16 weeks in CS (a), HCHF (b), CS + *t*-AUCB (c), HCHF + *t*-AUCB (d). Wright's staining of LV of the heart (×40) showing infiltration of inflammatory cells after 16 weeks in CS (e), HCHF (f), CS + *t*-AUCB (g), HCHF + *t*-AUCB (h). Wright's staining of LV of the heart (×100) showing infiltration of mast cells after 16 weeks in HCHF (i), HCHF + *t*-AUCB (j). Summary data of left ventricular interstitial collagen deposition (k) after 16 weeks in CS, HCHF, CS + *t*-AUCB, HCHF + *t*-AUCB. $^{*}P < 0.05$ versus CS-fed rats, $^{**}P < 0.05$ versus HCHF-fed rats.

significant amounts of endogenous EETs have been detected [31]. Also, EETs are potent mediators of insulin release in isolated rat islets [32]. In this study, we investigated whether *t*-AUCB treatment improves glucose and insulin intolerances and elevated plasma lipids induced by high-carbohydrate high-fat feeding. Oral glucose and intraperitoneal insulin tolerance tests showed improved tolerance to both glucose and insulin in rats treated with *t*-AUCB compared to untreated ones. A recent study assessed the role of sEH in glucose and insulin homeostasis in streptozotocin- (STZ-) treated mice using both sEH knockout and sEH inhibition using *t*-AUCB [33]. This study showed that both sEH knockout and *t*-AUCB treatment prevented hyperglycaemia in type I diabetes through enhanced islet glucose-stimulated insulin release by the alternate pathway and decreased islet cell apoptosis [33]. Further, a recent study supports our results and hypothesis by showing that inhibition of sEH restored glucose homeostasis and insulin signalling together with increased pancreatic islet size in a mice model of type II diabetes [34]. These could be the same mechanisms through which *t*-AUCB improved glucose and insulin abnormalities in our study.

In patients with type II diabetes mellitus, single nucleotide polymorphisms in the sEH gene were associated with an increased risk of cardiovascular disease [35] and hypertension [36]. Although it is well established that sEH inhibitors have beneficial effects in cardiovascular diseases [19–21], their therapeutic potential in metabolic syndrome-induced cardiovascular changes is unknown. In the present study,

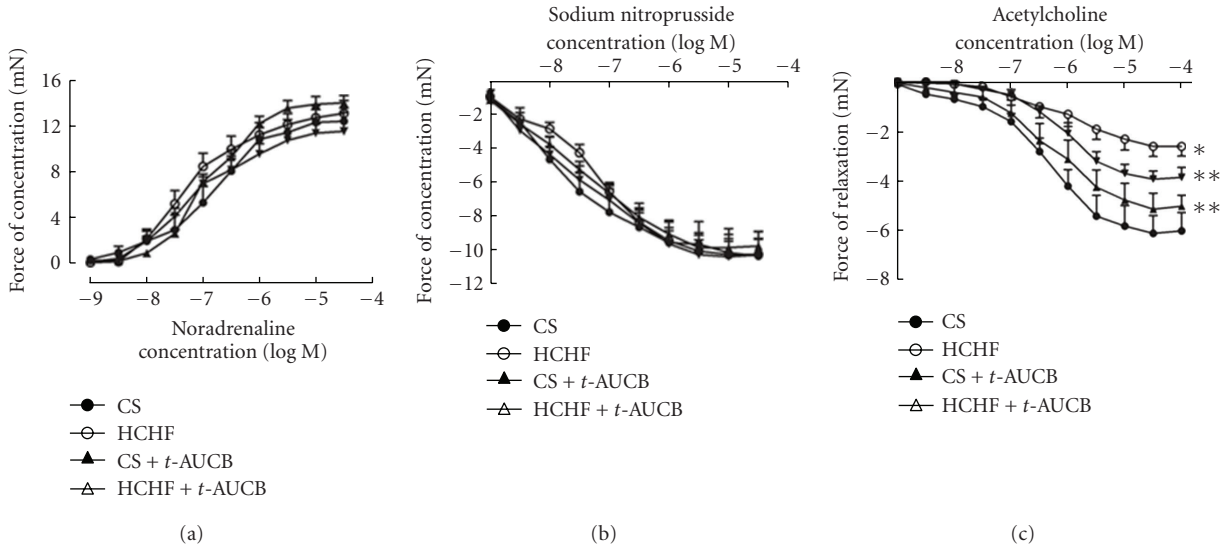

Figure 10: Cumulative concentration-response curves for noradrenaline (a), sodium nitroprusside (b), and acetylcholine (c) in thoracic aortic rings after 16 weeks from CS-, HCHF-, CS + *t*-AUCB-, HCHF + *t*-AUCB-treated rats. $^{*}P < 0.05$ versus CS-fed rats; $^{**}P < 0.05$ versus HCHF-fed rats.

t-AUCB treatment attenuated the cardiovascular changes induced by high-carbohydrate high-fat feeding including elevated systolic blood pressure and endothelial dysfunction fibrosis, hypertrophy, and increased stiffness in the left ventricle of the heart. Treatment with *t*-AUCB also showed improved plasma liver enzymes and decreased steatosis compared to untreated animals.

The sEH inhibitors show target engagement in many studies in terms of increasing the plasma concentrations of epoxy fatty acids and decreasing the concentrations of the corresponding diols [37]. The broad and usually beneficial biological activities observed with the sEH inhibitors may occur from several causes. One is that not only the 4 regioisomers of EETs are being stabilised but also the epoxides of other fatty acids are being stabilised including linoleate, eicosapentaenoic acid, and docosahexaenoic acid [38], but the concentrations of the sometimes inflammatory diols are being reduced [39]. Observing multiple biological effects following the administration of a drug that influences the arachidonate cascade is common in pharmacology with the nonsteroidal inflammatory drugs being a case in point. Although a receptor is not known for EETs, they are known to block nuclear translocation of NFκB [40] and to down-regulate induced iNOS and COX 2 [41], all of which influence many downstream biological activities. Further, the current paradigm in the origin of metabolic syndrome is that adipocyte dysfunction promotes the metabolic and cardiovascular symptoms of the metabolic syndrome [1, 5]. sEH activity in adipose tissue increases with adiposity and correlates with the associated metabolic dysfunction in obesity [29]. Thus, attenuating adiposity dysfunction by inhibiting the increased sEH activity in adipose tissue using *t*-AUCB may account for the beneficial responses seen in all the other metabolic systems.

The sEH inhibitor, *t*-AUCB, like any pharmacological probe could be producing off-target effects. However, the low nanomolar inhibitory concentrations and picomolar Ki of *t*-AUCB argue for selectivity. This class of compounds has shown few off-target effects with most of these being in the micromolar or nanomolar range. The same biological responses have been observed in multiple systems using radically different sEH inhibitor structures. Although not used in this study, the EET antagonist, 14,15-EEZE, reversed the responses to sEH inhibitors in other models while EET administration enhanced the responses. Some of these responses were also mimicked in rodent models of diabetes using genetic knockouts of the sEH [34].

In summary, the present study provides new information about the therapeutic potential of a selective sEH inhibitor in treating the signs of diet-induced metabolic syndrome, as well as the beneficial responses of sEH inhibition on metabolic syndrome-induced cardiovascular and liver abnormalities. The great diversity of biological effects resulting from increased concentrations of EETs suggests the presence of multiple receptors with the assumption that at least some G-protein-coupled receptors are involved [1]. Further research into identifying the receptors that mediate EET responses in metabolically relevant tissues would throw light on the mechanisms of action for their therapeutic responses. sEH inhibitors also synergise the anti-inflammatory actions of NSAIDs, which suggests that low doses of NSAIDs could be used in combination to reduce the symptoms of metabolic syndrome and adiposity possibly without compromising innate immunity [1, 42]. Thus, targeting sEH with selective inhibitors either alone or in combination with low doses of NSAIDs could reduce metabolic and cardiovascular dysfunction in metabolic syndrome.

Acknowledgments

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Review Article

Cardiac Insulin Resistance and MicroRNA Modulators

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Cardiac insulin resistance is a metabolic and functional disorder that is often associated with obesity and/or the cardiorenal metabolic syndrome (CRS), and this disorder may be accentuated by chronic alcohol consumption. In conditions of overnutrition, increased insulin (INS) and angiotensin II (Ang II) activate mammalian target for rapamycin (mTOR)/p70 S6 kinase (S6K1) signaling, whereas chronic alcohol consumption inhibits mTOR/S6K1 activation in cardiac tissue. Although excessive activation of mTOR/S6K1 induces cardiac INS resistance via serine phosphorylation of INS receptor substrates (IRS-1/2), it also renders cardioprotection via increased Ang II receptor 2 (AT2R) upregulation and adaptive hypertrophy. In the INS-resistant and hyperinsulinemic Zucker obese (ZO) rat, a rodent model for CRS, activation of mTOR/S6K1signaling in cardiac tissue is regulated by protective feed-back mechanisms involving mTOR↔AT2R signaling loop and profile changes of microRNA that target S6K1. Such regulation may play a role in attenuating progressive heart failure. Conversely, alcohol-mediated inhibition of mTOR/S6K1, down-regulation of INS receptor and growth-inhibitory mir-200 family, and upregulation of mir-212 that promotes fetal gene program may exacerbate CRS-related cardiomyopathy.

1. Introduction

The confluence of a constellation of interactive maladaptive factors such as hypertension, insulin (INS) resistance, metabolic dyslipidemia, obesity, microalbuminuria, and/or reduced renal function constitute the cardiorenal metabolic syndrome (CRS) [1–6]. Clustering the increasing numbers of these risk factors within an individual heightens metabolic perturbations which, in turn, promote development of cardiovascular diseases (CVD) and type 2 diabetes (T2DM) [5, 6]. The CRS affects more than one-third of the US population and is rising to pandemic proportions worldwide. Overnutrition caused by excessive consumption of diets rich in carbohydrates, largely from highly processed foods and sugar-sweetened beverages (Western diet), positively correlates with the rise of CRS risk factors and CVD [7–14]. Overnutrition results in chronic exposure of cardiovascular (CV) tissues to circulating nutrients, glucose, and INS. All of these factors promote attenuation of INS metabolic signaling and INS resistance independently, and therefore, collectively they exert significant stress on CV tissues [15–19]. Moreover, overnutrition induces activation of the renin-angiotensin system (RAS) which can elevate systemic and tissue angiotensin II (Ang II), a potent vasoconstrictive and proinflammatory hormone. Ang II-mediated activation of Ang II type 1 receptor (AT1R) promotes both INS resistance and CVD, and inhibition of Ang II generation, as well as blocking of AT1R signaling, has proven to be beneficial in treatment of INS resistance [20–32]. Overnutrition also alters adipocyte functions by reducing secretion of anti-inflammatory, anti-ischemic adiponectin and increasing secretion of proinflammatory, prothrombotic adipokines such as resistin [33–35]. Thus, in conditions of overnutrition, cardiac insult is exerted by a plethora of extracellular

signals (increased INS and Ang II, an adverse adipokine profile, and excessive glucose, amino acids and lipids) and hemodynamic/neuroendocrine stresses originating from hypertension, hypertrophy, and fibrosis.

In the initial stages of overnutrition related cardiac INS resistance, several compensatory mechanisms are activated in heart tissue to protect the functions of this vital organ by promoting adaptive compensatory signaling and remodeling. In this context, activation of the nutrient sensor kinase mammalian target for rapamycin (mTOR) in cardiac tissue, under conditions of overnutrition, is particularly noteworthy [36]. The mTOR Complex 1 (mTORC1) serves as a converging point for signals from nutrients, INS and Ang II, and is frequently activated in CV tissues in conditions of overnutrition and aging [36–44]. On the one hand, increased mTORC1-mediated signaling is implicated in left ventricular (LV) remodeling, myocardial infarction, hypertrophic cardiomyopathy, and atherosclerosis [45–48]. Conversely, mTOR is required for exercise-induced adaptive hypertrophy and remodeling [49]. Attenuation of mTORC1 signaling via cardiac specific ablation of Raptor, the scaffolding protein required for association of mTOR with its substrates p70 s6 kinase (S6K1) and eIF4E-binding protein (4E-BP), induces impairment of adaptive hypertrophy and causes heart failure in mice [50–54]. Given the fact that mTOR signaling is required for adaptive cardiac hypertrophy, it is conceivable that activation of mTORC1 in cardiac tissue in response to excess nutrients, Ang II, and INS can be a compensatory mechanism to help the heart cope with overnutrition-related stresses such as hemodynamic overload. In this paper, we describe regulation of overnutrition-related cardiac mTORC1 signaling by inherent protective feedback mechanisms that involve mTORC1-mediated activation of Ang II type 2 receptor (AT2R) and changes in microRNA profiles that, in turn, can potentially downregulate S6K1 expression.

Chronic alcohol consumption, in the context of overnutrition, is an additionally significant risk factor that promotes cardiac pathology and dysfunction [55]. Alcoholism may be the most common form of drug abuse. Moderate alcohol consumption has been associated with a reduced risk of CVD and improvement of INS resistance [56, 57]. However, chronic alcoholism (excessive and prolonged alcohol consumption with >80 g of ethanol a day for >10 years) can result in alcoholic cardiomyopathy [55–60]. Chronic alcohol treatment inhibits protein synthesis in cardiac muscles and cause protein loss [58–60]. This alcohol effect is mediated by an inhibition of stimulatory phosphorylation of mTOR and S6K1 in cardiac tissue and subsequent downregulation of protein synthesis [58]. We posit that alcohol-mediated inhibition of mTOR/S6K1 activation may contribute to attenuation of an important compensatory mechanism (mTORC1 signaling) that can promote adaptive hypertrophy under conditions of overnutrition. Moreover, chronic alcohol administration is shown to alter microRNA profiles in other tissues. A close examination of these alcohol-regulated microRNAs indicates that some of these microRNAs are also expressed in cardiac tissue. In this paper we explore the possible cardiac outcomes related to impaired myocardial INS metabolic signaling that can occur when alcohol-induced microRNA modulations are superimposed on overnutrition-induced adaptive compensatory signaling mechanisms in heart tissue.

2. Overnutrition-Induced Activation of mTORC1 and mTOR↔AT2R Signaling Loop in Cardiac Tissue and Cardiomyocytes

TOR is a 289-kDa serine/threonine protein kinase that is evolutionarily conserved from yeast to man (mTOR) and a member of the phosphatidylinositol 3-kinase- (PI3-K-) related kinase (PIKK) family. Signals that activate the canonical PI3-K-protein kinase B (Akt) pathway (growth factor receptors, Ang II, INS-mediated activation of IRS-1/PI3-K-Akt pathway) result in mTOR stimulation. Akt activates mTOR by directly phosphorylating mTOR at Ser^{2448} [37–42]. Akt also promotes mTORC1 complex formation indirectly since it phosphorylates proline-rich Akt substrate of 40 kDa (PRAS40), the negative regulator of Raptor, and promotes dissociation of PRAS40 from mTORC1 so that Raptor is free to associate with mTOR substrates S6K1 and 4E-BP. Additionally, phosphorylation of tuberous sclerosis complex 2 (TSC2) by Akt results in inhibition of its GTPase activity and thus promotes GTP loading on Rheb (Ras homolog enriched in brain) and Rheb-mediated mTORC1 activation [61]. Amino acids also activate mTORC1 via heterodimeric Rag GTPases that promote translocation of mTORC1 to a membrane-bound compartment that contains the mTORC1 activator, Rheb [62]. Moreover, these diverse mechanisms for activation of mTORC1 by amino acids and INS act in a cooperative manner and provide a physiological explanation for increased mTORC1 signaling in response to overnutrition-related increases in nutrients and INS [63]. However, mTORC1 activation contributes to heart and skeletal muscle INS resistance since mTOR substrate S6K1 is a serine (Ser)/threonine kinase that phosphorylates IRS-1, a critical INS signaling/docking molecule, on specific Ser residues. Excessive Ser phosphorylation of IRS-1 attenuates IRS-1 tyrosine phosphorylation and IRS-1-PI3-K association and subsequent INS metabolic signaling. In this context, mTORC1 activation can induce INS resistance in cardiac tissue. However, it is also conceivable that development of INS resistance in conditions of overnutrition and hyperinsulinemia is actually a compensatory mechanism that may serve to protect cardiac cells from excessive signaling generated by excess INS and nutrients.

Cardiac mTORC1 activation may play a significant role in increased protein synthesis required for adaptive hypertrophy. In this context, activation of mTORC1 leads to increased translation and cell growth by two mechanisms: first, phosphorylation of Thr^{389} of S6K1 by mTOR results in activation of S6K1 and subsequent phosphorylation of five evolutionarily conserved residues (Ser235, Ser236, Ser240, Ser244, and Ser247) of ribosomal protein S6 (RPS6) that activates RPS6. RPS6 increases translation of 5′TOPmRNAs and protein synthesis. Second, mTOR phosphorylates 4E-BP on Thr^{37} and Thr^{46} and relieves 4E-BP-mediated repression of translation

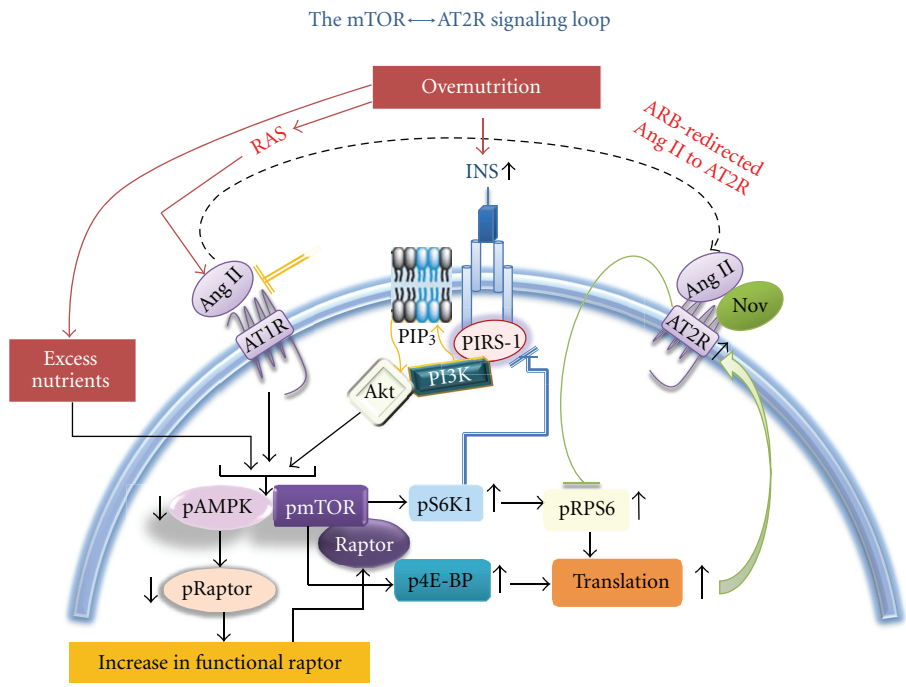

Figure 1: The mTORC1-mediated increase in AT2R expression can lead to AT2R-mediated inhibition of mTOR substrates and modulate mTORC1 signaling.

initiation factor eIF4E and thus enhances translation [39, 42, 50, 64–66]. It should be noted that cardiac overexpression of mTOR protects against cardiac dysfunction following LV pressure overload. Conversely, ablation of cardiac raptor results in impairment of adaptive cardiac hypertrophy and causes heart failure in mice [54]. Interestingly, mTOR also protects heart from pathological hypertrophy associated with inflammatory response [52, 54]. These observations imply a delicately balanced mTORC1 function that helps the heart cope with stress induced by exercise, pressure overload, and inflammation. Our recent findings support the notion that in the setting of overnutrition-induced impairment of cardiac INS metabolic signaling, mTORC1 signaling is carefully controlled by protective feedback mechanisms.

The CRS rodent model Zucker Obese (ZO) rat is polyphagic due to a mutation in the leptin receptor and serves as a model of overnutrition-induced INS resistance and diastolic heart failure with preserved ejection fraction [67–69]. Importantly, this genetic model manifests biochemical and functional cardiac abnormalities that are seen in obese humans; however, the ZO rat does not progress to overt diabetes until late in life. We observed activation of mTORC1 in the left ventricle of ZO rats; however, a concomitant increase in growth-inhibitory Ang II receptor AT2R was also observed [2]. It is conceivable that the hyperinsulinemic status of ZO rat may simultaneously promote mTORC1 activation since INS activates mTORC1 and increases AT2R expression [38, 70–72]. What is paradoxical is that accumulating evidence indicates that AT2R activation is cardioprotective [73–77]. AT2R inhibits cell growth and mediates the beneficial effects of AT1R blockade and PPAR-γ activation, reduces fibroblast growth and myocardial hypertrophy, and mediates the antihypertrophic and antifibrotic effects of AT1R blockade [76–80]. Since AT2R activates phosphatases, it is conceivable that AT2R expression in cardiac tissue in response to overnutrition/hyperinsulinemia can regulate mTORC1 kinase and down-stream signaling. Such regulation could play a significant role in maintaining the delicate balance of mTORC1 activation that promotes compensatory adaptive cardiac hypertrophy to cope with increased hemodynamic load associated with obesity. Our observation that Ang II- and INS-mediated activation of mTORC1 signaling in mouse cardiomyocytes is, in part, responsible for increased AT2R expression (that may, in turn, regulate mTORC1 signaling) supports this notion. Rapamycin treatment elevates glucose intolerance in obese sand rat. We observed that rapamycin-induced inhibition of mTORC1 and siRNA-mediated inhibition of S6K1 attenuated elevation of AT2R expression. Thus, treatments that ablate mTORC1 and inhibit AT2R do not seem to be cardioprotective in overnutrition conditions. Conversely, AT2R agonism in ZO rats by a nine-day infusion ($200\,\mu g \cdot kg^{-1} \cdot day^{-1}$) of Novokinin (Nov), an AT2R agonist, reduced the increase in mTORC1 signaling and yet improved myocardial performance. In brief, mTORC1 activation leads to the formation of an mTOR↔AT2R signaling loop that can serve as a protective feedback mechanism to balance enhanced mTORC1 signaling in cardiac tissue in conditions of overnutrition-induced INS resistance and RAS activation (Figure 1).

3. Regulation of S6K1 by microRNA in Cardiac Tissue

The microRNAs (miRNA) have emerged as an important group of translational regulators that target and regulate 60% of the mammalian genome [81–84]. Recent studies have shown that cardiac muscle-rich miRNAs or myomiRNAs such as mir-208 (mir-208a, b) play crucial roles in CVD [85–87]. The miRNAs are natural, single-stranded, small RNA molecules that are not translated into proteins and yet serve the pivotal function of regulating gene expression. It is estimated that only 1% of the genomic transcripts in mammalian cells encode miRNA. Genes encoding for miRNAs are transcribed from DNA to produce a primary transcript (pri-miRNA). The pri-mRNA is then processed into a shorter precursor miRNA (pre-miRNA), which undergoes further processing to form a mature, single-stranded miRNA that is 18 to 24 nucleotides long. A mature miRNA binds to its mRNA target at their complementary sequences and down-regulates gene expression by either inhibiting the mRNA translation to proteins or inducing mRNA degradation. Studies on experimental heart failure models have identified several miRNAs as differentially expressed (for a complete list of miRNAs that are differentially expressed in heart-failure models please see the review [88]). Thus a cumulative change in miRNA profile accompanies heart failure-associated cardiac pathology. In contrast, a detailed study by Naraba and Iwai has suggested that microRNA profiles of heart and kidney do not show significant changes in salt-sensitive hypertension [89]. These researchers constructed microRNA libraries using the kidneys of Dahl salt-sensitive and Lewis rats taking normal or high-salt diets (4 groups) and identified 91 previously reported and 12 new microRNAs and then compared their expression profiles in kidney and heart ventricles. They concluded that the microRNA system is unlikely to contribute to salt-sensitive hypertension in Dahl salt-sensitive rats. It has also been reported that plasma levels of some miRNAs (mir-1, mir-208, mir-133a, mir-423-5p, mir-499) can be used as biomarkers for myocardial injury [90–92]. The mir-143 has recently emerged as an obesity-induced miRNA that inhibits INS-stimulated Akt activation and impairs glucose metabolism [93].

Individual miRNAs can regulate several hundreds of genes and conversely a given gene can be regulated by multiple miRNAs. Knocking out the mTOR gene has shown to be embryonically lethal, however, knocking out S6K1 gene (RPS6KB1) seems to confer some beneficial health aspects to mice. Mice deficient in S6K1, though they have a small body size and reduced β-cell mass, are protected from INS resistance in conditions of overnutrition [94]. We observed that in ZO rat cardiac LV tissue, total S6K1 protein levels were significantly downregulated (Figure 2), and this prompted us to analyze whether or not a change in miRNA profile that modulates S6K1 translation and mRNA stability plays a role in reducing the protein levels of S6K1. To identify what miRNAs bind rat S6K1 mRNA, we performed a RegRNA analysis [95] of the 2287bp rat S6K1 mRNA. It was found that 298 putative miRNAs can bind rat S6K1 mRNA. We have initiated miRNA profiling studies of Zucker lean (ZL) and ZO cardiac tissues. The miRNA was isolated with mirVana mirNA isolation kit (Ambion Inc.) from fresh frozen tissues ($n = 3$ for each group), and was labeled with FlashTag Biotin HSR RNA Labeling Kit. Affymetrix miRNA GeneChip that carries 46,228 probes comprising 7,815 probe sets, including controls, was used for this study. The probes on this chip are derived from the Sanger miRBase miRNA database v11 (April 15, 2008, http://microrna.sanger.ac.uk) [96]. Data analysis was by miRNA QC tool and Significance Analysis of Microarrays (SAM) software (http://stat.stanford.edu/~tibs/SAM/). We compared the list of 298 miRNAs that can potentially modulate S6K1 expression with the list of statistically significant differentially expressed miRNAs in ZO LV tissue compared to ZL LV tissue. This analysis showed that only four of these S6K1-modulating miRNAs had a very modest, but statistically significant, increase in their expression compared to that of the ZL control cardiac tissues (Figure 2).

Three of these miRNAs, rno-let-7c, rno-mir-23a, and rno-mir-26a, were among the abundantly expressed miRNAs in ZO cardiac tissue. This was not surprising since these miRNAs are shown to be expressed in heart and upregulated in experimental models for heart failure [88]. In contrast, rno-mir-200c was expressed modestly in ZO cardiac tissue. The rno-mir-200c is located on chromosome 4 and interestingly, QTLs associated with mir-200c include heart rate QTL (Figure 3). The mir-200c has emerged as a cell growth inhibitor and targets apoptosis inhibitor FAP-1 [97]. The mir-200c also regulates stem cell factors, and it has been proposed that targeting the ZEB1-miR-200 feedback loop can lead to a promising treatment for fatal tumors, such as pancreatic cancer [98]. Therefore, it is conceivable that the modest mir-200c upregulation in cardiac tissue from an overnutrition model reflects a compensatory mechanism to delicately balance mTORC1 signaling and to control hypertrophy. While none of the S6K1 targeting miRNAs showed robust increase in ZO cardiac tissue compared to that of ZL, it is possible that a modest upregulation of multiple miRNAs that target different regions of S6K1mRNA (Figure 2) may achieve optimum regulation of S6K1 expression (by either reducing translation or mRNA degradation) without completely ablating S6K1 and inhibiting its beneficial downstream effects such as AT2R upregulation.

4. A Possible Molecular Explanation for Exacerbation of Cardiomyopathy by Chronic Alcohol Consumption under Conditions of Overnutrition

Although light to moderate alcohol consumption is cardioprotective, regular heavy ethanol consumption results in a form of dilated cardiomyopathy characterized by reduced contractility, ventricular dilatation, cardiomyocyte apoptosis, and fibrosis, often progressing to cardiac failure [99–102]. The risk of alcoholic cardiomyopathy is greater in women than men for any given life time amount of alcohol [103].

The "2008-2013 Action Plan" by World health Organization estimates that noncommunicable diseases (NCDs)

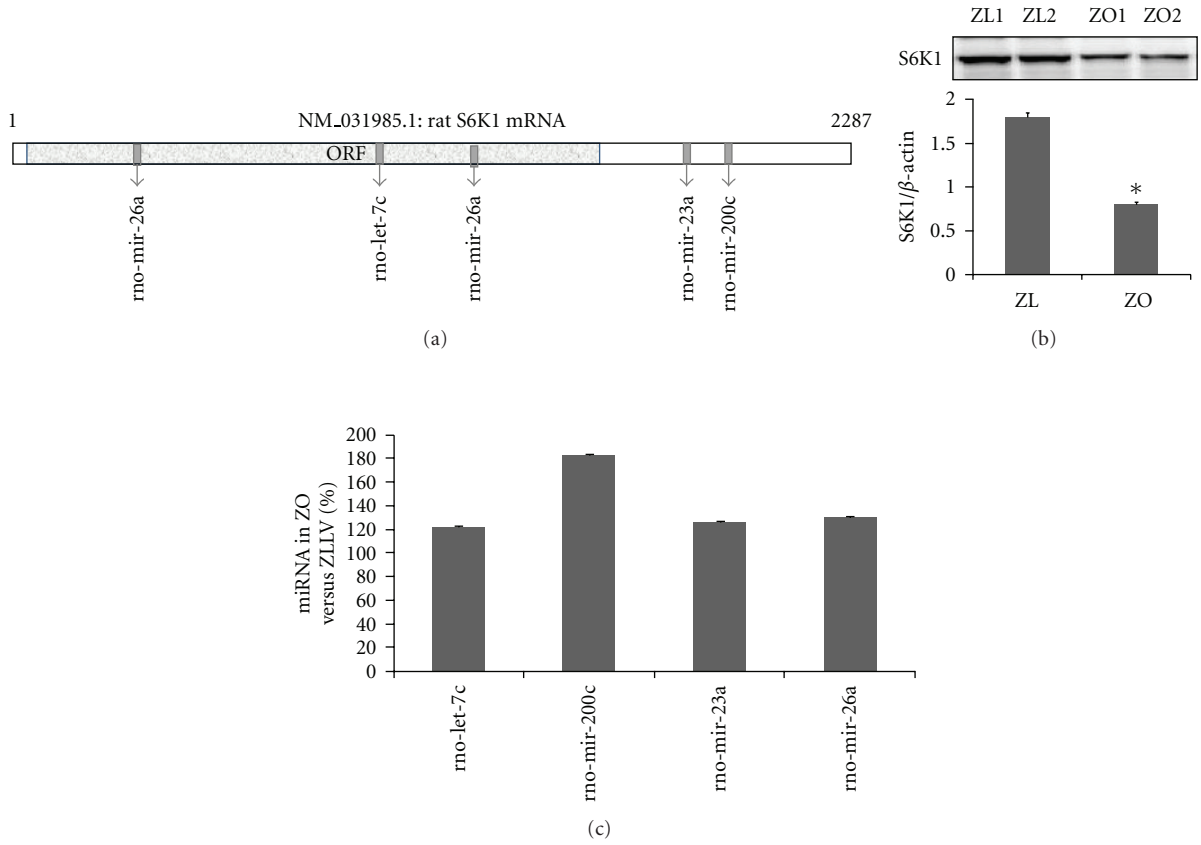

Figure 2: (a) Locations of miRNA binding sites on 2287bp S6K1 mRNA. Location of open reading frame (ORF) is marked. (b) Representative autoradiogram showing S6K1 protein levels in ZO and ZL LV tissues. $^*P < 0.05$ for ZO versus ZL LV tissue. (c) % increase in miRNA levels in ZO LV tissues versus ZL LV tissues ($n = 3$ for each group, P for ZO versus ZL < 0.05).

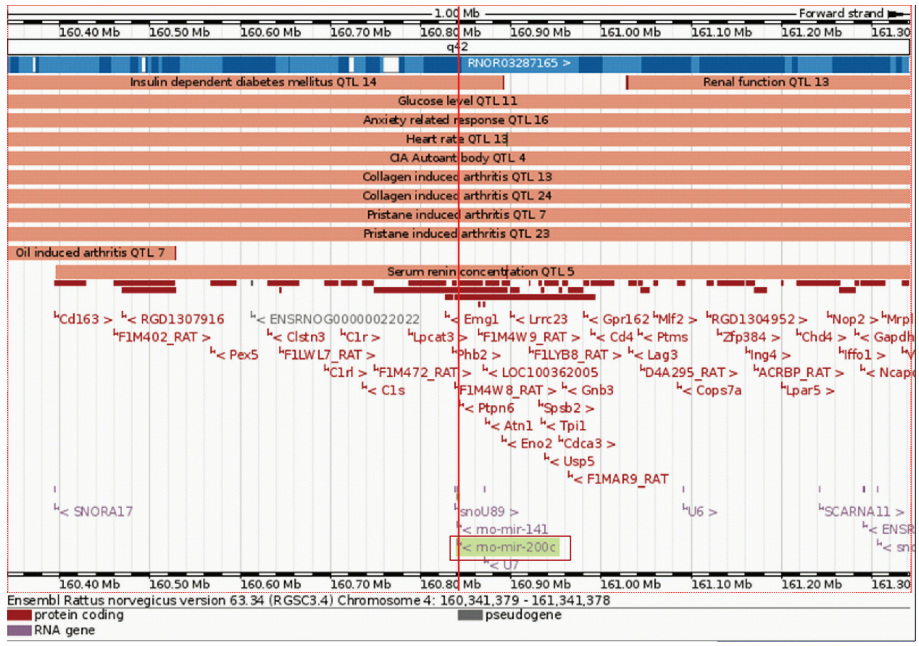

Figure 3: Location of rno-mir-200c on the chromosome 4 of rat with associated QTLs and protein coding regions as shown in Ensemble Rattus norvegicus version 63.34. rno-mir-200c is associated with Heart rate QTL 13.

including CVDs, diabetes, cancers, and chronic respiratory diseases, constitute 60% of mortality globally and can be prevented by eliminating the shared risk factors that include alcohol abuse [104]. Epidemiological studies show either an inverted U-shape or a positive linear relationship between alcohol consumption and INS sensitivity [105]. Chronic heavy alcohol consumption has been associated with the development of INS-resistant syndrome [106, 107]. A recent study on patterns of alcoholic consumption and Metabolic Syndrome has reported that "drinking in excess of the dietary guidelines was associated with an increased risk of impaired fasting glucose/diabetes mellitus, hypertriglyceridemia, abdominal obesity, and high blood pressure" [108].

Chronic ethanol ingestion in rats resulted in decreased expression of GLUT4 accompanied by downregulation of INS receptor-beta subunit, INS receptor substrate-1 (IRS-1) in rat cardiac tissues [109]. Chronic ethanol feeding (12 weeks) to FVB mice resulted in glucose intolerance, impaired cardiac glucose uptake, cardiac hypertrophy, and contractile dysfunction [110]. Ethanol feeding had no effect on either the expression of INS receptor β and IRS-1 with or without INS stimulation in cardiomyocytes or basal phosphorylated INS receptor (Tyr1146), basal tyrosine, and Ser phosphorylated IRS-1 [110]. However, chronic alcohol ingestion significantly impaired INS-stimulated tyrosine phosphorylation of INS receptor, IRS-1, and Akt, S6K1. In alcohol dehydrogenase (ADH), transgenic mice ethanol-induced decrease in tyrosine phosphorylation of IRS-1 was further increased without affecting the INS receptor. Alcohol ingestion significantly enhanced INS stimulated Ser phosphorylation of IRS-1, the effect of which was exaggerated in ADH transgenic mice [110]. In contrast, mitochondrial aldehyde dehydrogenase-2 (ALDH2) overexpression attenuated alcohol-induced decrease in tyrosine phosphorylation of INS receptor and IRS-1. Moreover, increased Ser phosphorylation of IRS-1 and decreased phosphorylation of Akt caused by alcohol ingestion were reversed by ALDH2 overexpression [111]. These results favor the role of ethanol metabolism and acetaldehyde in alcohol-induced myocardial INS resistance and myocardial dysfunction.

Recent studies have shown that chronic alcohol treatment results in inhibition of mTORC1 signaling in cardiac tissues [59], and this effect of alcohol is involved in reduced protein synthesis and cardiac muscle waste. Inhibition of mTORC1 signaling by chronic alcohol consumption has also been reported in cerebral cortex [112]. Interestingly, this effect of alcohol was independent of TSC2 or Akt phosphorylation status suggesting that other mechanisms are involved in alcohol-mediated mTORC1 inhibition. The observation that alcohol inhibits mTORC1 signaling in the heart is particularly noteworthy since this effect of alcohol opposes overnutrition-mediated signaling in heart. As discussed in the previous sections, mTORC1 signaling plays a pivotal role in regulating cardiac health and pathology. Activation of mTORC1 signaling in heart tissue and cardiomyocytes underlies initiation of compensatory mechanisms such as upregulation of the AT2R that has cardio-protective effects. A delicate balancing of mTORC1 signaling in cardiac tissues under overnutrition conditions is mediated by mTOR↔AT2R signaling loop and a moderate downregulation of total S6K1 protein by changes in miRNA profiles. Therefore, it is conceivable that alcohol-mediated inhibition of mTORC1 signaling may attenuate natural compensatory mechanisms that help the heart to cope with overnutrition (Figure 4).

Another effect of alcohol is its ability to downregulate mir-200a [113, 114] as shown by studies involving Lieber-DeCarli diet-induced alcoholic steatohepatitis mice models. The mir-200 family microRNAs are shown to function as inhibitors of growth in many cell types. Therefore, downregulation of mir-200 family microRNAs by alcohol can potentially increase growth and S6K1 protein levels. Alcohol-mediated upregulation of the micoRNA mir-212 is implicated in alcoholic liver disease [115]. Interestingly, mir-212 has emerged as an activator of fetal gene program [116, 117]. Chronic heart failure is characterized by LV remodeling and activation of the fetal gene program. The mir-212 is overexpressed in failing hearts. Additionally, transfection of isolated adult rat cardiomyocytes with a set of fetal miRNAs (miR-21, miR-129, and miR-212) induced cellular hypertrophy and activation of a fetal gene program [117]. Since alcohol is shown to up-regulate mir-212 in other cell types, it is conceivable that alcohol-mediated upregulation of mir-212 can be one of the mechanisms by which alcohol exacerbates cardiomyopathy.

Activation of RAS may contribute to progression of alcoholic cardiomyopathy. Binge mode ethanol consumption in chronic alcohol abuse patients and heavy alcohol consumption is associated with increased plasma angiotensin levels [118]. Studies on alcohol ingestion and cardiac injury in dogs showed that activation of RAS was followed by a progressive fall in LV contractility during six months of alcohol ingestion [119]. Moreover, angiotensin receptor blocker irbesartan prevented these alcohol-induced decreases in LV and myocyte contraction [119]. Recent animal studies in which chronic alcohol consumption with superimposed binge mode of ethanol administration has been associated with upregulation of RAS in the heart [120]. However, ethanol ingestion alone usually does not result in severe cardiac injury seen in humans and robust activation of RAS [118]. In this regard, gene polymorphism for angiotensin converting enzyme (ACE) is associated with increased vulnerability to alcoholic cardiomyopathy [121].

One signaling cascade activated by ethanol and angiotensin is activation of mitogen-activated protein kinases (MAPKs) including ERK1/2. The activation of RAS and cardiac injury caused by chronic ethanol were associated with activation of ERK1/2 in cardiac tissues, and both activation of RAS and activation of ERK1/2 were reduced by inhibition of ERK1/2 signaling through administration of MEK inhibitor PD98059 [120]. In this regard, it is interesting to note that ethanol potentiates ERK1/2 activation induced by angiotensin in hepatocytes [122]. Ang II inhibited INS-induced glucose uptake in vascular smooth muscle cells in an ERK1/2-dependent manner, and increased Ser307 phosphorylation of IRS-1 was also inhibited by MEK inhibitor PD98059 [123]. Ang II causes INS resistance in cardiomyocytes which is sensitive

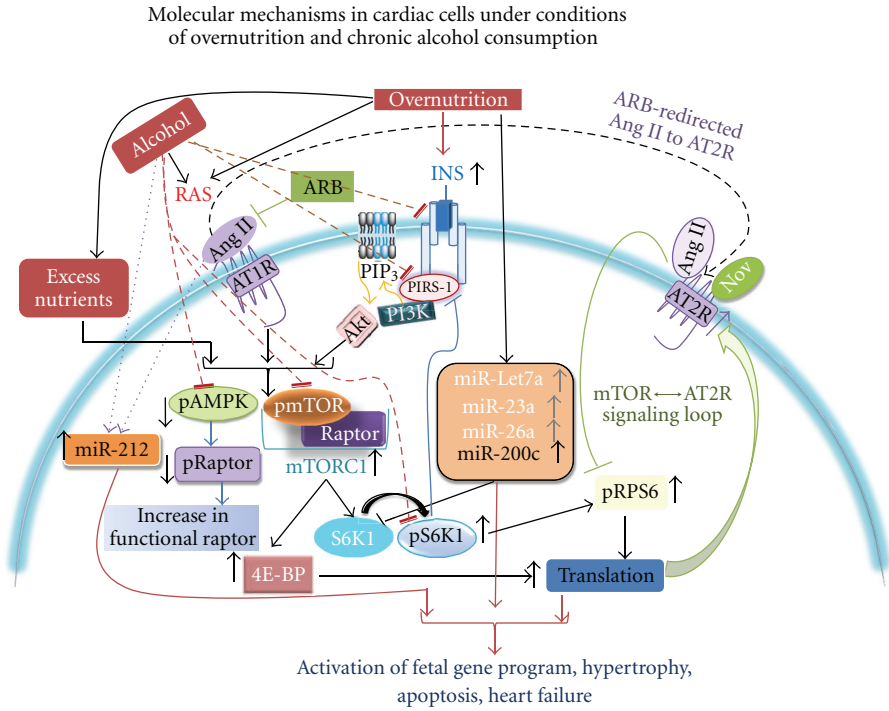

Figure 4: Established and putative signaling mechanisms activated by chronic alcohol consumption are superimposed on the signaling mechanisms activated by overnutrition to show how alcohol may exacerbate cardiac diseases in the setting of overnutrition. Dotted purple lines show alcohol mediated upregulation, and dotted orange lines show alcohol-induced inhibition of different signaling comonents.

to ERK1/2 inhibition [124]. In this regard, the role of mir-212 is noteworthy. Mir-212 has been shown to be upregulated by ethanol in intestinal cells [115] and Ang II in cardiac fibroblasts, which is associated with activation of ERK1/2 pathway [125]. Chronic ethanol ingestion also caused increased cardiac phosphorylation of CREB [126]. The significance of CREB phosphorylation emerges from a study reporting regulation of mir-212 in a CREB-dependent manner involving ERK1/2 activation [127]. Acetaldehyde causes activation of MAPKs including ERK1/2 in fetal human cardiac myocytes and ALDH2 transgene attenuated acetaldehyde-induced activation of phosphorylation of ERK1/2 and SAP/JNK [128]. Moreover, regulation of human microRNA-generating complex by MAPK/ERK pathway has also been reported [129]. These findings suggest possible interactions between alcohol and angiotensin on MAPK signaling and INS signaling through modulation of microRNA pathway in alcoholic cardiomyopathy.

Collectively, chronic alcohol consumption superimposed on overnutrition-mediated cardiac pathology can putatively induce the following signaling mechanisms: (1) inhibition of mTORC1 and attenuation of protective compensatory mechanisms; (2) inhibition of INS metabolic signaling via downregulation of INS receptor, IRS-1, GLUT4; (3) down-regulation of growth inhibitory mir-200 family microRNAs and (4) upregulation of mir-212 (Figure 4).

It is suggested that mTOR inhibits autophagy in myocardium, and this inhibition of autophagy by mTOR can result in accumulation of abnormal proteins that lead to enhanced endoplasmic reticulum stress, apoptosis, and cardiac dysfunction [130, 131]. Inhibition of mTOR by Everolimus (Rapamycin) seems to accentuate autophagy and improve cardiac function [130–132]. In this context, a recent study that demonstrates alcohol-induced autophagy in myocardium [133] is particularly noteworthy. This study shows that chronic alcohol treatment-induced cardiac dysfunction is associated with attenuation of mTOR stimulatory phosphorylation and induction of autophagy in mice; however, ALDH2 overexpressing mice were protected from these effects. Interestingly, ALDH2-mediated protection of heart in this model of chronic alcoholism seems to be mediated through Akt-mTOR-STAT3 (Signal Transducer and Activator of Transcription 3) signaling [133]. These data further support the notion that mTOR activation is cardioprotective [133, 134]. They also highlight the need for a cautious approach towards treatments involving ablation of mTOR-signaling in heart since enhanced autophagy can also deteriorate heart condition.

However, chronic alcohol intake also suppresses AMP kinase (AMPK) phosphorylation in myocardium [133]. Accumulating evidence suggests that AMPK is involved in GLUT4 translocation and glucose uptake [135–137]. Thus, inhibiting AMPK activation is an additional mechanism by which alcohol can dampen insulin metabolic signaling (Figure 4). Indeed, it is reported that in rats subjected to chronic alcohol consumption, the expression of AMPKalpha, and myocyte enhancer factor 2 (MEF2) is significantly reduced and this reduction is associated with GLUT4 decline [138].

5. Perspectives

In conditions of chronic alcohol intake and overnutrition, the potential compensatory mechanisms activated during overnutrition-related insulin resistance alone are overridden by alcohol-induced detrimental signaling (Figure 4). Moreover, since both overnutrition and alcohol activate RAS, and alcohol dampens insulin-signaling, insulin resistance is further exacerbated in cardiac tissue (Figure 4). In such conditions, upregulation of mir-212 by both alcohol and Ang II can lead to activation of fetal gene program and heart failure. Thus mir-212 can be a potential therapeutic target to protect the heart in conditions of overnutrition and chronic alcoholism. Recent advances in microRNA therapeutics are directed to develop effective strategies to block inappropriate expression of individual miRNAS that contribute to diseases [139–141]. In this context, the fact that miRNAs target multiple, functionally related genes (versus single genes), renders them powerful therapeutic tools. Antisense oligomers are known to work successfully in mammals [142–144]. A number of gene delivery systems have been developed to micromanage miRNAs by expressing such antisense oligonucleotides which include plasmids, and vectors based on adenoviruses, retroviruses, and lentiviruses. Cholesterol-conjugated antagomirs also provide an effective way to inhibit the activity of an miRNA. For example, the mir-21 antagomir/eraser is shown to be effective in alleviating cardiac fibrosis and hypertrophy since it derepresses the expression of mir-21target SPRY1 and increases myofibroblast apoptosis [144–146]. Therapeutic delivery of miRNAs can also be a very powerful strategy to regulate multiple genes at the same time. For example, therapeutic delivery of mir-200c is shown to ameliorate renal tubulointerstitial fibrosis [147]. However, a better understanding of the factors that regulate the rate and order of miRNA-mediated silencing of gene expression, colocalization of mRNA and miRNA, and miRNA turnover is crucial for optimizing the techniques for micromanipulating miRNAs as therapeutic targets.

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Research Article

Common Variants of Homocysteine Metabolism Pathway Genes and Risk of Type 2 Diabetes and Related Traits in Indians

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Hyperhomocysteinemia, a risk factor for cardiovascular disorder, obesity, and type 2 diabetes, is prevalent among Indians who are at high risk of these metabolic disorders. We evaluated association of common variants of genes involved in homocysteine metabolism or its levels with type 2 diabetes, obesity, and related traits in North Indians. We genotyped 90 variants in initial phase (2.115 subjects) and replicated top signals in an independent sample set (2.085 subjects). The variant *MTHFR*-rs1801133 was the top signal for association with type 2 diabetes (OR = 0.78 (95% CI = 0.67–0.92), $P = 0.003$) and was also associated with 2 h postload plasma glucose ($P = 0.04$), high-density lipoprotein cholesterol ($P = 0.004$), and total cholesterol ($P = 0.01$) in control subjects. These associations were neither replicated nor significant after meta-analysis. Studies involving a larger study population and different ethnic groups are required before ruling out the role of these important candidate genes in type 2 diabetes, obesity, and related traits.

1. Introduction

Homocysteine, a thiol containing amino acid, has emerged as a determinant for many complex metabolic disorders in the past few years. Hyperhomocysteinemia is a well-known independent risk factor for cardiovascular diseases [1, 2] and is also associated with diabetic complications [3–6], obesity, [7, 8] and metabolic syndrome [9]. Hyperhomocysteinemia may lead to any of the above metabolic disorders by elicitation of oxidative stress [10], systemic inflammation, [11] and/or endothelial dysfunction [12]. These factors are known to promote insulin resistance and β-cell dysfunction, two important underlying causes for type 2 diabetes [13]. Indians are at high risk for these metabolic disorders, as reflected by high prevalence of type 2 diabetes patients and metabolic syndrome [14, 15]. The observed trend of hyperhomocysteinemia among Indians [16] indicates the possibility of aberrant homocysteine metabolism with increased risk of metabolic disorders in Indian population. Hence, it becomes important to examine the association of variants of genes involved in homocysteine metabolism or modulating plasma homocysteine level with type 2 diabetes and related traits in Indians.

Previous studies suggested contribution of variants in homocysteine metabolism pathway genes in susceptibility to obesity, type 2 diabetes, or related traits [17–19]. Methylene tetrahydrofolate reductase (*MTHFR*) is shown to act synergistically with angiotensin-I-converting enzyme (*ACE*) to modulate type 2 diabetes risk [17]. The C677T polymorphism (rs1801133) of *MTHFR* is the most studied genetic variation and is associated with hyperhomocysteinemia [20]. Many studies have also reported association of C677T with type 2 diabetes and related complications [21–26]. Variants of *MTHFR* and other homocysteine metabolism pathway genes like *MTR* and *MTRR* have also been shown to be associated with obesity [27]. However, little insight is available in this regard from limited reports showing association of variants in homocysteine metabolism pathway

genes with plasma homocysteine levels in Indians [28, 29]. Here, we assessed association of 90 SNPs in 18 genes, from homocysteine metabolism pathway or those associated with homocysteine levels, with type 2 diabetes, obesity, and related traits in 4.200 North Indians.

2. Materials and Methods

2.1. Subjects. The study was performed in two phases: evaluation of 90 SNPs for association with type 2 diabetes and related traits in the first phase and replication of top signal in the second phase. All study participants were un-related, urban dwellers of Indo-European ethnicity from North India. All nondiabetic controls were recruited through "diabetes awareness camps" conducted in Delhi and National Capital Region, while type 2 diabetes patients were recruited from Endocrinology Clinic, All India Institute of Medical Sciences, New Delhi. Study population for the first phase consisted of 2,115 subjects comprising of 1.073 type 2 diabetes patients (from clinics) and 1.042 nondiabetic controls. Replication phase comprised of an independent sample set of 2.085 subjects including 1.047 type 2 diabetes patients and 1.308 nondiabetic controls. Subjects were characterized as type 2 diabetes patients and controls based on inclusion and exclusion criteria described previously [30]. Briefly, type 2 diabetes was defined by the WHO criteria [31], while criteria for non-diabetic controls were as follows: age ≥40 years, HbA1c < 6.0%, fasting plasma glucose <6.11 mmol/L, and no family history of diabetes.

Anthropometric and biochemical estimations were performed as described earlier [30]. The measured variables included height, weight, waist circumferences, hip circumferences, systolic and diastolic blood pressure, body mass index (BMI) and waist to hip ratio (WHR), glycosylated haemoglobin 1c (HbA1c), fasting plasma glucose (FPG), 2h postload plasma glucose (2h PPG), fasting plasma insulin (FPI), C-reactive protein (CRP), C-peptide, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglyceride (TG), blood urea, and creatinine. Subjects with BMI < 23 kg/m^2 were classified as normal weight (NW), whereas subjects with BMI ≥ 23 kg/m^2 were considered as overweight/obese (OW/OB), according to WHO Expert Committee recommendations for Asians [32]. Homeostasis model assessments of insulin resistance (HOMA-IR), β-cell function (HOMA-B), and insulin sensitivity (HOMA-S) were obtained using formulae as reported previously [33, 34].

The study was approved by ethics committee of participating institutions and was conducted in accordance with the principles of Helsinki Declaration. Written informed consent was obtained from each study subject.

2.2. Genotyping. We selected 79 SNPs from 15 genes which are involved in homocysteine metabolism pathway and 11 SNPs from 2 genes (*NOS3* and *ACE*) reported to be associated with homocysteine levels [17, 35] (see Table 1 in Supplementary Material available online at: 10.1155/2012/960318). SNPs in functional regions, with minor allele frequency (MAF) > 0.05, polymorphic in at least two populations and tag SNPs were preferred. Genotyping in the first phase was performed using Illumina Golden Gate assay. Stringent quality control (QC) criteria were applied as provided previously [36]. Also, 7.2% (147 samples) of total samples were genotyped in duplicate, and an error rate < 0.01 was figured. Of the 78 QC passed SNPs, those with MAF < 0.05 ($N = 9$) or deviating from Hardy-Weinberg equilibrium (HWE) in control subjects ($N = 2$) ($P < 6.41\times10^{-4}$, 0.05/78) were also excluded. Consequently, a total of 67 SNPs were considered for further association analyses.

In replication phase, we genotyped the top signal of first phase, rs1801133 (*MTHFR*), using iPLEX on a MassARRAY System (Sequenom, San Deigo, Calif, USA). The average genotyping success rate was 97% with >99.8% consistency rate in genotype calls in 5% duplicates.

2.3. Statistical Analysis. Genotype distributions were analyzed for deviation from HWE using χ^2 test. Association of SNPs with type 2 diabetes and obesity was determined by logistic regression assuming log additive model. Linear regression was performed under log additive model for association analysis of SNPs with quantitative traits in control subjects. The analyses were adjusted for sex, age, and BMI as appropriate. Odds ratios (ORs), β, and 95% confidence intervals (CI,) were calculated with respect to minor allele. To assess difference in allele frequency of the two study populations, equality of proportions Z-test was applied. Meta-analysis under fixed and random effect models was done by merging summary statistics of two phases. After correcting for multiple testing (Bonferroni correction), a P value $< 3.32 \times 10^{-5}$ ($\alpha = 0.05/(58 \times 26)$) was considered significant (58 independent SNPs and 26 traits). Power of study was calculated assuming 10% disease risk at $\alpha = 0.05$ under log additive model using Quanto (http://hydra.usc.edu/gxe/). Statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, Ill), Stata 10.1 (Stata Corporation, College Station, Tex, United States), and PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) unless specified otherwise.

3. Results

Clinical characters of the study subjects (phase 1 and phase 2) are provided in Table 1. The study had >82.9% power to detect association of variants with allele frequency of 0.20 and genetic effect size of 1.25. Allele frequency of controls and cases of the initial and replication phase for SNP rs1801133 did not differ significantly. The allelic and genotypic distribution of SNPs for type 2 diabetes patients and non-diabetic control subjects recruited in the first phase are provided in Supplementary Table 1, whereas details for normal weight (NW) and overweight/obese (OW/OB) subjects are presented in Supplementary Table 2.

Association analysis results of all the SNPs with type 2 diabetes are presented in Supplementary Table 3. *MTHFR* variant rs1801133 showed strongest association with type 2 diabetes in initial phase (OR = 0.78 (95% CI 0.67–0.92), $P = 0.003$) (Table 2). However, this could not be replicated in the second phase (OR = 1.01 (95% CI

Table 1: Anthropometric and clinical characteristics of the study populations.

Characteristics	Phase 1		Phase 2	
	Type 2 diabetes patients	Control Subjects	Type 2 diabetes Patients	Control Subjects
N (Men/Women)	1019 (592/427)	1006 (606/400)	1047 (619/428)	1038 (516/522)
Age (years)	53 (45–62)	50 (44–60)	55 (49–62)	54 (45–64)
BMI (Kg/m^2)				
Men	23.80 (22.00–26.00)	23.10 (20.10–5.70)	24.82 (22.68–27.76)	24.68 (22.35–27.39)
Women	26.70 (24.20–29.20)	25.00 (21.10–8.50)	27.39 (24.56–30.63)	26.37 (23.13–29.33)
WHR				
Men	1.00 (0.97–1.03)	0.97 (0.92–1.0)	0.98 (0.95–1.03)	0.97 (0.93–1.01)
Women	1.00 (0.97–1.03)	0.86 (0.82–0.92)	0.93 (0.87–0.97)	0.86 (0.82–0.90)
Systolic BP (mmHg)	130 (130–140)	120 (112–132)	130 (122–140)	130 (120–140)
Diastolic BP (mmHg)	80 (78–90)	80 (70–88)	82 (80–90)	80 (78–90)
HbA1c (%)	7.80 (6.50–9.40)	5.20 (4.90–5.60)	8.20 (6.90–9.60)	5.65 (5.33–5.89)
FPG (mmol/L)	7.90 (6.40–10.30)	4.90 (4.50–5.30)	7.79 (6.21–10.27)	4.87 (4.43–5.27)
2h PPG (mmol/L)		5.57 (4.83–6.30)		5.63 (4.88–6.22)
FPI (pmol/L)	82.80 (42.0–166.80)	32.40 (17.40–57.60)	74.40 (26.00–96.00)	43.80 (28.20–63.60)
HOMA-IR	5.20 (2.30–9.60)	1.20 (0.60–2.00)	4.30 (2.30–9.30)	1.60 (1.00–2.40)
HOMA-B	60.25 (25.3–154.51)	73.40 (40.70–138.60)	61.66 (25.61–131.86)	105.27 (65.13–167.32)
HOMA-S	0.20 (0.10–0.46)	0.86 (0.49–1.69)	0.23 (0.11–0.44)	0.64 (0.42–1.03)
C-peptide (nmol/L)	0.89 (0.56–1.36)	0.53 (0.36–0.73)	1.05 (0.69–1.60)	0.66 (0.50–0.86)
CRP (mg/L)	2.20 (0.90–4.70)	1.30 (0.60–3.00)	1.86 (0.90–3.44)	1.61 (0.90–3.04)
TC (mmol/L)	4.20 (3.50–5.00)	4.40 (3.7–5.10)	4.64 (3.86–5.42)	4.91 (4.22–5.52)
LDL-C (mmol/L)	2.57 (1.99–3.36)	2.79 (2.33–3.41)	2.73 (2.10–3.42)	3.01 (2.49–3.51)
HDL-C (mmol/L)	1.03 (0.88–1.22)	1.06 (0.88–1.28)	1.11 (0.94–1.34)	1.24 (1.06–1.46)
TG (mmol/L)	1.60 (1.10–2.20)	1.30 (1.00–1.80)	1.43 (0.98–2.13)	1.22 (0.86–1.64)

Values provided are median (interquartile range).
N: number of individuals, BMI: body mass index, WHR: waist to hip ratio, BP: blood pressure, HbA1c: glycosylated haemoglobin 1c, FPG: fasting plasma glucose, 2h PPG: 2h postload plasma glucose, FPI: fasting plasma insulin, HOMA-IR: homeostasis model assessments of insulin resistance, HOMA-B: homeostasis model assessments for β-cell function, HOMA-S: homeostasis model assessments for insulin sensitivity, CRP: C-reactive protein, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein-cholesterol, TG: triglyceride.

0.87–1.19), $P = 0.86$). Meta-analysis also failed to detect any association of rs1801133 with type 2 diabetes (P random effect (P_r) = 0.38, P fixed effect (P_f) = 0.05) (Table 3).

Our previous studies have indicated influence of obesity status on association of genetic variants with type 2 diabetes in North Indians [30, 37]. Thus, we stratified our subjects into NW and OW/OB groups and assessed association with type 2 diabetes in these subgroups. Among NW subjects, we observed nominal association of *MTHFR*-rs1801133, *MTHFR*-rs9651118, *CHDH*-rs4563403, *CBS*-rs706208, and *MTHFD1L*-rs1555179 with type 2 diabetes (P value range= 0.002–0.04) (Table 2). On the other hand, in OW/OB individuals, *MTHFD1*-rs1076991 (OR = 0.81 (95% CI 0.69–0.95) $P = 0.01$) and *TCN2*-rs1801198 (OR = 0.85 (95% CI 0.72–0.99), $P = 0.04$) showed nominal association with type 2 diabetes. In the second phase, association of *MTHFR*-rs1801133 with type 2 diabetes in NW subjects could not be replicated either in the second phase ($P = 0.53$) or upon Meta-analysis (P_r/P_f = 0.52/0.07) (Table 3).

Further, we investigated association of these SNPs with obesity status (OW/OB versus NW) in non-diabetic control subjects (Supplementary Table 4). Only a nominal association of *CHDH*-rs4563403 (OR = 0.69 (95% CI 0.52–0.92), $P = 0.01$), *TCN2*-rs1801198 (OR = 1.24 (95% CI 1.04–1.48), $P = 0.02$), and *MTR*-rs16834521 (OR = 0.82 (95% CI 0.68–0.99), $P = 0.04$) was observed in the first phase (Table 2). The top signal *MTHFR*-rs1801133 was not associated with obesity in both first and second phases or after meta-analysis (Tables 2 and 3).

We also assessed association of variants with quantitative traits related to type 2 diabetes and obesity. The first phase control subjects showed only nominal association with the quantitative traits as shown in Supplementary Table 5. Most significant association was observed at *AMD1*-rs1007274 with LDL-C levels ($\beta = -6.35$ (95% CI (-9.61)–(-3.1)), $P = 1.41 \times 10^{-4}$). *MTHFR* variant rs1801133 was nominally associated with 2h PPG ($\beta = -5.06$ (95% CI (-9.72)–(-0.40)), $P = 0.03$), HDL-C ($\beta = 2.14$ (95% CI 0.68–3.59), $P = 0.004$) and TC ($\beta = 5.61$ (95% CI 1.28–9.94), $P = 0.01$) in the first phase (Table 3). In the second phase, while none of the above associations were replicated, new associations were observed with FPI ($\beta = -0.77$ (95% CI -1.42–(-0.11)), $P = 0.02$), HOMA-IR ($\beta = -0.20$ (95% CI -0.36–(-0.04)), $P = 0.01$), HOMA-B ($\beta = -652.8$ (95% CI -1165–(-140.1)), $P = 0.01$) and HOMA-S ($\beta = 0.62$ (95% CI 0.13–1.11), $P = 0.01$). Meta-analysis revealed association of *MTHFR*-rs1801133 with LDL-C (P_r/P_f = 0.02/0.02, Q =

Table 2: Variants showing association (uncorrected *P*-value) with type 2 diabetes and obesity.

Trait	Gene	SNP	Allele (major/minor)	MAF (affected/unaffected)	OR (95% CI)	*P*
Type 2 diabetes (total study population: 1019 cases versus 1006 controls)†	*MTHFR*	rs1801133	C/T	0.18/0.21	0.78 (0.67–0.92)	0.003
Type 2 diabetes (Normal weight subjects: 290 cases versus 436 controls)†	*MTHFR*	rs1801133	C/T	0.18/0.21	0.62 (0.46–0.85)	0.002
	MTHFR	rs9651118	C/T	0.26/0.25	1.31 (1.01–1.68)	0.04
	CHDH	rs4563403	G/A	0.10/0.11	0.66 (0.45–0.95)	0.02
	CBS	rs706208	T/C	0.41/0.39	1.30 (1.03–1.63)	0.03
	MTHFD1L	rs1555179	C/T	0.26/0.23	1.36 (1.04–1.78)	0.03
Type 2 diabetes (Obese/over-weight subjects: 691cases versus 562 controls)†	*MTHFD1*	rs1076991	A/G	0.40/0.44	0.81 (0.69–0.95)	0.01
	TCN2	rs1801198	G/C	0.42/0.44	0.85 (0.72–0.99)	0.04
Obesity (562 Obese/over-weight versus 436 normal weights)‡	*CHDH*	rs4563403	G/A	0.09/0.13	0.69 (0.52–0.92)	0.01
	TCN2	rs1801198	G/C	0.46/0.41	1.24 (1.04–1.48)	0.02
	MTR	rs16834521	T/C	0.31/0.35	0.82 (0.68–0.99)	0.04

†Analyses adjusted for sex, age, and BMI. ‡Analysis performed only in control subjects and adjusted for sex and age, MAF: minor allele frequency, OR: odds ratio, CI: confidence interval.

Table 3: Association of rs1801133 with all traits in initial phase, replication phase, and meta-analysis.

Trait	Initial phase		Replication phase				Meta-analysis			
	OR/β (95% CI)	*P*	OR/β (95% CI)	*P*	P_f	P_r	OR_f/β_f	OR_r/β_r	*Q*	*I*
Type2 diabetes (all)†	0.78 (0.67–0.92)	0.003	1.01 (0.87–1.19)	0.86	0.05	0.38	0.90	0.89	0.024	80.44
Type2 diabetes (NW)†	0.62 (0.46–0.85)	0.002	1.11(0.80–1.54)	0.53	0.07	0.52	0.81	0.83	0.012	84.30
Type2 diabetes (OW/OB)†	0.86 (0.71–1.04)	0.13	0.98 (0.82–1.17)	0.85	0.25	0.25	0.93	0.93	0.32	0.25
Obesity (NW versus OW/OB)‡	1.07 (0.86–1.33)	0.56	1.11 (0.86–1.43)	0.42	0.33	0.33	1.09	1.09	0.82	0
BMI‡	0.32 (−0.19–(−0.84))	0.22	0.25 (−0.26–0.76)	0.34	0.12	0.12	0.29	0.29	0.84	0
Weight‡	1.41 (0.02–2.80)	0.05	0.43 (−0.95–1.81)	0.54	0.07	0.07	0.92	0.92	0.33	0
WC‡	0.47 (−0.76–1.70)	0.45	0.28 (−1.12–1.68)	0.70	0.41	0.41	0.39	0.39	0.84	0
WHR‡	−0.003 (−0.01–0.005)	0.46	−0.005 (−0.01–0.003)	0.24	0.18	0.18	−0.004	−0.004	0.73	0
FPG†	0.55 (−0.66–1.77)	0.37	-0.83 (−2.14–0.47)	0.21	0.84	0.86	−0.09	−0.12	0.13	56.98
2h PPG†	−5.06 ((−9.72)–(−0.40))	0.03	−1.13 (−4.84–2.58)	0.55	0.07	0.15	−2.65	−2.83	0.20	40.15
HbA1c†	0.02 (−0.03–0.07)	0.41	−0.03 (−0.07–0.01)	0.15	0.58	0.79	−0.009	−0.007	0.12	59.73
FPI†	0.43 (−0.44–1.30)	0.33	−0.77 ((−1.42)–(−0.11))	0.02	0.21	0.73	−0.33	−0.20	0.03	78.49
HOMA-IR†	0.11 (−0.09–0.31)	0.30	−0.20 (−0.36–−0.04)	0.01	0.21	0.73	−0.08	−0.05	0.02	81.77
HOMA-B†	70.25 (−34.99–175.50)	0.19	−652.8 (−1165–−140.1)	0.01	0.44	0.49	41.02	−245.94	0.0068	86.36
HOMA-S†	−0.09 (−0.26–0.07)	0.27	0.62 (0.13–1.11)	0.01	0.82	0.53	−0.02	0.23	0.0067	86.41
C-peptide†	0.01 (−0.10–0.12)	0.86	−0.07 (−0.17–0.04)	0.22	0.44	0.44	−0.03	−0.03	0.32	0
CRP†	−0.16 (−0.38–0.05)	0.14	−0.12 (−0.34–0.1)	0.28	0.07	0.07	−0.14	−0.14	0.79	0
HDL-C†	2.14 (0.68–3.59)	0.004	−0.05 (−1.31–1.21)	0.94	0.07	0.36	0.89	1.01	0.03	79.83
LDL-C†	3.25 (−0.32–6.81)	0.07	2.61 (−1.03–6.26)	0.16	0.02	0.02	2.94	2.94	0.81	0
TC†	5.61 (1.28–9.94)	0.01	2.02 (−2.43–6.47)	0.37	0.01	0.03	3.86	3.85	0.26	22.03
TG†	7.08 (−0.88–15.05)	0.08	−3.73 (−10.26–2.81)	0.26	0.81	0.79	0.62	1.43	0.04	76.37
Systolic BP†	1.53 (−0.41–3.48)	0.12	−0.77 (−2.58–1.03)	0.40	0.66	0.76	0.29	0.35	0.09	65.50
Diastolic BP†	0.77 (−0.30–1.85)	0.16	−0.74 (−1.82–0.35)	0.18	0.95	0.98	0.02	0.02	0.05	73.47
Creatinine†	0.02 (−0.004–0.04)	0.11	−0.01 (−0.05–0.02)	0.50	0.33	0.68	0.01	0.01	0.16	50.00
Urea†	0.28 (−0.63–1.20)	0.54	−0.12 (−1.14–0.9)	0.82	0.76	0.76	0.10	0.10	0.57	0
Uric acid†	0.37 (−0.02–0.76)	0.06	0.05 (−0.1–0.19)	0.55	0.22	0.31	0.09	0.15	0.13	56.68

†Analyses adjusted for sex, age, and BMI.
‡Analysis performed only in control subjects and adjusted for sex and age.
OR: odds ratio, CI: confidence interval, P_f: *P* value for fixed effect, P_r: *P* value for random effect OR_f: odds ratio for fixed effect, β_f: beta value for fixed effect, OR_r: odds ratio for random effect, β_r: beta value for random effect, *Q*: *P* value for Cochrane's *Q* statistic, and *I*: I^2 heterogeneity index (0–100).

0.81, $I^2 = 0$) and TC ($P_r/P_f = 0.01/0.03$, $Q = 0.26$, $I^2 = 22.03$). The association analysis results of *MTHFR*-rs1801133 with all traits (dichotomous and continuous) in the initial and replication phases and after meta-analysis are presented in Table 3. However, none of the associations observed in the present study were significant after correcting for multiple testing.

4. Discussion

Homocysteine plays an important role in cell metabolism as it is involved as an essential intermediate in the transfer of activated methyl groups in the activated methyl cycle. This cycle is responsible for global and gene promoter-specific DNA methylation, an important factor in regulating gene expression [38–40]. The biological relevance of homocysteine metabolism and its association with metabolic disorders make it an important candidate pathway for type 2 diabetes. To the best of our knowledge, the present study is first to comprehensively evaluate association of variants of homocysteine metabolism pathway genes as well as genes associated with homocysteine levels with type 2 diabetes, obesity, and related traits.

The C677T (rs1801133) variant of *MTHFR* is an established variant for plasma homocysteine levels [20, 41] and has also been reported to be associated with type 2 diabetes, its complications, and related traits like LDL-C levels [42, 43]. Association of *MTHFR*-rs1801133 with type 2 diabetes in different population has been inconsistent [13, 17, 44]. The association studies of *MTHFR*-rs1801133 with type 2 diabetes and LDL-C levels have also been limited to studies with small sample sizes. In the present study, *MTHFR*-rs1801133 showed association with type 2 diabetes and 2h PPG, HDL-C, and TC in initial phase but could not be replicated in the second phase. Interestingly, *MTHFR*-rs1801133 showed nominal association with LDL-C levels in meta-analysis as also suggested in a previous study though this association was not significant after correcting for multiple testing. The effect of *MTHFR*-rs1801133 variant on type 2 diabetes is most likely to be modulated by influencing levels of homocysteine. However, in the absence of levels of homocysteine in the present study population, this aspect could not be probed.

We also observed association of other investigated variants with quantitative traits. Earlier reports have suggested variants from *MTR* to be associated with obesity [27], similar to this, we also observed variants of *MTR* (rs16834521) to be associated with BMI. Variants from this gene were also associated with TC (rs1805087 and rs10737812) and HbA1c (rs1805087) levels. Interestingly, for the first time, we found association of variant rs1007274 of *AMD1* with plasma levels of LDL-C, CRP and TC. Though these associations could not reach the threshold after multiple testing corrections, they provide important leads for follow-up studies in larger study populations.

The present study is the largest among the studies investigating association of *MTHFR*-rs1801133 and was sufficiently powered to detect the previously reported effect sizes. However, we were unable to confirm the associations in Indian population. Differences in genetic architecture and LD pattern due to ethnic differences [17, 21, 44] could be one of the possible reasons for the observed inconsistency in the association of *MTHFR*-rs1801133 with type 2 diabetes. Previous studies by our group also observed inconsistency in association of variants of *TNF-LTA* [45] and *TNFRSF1B* [46] in Indians as compared to other ethnic groups indicating difference in genetic structure.

Population stratification may also give rise to spurious associations in a case-control study. However, cases and controls in our study populations were recruited from geographical locations that form a homogenous cluster as suggested by Indian genome variation consortium [47]. The multidimensional scaling (MDS) plot drawn from the information of 608 independent variants genotyped in the same set of samples indicates genetic homogeneity of the samples (Supplementary Figure 1). Study subjects of present study are also a part of an ongoing genome-wide association study of type 2 diabetes (unpublished data) and have been found to belong to single cluster after performing MDS using genome-wide SNP data. Hence, the subjects of two study populations were genetically similar, but phenotypic difference in terms of biochemical traits was observed between the two study groups which could have influenced association results. It is also known that dietary habits can influence levels of homocysteine and in Indians; individuals with vegetarian diet have been shown to have higher levels of homocysteine [48]. We observe difference in the number of individuals on vegetarian and nonvegetarian diets among both cases and controls of phase1 and phase2 (Supplementary Table 6). We believe this difference in status of vegetarian/nonvegetarian diet would have led to the difference in the association results of the two phases, but additional information on the frequency and kind of non-vegetarian diet along with other dietary information would have best answered this question.

5. Conclusions

In conclusion, variants from genes involved in homocysteine metabolism showed suggestive evidence for association with type 2 diabetes, obesity, and other related traits. However, these associations were not confirmed upon replication. Thus, studies involving a larger study population and different ethnic groups are required before completely ruling out the role of these important candidate genes in type 2 diabetes, obesity, and related traits.

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Research Article

Short-Chain Fatty Acid Propionate Alleviates Akt2 Knockout-Induced Myocardial Contractile Dysfunction

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Background and Aims. Dysregulation of Akt has been implicated in diseases such as cancer and diabetes, although little is known about the role of Akt deficiency on cardiomyocyte contractile function. This study was designed to examine the effect of Akt2 knockout-induced cardiomyocyte contractile response and the effect of dietary supplementation of short-chain fatty acid propionate on Akt2 knockout-induced cardiac dysfunction, if any. *Methods and Results*. Adult male wild-type (WT) and Akt2 knockout mice were treated with propionate (0.3 g/kg, p.o.) or vehicle for 7 days. Oral glucose tolerance test (OGTT) was performed. Cardiomyocyte contractile function and mitochondrial membrane potential were assessed. Expression of insulin-signaling molecules Akt, PTEN, GSK3β, and eNOS receptors for short-chain fatty acids GPR41, and GPR43 as well as protein phosphatase PP2AA, PP2AB, PP2C were evaluated using Western blot analysis. Our results revealed that Akt2 knockout led to overt glucose intolerance, compromised cardiomyocyte contractile function (reduced peak shortening and maximal velocity of shortening/relengthening as well as prolonged relengthening), loss of mitochondrial membrane potential, decreased GPR41 and elevated GPR43 expression, all of which, with the exception of glucose intolerance and elevated GPR43 level, were significantly attenuated by propionate. Neither Akt2 knockout nor propionate affected the expression of protein phosphatases, eNOS, pan, and phosphorylated PTEN and GSK3β. *Conclusions*. Taken together, these data depicted that Akt2 knockout may elicit cardiomyocyte contractile and mitochondrial defects and a beneficial role of propionate or short-chain fatty acids against Akt2 deficiency-induced cardiac anomalies.

1. Introduction

Cardiovascular disease, in particular heart disease, is the leading cause of morbidity and mortality in the USA and the rest of the world. Many risk factors have been confirmed for cardiovascular diseases including diabetes mellitus, insulin resistance, hypertension, and obesity [1–3]. Among these devastating factors, insulin resistance is the most recent attention due to the obesity pandemic [2, 4]. Although a number of scenarios have been postulated including dyslipidemia, inflammation, endoplasmic reticulum, and oxidative stress for insulin resistance-induced cardiovascular dysfunction [5–10], the precise mechanisms behind cardiac dysfunction in insulin resistance are still elusive thus making adequate clinical management somewhat ineffective. Although diet and exercise exhibit beneficial effects in retarding the progression of insulin resistance-associated cardiac anomalies, although the lifestyle remedies fail to eradicate the cardiac pathologies [11]. Therefore, recent attention has been geared towards identifying possible novel therapy to either increase insulin sensitivity or circumvent insulin resistance-induced impairment in the heart.

Insulin-receptor signaling plays an essential role in the regulation of myocardial oxidative phosphorylation and myocardial contractile function [3, 12]. This is supported by the fact that insulin-receptor knockout drastically dampens the oxidative phosphorylation and exacerbates cardiac dysfunction [13, 14]. Although insulin-receptor signaling is highly complex, involving a cascade of signaling molecules, the phosphatidylinositol 3-kinase (PI3K)-Akt pathway is

deemed the main player governing the majority of metabolic properties of insulin, and thus represents an important component in the insulin-signaling network [15, 16]. Akt is a serine/threonine kinase directly downstream of PI3K to mediate the metabolic actions of insulin [15]. Dysregulation of Akt has been documented in a number of diseases including cardiovascular diseases, cancer, and metabolic disorders [17–19]. As a matter of fact, the onset of insulin resistance and diabetes is often linked to changes in Akt phosphorylation. Akt2 knockout has been demonstrated to trigger global insulin resistance [20]. Nonetheless, a number of mechanisms independent or upstream of Akt may also contribute to the onset and progression of insulin resistance such as upregulation of TNF-α and protein-tyrosine phosphatase 1B [21].

Recent evidence has suggested a role of dietary fiber (nonstarch polysaccharides) and a resistant starch in the protection against insulin resistance and overall metabolic syndrome [22, 23]. Dietary fiber and resistance starch may escape digestion in the upper gastrointestinal tract and undergo anaerobic fermentation in the colon. This process produces short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, as the major by-products. SCFAs are known to possess significant biological effects on colonic epithelium both *in vivo* and *in vitro* [24]. Experimental evidence indicated that propionate is capable of stimulating insulin secretion. Supplementation of propionate in baboons was found to lower the postprandial blood-glucose responsiveness [25]. However, dietary supplementation with propionate also decreased fasting serum glucose and maximal insulin increments during a subsequent oral glucose tolerance test [25].

Given that cardiac dysfunction is a major complication in insulin resistance and that Akt-signaling anomalies have been implicated in cardiac dysfunction [17, 26, 27], this study was undertaken to examine the effect of Akt2 knockout on cardiomyocyte dysfunction, if any, and the impact of propionate treatment on Akt2 knockout-induced cardiomyocyte responses. In an effort to elucidate the mechanisms of action involved in Akt2 knockout and propionate-induced cardiomyocyte mechanical and mitochondrial responses, crucial protein markers of insulin signaling such as Akt, phosphatase and tensin homologue on chromosome 10 (PTEN), glycogen synthase kinase 3β (GSK3β), eNOS, and protein phosphatase, which usually negatively regulates insulin signaling [28], were examined in hearts from wild-type (WT) and Akt2 knockout mice.

2. Materials and Methods

2.1. Experimental Animals and Propionate Treatment. The experimental procedures described in this paper were approved by the University of Wyoming Animal Use and Care Committee (Laramie, Wyo, USA). In brief, 5-6-month-old adult male Akt knockout and the age-/gender-matched wild-type (WT) mice were used. Production of the Akt knockout mice was described in detail previously [20]. All mice were housed in a temperature-controlled room under a 12 hr/12 hr-light/dark and allowed access to tap water *ad libitum* in the School of Pharmacy Animal Facility. Akt knockout and wild-type mice were randomly assigned to receive either propionate (0.3 g/kg, p.o.) or vehicle (saline) for 7 days.

2.2. Oral Glucose Tolerance Test (OGTT). Oral glucose tolerance test was performed at the beginning and the end of the treatment-period following a 6-hr fasting period described previously [27]. Briefly, 2 g/kg glucose was given using gavage following which blood-glucose levels were measured using a glucometer (Accu-CheckII, model 792; Boehringer Mannheim Diagnostics, Indianapolis, Ind, USA) immediately before the glucose challenge or at 0, 30, 60, and 120 min thereafter.

2.3. Isolation of Murine Cardiomyocytes. Single cardiomyocytes were enzymatically isolated as described [27]. Briefly, hearts were removed and perfused (37°C) with oxygenated (5% CO_2 : 95% O_2) Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES, and 11.1 glucose. Hearts were subsequently perfused with a Ca^{2+}-free KHB-buffer that contains Liberase Blendzyme (10 mg/mL; Roche, Indianapolis, Ind, USA) for 15 min. After perfusion, left ventricles were removed and minced to disperse the individual ventricular myocytes in Ca^{2+}-free KHB-buffer. Extracellular Ca^{2+} was added incrementally to 1.25 mM. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were not used. Only rod-shaped myocytes with clear edges were selected for the recording of mechanical properties or intracellular Ca^{2+} transients.

2.4. Cell Shortening/Relengthening. Mechanical properties of cardiomyocytes were assessed using an SoftEdge MyoCam system (IonOptixCorp., Milton, Mass, USA) [27]. Myocytes were placed in a chamber mounted on the stage of an inverted microscope (Olympus IX-70) and superfused with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, at pH 7.4. The cells were field stimulated with a suprathreshold voltage and at a frequency of 0.5 Hz (3 ms duration) with the use of a pair of platinum wires placed on opposite sides of the chamber connected to an FHC Inc. stimulator (Frederick Haer & Co., Brunswick, Neb, USA). The soft-edge software (IonOptix) was used to capture changes in cell length during contraction. Cell shortening and relengthening were assessed including peak shortening (PS): peak contractility, time-to-PS (TPS): contraction duration, time-to-90% relengthening (TR_{90}): relaxation duration, and maximal velocities of shortening/relengthening ($\pm$dL/dt): maximal pressure development and decline.

2.5. Measurement of Mitochondrial Membrane Potential (MMP). Murine cardiomyocytes were suspended in HEPES-saline buffer, and mitochondrial membrane potential ($\Delta\Psi_m$) was detected as described [29]. Briefly, after incubation with JC-1 (5 μM) for 10 min at 37°C, cells were rinsed twice by sedimentation using the HEPES saline buffer

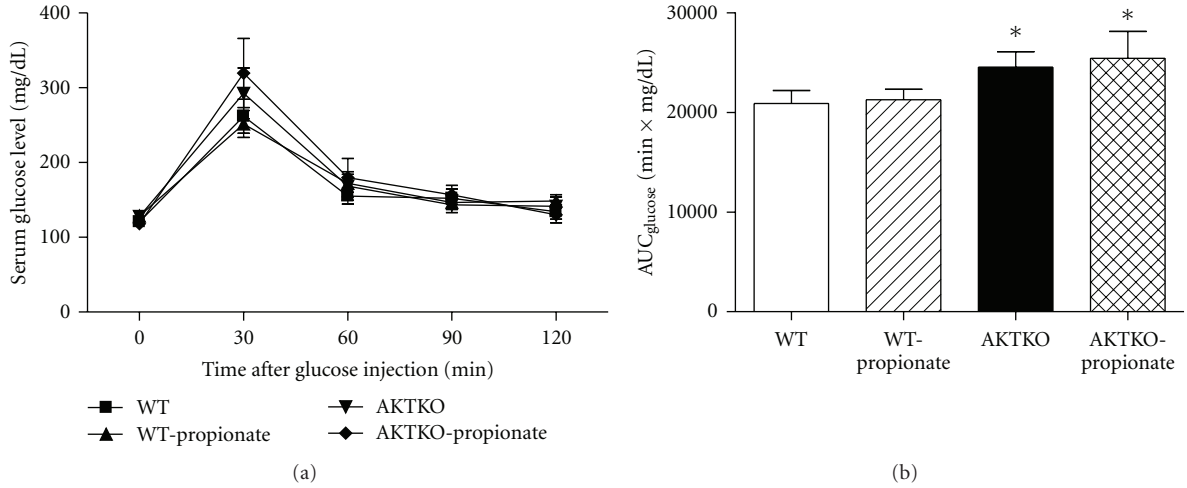

Figure 1: Oral glucose tolerance test (OGTT, 2 g/kg body weight) in WT and Akt2 knockout (AKTKO) mice treated with or without propionate (0.3 g/kg, p.o.) for 7 days. (a) OGTT curve; (b) Area underneath the curve plotted in panel A. Mean ± SEM, n = 7-8 mice per group, and $^*P < 0.05$ versus WT group.

free of JC-1 before being examined under a confocal laser-scanning microscope (Leica TCS SP2) at excitation wavelength of 490 nm. The emission of fluorescence was recorded at 530 nm (monomer form of JC-1, green) and at 590 nm (aggregate form of JC-1, red). Results in fluorescence intensity were expressed as 590-to-530-nm emission ratio.

2.6. Western Blot Analysis. Protein expressions of Akt, PTEN, PP2AA, PP2AB, PP2C, GSK3β, eNOS, GPR41, and GPR43 were examined by Western blot analysis. Left ventricular tissues were homogenized and centrifuged at 70,000 g for 20 min at 4°C. The supernatants were used for immunoblotting. The extracted proteins were separated on 10–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After being blocked, the membrane was incubated with anti-Akt (1 : 1,000), anti-phospho-Akt (pAkt, 1 : 1,000), anti-PTEN (1 : 1,000), anti-phospho-PTEN (pPTEN, 1 : 1,000), anti-PP2AA (1 : 1,000), anti-PP2AB (1 : 1,000), anti-PP2C (1 : 1,000), anti-GSK3β (1 : 1,000), anti-phospho-GSK3β (1 : 1,000), anti-eNOS (1 : 1,000), anti-GPR41 (1 : 1,000), anti-GPR43 (1 : 1,000), and anti-β-actin (loading control, 1 : 2,000) antibodies at 4°C overnight. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif, USA) or Cell Signaling Technology (Beverly, Mass, USA). After incubation with the primary antibodies, blots were incubated with horseradish peroxidase-linked secondary antibodies (1 : 5,000) for 60 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, Wis, USA). The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad) coupled with a Bio-Rad personal computer analysis software [27].

2.7. Statistical Analysis. Data were mean ± SEM. Statistical significance ($P < 0.05$) for each variable was determined by a one-way ANOVA (two-way for OGTT) followed by Tukey's *post hoc* test.

3. Results

3.1. Effect of Propionate Treatment on Whole-Body Glucose Tolerance. Oral-glucose tolerance was performed at the end of the 7-day propionate treatment. Following the oral-glucose challenge, serum-glucose levels in WT mice started to decline after peaking at 30 min and returned back to near baseline levels at 120 min. In contrast to the WT mice, Akt2 knockout mice displayed glucose intolerance as evidenced by a higher area under the curve (AUC) although neither basal- nor postchallenge glucose levels were significantly different between WT and Akt2 knockout mice. Propionate treatment failed to affect basal blood glucose or glucose-disposal rate following oral-glucose ingestion in either WT or Akt2 knockout mice. AUC in Akt2 knockout mice remains significantly greater compared to the WT group (Figure 1).

3.2. Effect of Propionate Treatment on Mechanical and Mitochondrial Properties of Cardiomyocytes. Mechanical properties revealed that the resting cell length was similar in cardiomyocytes from WT and Akt2 knockout mice with or without propionate treatment. Cardiomyocytes from Akt2 knockout mice displayed significantly reduced peak shortening and maximal velocity of shortening/relengthening (± dL/dt), prolonged time-to-90% relengthening (TR_{90}) associated with unchanged time-to-peak shortening (TPS) compared with those from the WT mice. Interestingly, these mechanical changes were ablated in Akt2 knockout mice receiving propionate while propionate itself failed to affect cardiomyocyte mechanical properties in cardiomyocytes from WT mice (Figure 2). In order to better understand the mechanism(s) underneath propionate-exerted beneficial effect against Akt2 knockout-induced myocardial contractile defect, JC-1 fluorescence was used to

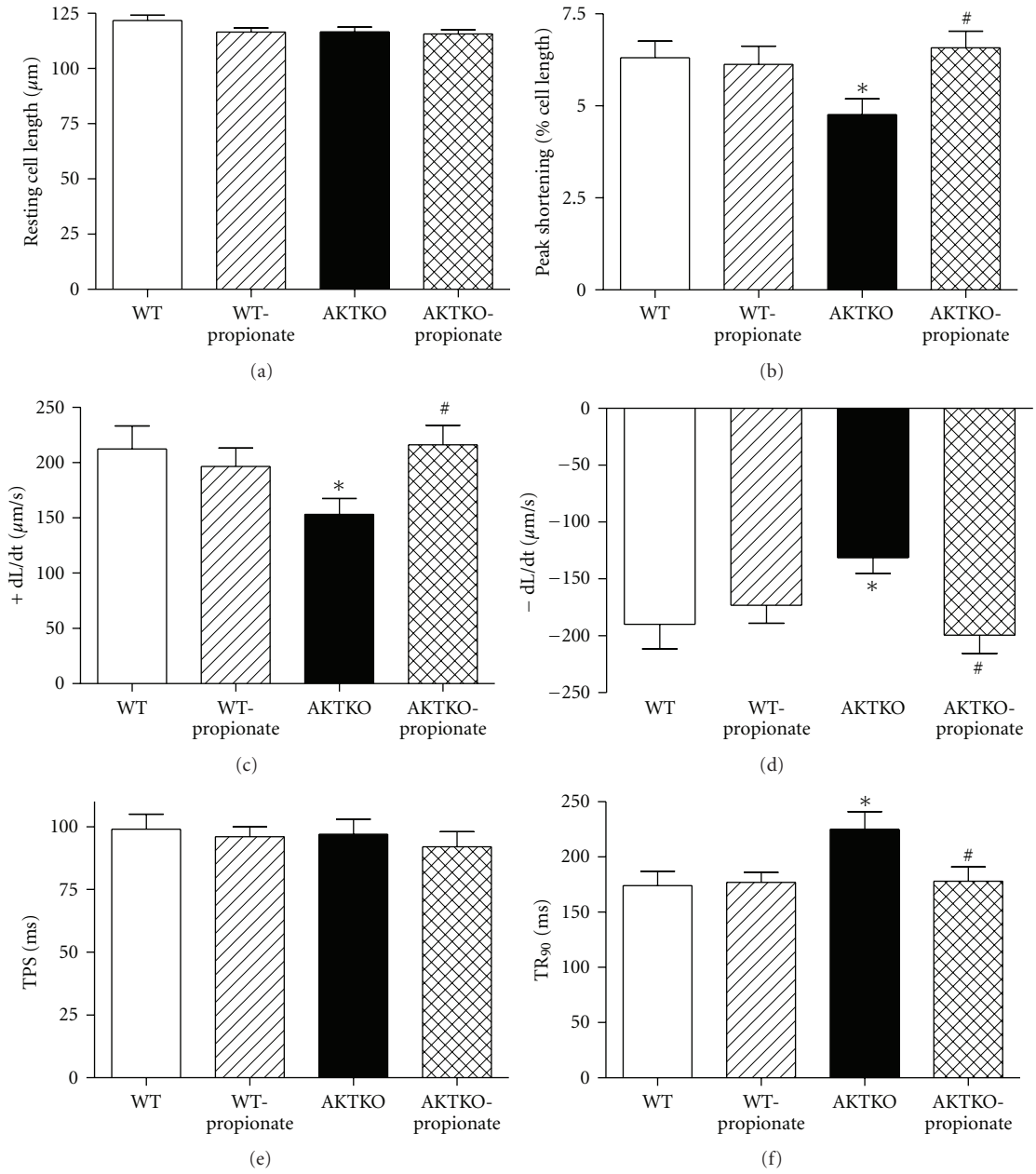

Figure 2: Cardiomyocyte mechanical function in WT and Akt2 knockout (AKTKO) mice treated with or without propionate (0.3 g/kg, i.p.) for 7 days. (a) Resting cell length; (b) peak shortening (PS, normalized to resting cell length); (c) maximal velocity of shortening (+ dL/dt); (d) maximal velocity of relengthening (− dL/dt); (e) time-to-PS (TPS); (f) time-to-90% relengthening (TR_{90}). Mean ± SEM, n = 103-104 cells per group, $^{*}P < 0.05$ versus WT group, and $^{\#}P < 0.05$ versus AKTKO group.

measure MMP. Cardiomyocytes from Akt2 knockout mice displayed significantly decreased MMP at both 30 min and 90 min, the effect of which was also reconciled by propionate treatment. Propionate itself did not alter MMP levels in WT mice (Figure 3).

3.3. Effects of Propionate Treatment on Akt and PTEN Signaling. Western blot analysis revealed an overtly reduced expression of Akt in Akt2 knockout mice, validating the knockout model. Phosphorylation of Akt was significantly reduced in Akt2 knockout mice (although the normalized Akt phosphorylation was unchanged after normalizing to pan Akt level). Propionate treatment failed to alter pan or phosphorylated Akt levels in Akt2 knockout mice, although it significantly attenuated Akt activation (absolute or normalized values) without affecting pan Akt level in WT mice. Neither Akt2 knockout nor propionate treatment, or both, affected the levels of pan and phosphorylated PTEN (Figure 4).

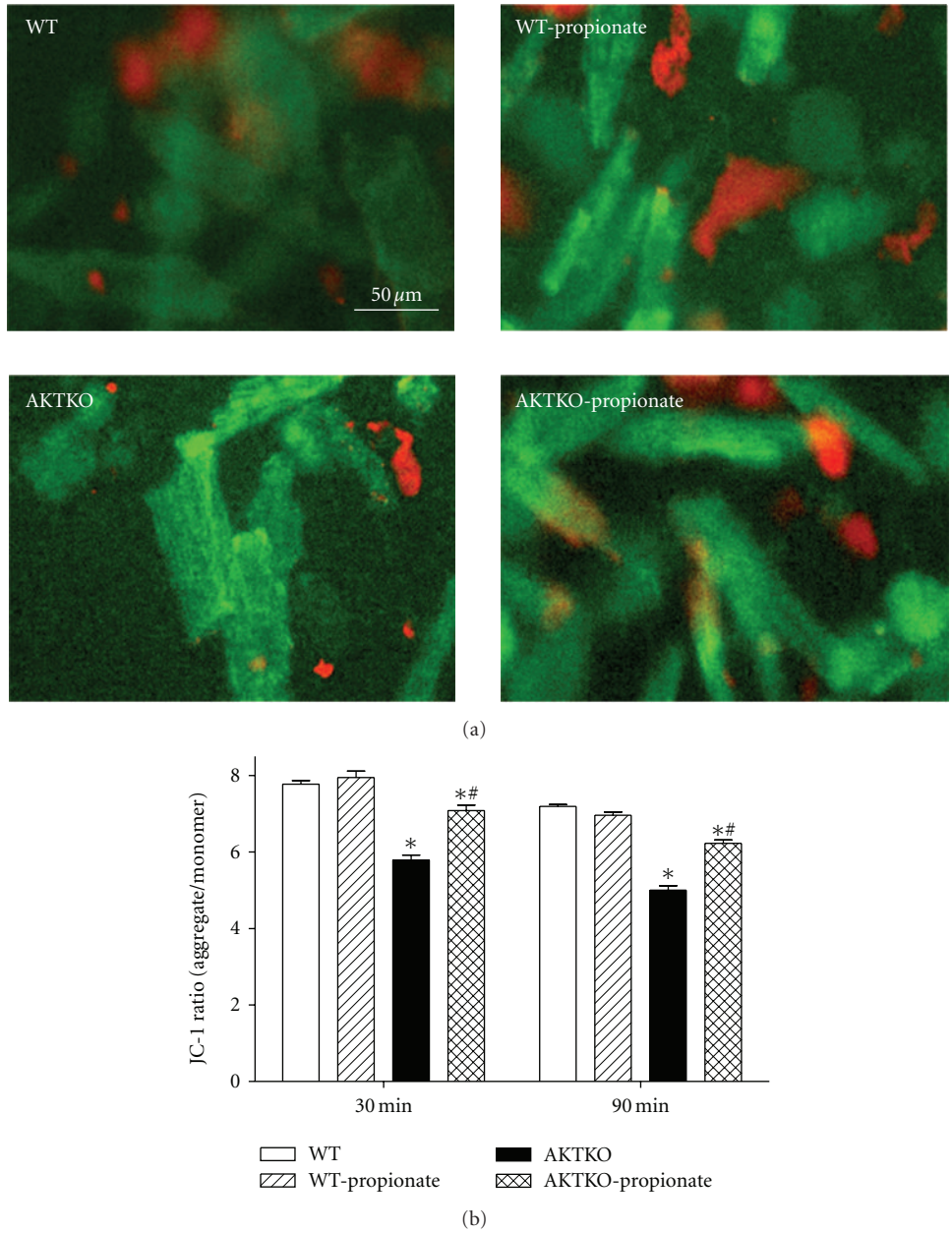

Figure 3: Cardiomyocyte mitochondrial membrane potential (MMP) in WT and Akt2 knockout (AKTKO) mice treated with or without propionate (0.3 g/kg, i.p.) for 7 days. MMP was measured using JC-1 fluorochrome (ratio of red to green fluorescence). (a) MMP at 30 min; (b) MMP at 90 min. Mean ± SEM, n = 6-7 isolations, $^{*}P < 0.05$ versus WT group, and $^{\#}P < 0.05$ versus AKTKO group.

3.4. Effects of Propionate on Protein Phosphatase Expression. To explore if protein phosphatase plays a role in Akt knockout or propionate-induced cardiac responses, expression of protein phosphatases PP2AA, PP2AB, and PP2C was evaluated. The results shown in Figure 5 demonstrate that neither Akt2 knockout nor propionate treatment, or both, affected the expression of PP2AA, PP2AB, and PP2C with the exception of downregulated PP2AA levels in response to the combination of Akt2 knockout and propionate.

3.5. Effects of Propionate on GSK3β, eNOS, GPR41, and GPR43 Expression. As depicted in Figure 6, Western blot analysis further revealed that neither Akt2 knockout nor propionate altered the expression of eNOS, pan and phosphorylated GSK3β (or the pGSK3β-toGSK3β ratio). Akt2 knockout significantly down- and upregulated the expression of GPR41 and GPR43, respectively. Although propionate treatment itself did not affect the expression of GPR41 and GPR43 in WT mice, it abolished the downregulated GPR41 without affecting the upregulated GPR43 under Akt2 deficiency.

4. Discussion

Our results reveal that propionate attenuated Akt2 knockout-induced cardiac contractile and mitochondrial dysfunction.

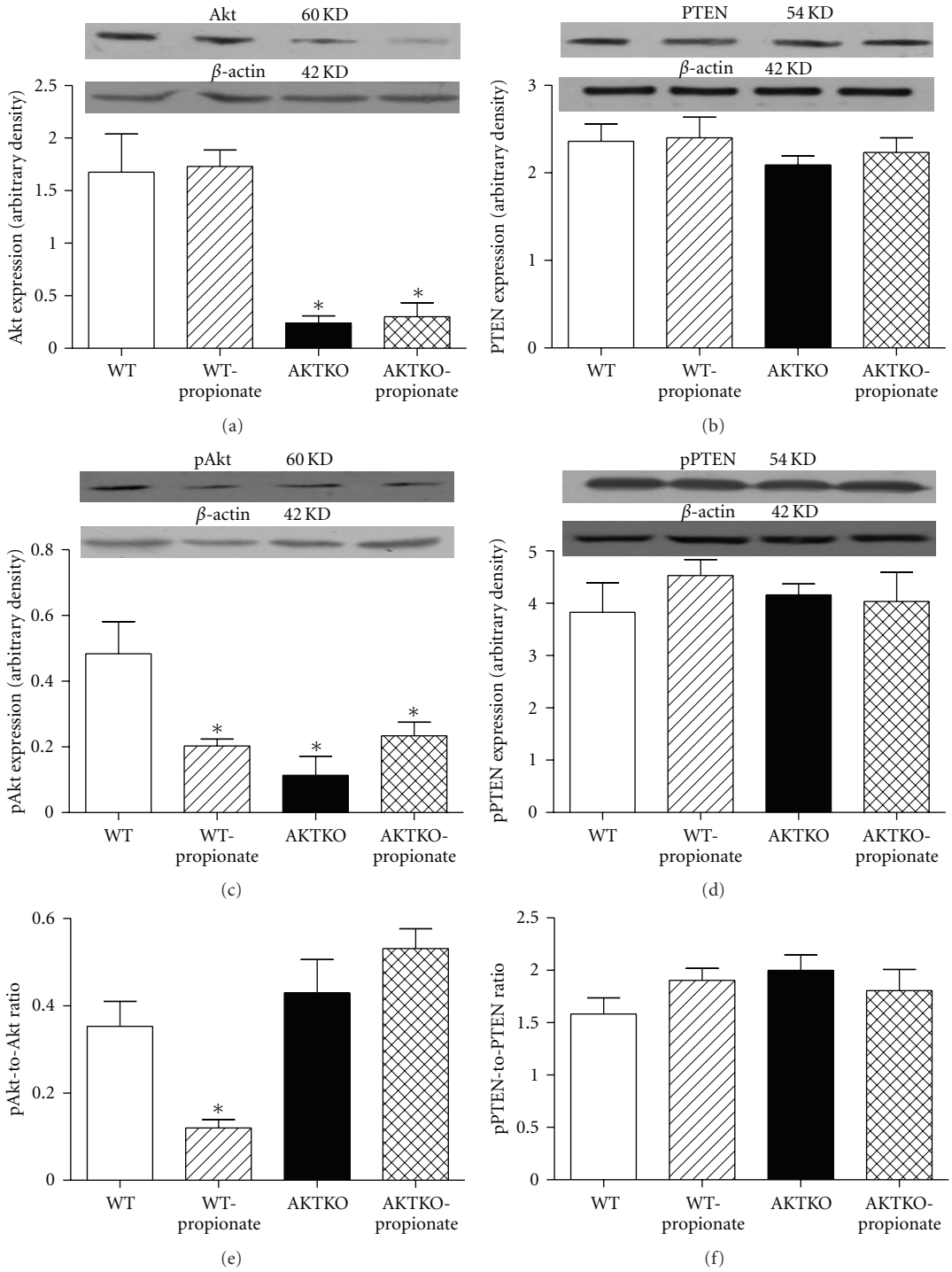

Figure 4: Effect of propionate treatment (0.3 g/kg, i.p. for 7 days) on Akt knockout-induced change in pan and phosphorylated Akt and PTEN. (a) Pan Akt; (b) pan PTEN; (c) phosphorylated Akt (pAkt); (d) phosphorylated PTEN (pPTEN); (e) pAkt-to-Akt ratio; (f) pPTEN-to-PTEN ratio. Insets: representative gel blots depicting expression and phosphorylation of these proteins using specific antibodies. β-Actin was used as the loading control. Mean ± SEM, n = 4-5 mice per group, and $^*P < 0.05$ versus WT group.

Propionate significantly improved cardiomyocyte contractile dysfunction including reduced peak shortening, maximal velocity of shortening/relengthening, and prolonged TR_{90} in Akt2 knockout mice. Furthermore, the Akt2 knockout-triggered loss in MMP and reduced expression of GPR41 were ameliorated by propionate. Neither Akt2 knockout nor propionate significantly affected the levels of protein phosphatases, eNOS, both pan and phosphorylated forms of PTEN and GSK3β. These data depicted that Akt2 knockout may elicit cardiomyocyte contractile and mitochondrial defects and a beneficial role of propionate against Akt2 deficiency-induced cardiac mechanical anomalies. Our

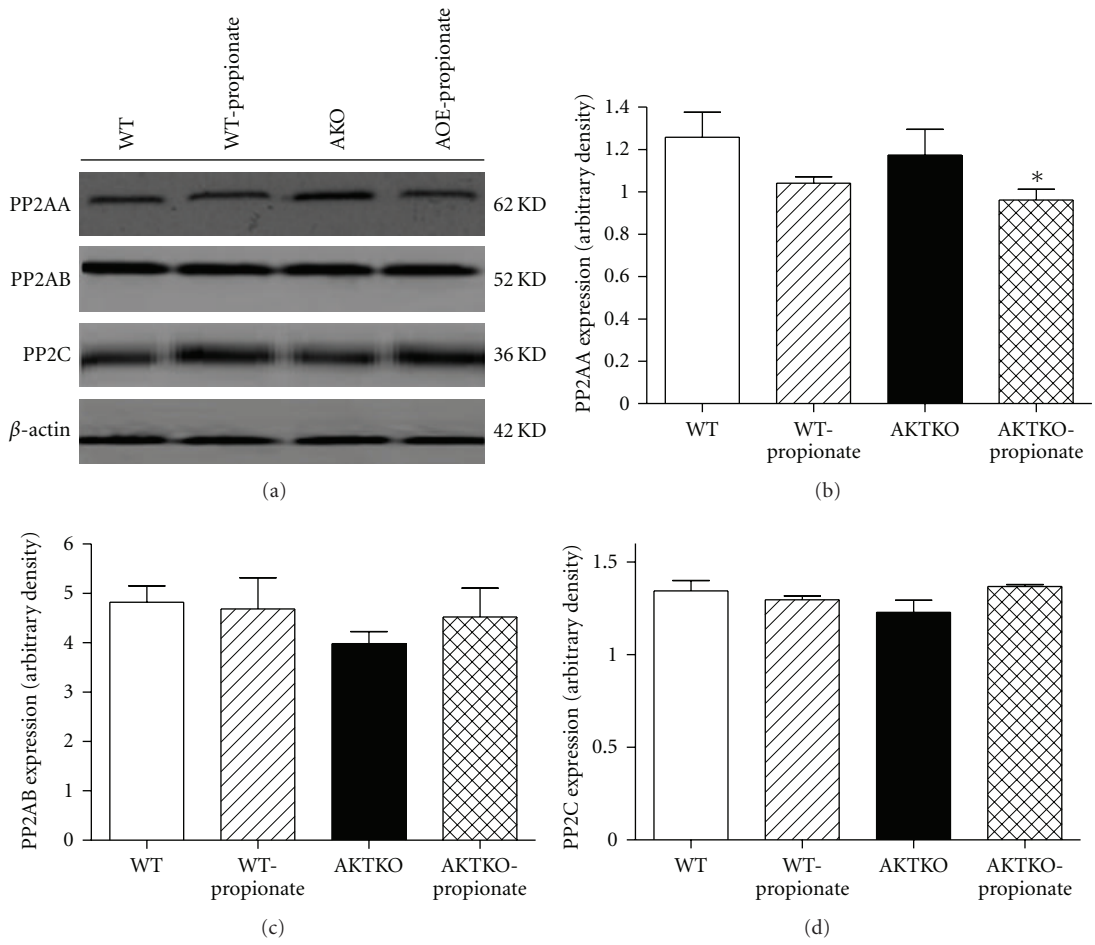

Figure 5: Effect of propionate treatment (0.3 g/kg, i.p. for 7 days) on Akt knockout-induced change in protein phosphatase levels. (a) Representative gel blots depicting expression of PP2AA, PP2AB, PP2C, and β-actin (loading control) using specific antibodies; (b) PP2AA; (c) PP2AB; (d) PP2C. Mean ± SEM, n = 4-5 per group, and $^*P < 0.05$ versus WT group.

findings have revealed therapeutic potential of propionate and other SCFAs in insulin resistance-associated cardiac dysfunction.

Our data confirmed that the Akt2 knockout model may serve as an insulin resistance model. These findings support the pivotal role of Akt in the regulation of glucose metabolism and myocardial function [17–19]. Although the baseline glucose levels from Akt2 knockout mice were somewhat similar to those from the WT mice, the area underneath OGTT curve was significantly greater in the Akt2 knockout mice. These data validated the presence of insulin resistance in Akt2 knockout model, consistent with the notion of insulin resistance in this murine model [20]. Along the same line, cardiomyocytes from Akt2 knockout mice exhibited reduced peak shortening, maximal velocity of shortening/relengthening (± dL/dt) as well as prolonged relengthening duration (TR_{90}) associated with comparable resting cell length and duration of shortening (TPS) compared WT group. These findings are somewhat consistent with our earlier findings using a sucrose or high fat diet-induced insulin resistance model [26, 27, 30]. More importantly, our data revealed that propionate treatment for 7 days was effective in reversing cardiomyocyte mechanical myocardial dysfunction in Akt2 knockout mice. These compromised cardiomyocyte mechanical parameters are reconciled by propionate treatment.

A number of mechanisms may be speculated for Akt2 knockout-induced cardiomyocyte anomalies and propionate-offered cardioprotection. First, emerging evidence has indicated a role of mitochondrial function in the pathogenesis and management of cardiac dysfunction in insulin resistance [3]. Using mice with cardiac-specific overexpression of the heavy metal scavenger metallothionein, we found that reduction of cardiac oxidative stress by metallothionein protects against high fat diet-induced cardiac contractile dysfunction through protection against mitochondrial damage [31]. Our current findings exhibited that treatment with propionate is capable of alleviating Akt2 knockout-induced loss of MMP. It is plausible to speculate that propionate-elicited beneficial effects against Akt2 knockout-induced cardiomyocyte functional defects may be mediated via alleviation of mitochondrial anomalies. Mitochondria play a central role in the control of energy metabolism, cell survival, and myocardial function [3]. Among many

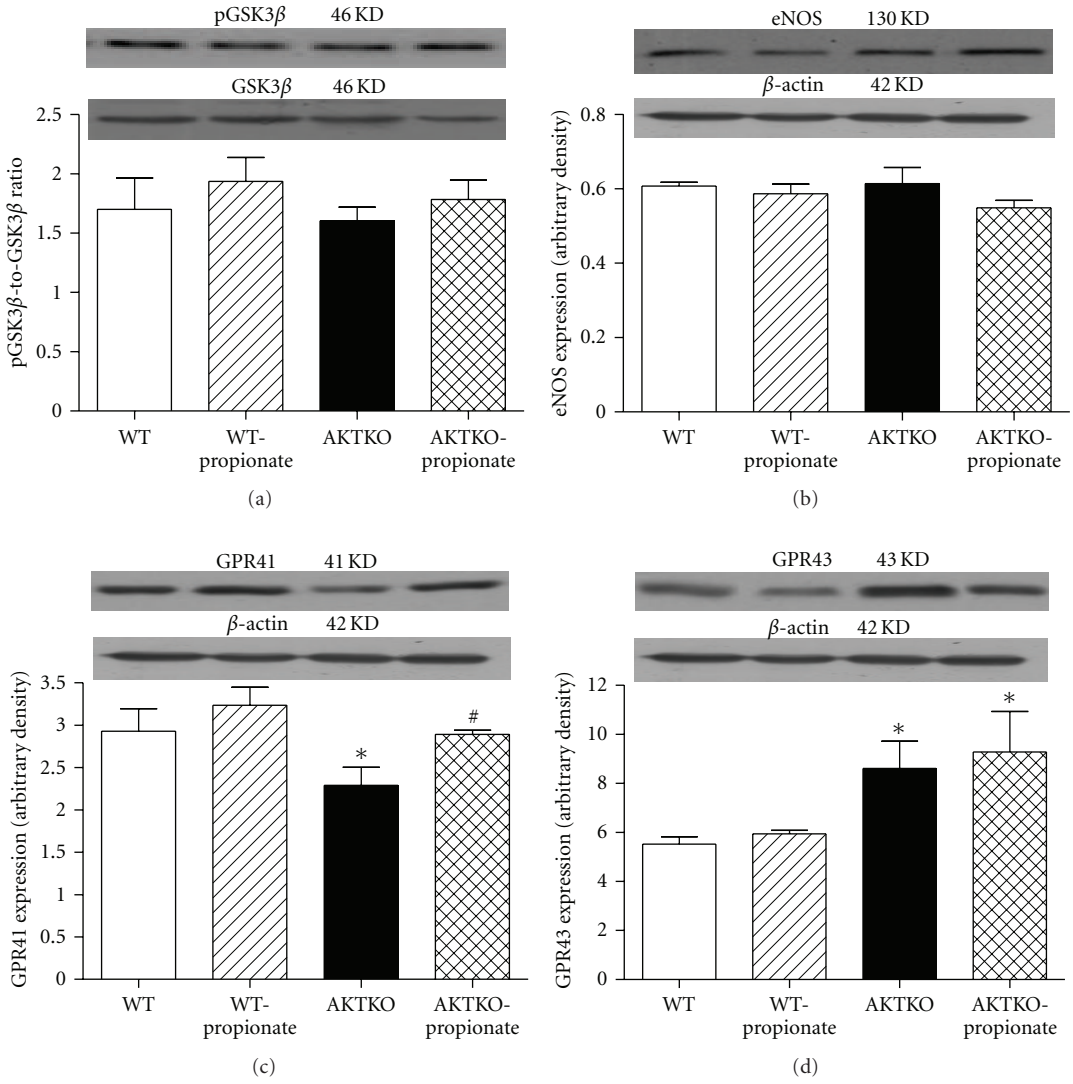

Figure 6: Effect of propionate treatment (0.3 g/kg, i.p. for 7 days) on Akt knockout-induced change in pan and phosphorylated GSK3β, eNOS, GPR41, and PGR43 levels. (a) pGSK3β-to-GSK3β ratio; (b) eNOS expression; (c) GPR41 expression; (d) GPR43 expression. Insets: representative gel blots depicting expression and/or phosphorylation of these proteins using specific antibodies. β-Actin was used as the loading control. Mean ± SEM, n = 4-5 mice per group, $^{*}P < 0.05$ versus WT group, and $^{\#}P < 0.05$ versus AKTKO group.

cell signaling cascades involved in the regulation of cardiac structure and function, Akt is known to exert a wide range of regulatory responses on mitochondrial biology [32]. Emerging research continues to unveil novel mechanisms governing the protective effects of Akt signaling in the context of cardiac mitochondria. For example, recent findings have suggested pivotal roles of hexokinase and Pim-1 kinase in the preservation of mitochondrial function downstream of Akt [32]. Moreover, Akt is capable of suppressing mitochondrial permeation pore opening, thus protecting mitochondrial integrity via phosphorylation of GSK-3β [33]. Second, our data revealed that propionate reconciled Akt2 knockout-induced loss in the receptors for short-chain fatty acids GPR41. This is somewhat consistent with the recent report that SCFAs regulate sympathetic nervous system thus to control body energy expenditure and metabolic homeostasis via GPR41 [34]. On the contrary, Akt2 knockout significantly upregulated the level of another receptor for SCFAs, namely, GPR43, the effect of which was unaffected by propionate. These findings suggested a role of GPR41 but not GPR43 in propionate-offered beneficial cardiac effects. GPR41 and GPR43 are deemed as orphan G-protein coupled receptors that vary in specificity for individual SCFAs, intracellular signaling, and tissue localization [35, 36]. GPR43 exhibits comparable agonist activities for acetate, propionate, and butyrate, whereas GPR41 displays remarkable discrepancies in the rank order for receptor affinity (propionate ≥ butyrate > acetate) [35, 36]. Our findings revealed that both receptors may play a role in Akt2 knockout-induced cardiomyocyte contractile dysfunction, although only GPR41 (with a much higher affinity for propionate) may be responsible for the beneficial

effect elicited by propionate. Further study is warranted to better elucidate the mechanism of action behind GPR41- and GPR43-elicited metabolic and functional regulation in the heart. Our data failed to identify any changes in the levels of protein phosphatases, eNOS, pan and phosphorylated forms of PTEN and GSK3β in either Akt2 knockout or propionate treatment group, thus not favoring a role of these signaling molecules in our current experimental settings. The levels of Akt were greatly diminished in Akt2 mice, validating the murine model. However, the Akt phosphorylation (ratio between pAkt and Akt) remains unchanged, possibly due to the remaining Akt2 or Akt1 levels in these hearts. To our surprise, propionate treatment itself overtly dampened phosphorylation of Akt although the precise mechanism of action involved is still elusive at this time. Together with the notion that dietary fiber and SCFAs may be beneficial to insulin resistance and metabolic syndrome [22, 23], our findings have unveiled promises of SCFAs in the treatment of cardiac pathologies in insulin resistance and other metabolic disturbances.

Epidemiological evidence has depicted the beneficial effect of dietary fiber in the clinical management of obesity, diabetes, cancer, and cardiovascular diseases [37]. In particular, dietary fibers improve fecal bulking and satiety, viscosity and SCFA production, and reduce glycemic response. The mechanism of action most likely responsible for the beneficial role of SCFAs involves the interference with lipid digestion, cholesterol, and bile acid absorption, or with lipid transport and deposition [37]. Moreover, although glucose may serve as the main metabolic fuel, SCFAs produced by colonic bacterial fermentation of dietary fiber furnish a significant portion of daily energy requirement [34, 38]. Maintenance of energy homeostasis is critical for life, and the dysregulation of which results in metabolic disorders [34], depicting an important role of SCFAs in the management of metabolic diseases. Nonetheless, the nutritional impact of SCFAs may be easily confounded by changes in the relative glycemic index of the test diets, thus the results may not truly reflect the sole impact of fermentation or changes in SCFAs [37]. Further in-depth study is warranted to explore the complex nutritional and physiological effects of dietary fibers and SCFAs.

In conclusion, results from our current study offers evidence, for the first time, that Akt2 knockout compromised cardiomyocyte contractile and mitochondrial dysfunction, the effects of which may be alleviated by short-term treatment of propionate. In light of the present dismal status for successful management against insulin resistance-associated cardiac anomalies, it is tempting to speculate that propionate and SCFAs may be of some particular clinical value in the treatment and prevention of cardiac diseases associated with insulin resistance.

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Clinical Study

Low Levels of Serum Paraoxonase Activities are Characteristic of Metabolic Syndrome and May Influence the Metabolic-Syndrome-Related Risk of Coronary Artery Disease

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Low concentrations of plasma high-density lipoprotein (HDLs) are characteristic in metabolic syndrome (MS). The antioxidant ability of HDLs is, at least in part, attributable to pleiotropic serum paraoxonase (PON1). Different PON1 activities have been assessed in 293 subjects with ($n = 88$) or without MS ($n = 205$) and with ($n = 195$) or without ($n = 98$) angiographically proven coronary artery disease (CAD). MS subjects had low PON1 activities, with a progressively decreasing trend by increasing the number of MS abnormalities. The activity versus 7-O-diethyl phosphoryl,3-cyano,4-methyl,7-hydroxycoumarin (DEPCyMC), which is considered a surrogate marker of PON1 concentration, showed the most significant association with MS, independently of both HDL and apolipoprotein A-I levels. Subjects with MS and low DEPCyMCase activity had the highest CAD risk (OR 4.34 with 95% CI 1.44–13.10), while no significant increase of risk was found among those with MS but high DEPCyMCase activity (OR 1.45 with 95% CI 0.47–4.46). Our results suggest that low PON1 concentrations are typical in MS and may modulate the MS-related risk of CAD.

1. Introduction

Metabolic syndrome (MS) defines a well-known cluster of metabolic disturbances associated with an increased risk of cardiovascular disease and diabetes [1–3]. Insulin resistance is thought to be the core of MS [2, 3]. Nonetheless, oxidative stress pathways have been also proposed to play a role in MS. An increased oxidative stress, as well as a reduction of antioxidant defences, may impair insulin signalling, therefore leading *per se* to insulin resistance [4]. Moreover, the crucial role of oxidative damage is well documented in both endothelial dysfunction and atherosclerosis processes as it is also accepted that the imbalance of reduction-oxidation (redox) homeostasis may contribute to the development of cardiovascular diseases in MS [4, 5].

Low levels of high-density lipoprotein (HDL) are typical of the biochemical cluster defining MS. HDLs are one of the most important antioxidant defence systems in plasma. They are well known to prevent low-density lipoprotein (LDL) oxidation and protect against LDL-induced cytotoxicity [6–9]. HDLs also possess anti-inflammatory properties, including the ability of suppressing cytokine-induced endothelial cell adhesion molecules function [10–12]. The antioxidant properties of HDLs are, at least to some extent, attributable to serum paraoxonase (PON1). PON1 is a 45 kDa, 355-amino-acid glycoprotein which is synthesized essentially by the liver and then secreted into the blood, where it links to HDLs [13, 14]. PON1 is a pleiotropic enzyme, whose name originally derives from its capacity to neutralize highly toxic, xenobiotic compounds, such as paraoxon. The physiological

substrates of PON1, however, are yet partially undefined, even if convincing evidence points to a principal role of PON1 as lactonase, with lipophylic lactones as the primary substrates [15–17]. Previous studies by measuring the rate of paraoxon hydrolysis have shown that subjects with MS had lower PON1 activity [18, 19]. However, it is not clear to what extent the classical, but not physiological, assay, such as the paraoxonase activity, reflects the actual antioxidant capacity of the enzyme. Recently, novel PON1 assays have been developed: 5-thiobutyl butyrolactone (TBBL) and 7-O-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC) [20]. Both TBBLase and DEPCyMCase activities in sera are highly specific to PON1. TBBL is a chromogenic lactone that resembles the most favourable PON1 lactone substrates, allowing a specific evaluation of PON1 lactonase activity. The DEPCyMC is a chromogenic phosphotriester substrate that, differently from the other methods for PON1 activities, allows estimating total PON1 concentration, the DEPCyMCase assay being not influenced by the degree of catalytic stimulus by HDL. The ratio between these two activities (TBBL-to-DEPCyMC activity ratio) has been suggested to provide the so-called "normalized lactonase activity" (NLA), which may reflect the level of PON1 lactonase catalytic stimulation by HDL [20]. In a previous work, we showed for the first time that these novel PON1 activity assays may be associated with coronary artery disease (CAD). More precisely, we observed that CAD patients had low PON1 concentration, defined by low DEPCyMCase activity, but presented high stimulation of PON1 lactonase activity, as indicated by high NLA values [21].

The aim of this study was to evaluate PON1 activities by means of both traditional (i.e., paraoxonase and arylesterase activity) and new assays (i.e., TBBLase and DEPCyMCase activity) in a population of subjects with or without angiographically confirmed CAD, according to either the presence or absence of MS diagnosis. The potential interaction between MS and PON1 activity as a determinant of CAD risk was also assessed.

2. Materials and Methods

2.1. Study Population. This study was performed within the framework of the Verona Heart Project, a regional survey designed for identification of new risk factors for CAD in subjects with objective angiographic documentation of their coronary vessels. Details about enrolment criteria have been described in detail elsewhere [22]. As previously reported, a total of 300 subjects were selected and divided into three subgroups matched for sex and age: CAD-free, CAD without myocardial infarction (MI), and CAD with MI [21]. CAD-free ($n = 100$) group had completely normal coronary arteries, being submitted to coronary angiography for reasons other than CAD, mainly valvular heart disease. These controls were also required to have neither history nor clinical or instrumental evidence of atherosclerosis in vascular districts beyond the coronary bed. Two-hundred subjects had angiographically proven CAD with at least one of the main epicardial coronary arteries affected (left anterior descending, circumflex, or right) with ≥1 significant stenosis (≥50%). CAD patients were classified into MI ($n = 100$) and non-MI ($n = 100$) subgroups on the basis of a thorough review of medical records including history, electrocardiogram, enzyme changes, and/or the typical sequelae of MI on ventricular angiography. The angiograms were assessed by cardiologists who were unaware that the patients were to be included in the study. All participants came from the same geographical area (Northern Italy). At the time of blood sampling, a complete clinical history was collected, including the assessment of cardiovascular risk factors such as obesity, smoking, hypertension, and diabetes. From these 300 subjects we further selected 293 subjects (195 CAD and 98 CAD-free) for whom complete data for MS diagnosis were available. According to the established criteria [23], patients were classified as having MS when at least three of the following conditions were present: body mass index >30 kg/m^2; documented history of hypertension or blood pressure >140/90 mmHg; fasting glucose >6.1 mmol/L; plasma triglycerides >1.7 mmol/L; HDL <1.03 mmol/L for males or <1.29 mmol/L for females.

The study was approved by the Ethic Committee of our Institution (Azienda Ospedaliera, Verona). A written informed consent was obtained from all the participants after a full explanation of the study.

2.2. Biochemical Analysis. Samples of venous blood were withdrawn from each subject, after an overnight fast. Serum lipids and the other common biochemical parameters were determined by routine methods. Apolipoprotein A-I (Apo A-I) and Apolipoprotein B (Apo B) were measured by commercially available nephelometric immunoassays; antisera, calibrators, and the BNII nephelometer were from Dade Behring [21]. LDL cholesterol/Apo B ratio was calculated as surrogate marker of small and dense LDL particles [19].

2.3. PON1 Activity Assays. PON1 activity assays were performed as previously described [20]. TBBL and DEPCyMC were kindly provided by Dan Tawfik (Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel).

Lactonase activity was measured in activity buffer (50 mM Tris pH 8.0, 1 mM $CaCl_2$) containing 0.25 mM of TBBL and 0.5 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) by monitoring the absorbance at 412 nm in a final volume of 200 μL (€ = 7,000 OD/M), using an automated microplate reader. The serum was diluted 400-fold in 100 μL of activity buffer complemented with 1 mM DTNB. DTNB was used from 100 mM stock in DMSO. TBBL was used from 250 mM stock in acetonitrile. TBBL was diluted 500-fold in activity buffer containing 2% acetonitrile. The reaction was initiated by adding 100 μL of TBBL (0.5 mM) to 100 μL of sera dilution. The final sera dilution was 800-fold. All the reaction mixtures contained a final 1% acetonitrile. Rates of spontaneous hydrolysis of TBBL in buffer were subtracted from all the measurements. Activities were expressed as U/mL (1 unit = 1 μmol of TBBL hydrolyzed per minute per 1 mL of undiluted serum).

Total PON1 concentrations in human sera were assessed by measuring the activity with DEPCyMC. For the enzymatic measurements, DEPCyMC was used from 100 mM stock in DMSO, and all the reaction mixtures contained a final 1% DMSO. The activity was measured with 10 μL of serum and 1 mM substrate in 50 mM bis-tris-propane, pH 9.0, with 1 mM $CaCl_2$, by monitoring the absorbance at 400 nm in a final volume of 200 μL (€ = 22,240 OD/M). Activities were expressed as mU/mL (1 milliunit = 1 nmol of DEPCyMC hydrolyzed per minute per 1 mL of undiluted serum).

The normalized lactonase activity (NLA) was calculated by dividing TBBLase activity of each sample by its DEPCyMC activity.

Paraoxonase activity in sera samples was measured in activity buffer with 1mM paraoxon by monitoring the absorbance at 405 nm (€ = 10,515 OD/M). Arylesterase activity was measured in activity buffer with 1 mM phenyl acetate by monitoring the absorbance at 270 nm (€ = 700 OD/M). Activities were expressed as U/L for paraoxon and kU/L for phenyl acetate (1 unit = 1 nmol of paraoxon or 1 μmol of phenyl acetate hydrolyzed per minute per 1 mL of undiluted serum).

All the measures of PON1 activity were performed in duplicate, and all the coefficients of variations were less than 5%.

2.4. PON1 $Gln_{192}Arg$ and $Leu_{55}Met$ Polymorphism Analysis. Genomic DNA was extracted from whole-blood samples by a phenol-chloroform procedure, and subjects were genotyped according to a previously described multilocus assay [24]. PON1 genotypes were available for 264/293 (90.1%) subjects.

2.5. Statistics. Calculations were performed with SPSS 17.0 statistical package (SPSS Inc., Chicago, Ill). Distributions of continuous variables in groups were expressed as means ± standard deviation. Statistical analysis on skewed variables, like paraoxonase activity, was computed on the corresponding log-transformed values. However, for the sake of clarity, nontransformed data are reported in the results. Quantitative data were assessed using Student's t-test or analysis of variance (ANOVA). Correlations between quantitative variables were assessed using Pearson's correlation test. Qualitative data were analyzed with the χ^2-test and with χ^2 for linear trend analysis when indicated. A value of $P < 0.05$ was considered statistically significant. Statistical power was estimated by means of Altmann nomogram.

Within each group examined, the frequencies of the genotypes associated with each of the polymorphisms were compared by the χ^2-test with the values predicted on the basis of the Hardy-Weinberg equilibrium.

The strength of association of PON1 activity with MS was evaluated by including all the four assessed activities in a multiple regression model with a forward-stepwise variable selection that lastly was adjusted also for HDL and Apo A-I concentrations. The combined effect of MS and DEPCyMCase activity in determining CAD risk was estimated calculating the odds ratios with 95% CIs by multiple logistic regression after adjustment for traditional cardiovascular risk factors not included in MS cluster (i.e., age, sex, smoke, and LDL cholesterol). Finally, the correlation between DEPCyMCase activity and LDL cholesterol/Apo B ratio was evaluated by means of a linear regression analysis model adjusted for age, sex, and HDL concentration.

3. Results and Discussion

3.1. Results. The clinical characteristics of the study population divided on the basis of MS diagnosis are reported in Table 1. Subjects with ($n = 88$) or without MS ($n = 205$) differed for several characteristics, not only related to MS cluster. As expected, MS was more represented among CAD patients. Moreover, MS subjects presented a lower LDL cholesterol/Apo B ratio. All the four investigated PON1 activities (TBBLase, DEPCyMCase, arylesterase, and paraoxonase activities) were significantly lower in MS subjects, while no significant difference was found for NLA, nor for PON1 genotypes distribution, which respected the Hardy-Weinberg equilibrium (Table 1). No significant difference was found between CAD subjects with or without MI for PON1 activities nor for MS distribution (data not shown).

Ranking the study population on the basis of MS abnormalities, PON1 activity levels, as well as HDL and Apo A-I concentrations, and LDL-cholesterol/Apo B ratio decreased progressively by increasing the number of metabolic disturbances (Table 2). In particular, DEPCyMCase activity presented a very high significant association with both MS diagnosis (20.60 ± 6.05 versus 23.8 ± 5.6 mU/mL in subjects with or without MS, resp., $P = 1.58 \times 10^{-5}$) and the number of metabolic disturbances (P for linear trend $= 3.84 \times 10^{-6}$). Including all the PON1 activities in a regression model with a forward-stepwise variable selection, only DEPCyMCase activity remained a significant predictor of MS (OR for 1 mU/mL increase = 0.90 with 95% CI 0.86–0.95; $P < 0.001$). Noteworthy, this association was independent of HDL and Apo A-I levels (OR for 1 mU/mL increase = 0.93 with 95% CI 0.88–0.98; $P = 0.004$) and of both PON1 genotypes (OR for 1 mU/mL increase = 0.92 with 95% CI 0.86–0.99; $P = 0.017$).

Consistently with the last outcome, by stratifying the study population on the basis of MS diagnosis and HDL levels, which were considered as a dichotomic variable on the basis of MS-related threshold level, subjects with low HDL levels but without MS had a higher DEPCyMCase activity than subjects with equally low HDL levels and MS (Figure 1(a)). Interestingly, subjects with low HDL levels but without MS presented a DEPCyMCase activity comparable with that of subjects with high HDL levels and without MS (Figure 1(a)). On the other hand, subjects with high HDL levels but with MS showed a trend versus lower DEPCyMCase activity compared to those with high HDL levels and without MS, even if this difference did not reach statistical significance. Noteworthy, no difference in HDL or Apo A-I concentration appeared to justify such results in the above-mentioned four subgroups. Indeed, within the groups with either low or high HDL levels, MS diagnosis was not associated with additional differences in HDL and Apo A-I concentrations (Figures 1(b) and 1(c)), thus supporting an HDL-independent, Apo A-I-independent association of DEPCyMCase activity with MS.

Table 1: Characteristics of the study population, with or without metabolic syndrome (MS).

Characteristics	MS-free ($n = 205$)	MS ($n = 88$)	P
Age (years)	60.9 ± 9.3	60.8 ± 9.6	NS*
Male sex (%)	59.5	42.0	0.006†
Coronary Artery Disease (%)	61.0	79.5	0.002†
BMI (kg/m^2)	25.5 ± 2.9	28.7 ± 5.1	<0.001*
Hypertension (%)	57.1	89.8	<0.001†
Smoking (%)	52.8	54.9	NS †
Diabetes (%)	7.8	46.5	<0.001†
Glucose (mmol/L)	5.40 ± 0.98	7.01 ± 2.29	<0.001*
Creatinine (¯mol/L)	90.1 ± 64.2	89.4 ± 21.9	NS*
Total cholesterol (mmol/L)	5.16 ± 1.13	5.29 ± 1.19	NS*
LDL-cholesterol (mmol/L)	3.18 ± 0.95	3.33 ± 1.11	NS*
HDL-cholesterol (mmol/L)	1.35 ± 0.34	1.07 ± 0.31	<0.001*
Triglycerides (mmol/L)	1.47 ± 0.65	2.33 ± 0.98	<0.001*
Apo A-I (g/L)	1.30 ± 0.26	1.13 ± 0.23	<0.001*
Apo B (g/L)	0.98 ± 0.24	1.08 ± 0.28	0.003*
TBBLase activity (U/mL)	3.45 ± 0.98	3.02 ± 1.02	0.001*
DEPCyMCase activity (mU/mL)	23.83 ± 5.65	20.60 ± 6.05	<0.001*
Paraoxonase activity (U/L)	120.8 ± 79.1	102.6 ± 71.5	0.030*
Arylesterase activity (kU/L)	101.3 ± 31.4	86.8 ± 32.5	<0.001*
Normalized lactonase activity	145.0 ± 24.3	146.1 ± 35.0	NS*
PON1 $Gln_{192}Arg$ ‡			
Gln/Gln	50.3	45.6	
Gln/Arg	40.0	49.4	NS†
Arg/Arg	9.7	5.1	*
PON1 $Leu_{55}Met$‡			
Leu/Leu	38.0	38.0	
Leu/Met	48.9	44.3	NS†
Met/Met	13.1	17.7	

* by t-test; † by χ^2 test; ‡ PON1 genotype data were available for 264/293 (90.1%) subjects, that is, 185 MS-free and 79 with MS; NS: no significant.

Table 2: Serum paraoxonase (PON1) activities, HDL cholesterol, and apolipoprotein A-I (Apo A-I) concentrations and LDL cholesterol/Apo B ratio according to the number of metabolic syndrome (MS) abnormalities.

	0 MS element ($n = 35$)	1 MS element ($n = 78$)	2 MS elements ($n = 92$)	3 MS elements ($n = 50$)	4 MS elements ($n = 31$)	5 MS elements ($n = 7$)	P*
TBBLase activity (U/mL)	3.60 ± 1.38	3.46 ± 0.95	3.38 ± 0.80	3.08 ± 0.99	2.98 ± 1.13	2.69 ± 0.56	<0.001*
DEPCyMCase activity (mU/mL)	24.49 ± 7.62	23.97 ± 5.90	23.45 ± 4.49	21.74 ± 5.96	19.36 ± 6.23	17.92 ± 4.37	<0.001
Paraoxonase activity (U/L)	128.4 ± 103.7	118.8 ± 69.2	119.5 ± 77.1	102.2 ± 63.0	103.6 ± 89.7	101.4 ± 38.3	NS
Arylesterase activity (kU/L)	106.3 ± 35.7	101.3 ± 31.1	99.3 ± 30.0	89.1 ± 34.0	85.6 ± 32.3	76.0 ± 21.0	<0.001
Normalized Lactonase Activity	145.4 ± 23.3	145.3 ± 24.4	144.7 ± 24.9	140.8 ± 31.9	152.8 ± 39.1	154.3 ± 34.7	NS
HDL cholesterol (mmol/L)	1.25 ± 0.26	1.19 ± 0.25	1.14 ± 0.25	1.05 ± 0.23	0.97 ± 0.15	0.83 ± 0.19	<0.001
Apo A-I(g/L)	1.38 ± 0.25	1.31 ± 0.27	1.27 ± 0.26	1.18 ± 0.25	1.09 ± 0.17	0.93 ± 0.21	<0.001
LDL cholesterol-to-Apo B ratio (mmol/g)	3.32 ± 0.43	3.31 ± 0.73	3.21 ± 0.65	3.04 ± 0.65	2.97 ± 0.45	2.88 ± 0.57	0.001

* by ANOVA with polynomial contrast for linear trend.

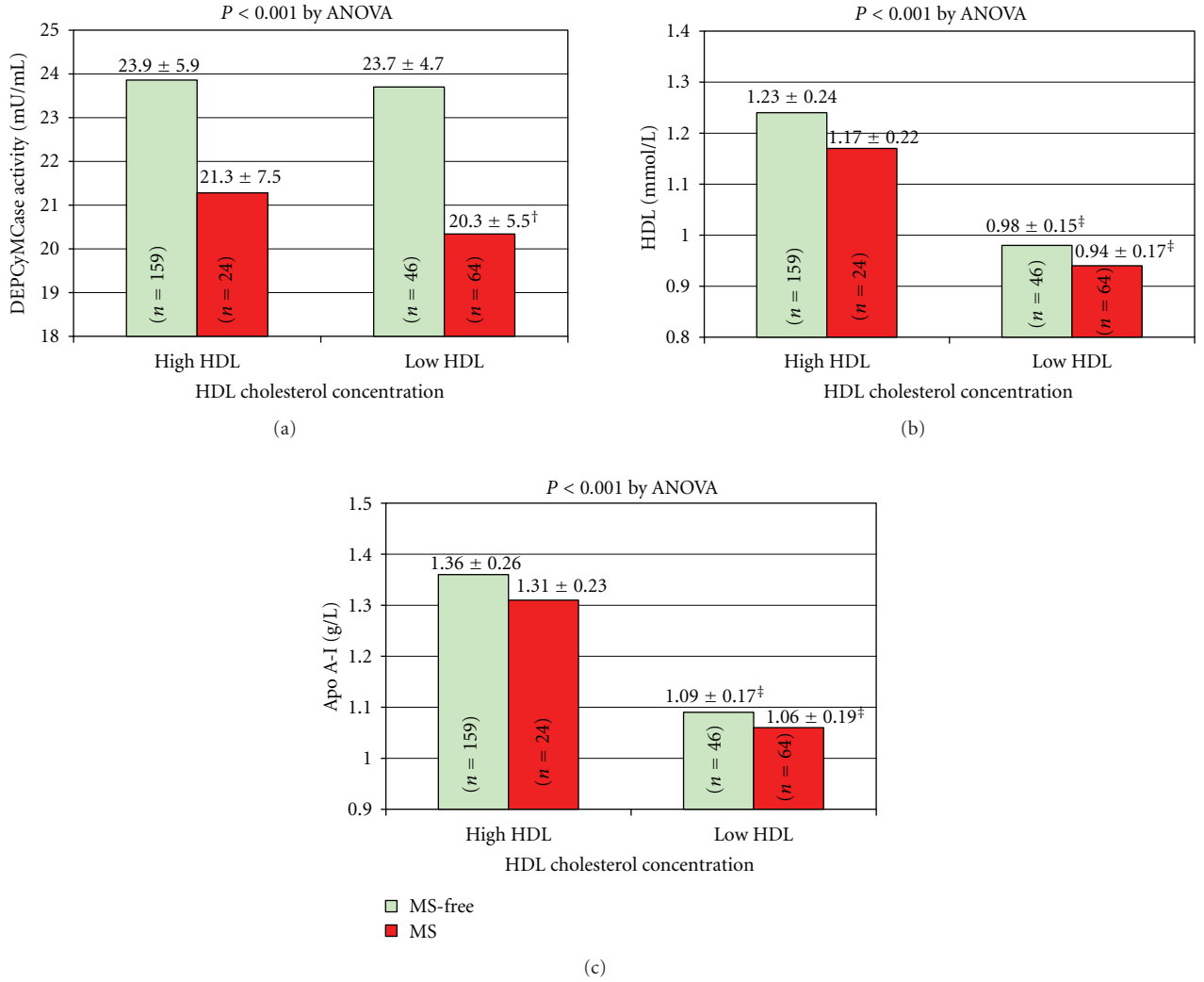

Figure 1: DEPCyMCase activity (a), HDL cholesterol (b), and apolipoprotein A-I concentration (c) according to high/low HDL cholesterol levels and metabolic syndrome (MS) diagnosis (b). Low HDL-cholesterol concentrations are defined on the basis of ATP-III criteria for MS-diagnosis, that is, <1.03 mmol/L for males or <1.29 mmol/L for females. †Significantly lower than no-MS with high or low HDL ($P < 0.01$ by Tukey post-hoc comparison). ‡Significantly lower than high HDL with or without MS ($P < 0.01$ by Tukey post-hoc comparison).

Considering that MS is linked to an increased risk of CAD and that an association of low DEPCyMCase activity with CAD has been previously found in the same study population [21], an analysis about potential combined effects on CAD risk was performed. In a multiple logistic regression model, both MS and DEPCyMCase activity remained significantly associated with CAD (OR for MS = 2.17 with 95% CI 1.19–3.97, $P = 0.012$; OR for 1 mU/mL increase of DEPCyMCase activity = 0.95 with 95% CI 0.91–0.99, $P = 0.033$). After stratifying the study population on the basis of MS diagnosis and DEPCyMCase activity tertiles, the statistical analysis highlighted a progressive increase of CAD diagnosis prevalence among subjects without MS but within the highest DEPCyMCase activity tertile group, compared to those with MS and within the lowest DEPCyMCase activity tertile (Figure 2(a)). Considering subjects without MS and with the highest DEPCyMCase activity tertile as reference group, subjects with MS and low DEPCyMCase activity presented a marked increase of CAD risk, even after adjustments for the classical cardiovascular risk factors not included in MS cluster (OR 4.34 with 95% CI 1.44–13.10, Figure 2(b)), while no significant increase of CAD risk was found for those with MS but high DEPCyMCase activity (OR 1.45 with 95% CI 0.47–4.46). Such results were confirmed also by including PON1 genotypes in the regression model (for subjects with MS and low DEPCyMCase activity: OR 4.67 with 95% CI 1.12–15.15).

An analysis on LDL cholesterol/Apo B ratio was also performed. The LDL cholesterol/Apo B ratio was used as a surrogate marker for small and dense LDLs, where the lower ratios suggested a higher prevalence of this type of LDL particles. A low LDL cholesterol/Apo B ratio was associated with both CAD (3.09 ± 0.57 versus 3.36 ± 0.60 mmol/g in CAD-free, $P < 0.001$) and MS (3.00 ± 0.57 versus 3.26 ± 0.59 mmol/g in MS-free, $P = 0.001$). Furthermore, the ratio progressively decreased with the consensual increase of the

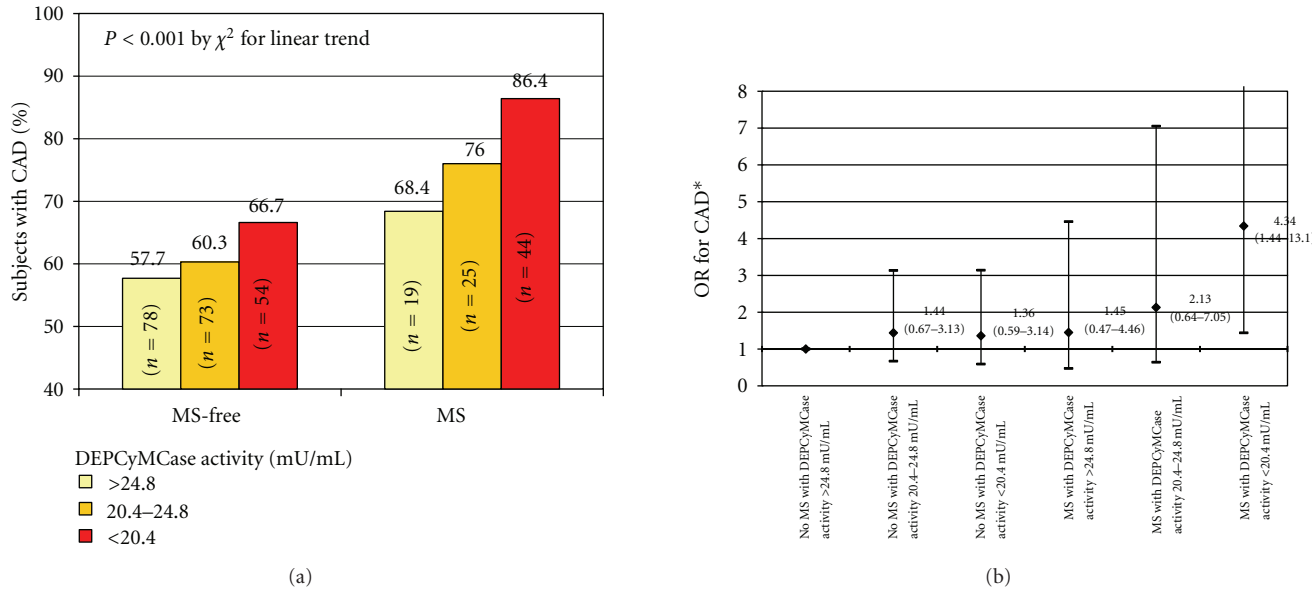

Figure 2: Prevalence of subjects with coronary artery disease (CAD) according to metabolic syndrome (MS) diagnosis and DEPCyMCase activity (a) and the relative ORs for CAD in a multiple adjusted regression model considering subjects without MS and within the highest DEPCyMCaseactivity tertile as reference group (b). *B y multiple logistic regression adjusted for classical CAD risk factors not included in MS definition, that is, sex, age, smoke and LDL cholesterol.

number of metabolic disturbances (Table 2). DEPCyMCase activity showed also a positive correlation with LDL cholesterol/Apo B ratio (Pearsons'coefficient 0.165, $P = 0.008$) that remained significant even after a regression model adjusted for age, gender, and HDL levels (standardized beta-coefficient 0.158, $P = 0.014$) was performed. By stratifying the study population on the basis of MS diagnosis and DEPCyMCase activity tertiles, there was a progressive decrease of LDL cholesterol/Apo B ratio from subjects without MS and within the highest DEPCyMCase activity tertile toward those subjects who instead had MS and were within the lowest DEPCyMCase activity tertile. These findings were observed both when the analysis was performed in the whole study population (Figure 3(a)) and even after the exclusion of subjects taking lipid-lowering therapy (Figure 3(b)).

4. Discussion

The current study, consistently with some previous observations [18, 19], shows a substantial impairment of PON1 activities in patients affected by MS.

MS is characterized by a constellation of metabolic abnormalities that altogether lead to an increased risk of cardiovascular diseases. Although there is a substantial disagreement over the terminology and diagnostic criteria of MS and even some controversies exist about whether MS is a true syndrome or a mixture of various phenotypes [1–3], it is undeniable that the clustering of MS abnormalities occurs jointly more often than by chance alone, suggesting the possibility of an underlying, common pathogenesis [1]. A biologically plausible hypothesis proposes that MS presents when an excess of body fat accumulates in subjects with a specific metabolic susceptibility which seems likely represented by insulin resistance [2]. On the other hand, MS is also known to be associated with a prooxidant and proinflammatory status. Moreover, oxidative stress is considered to play a pivotal role in MS pathophysiology, favouring atherosclerotic damage and increasing CAD risk [4].

PON1 is a HDL-associated, pleiotropic enzyme and may play a role in several different pathways: from the protection against oxidative damage and lipid peroxidation to the contribution to innate immunity processes and from the detoxification of reactive molecules and/or xenobiotic compounds to drug bioactivation (e.g., clopidogrel) [14, 25–27]. More specifically, PON1 is capable of protecting lipoproteins from lipid peroxidation by degrading specific oxidized cholesteryl esters and phospholipids, and antioxidant properties of HDL have been attributed, at least partially, to PON1 [25, 28, 29]. On the other hand, PON1 can be, in turn, inactivated by oxidative stress and oxidized lipids [30]. Thus, there are many biologically plausible reasons linking the pro-oxidant, HDL-poor MS with the anti-oxidant, HDL-associated PON1.

In the first study linking PON1 and MS, Senti and coworkers observed a progressive decrease of paraoxonase activity by increasing the number of MS disturbances [18]. Concomitantly, a progressive increase of lipid peroxides concentration was observed, so that the authors hypothesized that a greater degree of severity of MS is associated with an increased oxidative stress which inactivates PON1 function. On the other hand, they emphasized also the possibility that low PON1 function fails an efficient protection against MS-related oxidative damage that cannot be excluded [18]. Later on, those results were confirmed by Blatter Garin and colleagues, who found a low paraoxonase activity and a reduced

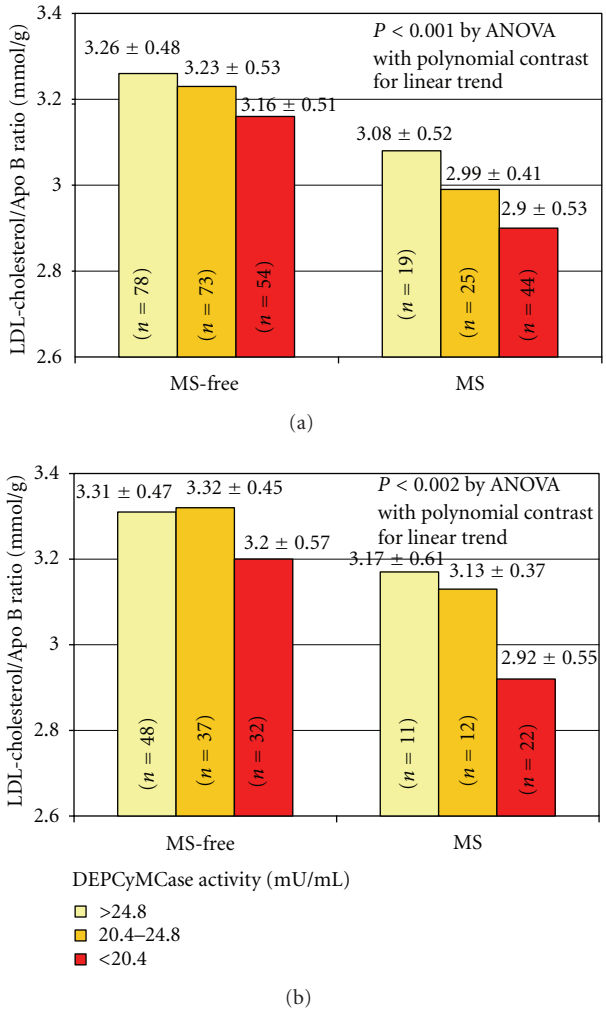

Figure 3: LDL cholesterol/Apo B ratio according to metabolic syndrome (MS) diagnosis and DEPCyMCase activity in the whole population (a) ($n = 293$) and in subjects without lipid-lowering therapy (b) ($n = 162$).

PON1 mass in patients with MS [19]. These authors also found that MS patients had a decreased LDL cholesterol/Apo B ratio, indicative of the presence of small, dense, oxidized, and proatherogenic lipoprotein particles [19].

Our study extends such findings also for the novel PON1 assays utilized, the DEPCyMCase and TBBLase activity. Moreover, the most significant association found in our analysis was that with DEPCyMCase activity, which is considered the best surrogate marker of PON1 concentration, since, differently from the other assay to test PON1 activities, it is not influenced by PON1 genotype nor is it stimulated by HDL binding [20].

Although it could be argued that MS-related, low PON1 concentration is merely an epiphenomenon of low level of HDLs (i.e., the plasma carriers of PON1), our analysis does not support this point of view, the association of DEPCyMCase activity with MS being independent of both HDL and Apo A-I levels. This HDL-independent association is further emphasized by observing that subjects with low HDL level but without MS appeared to have a "normal" not reduced DEPCyMCase activity, while those with high HDL level but with MS tended to have a reduced DEPCyMCase activity (Figure 1(a)). Taking all together, these results suggest that a low DEPCyMCase activity, that is, a low PON1 concentration, is characteristic of MS, independently of low HDL concentration. Moreover, they invite to take into account not only HDL quantity but also HDL quality, which could be reflected, at least in part, by PON1 concentration/activity. It is noted, in this regard, that subjects with low HDL but without MS may have a small amount of "high-quality" HDLs, while subjects with high HDL levels but with MS may have a large amount of "low-quality" HDLs. The relevance of considering HDL quality and not only its quantity has been indirectly emphasized by the failure of the cholesteryl ester transfer protein (CETP) inhibitor, torcetrapib, to improve cardiovascular clinical end points. It has been in fact reported that torcetrapib, in spite of its demonstrated efficiency to elevate HDL concentration, eventually leads instead to an increased cardiovascular morbidity, even if also other off-target adverse effects for CAD risk (e.g., blood pressure elevation) have been related to torcetrapib use [31, 32]. However, in any case, these results have underscored the intricacy of HDL metabolism, with functional quality perhaps being more important than the circulating quantity of HDL [33, 34]. Indeed, both quantitative and qualitative changes to lipoprotein profiles may lead to an increased CAD risk and PON1 could be considered as a potential marker of HDL quality, being linked to the antioxidant, anti-inflammatory properties of HDL.

Accordingly with this last consideration, in our study PON1 appears to influence the MS-related risk of CAD. Certainly, an additive effect between MS and PON1 levels (marked by DEPCyMCase activity) should be considered in determining CAD risk, which increased progressively from subjects without MS and with high PON1 levels to those with MS and low PON1 levels. Remarkably, subjects with MS but still high PON1 levels did not present a significant increase of CAD risk. This result is consistent with a previous study showing that an increased expression of human PON1 in a mouse model of MS inhibited the development of atherosclerosis, probably by reducing the amount of oxidized LDL in both plasma and atherosclerotic plaque [35]. Moreover, it is worthy of note that, in our study population, there was a significant, positive correlation between DEPCyMCase activity and LDL cholesterol/Apo B ratio, suggestive of high PON1 levels associated with low levels of small, dense, and oxidized LDL. Remarkably, LDL cholesterol/Apo B ratio trend across the MS-DEPCyMCase activity stratification groups (Figure 3) was impressively consistent with that of CAD risk (Figure 2). Taken together these results, we are tempting to speculate that an adequate function of PON1 may counterbalance some of MS-related, harmful effects at vascular level, probably by reducing oxidative stress and lipid peroxidation, and thus protect against MS-associated risk of CAD. If this hypothesis is true, by restoring PON1 function, there could be a possibility for a new therapeutic tool for cardiovascular diseases, with particular advantageous effects in high-risk patients, such subjects with MS. However, PON1

reduction may be merely the result of a more extensive PON1 consumption/inactivation during conditions characterized by increasing severity of oxidative stress, like a more advanced MS [19] or a clinically evident CAD. Therefore, further studies are needed to address the issue whether PON1 reduction could be a pathogenic, contributory factor to increased risk of CAD in MS or that reduction would be simply the consequence of ancillary enzyme consumption/inactivation due to CAD/MS-associated oxidative stress.

4.1. Study Limitations. There are some significant caveats to the present study. The retrospective case-control design, the small number of enrolled subjects, and the lack of some clinical data, such as the waist circumference value, are possible limitations of this study. However, despite the relatively low sample size of our study population, the statistical power of the analysis for DEPCyMCase activity difference between MS and MS-free subjects was >90% by Altmann nomogram with a significance level at 0.05. As further experimental limitation, it should be underlined that the DEPCyMCase activity is only a surrogate marker of PON1 concentration and that we did not perform a direct PON1 quantification by ELISA. However, in a previous study the DEPCyMCase assay has been shown to provide information similar to PON1 ELISA assay [20]. Also LDL cholesterol/Apo B ratio is regarded as a surrogate marker of small and dense LDL and, in addition, Apo B was measured in whole serum, although it is known to essentially reflect Apo B in LDL. On the other hand, a remarkable strength of this study is represented by the angiography evaluation of the coronary artery bed, which allows a clear-cut definition of the clinical phenotype and avoids the possibility to include in the control group subjects with subclinical, but significant CAD.

5. Conclusions

In summary, our results show that PON1 activities, including those evaluated by means of novel substrate assays (TBBL and DEPCyMC), are impaired in MS. In particular, these assays suggest that a low PON1 concentration, as indicated by the low DEPCyMCase activity, is characteristic of MS cluster, independently of HDL concentration. Moreover, DEPCyMCase activity appears to interact with MS in determining the risk of CAD, with data suggesting that this association may be also related to the prevalence of small, dense, and oxidized LDL. More precisely, a high DEPCyMCase activity (i.e., a high PON1 concentration) seems protective for CAD risk in subjects with MS, while the highest risk for CAD was observed among subjects with MS and concomitant low levels of DEPCyMCase activity (i.e., a low PON1 concentration). Certainly, a statistically significant association does not mean a link of causality. Nonetheless, PON1 assays seem to add more important information than the simple HDL quantity assessment. According to this hypothesis of heterogeneous HDL-related effects, a recent study demonstrated that in contrast to HDL from healthy subjects, HDL from patients with CAD does not have endothelial anti-inflammatory effects and does not stimulate endothelial repair because it fails to induce endothelial NO production [36]. Mechanistically, the reduction of HDL-associated PON1 activity appears to lead to inhibition of eNOS activation and the subsequent loss of the endothelial antiinflammatory and endothelial repair-stimulating effects of HDL [36], thus supporting the concept that the cardiovascular impact of HDL is not simply related to its abundance [37]. Indeed, HDL cholesterol is only an integrative but nonfunctional measure of lipoproteins, and, therefore, novel biomarkers reflecting the functionality of HDL particles are needed to assess and better monitor cardiovascular risk [34], in particular in the presence of conditions at high CAD risk such as the case of MS. PON1 assays could be potentially considered as such new diagnostic tools while further studies are needed to address this intriguing issue.

Acknowledgments

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Clinical Study

Stepwise Increases in Left Ventricular Mass Index and Decreases in Left Ventricular Ejection Fraction Correspond with the Stages of Chronic Kidney Disease in Diabetes Patients

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Aims. Patients with diabetic nephropathy are reported to have a high prevalence of left ventricular structural and functional abnormalities. This study was designed to assess the determinants of left ventricular mass index (LVMI) and left ventricular ejection fraction (LVEF) in diabetic patients at various stages of chronic kidney disease (CKD). *Methods*. This cross-sectional study enrolled 285 diabetic patients with CKD stages 3 to 5 from our outpatient department of internal medicine. Clinical and echocardiographic parameters were compared and analyzed. *Results*. We found a significant stepwise increase in LVMI ($P < 0.001$), LVH ($P < 0.001$), and LVEF < 55% ($P = 0.013$) and a stepwise decrease in LVEF ($P = 0.038$) corresponding to advance in CKD stages. *Conclusions*. Our findings suggest that increases in LVMI and decreases in LVEF coincide with advances in CKD stages in patients with diabetes.

1. Introduction

Diabetic nephropathy is one of the major complications of diabetes mellitus (DM) and one of the major reasons for renal replacement therapy [1]. The leading cause of morbidity and mortality in patients with diabetic nephropathy is cardiovascular disease [2]. Cardiovascular risk in this population can partially be attributed to an increase of traditional risk factors among people with DM but may also be related to the risk factors for coexisting chronic kidney disease (CKD), such as proteinuria, fluid retention, anemia, oxidative stress, and chronic inflammatory state [2–4].

There are a number of hemodynamic and metabolic disturbances that affect the structure and function of heart in patients with diabetic nephropathy. The major factors that contribute to further heart failure in diabetic patients include cardiac microangiopathy, neuropathy of the cardiac autonomous nervous system, disturbed metabolism, and fatty degeneration of the myocardium [5]. These patients are reported to have a high prevalence of decreased left ventricular systolic function and increased left ventricular mass index (LVMI) resulting from pressure and volume overload [6, 7]. Echocardiographic measures of left ventricular function and structure have been reported to predict adverse cardiovascular outcomes in a variety of populations [8, 9]. Therefore, it is important to detect and treat abnormal geometry and dysfunction of heart early. However, little is known about the relation between the severity of left ventricular geometry and dysfunction and renal function impairment in diabetic patients. The aim of this study was to compare the LVMI and left ventricular ejection fraction (LVEF) among diabetic patients with various degrees of

renal insufficiency and identify the independent risk factors associated with increased LVMI and decreased LVEF in this population.

2. Subjects and Methods

2.1. Study Patients and Design. The study was conducted in a regional hospital in southern Taiwan. In total, 285 diabetic patients with CKD stages 3 to 5 were enrolled consecutively from our outpatient department of internal medicine from January 2007 to May 2010. Patients with evidence of kidney damage lasting for more than 3 months were classified into CKD stage 3, 4, or 5 groups based on estimated glomerular filtration rate (eGFR) level (mL/min/1.73 m^2) of 30 to 59, 15 to 29, and <15, respectively, as recommended in the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines [10]. Patients with significant mitral valve disease and inadequate image visualization were excluded. The protocol for this study was approved by our institutional review board, and all enrolled patients gave written informed consent.

2.2. Evaluation of Cardiac Structure and Function. The echocardiographic examination was performed using VIVID 7 (General Electric Medical Systems, Horten, Norway), with the participant respiring quietly in the left decubitus position. The echocardiographers were blind to patient data. Two-dimensional and two-dimensional guided M-mode images were recorded from the standardized view points. The echocardiographic measurements included aortic root diameter, left atrial diameter (LAD), left ventricular internal diameter in diastole (LVIDd), and left ventricular internal diameter in systole (LVIDs), LVEF, peak early transmitral filling wave velocity (E), and peak late transmitral filling wave velocity (A). Left ventricular mass was calculated using the Devereux-modified method [11]. LVMI was calculated by dividing left ventricular mass by body surface area. Left ventricular hypertrophy (LVH) was defined when LVMI exceeded 134 g/m^2 and 110 g/m^2 for men and women, respectively [12]. Systolic function was assessed by measuring ejection fraction of left ventricle. Systolic dysfunction was defined as LVEF <55%. Diastolic function was estimated by measuring the E/A ratio; a value of <1.0 was considered diastolic dysfunction.

2.3. Collection of Demographic, Medical, and Laboratory Data. Demographic and medical data, including age, gender, smoking history (ever versus never), and comorbid conditions, were garnered from medical records or interviews with patients. Study subjects were defined as having DM if their fasting blood glucose levels were greater than 126 mg/dL or they were taking hypoglycemic agents to control blood glucose levels. Similarly, participants were defined as having hypertension if their systolic blood pressures were ≥140 mmHg or diastolic blood pressure ≥90 mmHg or they were taking antihypertensive drugs. Coronary artery disease was defined if they had a history of typical angina with positive stress test, angiographically documented coronary artery disease, and old myocardial infarction or they had undergone coronary artery bypass surgery or angioplasty. Cerebrovascular disease was defined if they had a history of cerebrovascular incidents such as cerebral bleeding and infarction. Congestive heart failure was defined based on the Framingham criteria. Body mass index was calculated as the ratio of weight in kilograms divided by square of height in meters. Blood and urine samples were obtained within 1 month of enrollment. Laboratory data were measured from fasting blood samples using an autoanalyzer (Roche Diagnostics GmbH, D-68298 Mannheim COBAS Integra 400). Serum creatinine was measured by the compensated Jaffé (kinetic alkaline picrate) method in a Roche/Integra 400 Analyzer (Roche Diagnostics, Mannheim, Germany) using a calibrator traceable to isotope-dilution mass spectrometry [13]. The value of eGFR was calculated using the 4-variable equation in the Modification of Diet in Renal Disease (MDRD) study [14]. The HbA1c was measured by Prismus CLC 385 automated analyzer. Proteinuria was examined by dipsticks (Hema-Combistix, Bayer Diagnostics). A test result of 1+ or more was defined as positive. In addition, information regarding patient medications including aspirin, angiotensin converting enzyme inhibitors (ACEIs), angiotensin II receptor blockers (ARBs), non-ACEI/ARB antihypertensive drugs, and HMG-CoA reductase inhibitors (statins) during the study period was obtained from medical records.

2.4. Statistical Analysis. Data are expressed as percentages or mean ± standard deviation or median (25th–75th percentile) for triglyceride. Multiple comparisons among the study groups were performed by one-way analysis of variance (ANOVA) followed by post hoc test adjusted with a LSD correction. The relationship between two continuous variables was assessed by a bivariate correlation method (Pearson's correlation). Linear regression analysis was used to identify the factors associated with LVMI and LVEF. Significant variables in univariate analysis were selected for multivariate analysis. P value less than 0.05 was considered significant. All statistical operations were performed using SPSS 12.0 for Windows (SPSS Inc. Chicago, USA).

3. Results

As can be seen in Table 1, a summary of clinical characteristics organized by CKD stage, we studied 285 nondialyzed CKD patients (174 men and 111 women, mean age 66.4 ± 11.6 years). The prevalence of LVH and LVEF < 55% was 62.5% and 10.5%, respectively. Stepwise increases in the prevalence of a history of hypertension, cerebrovascular disease, and congestive heart failure, pulse pressure, uric acid, phosphorous, calcium-phosphorous product, proteinuria, and percentage of non-ACEI/ARB antihypertensive drug use and stepwise decreases in the diastolic blood pressure, albumin, hemoglobin, eGFR, and calcium corresponded to advancement in CKD from stage 3 to 5. In addition, there was a significant trend for a stepwise increase in the LAD, LVIDd, LVIDs, LVMI, and the prevalence of LVH and LVEF < 55% and a stepwise decrease in the LVEF corresponding to advancement in CKD from stage 3 to 5. Figure 1 shows

Table 1: Clinical characteristics of patients among different stages of CKD.

Characteristics	Stage 3 ($n = 99$)	Stage 4 ($n = 99$)	Stage 5 ($n = 87$)	P for trend	All patients ($n = 285$)
Age (year)	66.3 ± 12.4	68.4 ± 10.7	64.1 ± 11.5†	0.039	66.4 ± 11.6
Male gender (%)	75.8	58.6*	47.1*	<0.001	61.1
Smoking history (%)	32.3	36.4	28.7	0.540	32.6
Hypertension (%)	79.8	81.8	97.7*†	0.001	86.0
Coronary artery disease (%)	13.1	13.1	18.4	0.514	14.7
Cerebrovascular disease (%)	10.1	21.2*	27.6*	0.009	19.3
Congestive heart failure (%)	10.1	15.2	28.7*†	0.003	17.5
Systolic blood pressure (mmHg)	144.5 ± 21.2	141.2 ± 20.0	148.3 ± 23.7†	0.089	144.6 ± 21.8
Diastolic blood pressure (mmHg)	82.4 ± 12.3	77.4 ± 11.9*	76.7 ± 14.1*	0.005	78.9 ± 13.0
Pulse pressure (mmHg)	62.1 ± 16.7	63.8 ± 11.7	71.7 ± 19.9*†	0.001	65.7 ± 18.2
Body mass index (kg/m^2)	26.2 ± 4.0	26.3 ± 3.5	25.0 ± 3.8*†	0.043	25.9 ± 3.8
Laboratory parameters					
Albumin (g/L)	41.3 ± 3.5	39.9 ± 3.9*	37.3 ± 4.5*†	<0.001	39.6 ± 4.3
Fasting glucose (mmol/L)	8.2 ± 3.2	8.0 ± 3.8	8.3 ± 4.7	0.896	8.1 ± 3.9
HbA1c (%)	7.5 ± 1.4	8.1 ± 2.1*	7.4 ± 1.8†	0.032	7.7 ± 1.8
Triglyceride (mmol/L)	1.8 (1.1–2.4)	1.8 (1.4–2.6)	1.8 (1.2–2.7)	0.173	1.8 (1.2–2.6)
Total cholesterol (mmol/L)	5.0 ± 1.1	5.1 ± 1.3	5.2 ± 1.4	0.544	5.1 ± 1.3
Hemoglobin (g/L)	128.8 ± 18.7	115.4 ± 19.2*	93.1 ± 13.4*†	<0.001	113.2 ± 22.7
Baseline eGFR (mL/min/1.73 m^2)	40.5 ± 6.6	23.1 ± 4.5*	10.3 ± 3.0*†	<0.001	25.2 ± 13.3
Calcium (mmol/L)	2.4 ± 0.2	2.4 ± 0.2	2.3 ± 0.2*†	<0.001	2.4 ± 0.2
Phosphate (mmol/L)	1.1 ± 0.2	1.3 ± 0.2*	1.6 ± 0.4*†	<0.001	1.3 ± 0.3
Calcium-phosphorous product (mmol^2/L^2)	2.8 ± 0.5	3.1 ± 0.6*	3.6 ± 0.8*†	<0.001	3.1 ± 0.7
Uric acid (μmol/L)	456.7 ± 113.3	505.4 ± 138.6*	530.8 ± 143.3*	0.001	496.5 ± 135.1
Proteinuria (%)	47.5	75.5*	98.9*†	<0.001	72.9
Medications					
Aspirin use (%)	30.2	32.3	34.5	0.826	32.2
ACEI and/or ARB use (%)	80.2	83.3	63.1*†	0.003	76.1
Non-ACEI/ARB antihypertensive drug use (%)	67.7	80.8*	94.3*†	<0.001	80.4
Statin use (%)	36.5	29.2	31.0	0.532	32.2
Echocardiographic data					
Aortic root diameter (cm)	3.3 ± 0.4	3.3 ± 0.4	3.2 ± 0.4	0.262	3.3 ± 0.4
LAD (cm)	3.7 ± 0.6	3.9 ± 0.6	4.1 ± 0.6*†	< 0.001	3.9 ± 0.6
LVIDd (cm)	4.8 ± 0.7	4.9 ± 0.8	5.1 ± 0.7*†	0.005	4.9 ± 0.8
LVIDs (cm)	2.9 ± 0.7	3.1 ± 0.8	3.3 ± 0.8*†	0.002	3.1 ± 0.8
LVMI (g/m^2)	129.5 ± 43.5	139.1 ± 52.4	167.1 ± 45.9*†	<0.001	144.3 ± 49.8
LVH (%)	44.4	61.6*	83.9*†	<0.001	62.5
LVEF (%)	69.0 ± 11.1	67.0 ± 11.7	64.5 ± 13.2*	0.038	66.9 ± 12.1
LVEF < 55% (%)	4.0	11.1*	17.2*	0.013	10.5
E/A < 1 (%)	78.9	84.9	75.0	0.250	79.8

CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin II receptor blocker; LAD: left atrial diameter; LVIDd: left ventricular internal diameter in diastole; LVIDs: left ventricular internal diameter in systole; LVMI: left ventricular mass index; LVH: left ventricular hypertrophy; LVEF: left ventricular ejection fraction; E: peak early transmitral filling wave velocity; A: peak late transmitral filling wave velocity.

*$P < 0.05$ compared to stage 3; †$P < 0.05$ compared to stage 4.

the significant trend for a stepwise increase in LVMI (a) and the prevalence of LVH (b) corresponding to the advancement in CKD from stage 3 to 5. Figure 2 shows the significant trend for a stepwise decrease in LVEF (a) and a stepwise increase in the prevalence of LVEF < 55% (b) corresponding to the advancement in CKD from stage 3 to 5.

As seen in Table 2 which summarizes our findings on the possible determinants of LVMI in our study patients, univariate analysis showed a significant positive correlation between LVMI and being male, a history of smoking, coronary artery disease, and congestive heart failure, advanced CKD stages, systolic blood pressure, pulse pressure,

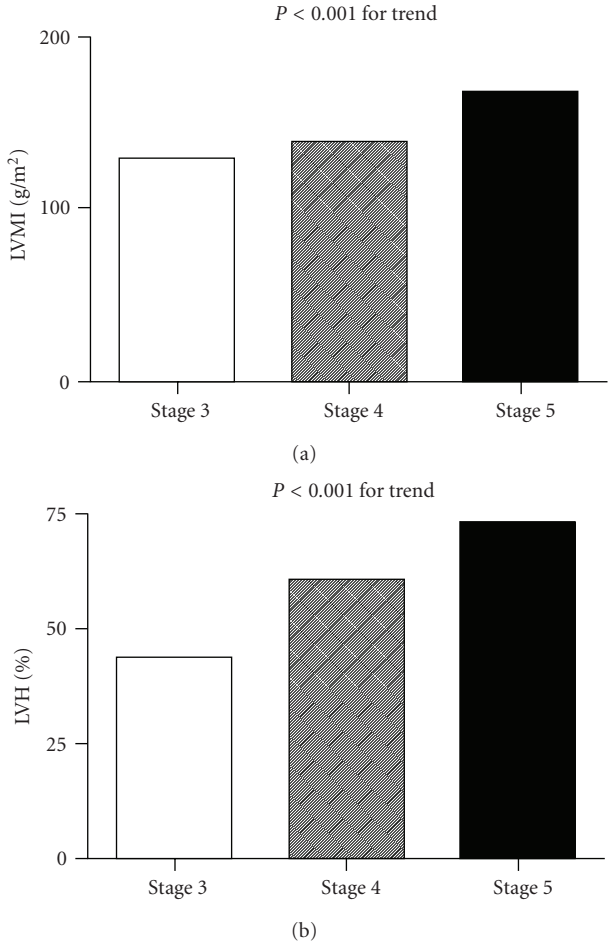

Figure 1: There was a significant trend for a stepwise increase in left ventricular mass index (LVMI) ($P < 0.001$ for trend) (a) and the prevalence of left ventricular hypertrophy (LVH) (44.4%, 61.6%, and 83.9%, resp.; $P < 0.001$ for trend) (b) corresponding to the advancement in chronic kidney disease from stage 3 to 5.

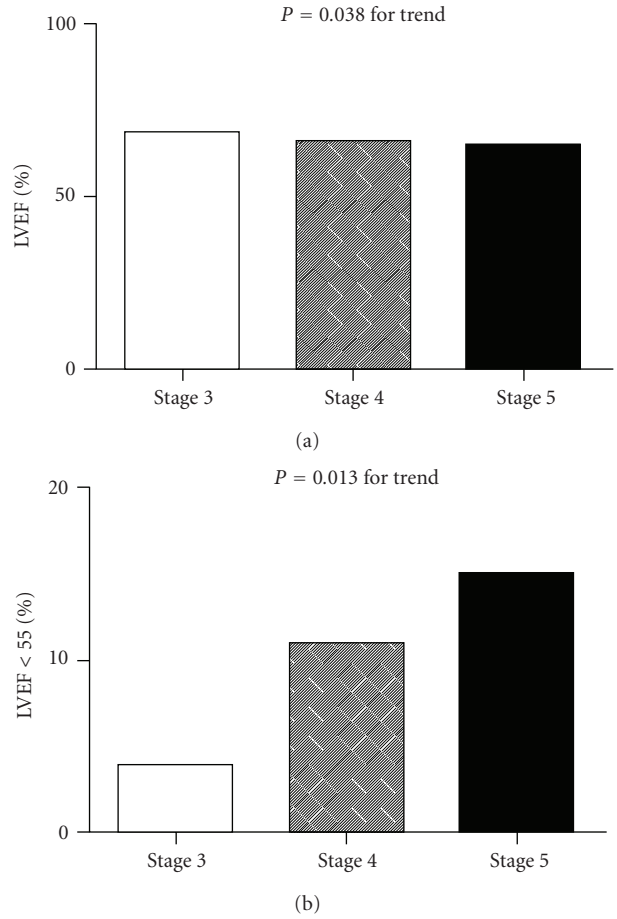

Figure 2: There was a significant trend for a stepwise decrease in left ventricular ejection fraction (LVEF) ($P = 0.038$ for trend) (a) and stepwise increase in the prevalence of LVEF < 55% (4.0%, 11.1%, and 17.2%, resp.; $P < 0.013$ for trend) (b) corresponding to the advancement in chronic kidney disease from stage 3 to 5.

phosphorous, proteinuria, aspirin use, and non-ACEI/ARB antihypertensive drug use and negative correlation between LVMI and albumin, hemoglobin, calcium, and ACEI and/or ARB use. Further forward multivariate analysis revealed a significant correlation between increases in LVMI and being male, a history of congestive heart failure, advanced CKD stages, high systolic blood pressure, and low serum albumin level.

Table 3 summarizes the results of our analysis of possible determinants of LVEF in our study patients. Univariate analysis showed a positive correlation between LVEF and albumin, calcium, and ACEI and/or ARB use and a negative correlation with being male, a history of coronary artery disease and congestive heart failure, advanced CKD stages, uric acid, phosphorous, aspirin use, and non-ACEI/ARB antihypertensive drug use. Further forward multivariate analysis revealed a correlation between decreased LVEF and being male, a history of coronary artery disease, advanced CKD stages, low serum albumin level, and ACEI and/or ARB use.

4. Discussion

In the present study, we evaluated the determinants of LVMI and LVEF in diabetic patients with various stages of CKD. We found a significant trend for a stepwise increase in LVMI and the prevalence of LVH and LVEF < 55% and a stepwise decrease in LVEF corresponding to advancement in CKD stage.

Patients with diabetic nephropathy have a high prevalence of LVH and left ventricular systolic dysfunction, two disorders that contribute majorly to increased risk of cardiovascular death [2–4]. Structural and functional abnormalities of the heart are common in patients with diabetic nephropathy because of pressure and volume overload [15, 16]. The prevalence of LVH ranges from 17% to 42% in patients with hypertension, 22% to 47% in patients with CKD, and 68.5% of dialysis patients, and LVH occurs in only 3.2% of the general population [8, 17, 18]. However, the prevalence of LVH in our study patients was relatively high (62.5%), which might be explained by the fact that all of the patients included in our study had diabetic nephropathy.

Table 2: Determinants of left ventricular mass index (LVMI) in study patients.

Characteristics	Univariate		Multivariate (forward)	
	Standardized coefficient β	P	Standardized coefficient β	P
Age (year)	0.012	0.842	—	—
Male *versus* female	0.117	0.048	0.211	<0.001
Smoking(ever *versus* never)	0.126	0.033	—	—
Coronary artery disease	0.147	0.013	—	—
Cerebrovascular disease	0.024	0.681	—	—
Congestive heart failure	0.262	<0.001	0.196	0.001
CKD stage	0.301	<0.001	0.262	<0.001
Systolic blood pressure (mmHg)	0.225	<0.001	0.203	<0.001
Diastolic blood pressure (mmHg)	0.095	0.118	—	—
Pulse pressure (mmHg)	0.202	0.001	—	—
Body mass index (kg/m^2)	0.049	0.413	—	—
Laboratory parameters				
Albumin (g/L)	−0.321	<0.001	−0.132	0.032
Fasting glucose (mmol/L)	0.027	0.653	—	—
HbA1c (%)	−0.052	0.389	—	—
Triglyceride (Log mmol/L)	−0.034	0.570	—	—
Cholesterol (mmol/L)	0.047	0.436	—	—
Hemoglobin (g/L)	−0.212	<0.001	—	—
Calcium (mmol/L)	−0.185	0.002	—	—
Phosphate (mmol/L)	0.182	0.002	—	—
Calcium-phosphorous product (mmol^2/L^2)	0.114	0.060	—	—
Uric acid (μmol/L)	0.117	0.052	—	—
Proteinuria	0.216	<0.001	—	—
Medications			—	—
Aspirin use (%)	0.190	0.001	—	—
ACEI and/or ARB use (%)	−0.167	0.005	—	—
Non-ACEI and/or ARB antihypertensive drug use (%)	0.188	0.001	—	—
Statin use (%)	−0.041	0.502	—	—

Values expressed as standardized coefficient β. Abbreviations are the same as Table 1.

The prevalence of left ventricular systolic dysfunction in patients with chronic renal insufficiency is approximately 7.6%–22% [8, 19]. In our patients, the prevalence of LVEF < 55% was 10.5%, which is compatible with previous findings.

DM, hypertension, and dyslipidemia are traditional cardiovascular risk factors. In addition to these traditional risk factors, patients with CKD may have other risk factors for increase cardiovascular risk such as inflammation, oxidative stress, anemia, metabolic disorders, calcium-phosphorous disorders, hypervolemia, and structural and functional abnormalities of heart, which may help to explain the high cardiovascular morbidity and mortality in such patients [20–25]. LVH, a common finding in patients with CKD, has been reported to advance with decreases in glomerular filtration rate [3]. Hillege et al. found that there was also a significant correlation between the deterioration of congestive heart failure and the progression of renal failure [26]. Our study found that, with the decrease of renal function, there was a significant trend for a stepwise increase in LVMI and the prevalence of LVH and LVEF < 55% and a stepwise decrease in LVEF in patients with diabetic nephropathy, which is consistent with the previous findings.

Low serum albumin level has been regarded as indicator of malnutrition. Malnutrition may worsen the outcome of CKD by aggravating existing inflammation and heart failure [27]. Hypoalbuminemia has been correlated with left ventricular structure and function [25, 28, 29]. Kursat et al. [25], evaluating the relationship between the degree of malnutrition and echocardiographic parameters in 72 hemodialysis patients, found that the malnutrition index, calculated using Subjective Global Assessment, had a positive correlation with left ventricular mass and index. They cited inadequate volume control as an explanation for their findings. Volume overload may substantially decrease energy and protein intake, suggesting a possible relation between volume overload and malnutrition. In addition, volume overload may increase the diastolic wall stress and in turn cause the development of LVH [25]. Trovato et al. [29],

Table 3: Determinants of left ventricular ejection fraction (LVEF) in study patients.

Characteristics	Univariate		Multivariate (forward)	
	Standardized coefficient β	P	Standardized coefficient β	P
Age (year)	0.062	0.297	—	—
Male *versus* female	−0.223	<0.001	−0.227	<0.001
Smoking(ever *versus* never)	−0.090	0.130	—	—
Coronary artery disease	−0.169	0.004	−0.153	0.008
Cerebrovascular disease	−0.071	0.235	—	—
Congestive heart failure	−0.155	0.009	—	—
CKD stage	−0.151	0.011	−0.173	0.007
Systolic blood pressure (mmHg)	−0.038	0.528	—	—
Diastolic blood pressure (mmHg)	−0.092	0.130	—	—
Pulse pressure (mmHg)	0.020	0.747	—	—
Body mass index (kg/m^2)	0.010	0.871	—	—
Laboratory parameters				
Albumin (g/L)	0.258	< 0.001	0.188	0.003
Fasting glucose (mmol/L)	−0.097	0.107	—	—
HbA1c (%)	−0.040	0.509		
Triglyceride (Log mmol/L)	−0.039	0.518	—	—
Cholesterol (mmol/L)	−0.069	0.247	—	—
Hemoglobin (g/L)	0.103	0.083	—	—
Calcium (mmol/L)	0.122	0.044		
Phosphate (mmol/L)	−0.168	0.005		
Calcium-phosphorous product (mmol^2/L^2)	−0.103	0.088	—	—
Uric acid (μmol/L)	−0.146	0.015	—	—
Proteinuria	−0.096	0.106	—	—
Medications				
Aspirin use (%)	−0.132	0.028	—	—
ACEI and/or ARB use (%)	0.203	0.001	0.143	0.014
Non-ACEI and/or ARB antihypertensive drug use (%)	0.048	0.422	—	—
Statin use (%)	0.004	0.951	—	—

Values expressed as standardized coefficient β. Abbreviations are the same as Table 1.

also investigating the correlation between heart failure and nutritional status in hemodialysis patients, reported an association between low serum albumin level and decreased LVEF. Our results consistently demonstrate independent association between low serum albumin levels and increased LVMI and decreased LVEF in patients with diabetic nephropathy.

One limitation of this study was that it had a cross-sectional design, and thus the predictors of cardiovascular events could not be evaluated. Further prospective studies are needed to confirm our findings.

In conclusion, our results found a significant trend for a stepwise increase in LVMI and the prevalence of LVH and LVEF < 55% and a stepwise decrease in LVEF corresponding to advancement in CKD stage in diabetic patients.

Disclosure

The authors have no financial interest in the information contained in the paper.

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Clinical Study

Epidemiological Survey on the Prevalence of Periodontitis and Diabetes Mellitus in Uyghur Adults from Rural Hotan Area in Xinjiang

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Background and Aims. This study was designed to explore the relationship between periodontitis and diabetes mellitus (DM) in Uygur adults from Xinjiang. *Methods and Results*. Data were obtained using questionnaire and oral examination. Participants (48.87 ±13.72 yr) were categorized into periodontitis and non-periodontitis groups in accordance with the chronic periodontitis diagnostic criteria. Based on gum inflammation, bleeding on probing, periodontal pocket depth and attachment loss, patients were further divided into mild, moderate and severe periodontitis groups. Among 962 subjects, 453 (47.1%) suffered from chronic periodontitis with a prevalence of type 2 DM and impaired fasting glucose of 9.5% and 11.4%, respectively. In the periodontitis group, the prevalence of type 2 DM was 75.6% compared with 22.4% in the non-periodontitis group. Likewise, the prevalence of impaired fasting glucose was 71.3% and 28.7% in periodontitis and non-periodontitis groups, respectively. The univariate logistic regression analysis revealed moderate and severe periodontitis as risk factors for DM (OR = 3.4, OR = 2.9). Multivariate logistic regression analysis showed that moderate periodontitis is independently associated with DM (OR = 4). *Conclusions*. Our data revealed that prevalence of DM is overtly higher in periodontitis patients than in individuals without periodontitis. Furthermore, moderate periodontitis is considered an independent risk factor for type 2 DM.

1. Introduction

Diabetes mellitus (DM), a chronic metabolic disease characterized by hyperglycemia, is often attributed to environmental and genetic factors. The prevalence of DM has risen dramatically in recent years, resulting in a rapid increase of diabetic patients. Asia in particular has the highest prevalence of diabetes in the world. Countries exhibiting the fastest rate in diabetic population growth include India and China, among many other developing countries [1]. Hyperglycemia triggers a wide variety of long-term complications in diabetics such as large vessel diseases, cardiomyopathy, and kidney and eye impairments [2, 3]. Periodontal diseases can be divided into gingivitis and periodontitis. Periodontitis is known as a chronic infectious disease of tissues surrounding the teeth which is induced by microorganisms. Periodontitis is a disease characterized by periodontal pocket formation, loss of connective tissue attachment, alveolar bone resorption, and gingival inflammation, ultimately resulting in tooth loss. When oral hygiene is compromised, oral bacteria may form a plaque biofilm, which is resistant to chemicals and immune cells [4, 5]. Without mechanical debridement, the plaque biofilm matures and causes gingivitis in a few days. Gingivitis represents chronic but reversible inflammation and can be usually treated by proper plaque control. Gingivitis typically extends to irreversible periodontitis for months or years [4–6]. Intriguingly, interaction and mutual influences between diabetes and periodontitis have been indicated [7, 8]. In fact, an ongoing longitudinal population-based observational study, the Hisayama study, reported a positive relationship

between body mass index (BMI)/waist-hip ratio and the incidence of periodontal disease although neither impaired glucose tolerance nor diabetes was closely associated with the probing pocket depth [9]. Nonetheless, convincing evidence is still lacking on whether the therapeutic remedy for periodontal disease (such as antibiotic treatment) may achieve optimal glycemic control in diabetic patients [9]. Although preliminary study has been carried out on this important dental health issue [9], the precise underlying mechanisms remain elusive. To better understand the correlation between DM and periodontal disease, an epidemiological study was conducted in Uyghur adults from the town Cele in the Xinjiang Hetian region in 2010.

2. Methods

2.1. Study Subjects. The survey described here represents a cross-sectional study belonging to a sampling survey. Convenience sampling was used, and study subjects were recruited through television advertisements on the local television station. Inclusion criteria were as follows: willing citizens of Cele. The study protocol was approved by our institutional IRB committee, and written informed consent was obtained from all participants.

2.2. Study Protocols

2.2.1. Questionnaire. The questionnaire was designed by an experienced research team. Following professional training, a group of Uyghur medical students fluent in the Chinese and Uyghur languages served as investigators and assisted in filling out the questionnaires. Contents of questionnaire include demographic information (gender, age), medical history (hypertension, diabetes, coronary heart disease, chronic renal disease, chronic respiratory diseases, etc.) behavior and personal habits (e.g., smoking), and family history. Results from the subsequent physical and blood tests were also included.

2.2.2. Physical Examination. Participants were examined by trained medical students using uniform procedures and standards. Physical examination encompassed height, weight, waist circumference, abdominal/hip circumference, and blood pressure. Blood pressure was recorded three times, in accordance with the JNC 7 recommended standard [10]. Prior to each measurement, participants were asked to remain resting for at least 5 min.

2.2.3. Collection of Blood Sample. Five mL fasting blood was collected from each participant. The samples were centrifuged, and the serum were placed in sodium fluoride tubes and kept frozen in individual containers. Serum sodium fluoride tubes were used to test the fasting blood glucose within 2 hours using a Johnson blood glucose meter. Serum was stored at -80°C in frozen containers in liquid nitrogen tanks.

2.2.4. Oral Examination. According to the basic method issued by WHO oral health survey [11], gingival bleeding, probing depth (PD), and periodontal attachment loss (AL) were monitored using the plane mouth mirror, tweezers, and CPI periodontal probe, in conjunction with the probing method (probe's power <25 g) and visual examination. The mouth was divided into six sections, with index teeth 11, 16, 17, 26, 27, 31, 36, 37, 46, and 47 representing each section. Typically, testing results of index teeth represented the periodontal health for each section. All examinations were performed by an experienced dental specialist.

2.3. Diagnosis Criteria

2.3.1. Diagnostic Criteria of Hyperglycemia. Diabetes mellitus (DM) and hyperglycemia diagnostic criteria are in line with the WHO standards issued in 1999 and American Diabetes Association standards [12, 13]. Impaired fasting glucose (IFG) was diagnosed with a fasting venous blood glucose level between 6.1 and 7.0 mmol/L and no history of DM. Hyperglycemia was diagnosed with a fasting venous blood glucose at 7.0 mmol/L or higher. A level of 7.0 mmol/L or above confirmed by repeated test at different days indicates the diagnosis of diabetes mellitus.

2.3.2. Diagnosis of Periodontitis. Chronic periodontitis was categorized into the following categories. Mild periodontitis: gum inflammation and bleeding on probing, periodontal pocket depth ≤4 mm, and attachment loss of 1~2 mm. Moderate periodontitis: gingival inflammation and bleeding on probing, presence of pus, periodontal pocket depth ≤6 mm, attachment loss of 3~4 mm, and possible presence of slight loose teeth. Severe periodontitis: obvious inflammation or occurrence of periodontal abscess, periodontal pocket depth >6 mm, attachment loss ≥5 mm, and more than one loose tooth [14].

2.3.3. Diagnosis of Metabolic Syndrome. Based on the 1999 WHO criteria [15], metabolic syndrome was diagnosed with at least 3 of the following components: (1) overweight and (or) obesity: BMI ≥ 30 Kg/m^2; [16] (2) high blood glucose: FPG ≥ 6.1 mmol/L and (or) 2hPG ≥ 7.8 mmol/L, and (or) diagnosis of diabetes; [17] (3) hypertension: SBP/DBP ≥ 140/90 mmHg, and (or) diagnosis of high blood pressure and treatment of persons; (4) dyslipoidemia: fasting TG ≥ 1.7 mmol/L, and (or) fasting HDL-C < 0.9 mmol/L (male) or < 1.0 mmol/L (female).

2.3.4. Diagnostic Criteria of Hypertension. Hypertension is defined as systolic blood pressure (SBP) ≥ 140 mmHg and (or) diastolic blood pressure (DBP) ≥ 90 mmHg, or taking hypotensors.

2.3.5. Statistical Analysis. All data input is in duplicate using EpiData3.1 software with the logic and consistency checks. Data were analyzed using an SPSS software for Windows version 13.0. Measurement data was indicated with $x \pm s$. Chi-square test was used to compare the percentage or count data. A logistic regression analysis was performed, using the forward Wald method with $\alpha = 0.05$.

3. Results

3.1. General and Health Information. The Uyghur nationality in the town of Cele accounts for 95% of the total population of 140,000. The survey examined a total of 1099 cases of Uyghur adults. We received 1043 valid and complete questionnaires. 62 subjects failed to take the oral examination, and 19 cases lacked a full set of teeth; thus, 962 cases remained with complete data to be included. This study covered a population of 20 years old or older. The age proportional ratio of the total sample population is shown in Figure 1. The proportional ratio of different age groups is not the same, with the majority of the sample population represented by the 40~49 age group (25.4%). Sex ratio of the participants was male: female = 42.7%: 57.3%. General information of the survey is displayed in Table 1 (due to unfilled questionnaires, the number of effective response cases was not always consistent). General information about the periodontitis group and the non-periodontitis group is shown in Table 2. Table 2 indicates that in the elderly and male patients, the prevalence of impaired fasting glucose, diabetes, hyperlipidemia, metabolic syndrome, and hypertension is significantly higher in the periodontitis group than in the non-periodontitis group.

3.2. The Prevalence of Diabetes Mellitus and Periodontitis. In the survey, the prevalence of periodontitis was 47.1% (453 cases) in 962 individuals. The prevalence of mild, moderate, and severe periodontitis was 28.9% (278 cases), 10.2% (98 cases), and 8.0% (77 cases), respectively. The oral health conditions of the surveyed population are shown in Table 3. In the survey, prevalence of diabetes 9.0% (99/1043) and prevalence of impaired fasting glucose was 11.4% (101/880). The prevalence of DM in the periodontitis group was significantly higher than in the non-periodontitis group (75.6% versus 22.4%, $\chi^2 = 32.300$, $P = .000$). Furthermore, the prevalence of impaired fasting blood glucose in individuals with periodontitis was significantly higher than in individuals without periodontitis (71.3% versus 28.7%, $\chi^2 = 25.322$, $P = .000$), (Table 2).

3.3. Regression Analysis of DM-Related Risk Factors

3.3.1. Univariate Logistic Regression. A further logistic regression analysis was performed. The variables included age, gender, smoking, BMI, hyperlipidemia, hypertension, metabolic syndrome, and periodontitis. Classification valuation of independent variables is shown in Table 4. Results of the logistic regression analysis of DM risk factors are shown in Table 5. Our data revealed that age, BMI, hyperlipidemia, hypertension, metabolic syndrome, moderate periodontitis, and severe periodontitis are major risk factors to this group, indicating a likely correlation between DM and the above-mentioned risk factors. Without considering the other risk factors, the risks of moderate or severe periodontitis patients with diabetes were 2.9- or 3.4-fold higher than the nonmoderate or nonsevere periodontitis patients.

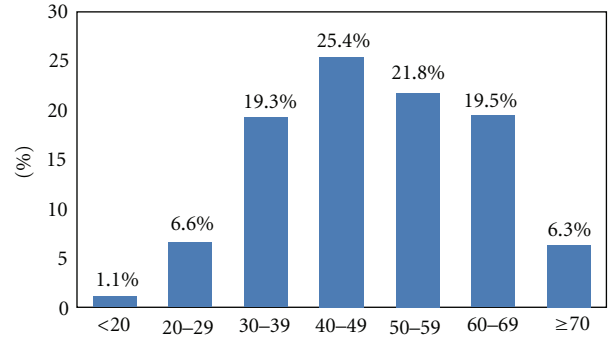

Figure 1: Age proportional ratio of the total sample population.

3.3.2. Multivariate Logistic Regression Analysis. Using the independent variables of age, BMI, hyperlipidemia, hypertension, metabolic syndrome, moderate periodontitis and severe periodontitis, and diabetes as the dependent variable, a multivariate logistic regression analysis was performed using the forward Wald method with the thresholds of 0.05 for lead into and reject. Results of logistic regression analysis of DM risk factors are shown in Table 6. Our findings indicated that age, BMI, metabolic syndrome, and moderate periodontitis and severe periodontitis may serve as independent risk factors for diabetes. The risk of the moderate periodontitis patients with diabetes was 4-fold higher than those patients without moderate periodontitis.

4. Discussion

DM and periodontitis are common multigenetic and multifactorial chronic diseases with a higher incidence at increased age. Both of the morbidities negatively affect periodontal health and systemic health, thus affecting the quality of life [18]. An abundance of recent evidence has consolidated a bidirectional correlation between diabetes and periodontitis. While diabetes is an independent risk factor for periodontitis [19], periodontitis as a chronic inflammation has a negative impact on the metabolic control of diabetes [20]. In particular, periodontitis ranks sixth among all complications of diabetes [21].

The majority (76%) of Uyghur popultion from Xinjiang region reside around the Taklimakan Desert Oasis, among which 70% of the residents live in rural areas. Cele, located at the Southern edge of Taklimakan Desert, is a typical Uyghur rural area. Epidemiological data of periodontitis from adults living in the Hotan region display a much higher prevalence of periodontitis in Uyghur adults than the average among all age groups, according to the second national oral health epidemiological survey [22] and the United States NHANES1999~2000 [23]. Interestingly, the Uyghur adults are a high-risk group for periodontitis in the Hotan region. The current survey revealed a 47.8% prevalence of periodontitis in rural Uyghur adults in Cele, probably due to their unique ethnic lifestyle, oral hygiene habits, and economic conditions in the region. A survey for diabetes was performed in Tianjin regions, reporting a 51.41% prevalence

Table 1: General information of survey population.

Parameter	Effective response	Female ($n = 598$)	Male ($n = 445$)	Total ($n = 1043$)
Age	1043	47.24 ± 12.88	51.05 ± 14.50	48.87 ± 13.72
Smoking	1019	5	125	130
BMI	966	25.57 ± 4.40	26.19 ± 3.92	25.84 ± 4.21
Total cholesterol (mmol/L)	880	4.85 ± 1.80	4.86 ± 1.05	4.85 ± 1.53
Triglyceride (mmol/L)	880	1.86 ± 1.52	2.10 ± 1.62	1.96 ± 1.57
HDL-C (mmol/L)	879	1.30 ± 0.40	1.31 ± 0.57	1.30 ± 0.48
LDL-C (mmol/L)	877	1.97 ± 0.61	2.11 ± 0.59	2.03 ± 0.60
Mean SBP (mmHg)	970	118.72 ± 24.12	124.30 ± 21.02	121.10 ± 23.00
Mean DBP (mmHg)	970	75.56 ± 15.54	77.70 ± 14.46	76.59 ± 15.11
Fasting blood glucose (mmol/L)	880	5.06 ± 1.75	5.24 ± 2.53	5.14 ± 2.12
Metabolic syndrome	880	268	232	500
Hypertension	970	133	94	227
Diabetes mellitus	1043	48	51	99
Periodontitis	962	216	237	453

of diabetes in patients with periodontitis ($\chi^2 = 7.363$, $P = .007$) and a 27.68% prevalence of severe periodontitis ($\chi^2 = 4.967$, $P = .033$) [9]. The data showed that the prevalence of diabetes patients with periodontitis was 75.6% ($\chi^2 = 32.300$, $P = .000$), much higher than in the Tianjin study. Nonetheless, the prevalence of severe periodontitis (8.0%) was significantly lower than in the Tianjin study (27.68%).

In this survey, the overall prevalence of diabetes was 9.3%. The prevalence of diabetes in patients with periodontitis (17.7%) (453) was significantly higher than that of the non-periodontitis group (4.5%). In the periodontitis group with advanced age, impaired fasting glucose, hyperlipidemia, metabolic syndrome, hypertension, and other features, compared with the non-periodontitis group, there was a statistically significant difference. The prevalence of impaired fasting glucose, high blood lipids, metabolic syndrome, and hypertension was significantly higher than the non-periodontitis group. With the univariate logistic regression analysis for diabetes mellitus, and the related risk factors, our data depicted that age, BMI, hyperlipidemia, hypertension, metabolic syndrome, and moderate-to-severe periodontitis were risk factors for DM in the survey group. Notably, the univariate analysis showed that moderate to severe periodontitis was a risk factor for diabetes. The risks of moderate or severe periodontitis patients with diabetes were 2.4- or 1.9-fold greater than the nonmoderate or severe periodontitis patients. Given that multiple risk factors may affect the population prevalence of DM, our finding indicated that moderate-to-severe periodontitis was a risk factor in the survey group through multivariate regression analysis. The risks of moderate or severe periodontitis patients with diabetes were 3.0- or 1.3-fold greater than the patients without moderate or severe periodontitis.

Ample evidence has suggested that periodontitis may lead to cardiovascular disease through bacteremia of periodontal pathogens and the corresponding antigen-mediated chronic inflammation or immune response [24, 25]. Meanwhile, inflammation is known to promote the onset and development of insulin resistance [26–29] and, subsequently, type 2 diabetes. Periodontitis can easily turn periodontal tissue into a proinflammatory environment through increased levels of inflammatory mediators. The accumulated proinflammatory mediators play a pivotal role in reducing the sensitivity of insulin signaling and glucose metabolism [30–34]. Inflammatory cytokines such as TNF-α and IL-6 are known to promote insulin resistance [35]. Challenge of adipocytes with proinflammatory cytokines such as TNF-α phosphorylates insulin-receptor substrate-1 (IRS-1) at Serine residue and impairs insulin receptor tyrosine kinase [33]. Uysal and colleagues reported that mice lacking TNF-α were resistant to obesity-induced insulin resistance [9]. Administration of IL-6 to otherwise healthy volunteers led to a dose-dependent increase in the fasting blood glucose [9]. These results suggest that inflammatory cytokines, which may promote both insulin resistance and chronic inflammatory diseases including periodontitis, are expected to augment insulin resistance and risk of cardiovascular diseases through production of proinflammatory cytokines

Table 2: General information of periodontitis group and the non-periodontitis group.

	Non-periodontitis		Periodontitis		χ^2	P
	$N = 509$	%	$N = 453$	%		
Age						
20–44	308	78.2	86	21.8	195.394	.000
45–59	146	44.4	183	55.6		
≥60	55	23.1	183	76.9		
Gender						
female	340	61.2	216	38.8	35.900	.000
male	169	41.6	237	58.4		
Smoking						
no	428	52.2	392	47.8	0.245	.620
yes	65	54.6	54	45.4		
BMI (Kg/m^2)						
<25	219	52.4	199	47.6	0.160	.689
≥25	283	53.7	244	46.3		
Impaired fasting glucose						
no	420	55.3	339	44.7	25.322	.000
yes	29	28.7	72	71.3		
Diabetes						
no	487	55.8	385	44.2	32.300	.000
yes	22	22.4	68	75.6		
Hyperlipidemia						
no	234	57.9	170	42.1	9.961	.002
yes	215	47.1	241	52.9		
Metabolic syndrome						
no	227	61.0	145	39.0	20.403	.000
yes	222	45.5	266	54.5		
Hypertension						
no	414	55.6	330	44.4	9.854	.002
yes	95	43.6	123	56.4		

Table 3: Oral health conditions of survey populations.

Oral condition	Case	Ratio (%)
Normal	49	5.1
Gingivitis	460	47.8
Mild periodontitis	278	28.9
Moderate periodontitis	98	10.2
Severe periodontitis	77	8.0
Total	962	100.0

Table 4: Classification valuation of independent variables.

Variable number	Number
X1 (age)	18–39 years = 1, 40–59 years = 2, ≥60 years = 3
X2 (gender)	female = 0, male = 1
X3 (smoking)	no = 0, yes = 1
X4 (BMI)	BMI ≤ 25 = 0, BMI > 25 = 1
X5 (hyperlipidemia)	no = 0, yes = 1
X6 (hypertension)	no = 0, yes = 1
X7 (high blood glucose)	no = 0, yes = 1
X8 (metabolic syndrome)	no = 0, yes = 1
X9 (mild periodontitis)	no = 0, yes = 1
X10 (moderate periodontitis)	no = 0, yes = 1
X11 (severe periodontitis)	no = 0, yes = 1

within the lesion site. In addition, both TNF-α and IL-6 are produced in adipose tissues (e.g., one-third of circulating IL-6 is derived from adipose tissues) [9]. These lines of evidence suggest that obesity, diabetes, and chronic periodontitis are mutually related to one another. At this time, little evidence is available to confirm a solid and direct link between periodontitis and insulin resistance, apparently due to the lack of epidemiological and experimental evidence. Our study in Uygur adults has shown that Uyghur adults are a high risk group for periodontitis. Interestingly, the prevalence of diabetes in patients with periodontitis was much higher than the non-periodontitis groups. Our survey

Table 5: Univariate logistic regression analysis of DM risk factors.

	B	Wald	S.E.	OR	95.0% CI		*P*
					Lower	Upper	
Age	0.862	37.137	0.141	2.368	1.795	3.125	.000
Gender	−0.394	3.469	0.212	0.674	0.445	1.021	.063
Smoking	−0.186	0.368	0.306	1.204	0.661	2.193	.544
BMI	0.991	15.176	0.254	2.695	1.636	4.437	.000
Hyperlipidemia	0.767	9.722	0.246	2.152	1.329	3.485	.002
Metabolic syndrome	2.9	31.449	0.517	18.172	6.595	50.067	.000
Hypertension	0.494	4.280	0.239	1.639	1.026	2.619	.039
Mild periodontitis	0.241	1.054	0.235	1.273	0.803	2.019	.305
Moderate periodontitis	1.228	20.292	0.273	3.415	2.001	5.827	.000
Severe periodontitis	1.064	12.127	0.306	2.899	1.593	5.278	.000

Table 6: Multivariate logistic regression analysis of DM risk factors.

	B	Wald	S.E.	OR	95.0% CI		*P*
					Lower	Upper	
Age	0.474	6.136	0.191	1.607	1.104	2.338	.013
BMI	0.968	9.022	0.322	2.633	1.400	4.952	.003
Metabolic syndrome	4.874	57.121	0.645	130.903	36.98	463.375	.000
Moderate periodontitis	1.394	16.76	0.341	4.033	2.069	7.861	.000
Severe periodontitis	1.839	4.246	0.407	2.313	1.042	5.137	.039

favors the notion that moderate and severe periodontitis should be considered independent risk factors for diabetes. Periodontitis displays a close relationship to diabetes mellitus in many ethnic groups [36, 37] although there is no direct epidemiologic evidence consolidating the positive correlation between diabetes and/or glucose intolerance and periodontal diseases. Further scrutiny is warranted with regard to the relationship between diabetes and periodontitis in large population scales.

Conflict of interests

The authors declared that no conflict of interests exists.

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Review Article

Diabetic Inhibition of Preconditioning- and Postconditioning-Mediated Myocardial Protection against Ischemia/Reperfusion Injury

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Ischemic preconditioning (IPC) or postconditioning (Ipost) is proved to efficiently prevent ischemia/reperfusion injuries. Mortality of diabetic patients with acute myocardial infarction was found to be 2–6 folds higher than that of non-diabetic patients with same myocardial infarction, which may be in part due to diabetic inhibition of IPC- and Ipost-mediated protective mechanisms. Both IPC- and Ipost-mediated myocardial protection is predominantly mediated by stimulating PI3K/Akt and associated GSK-3β pathway while diabetes-mediated pathogenic effects are found to be mediated by inhibiting PI3K/Akt and associated GSK-3β pathway. Therefore, this review briefly introduced the general features of IPC- and Ipost-mediated myocardial protection and the general pathogenic effects of diabetes on the myocardium. We have collected experimental evidence that indicates the diabetic inhibition of IPC- and Ipost-mediated myocardial protection. Increasing evidence implies that diabetic inhibition of IPC- and Ipost-mediated myocardial protection may be mediated by inhibiting PI3K/Akt and associated GSK-3β pathway. Therefore any strategy to activate PI3K/Akt and associated GSK-3β pathway to release the diabetic inhibition of both IPC and Ipost-mediated myocardial protection may provide the protective effect against ischemia/reperfusion injuries.

1. Introduction

Acute myocardial infarction (AMI) is a worldwide problem that threatens the human's health both in the developed and developing countries. AMI is often induced by the complete thrombotic occlusion of coronary arteries at the site of a ruptured atherosclerotic plaque. Prompt reperfusion is a definitive treatment to salvage ischemic myocardium from inevitable death. Experimental and clinical investigations suggest that although reperfusion can salvage the ischemic myocardium, it can also induce side effect, called as ischemia/reperfusion injuries. It is appreciated now that lethal myocardial injury caused by ischemia/reperfusion accounts for up to 50% of the final infarct size of a myocardial infarct [1].

Myocardial ischemia/reperfusion injury is a complex pathophysiological event, resulting in serious acute and chronic myocardial damage. It is characterized by a cascade of acutely initiated local inflammatory responses, metabolic disorder, and cell death, leading to myocardial ultrastructural changes and remodeling and subsequently myocardial systolic and diastolic dysfunction [2–4]. Myocardial ischemia/reperfusion injury also induces ventricular arrhythmias, resulting in circulation collapse and sudden death [5, 6].

Numerous studies have demonstrated that inflammation following ischemia/reperfusion injury exacerbates myocardial injury [4, 7]. In addition to inflammation, profound alterations in myocardial metabolism, such as the disarrangement of glycolysis and fatty acid oxidation, also significantly

impact on the cell integrity and functional recovery of the myocardium [8]. Evidence from previous studies suggests that reactive oxygen or nitrogen species (ROS or RNS), including superoxide radicals, hydrogen peroxide, hydroxyl radicals, singlet oxygen, nitric oxide, and peroxynitrite play major contribution to myocardial ischemia/reperfusion injury [9, 10]. These ROS and/or RNSs, which are formed within the ischemic myocardial cells and in the first few moments of reperfusion, are known to be cytotoxic to surrounding cells. In addition, it is also widely accepted that apoptotic cell death is involved in the development of ischemic myocardial damage [11]. Therefore, how to protect the ischemic myocardium from reperfusion injury is the key issue for cardiologist and cardiovascular physicians. This review briefly overviews the status of ischemic preconditioning (IPC) and ischemic postconditioning (Ipost) with an emphasis of the diabetic effects on the myocardial protection of IPC and Ipost as well as possible mechanisms.

2. Ischemic Preconditioning, Postconditioning, and Their Myocardial Protective Mechanisms

2.1. Ischemic Preconditioning and Its Myocardial Protection. Murry et al. (1986) first found the potent myocardial protection by preconditioning the ischemic myocardium when they gave transient and repeat ischemia and reperfusion before the occlusion of the coronary artery in dog heart [12]. They found that multiple brief ischemic episodes actually protected the myocardium from a subsequent sustained ischemic insult. They called this protective effect as IPC (Figure 1). IPC is a well-described adaptive response by which brief exposure to ischemia/reperfusion before sustained ischemia markedly enhances the ability of the myocardium to withstand a subsequent ischemic insult [13]. The protection of IPC is displayed as the reduction of ischemia/reperfusion-induced infarct size, arrhythmia, and the improvement of contractile and diastolic function of the myocardial muscle. Consequently, many studies indicated that IPC was an endogenous protection for AMI, by inducing phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC) and JAK/STAT pathways [12, 14–17]. Among these, the activation of PI3K/protein kinase B (Akt) pathway was found to play an important role in protecting myocardial ischemia/reperfusion injury [15, 16, 18]. The PI3K/Akt pathway affects cell survival by a variety of substrates, including apoptotic proteins, endothelial nitric oxide synthase (eNOS), and PKC [19, 20]. More recent interest has focused on glycogen synthase kinase-3β (GSK-3β) as a distal kinase, phosphorylated (and hence inactivated) by other kinases, including Akt and p42/p44 MAPK/ERK [21, 22]. GSK-3β is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. GSK-3 activity has been associated with many cell processes, including the regulation of multiple transcription factors, the Wnt pathway, nuclear factor κB, endoplasmic reticulum stress, embryogenesis, apoptosis and cell survival, cell cycle progression, cell migration, and so on [23, 24].

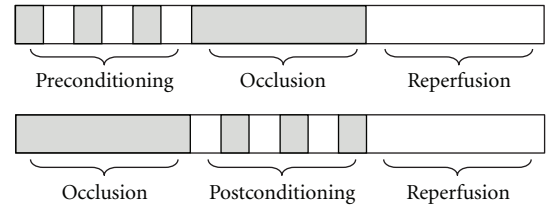

Figure 1: The illustration of IPC and Ipost. IPC means that transient and repeat ischemia and reperfusions were given before the occlusion of the coronary artery. Ipost means that transient and repeat ischemia and reperfusions were given after the occlusion and before the reperfusion of coronary artery.

IPC produces myocardial protection by phosphorylating and consequently inactivating GSK-3β [21]. However, since ischemic event is unpredictable and IPC is also invasive, myocardial protection by IPC is difficult to be used in clinics. In this review, we do not introduce the detail status of IPC myocardial protection and possible mechanisms since these issues have been extensively discussed in a few recent excellent reviews [25–28].

2.2. Ischemic Postconditioning and Its Myocardial Protection. The Ipost came into notice of Zhao et al. (2003) when they moved the transient and repeat ischemia/reperfusions to after the occlusion and before the reperfusion, as illustrated in Figure 1 [2]. Subsequently, a lot of researchers reported the same protective effects [29, 30]. They found that cycles of brief reperfusion and ischemia performed immediately at the onset of reperfusion following a prolonged ischemic insult markedly limited reperfusion injury. Like IPC, the Ipost is also a powerful approach to protect the ischemic myocardium from reperfusion-induced damage [31–33]. In clinics, with the development of percutaneous coronary intervention emerged as an exciting innovative treatment strategy, it makes Ipost possible to intervene AMI. A recent analysis of data on infarct size and ischemic zone size indicates that current reperfusion therapy salvages more than 50% of the ischemic myocardium in approximately half of the patients with AMI [34].

It has been supported by several studies that Ipost protected the myocardium against the detrimental effects of lethal myocardial reperfusion injury by limiting oxidative stress, reducing calcium accumulation, maintaining endothelial function, and reducing inflammation [35–37]. Subsequent studies have identified a number of signaling pathways which are activated by Ipost, and involve in the myocardial protection of Ipost. Among these pathways, reperfusion injury salvage kinase (RISK) pathway was the first signaling cascade to be linked to Ipost [21], which showed that Ipost was capable of recruiting prosurvival signal cascades including PI3K/Akt, PKC, GSK-3β, eNOS, and guanylyl-cyclase, as disclosed for the mechanisms of IPC myocardial protection (see the above discussion).

The discovery of IPC and Ipost, including pharmacological preconditioning and postconditioning, as the two major forms of endogenously protective mechanisms in the

myocardium have encouraged us to explore new ways to protect the myocardium from ischemia/reperfusion and have enriched our knowledge of the molecular basis of injury and survival during ischemia/reperfusion [13]. In both IPC- and Ipost-mediated myocardial protections, PI3K/Akt activation is considered as an initial step that induces phosphorylation of downstream kinases to inhibit the several pro-apoptotic factors and mitochondrial permeability transition pore (mPTP)'s opening at reperfusion, as illustrated in Figure 2 [23, 38–44]. One of the downstream targets of the RISK pathway is GSK-3β that plays important roles in necrosis and apoptosis of cardiomyocytes [23]. GSK-3β links to the regulation of a variety of cellular functions including glycogen metabolism, gene expression, and cellular survival. Experimental studies have demonstrated that the phosphorylation or inactivation of GSK-3β confers myocardial protective effects through its potential mitochondrial effects that include the inhibition of mPTP's opening and the control of mitochondrial adenine nucleotide transport through the outer mitochondrial membrane [35–37]. The mPTP is a nonselective large conductance channel in the mitochondrial inner membrane, which is physiologically closed. The mPTP remains closed during ischemia but opens at the onset of reperfusion [45], and modulation of the mPTP opening at early reperfusion can protect the myocardium from reperfusion injury [46, 47].

Opening of mPTPs is involved in cell death induced by a variety of causes, including ischemia/reperfusion, alcohol, endotoxin, and anticancer agents [48]. In addition to Ca^{2+}, ROS and/or RNS-caused accumulation of inorganic phosphate and depletion of ATP all can open mPTPs [49, 50]. It is also clear that all of these mPTP opening stimuli are induced in cardiomyocytes subjected to long-sustained ischemia/reperfusion. Ipost significantly elevated the threshold of mPTP's opening in myocardial mitochondria [23]. The inhibition of mPTP's opening plays a critical end effector for the myocardial protective effects of Ipost. Juhaszova et al. [21] first reported that GSK-3β activity is a determinant of the threshold for mPTP's opening in cardiomyocytes. Therefore, GSK-3β plays a critical role in IPC- and Ipost-mediated myocardial protection.

So far, there were two studies that have examined the role of GSK-3β as an obligatory mediator of Ipost using transgenic mice and showed different results. Gomez et al. [35] found that mice containing a mutant form of GSK-3β (which cannot be phosphorylated and inhibited) were resistant to the myocardial infarct-limiting effects of Ipost in situ, suggesting that GSK-3β inactivation is required for Ipost's myocardial protection. Contrast to the study of Gomez et al., Nishino et al. [51] have reported that mice with a mutant form of both GSK-3β and GSK-3α in which the Akt phosphorylation sites were changed, thereby rendering them to resistant to inactivation, were still amenable to the myocardial infarct-limiting effects of both IPC and Ipost. This study suggests that GSK-3β and GSK-3α inactivation are not necessary for myocardial protection in these settings. Therefore, the exact role of GSK-3β in the setting of Ipost remains further investigation, particularly under different conditions.

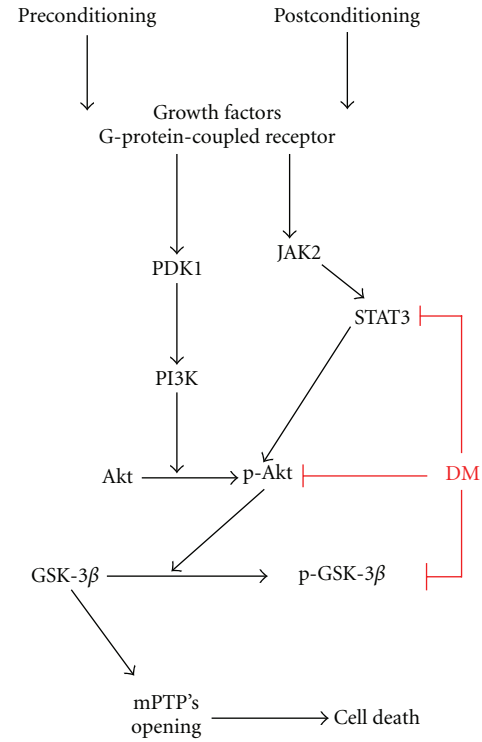

Figure 2: Major signaling pathways of IPC- and Ipost-mediated protection against cardiac cell death. Myocardial protection of IPC and Ipost were proposed to be mediated by stimulation of the prosurvival signaling pathway—PI3K/Akt pathway to inhibit the GSK-3β activation either via PI3K pathway or JAK2/STAT3 pathway. Diabetes (DM) can inhibit the activation of STAT3 or Akt to consequently activate GSK-3β that in turn induces mitochondrial cell death that is the critical cellular event for ischemia/reperfusion-induced myocardial infarction.

3. Diabetic Inhibition of Ischemic Preconditioning- and Postconditioning-Mediated Myocardial Protection against Ischemia/Reperfusion Injury

Epidemiological data show that diabetes is a major risk for cardiovascular morbidity and mortality [52, 53]. Coronary artery diseases leading to myocardial infarction and myocardium failure are one of the major chronic complications of diabetes, accounting for >75% of hospitalizations in diabetic patients. The mortality rate of diabetic patients after AMI is 2–6 folds higher than that of nondiabetic patients [54, 55]. Increased mortality or increased myocardial injury following AMI in diabetes is thought probably because of the high prevalence of other risk factors, that is, hypertension, hyperlipidemia, and advanced coronary artery diseases [56]. The poor prognosis may be also in part because of an increase in the myocardial injury in response to ischemia and reperfusion [57].

It is well known that insulin regulates metabolism in the myocardium by modulating glucose transport, glycolysis, glycogen synthesis, lipid metabolism, protein synthesis,

growth, contractility, and apoptosis in cardiomyocytes [58, 59]. Myocardial insulin resistance develops in animal models of both type 1 and type 2 diabetes [59]. These insulin-stimulated effects have been shown to be reduced in the myocardium and cardiomyocytes of diabetic rats [60], which may be the main reason for the increase in myocardial injury in response to ischemia and reperfusion in diabetic subjects.

In normal physiological status, insulin can regulate the metabolism of glucose through PI3K/Akt pathway. Insulin binds to its receptor and phosphorylates insulin receptor's substrates (IRS) such as IRS protein 1–4, Shc, Grb-2 associated binder-1, and APS adapter protein. These substrates have the SH2 structural domain and can provide the orientation sites for other signaling protein molecules, including the downstream signaling molecules of PI3K [61, 62]. Activated PI3K can phosphorylate the PI's substrates specifically to produce PIP2 and PIP3. PIP1 and PIP2 can translocate the PI3K-dependent kinase (PDK1) and Akt from the cytoplasm to plasma membrane. Under these conditions, Akt can be phosphorylated at Thr308 and Ser473, and the activated Akt then phosphorylates GSK-3β. The phosphorylation of GSK-3β inactivates its activity, which will release its inhibition of the synthesis of glycogen, as shown in Figure 2. The activity of GSK-3β is two-fold higher in diabetes than that of nondiabetes. Hyperglycemia and hyperinsulinemia can both activate the GSK-3β [43, 44, 63]. The activated GSK-3β can inhibit the myocardial transduction of insulin signaling and the utilization of glucose through the phosphorylation of IRS-1.

We have recently reported for the first time that the activation of GSK-3β played the pivotal role in diabetes-induced energy disarrangement and consequently pathological remodeling in the myocardium [63]. This study suggests that the activation of GSK-3β plays an important role in the development of diabetic cardiomyopathy.

Diabetes is an independent risk factor for ischemic myocardium disease; therefore, whether diabetes could decrease the IPC and/or Ipost protection against ischemia/reperfusion-induced myocardial damage has been questioned. Tosaki et al. found that IPC did not afford protection against ischemic damage in diabetic subjects [76]. Other studies also showed that STZ-induced diabetes significantly aggravated myocardial ischemia/reperfusion injury and blunted the protective effects of IPC [77, 78]. However, whether diabetes abrogates IPC- or Ipost-mediated myocardial protection depends on IPC times or the periods of diabetes. For instance, Tsang et al. [15] discovered that in normal Wistar rats, one, two, and three cycles of IPC significantly reduced infarct size induced by ischemia/reperfusion; however, in diabetic Goto-Kakizaki (GK) rats, only three cycles of IPC reduced infarct size induced by ischemia/reperfusion, compared with GK control hearts. Both one and two cycles of IPC failed to afford reductive effect on the infarct, suggesting that the diabetic heart has a high threshed to IPC stimulus-induced myocardial protection. In addition, Shi-Ting et al. [79] also showed that mice with diabetes for 4 weeks showed a tolerance to ischemia/reperfusion-induced damage as compared to normal rats; IPC of these diabetic mice remained affording partial myocardial protection. In contrast, mice with diabetes for 8 weeks showed a low tolerance to ischemia/reperfusion damage as compared to normal mice, and the IPC-induced myocardial protection was not evident. These findings suggest that short-term diabetes makes the myocardium more tolerant, like an adaptive response, but long-term diabetes makes the myocardium more susceptible to ischemia/reperfusion-induced damage, like a decompensated response.

Recently, Przyklenk et al. [80] have assessed the consequences of a major risk factor—diabetes on the infarct-sparing effect of stuttered reflow using type 1 and type 2 diabetic mouse models. They gave the isolated buffer-perfused myocardium for 30 min ischemia, and the myocardium received either standard reperfusion or three to six 10s cycles of stuttered reflow as Ipost. They found that Ipost-reduced infarct size via upregulation of extracellular signal-regulated kinase 1/2 (ERK1/2) in normoglycemic mice, but diabetic myocardium was refractory to Ipost-induced cardioprotection. They also found that in the type-1 diabetic model, Ipost's protective effects were reversed by the restoration of normoglycemia. Therefore, this study provided strong evidence for a profound, but potentially reversible, defect induced by diabetes in the myocardial protection of Ipost. In a study by Drenger et al. [81], however, the protective effects of Ipost were found to be inhibited in the diabetes rats, and the diabetic inhibition of Ipost's myocardial protection was not relieved by insulin-induced normoglycemia. The discrepancy between these two studies may be also due to hyperglycemic times; as the hyperglycemic time increases, the inhibited protective function of Ipost by diabetes may become irreversible.

As the myocardial protection of IPC and Ipost is mediated by a number of signaling pathways, the blunted myocardial protection mediated by IPC in diabetes may be related to the impairment in myocardial protective signaling pathways such as the PI3K/Akt pathway, as illustrated in Figure 2 [2, 15, 77]. Since signal transducer and activator of transcription (STAT) 3-mediated signaling pathway has been found to play an important role in the cardiac protection induced by IPC [14] and Ipost [69]. Downregulation of STAT3 was found to be a causative of abolishment of the cardiac protection mediated by IPC [17] and Ipost [81–83] under several conditions. Therefore, STAT3 downregulation may be one of the mechanisms for diabetic inhibition of Ipost-mediated cardiac protection, as discussed by Drenger et al. [81]. Reportedly erythropoietin (EPO) has an IPC-like effect to show myocardial protection against ischemia/reperfusion-induced damage [73, 74]. However, Ghaboura et al. have shown the attenuation of EPO-mediated myocardial protection under diabetic condition [43].

4. Diabetic Activation of GSK-3β Plays a Critical Role in Diminishing IPC- and Ipost-Mediated Myocardial Protective Function

In the above sections, we mentioned that there are several signaling pathways that may involve in the myocardial

Table 1: Potential candidates that may have protective effect against ischemia reperfusion injury related with Akt/GSK-3β pathway.

Potential candidates	Target of signaling pathway	Reference
Lithium chloride	GSK-3β inhibitor	[44]
Indirubin-3 monooxime	GSK-3β inhibitor	[44]
SB216763	GSK-3β inhibitor	[44]
Zinc	Inactivation of GSK-3β directly or indirectly	[42, 64–67]
Adenosine	Activation/translocation of PKC, PI3K, and MAPK	[68]
Endogenous opioids	JAK-STAT pathway and then inactivation of GSK-3β	[69–72]
Erythropoietin	Activate Akt and inhibit GSK-3β	[43, 73, 74]
Sevoflurane	Phosphorylates Akt and then GSK-3β	[75]

Except for the GSK-3β inhibitors, most of other potential candidates may exert their protective effect against ischemia reperfusion injury through activation of Akt and then inactivation of GSK-3β.

protection mediated by IPC or Ipost. As shown in Figure 2, however, inactivation of GSK-3β has been considered as the pivotal step for both IPC and Ipost's myocardial protection. Furthermore, studies have demonstrated that diabetes-induced activation of GSK-3β and impairment of RISK play critical roles in diabetes-induced myocardial oxidative damage and remodeling [43, 63]; other studies also reported that the activity of GSK-3β is twice in diabetic patients compared to that of nondiabetic patients [84]. Therefore, whether diabetic activation of GSK-3β blunts IPC and Ipost's myocardial protection really needs to be investigated [83, 85, 86].

To date, studies have demonstrated a decreased protective effect of IPC on AMI in diabetic subjects [43, 44, 83, 85–87]. It is clear that IPC produces myocardial protection by phosphorylation of GSK-3β that inhibited the opening of mPTP, but the activity of GSK-3β was found to be elevated during diabetes [21, 23, 35, 63]. Yadav et al. [44] investigated the role of GSK-3β in attenuating the cardioprotective effect of IPC using a type-1diabetic rat model. They found that IPC had protective effect on normal rat myocardium, but this cardioprotective effect of IPC was significantly attenuated in diabetic rat. At the same time, they found that GSK-3β inhibitors, including lithium chloride, indirubin-3 monooxime, and SB216763, significantly reduced the myocardial damage and decreased infarct size in diabetic rat myocardium. This study suggests that diabetes-induced attenuation of myocardial protection mediated by IPC involves in the activation of GSK-3β. In addition, Ghaboura et al. [43] also demonstrated that the attenuation of EPO-mediated myocardial protection from ischemia/reperfusion under diabetic condition was related to the decrease in EPO-stimulated GSK-3β phosphorylation. The administration of GSK-3β inhibitor SB216763 protected the hearts from ischemia/reperfusion-induced damage in control and diabetic groups [43]. Therefore, the inhibition of IPC myocardial protection in the diabetes is most likely related to the activation of GSK-3β [43, 44].

Because Ipost and IPC share some common signal transduction cascades proposed above (Figure 2), which include the activation of survival protein kinase pathways [13]. In the study by Drenger et al. [81], they demonstrated that diabetes can impair the protective effect of Ipost on myocardial damage or infarction through inhibition of STAT 3-mediated PI3K/Akt pathways. Up to now, there remains no proof to indicate that diabetes can inhibit the protective effect of Ipost on the myocardium; therefore, it remains to be further explored.

5. Is It Possible to Prevent the Diabetic Inhibition of IPC or Ipost Myocardial Protection against Ischemia/Reperfusion Injury?

We have demonstrated that diabetes-induced myocardial oxidative damage and inflammation mainly due to the activation of GSK-3β. When we inactivated GSK-3β activity with its inactivator in diabetic mice, diabetes-induced myocardial damage were almost completely prevented [63]. In addition, we have discussed above that inactivation of GSK-3β with its specific inactivators can also directly afford the myocardial protection in diabetic animals treated with GSK-3β inactivators [43, 44]. Therefore, any reagents that can inactivate GSK-3β may have the potential to be applied for the prevention of diabetic inhibition of IPC- and/or Ipost-mediated myocardial protection. Except for the consideration of GSK-3β inhibitors as discussed above and also listed in the Table 1, the following reagents (Table 1) may also have such potential.

Zinc (Zn) is an interesting candidate because Zn is an important trace element found in most body tissues as bivalent cations and has essential roles in human health. Zn has also an insulin-like function that was found also to be related to its inactivation of GSK-3β [88]. We have demonstrated that Zn supplementation to diabetic mice could significantly prevent the development of myocardial oxidative damage, remodeling, and dysfunction in these diabetic mice [64]. Although we did not explore whether the myocardial protection by Zn supplementation in these diabetic mice is mediated by the inactivation of GSK-3β by supplied Zn, other studies have reported that Zn also inactivated GSK-3β in several conditions. In the experiment from Chanoit et al. [42], for instance, they found that the treatment of myocardial H9c2 cells with $ZnCl_2$(10 μM) for 20 min significantly enhanced GSK-3β phosphorylation at

Ser9, indicating that exogenous Zn can inactivate GSK-3β in H9c2 cells. Other experiments [41] also demonstrated that Zn also increased mitochondrial GSK-3β phosphorylation. This may indicate an involvement of the mitochondria in the action of Zn.

Zn applied at reperfusion period reduced cell death in the cells subjected to ischemia/reperfusion, which confirmed that Zn may act as an inactivator of GSK-3β to provide a myocardial protection at reperfusion [41, 42, 89]. Besides the direct inactivation of GSK-3β, Zn was also reported to stimulate Akt phosphorylation by inhibiting Akt negative regulators, including phosphatase and tensin homologue on chromosome 10 (PTEN) and protein tyrosine phosphatase 1B (PTP1B) [65–67]. Inactivation of PTEN and/or PTP1B may also contribute to Zn's inactivation of GSK-3β via Akt activation [41]. Therefore, Zn may inhibit GSK-3β by direct and indirect mechanisms to protect the myocardium from diabetic activation of GSK-3β-mediated pathogenic effects.

In addition to Zn protective effects, other substrates are also reported to exert their protective effect, as IPC and Ipost, on ischemia/reperfusion-induced cardiac damage. For instance, adenosine leads to the activation and/or translocation of PKC, PI3K, and mitogen-activated protein kinase (MAPK) and, subsequently, affords IPC- or Ipost-like myocardial protection at the level of mitochondrial targets [68]. Endogenous opioids have also been documented to be involved in protective effects of Ipost [69, 70]. The administration of EPO at the time of reperfusion afforded a beneficial effect on Ipost myocardial protection in rabbits [90] and mice [73]. EPO administration just prior to reperfusion has reduced infarct size in isolated rat and dog hearts, and even in canine hearts. Furthermore, EPO administration even 5 min after the reperfusion has also provided protective responses [74, 91]. Lamont et al. reported that both melatonin and resveratrol, as found in red wine, protected the myocardium in an experimental model from myocardial infarction via the survivor activating factor enhancement pathway [92]. Fang et al. demonstrate that sevoflurane administered immediately during early reperfusion prevented the myocardial infarction [75].

Although all these substances can afford myocardial protective effects on ischemia/reperfusion in the models without diabetes, whether these substances can modify diabetic individuals to maintain the myocardial protection of IPC and Ipost remains to be explored in the future studies.

6. Conclusion

Epidemiological data show that diabetes is a major risk for cardiovascular diseases and the mortality of diabetic patients with acute myocardial infarction is 2–6 folds higher than that of nondiabetic patients with the same myocardial infarction. The poor prognosis may be at least in part because of diabetic inhibition of IPC- and Ipost-mediated protective mechanisms against ischemia/reperfusion injuries. Emerging evidence indicates that both IPC- and Ipost-mediated myocardial protection predominantly be mediated by stimulating PI3K/Akt and associated GSK-3β pathway while diabetes-mediated pathogenic effects are found to be mediated by inhibiting PI3K/Akt and associated GSK-3β pathway. Therefore, diabetic inhibition of IPC- and Ipost-mediated myocardial protection may be mediated by the activation of GSK-3β pathway, which suggests a possibility that we may activate PI3K/Akt indirectly to inactivate GSK-3β pathway or use GSK-3β inactivator directly to inactive GSK-3β pathway to preserve IPC- and/or Ipost-mediated myocardial protection under diabetic conditions. Although there is not enough experimental and epidemiological evidence to support our assumption, it was worthy to be explored in the future studies.

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