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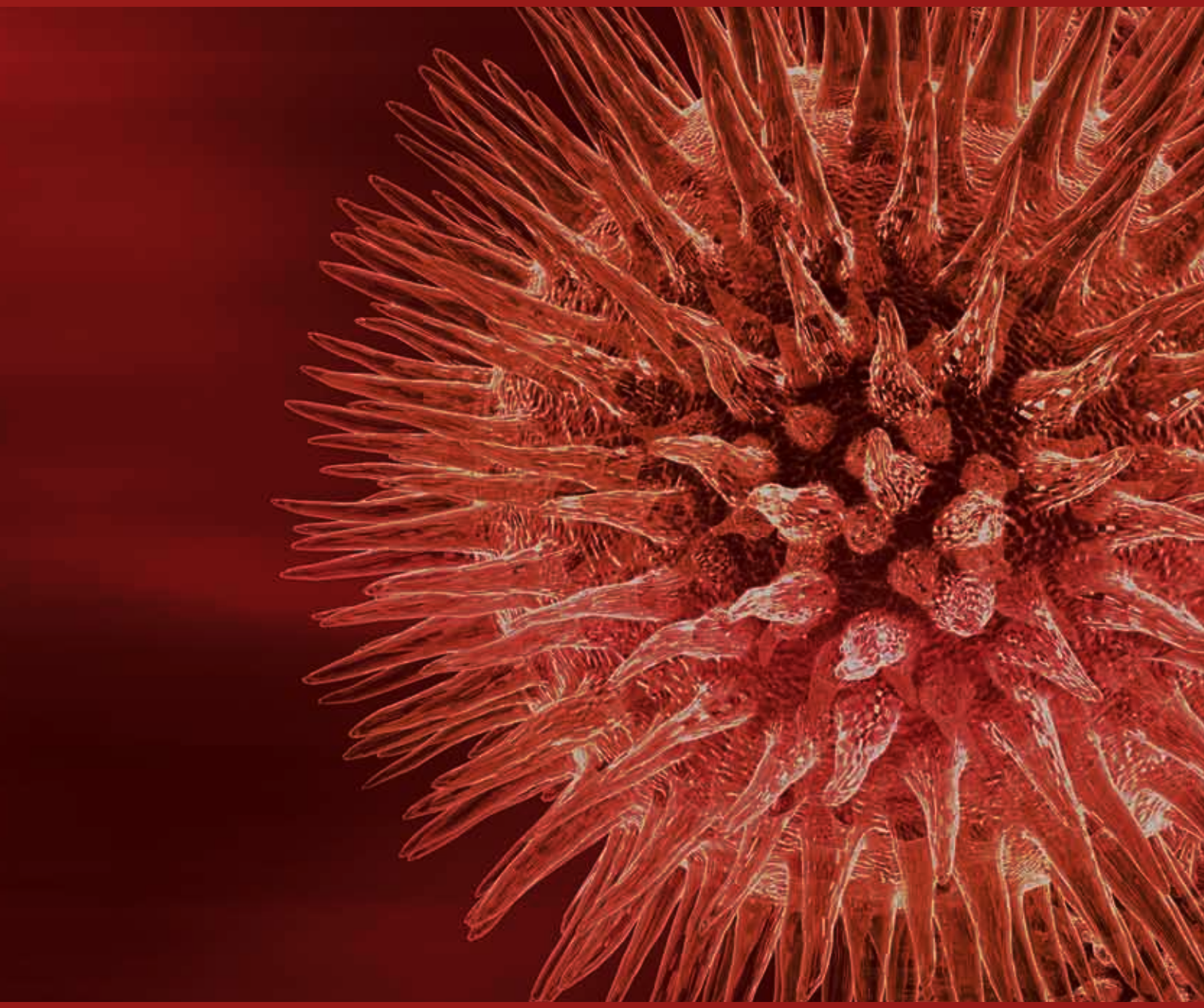
Cardiometabolic Diseases and Related Complications: Current Status and Future Perspective

Guest Editors: Joseph Fomusi Ndisang and Sharad Rastogi



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Contents

Cardiometabolic Diseases and Related Complications: Current Status and Future Perspective, Joseph Fomusi Ndisang and Sharad Rastogi
Volume 2013, Article ID 467682, 3 pages

A Novel Technique for the Assessment of Preoperative Cardiovascular Risk: Reactive Hyperemic Response to Short-Term Exercise, Robert Schier, Jochen Hinkelbein, Hanke Marcus, Ashley Smallwood, Arlene M. Correa, Reza Mehran, Randa El-Zein, and Bernhard Riedel
Volume 2013, Article ID 837130, 7 pages

A Systematic Review of Effects of Concurrent Strength and Endurance Training on the Health-Related Quality of Life and Cardiopulmonary Status in Patients with HIV/AIDS, Mansueto Gomes Neto, Cecília Ogalha, Antônio Marcos Andrade, and Carlos Brites
Volume 2013, Article ID 319524, 8 pages

Chronic Inhibition of 11β-Hydroxysteroid Dehydrogenase Type 1 Activity Decreases Hypertension, Insulin Resistance, and Hypertriglyceridemia in Metabolic Syndrome, Christine G. Schnackenberg, Melissa H. Costell, Daniel J. Krosky, Jianqi Cui, Charlene W. Wu, Victor S. Hong, Mark R. Harpel, Robert N. Willette, and Tian-Li Yue
Volume 2013, Article ID 427640, 10 pages

Transfected Early Growth Response Gene-1 DNA Enzyme Prevents Stenosis and Occlusion of Autogenous Vein Graft *In Vivo*, Chengwei Liu, Xuesong Zhang, Shi Wang, Mingxun Cheng, Chuanyu Liu, Shuqing Wang, Xinhua Hu, and Qiang Zhang
Volume 2013, Article ID 310406, 10 pages

A Case-Control Study between Gene Polymorphisms of Polyunsaturated Fatty Acid Metabolic Rate-Limiting Enzymes and Acute Coronary Syndrome in Chinese Han Population, Zikai Song, Hongyan Cao, Ling Qin, and Yanfang Jiang
Volume 2013, Article ID 928178, 7 pages

The Use of Continuous Glucose Monitoring Combined with Computer-Based eMPC Algorithm for Tight Glucose Control in Cardiosurgical ICU, Petr Kopecký, Miloš Mráz, Jan Bláha, Jaroslav Lindner, Štěpán Svačina, Roman Hovorka, and Martin Haluzík
Volume 2013, Article ID 186439, 8 pages

Human Resistin Inhibits Myogenic Differentiation and Induces Insulin Resistance in Myocytes, Chun Hua Sheng, Zhen Wu Du, Yang Song, Xiao Dong Wu, Yu Cheng Zhang, Mei Wu, Qian Wang, and Gui Zhen Zhang
Volume 2013, Article ID 804632, 8 pages

Mulberry Leaf Reduces Oxidation and C-Reactive Protein Level in Patients with Mild Dyslipidemia, Pornanong Aramwit, Ouppatham Supasyndh, Tippawan Siritienthong, and Nipaporn Bang
Volume 2013, Article ID 787981, 7 pages

Possible Role of Hyperinsulinemia and Insulin Resistance in Lower Vitamin D Levels in Overweight and Obese Patients, Giovanni De Pergola, Alessandro Nitti, Nicola Bartolomeo, Antonella Gesuita, Vito Angelo Giagulli, Vincenzo Triggiani, Edoardo Guastamacchia, and Franco Silvestris
Volume 2013, Article ID 921348, 6 pages

Serum Fetuin-A Levels Related with Microalbuminuria in Diet-Induced Obese Rats, Yanyan Li, Xiaodong Sun, and Yerong Yu
Volume 2013, Article ID 795103, 9 pages

Lipoprotein(a) in Cardiovascular Diseases, Michele Malaguarnera, Marco Vacante, Cristina Russo, Giulia Malaguarnera, Tijana Antic, Lucia Malaguarnera, Rita Bella, Giovanni Pennisi, Fabio Galvano, and Alessandro Frigiola
Volume 2013, Article ID 650989, 9 pages

Sabiporide Reduces Ischemia-Induced Arrhythmias and Myocardial Infarction and Attenuates ERK Phosphorylation and iNOS Induction in Rats, Henri Doods and Dongmei Wu
Volume 2013, Article ID 504320, 9 pages

High Insulin and Leptin Increase Resistin and Inflammatory Cytokine Production from Human Mononuclear Cells, Panayoula C. Tsiotra, Eleni Boutati, George Dimitriadis, and Sotirios A. Raptis
Volume 2013, Article ID 487081, 10 pages

A Retrospective Longitudinal Cohort Study of Antihypertensive Drug Use and New-Onset Diabetes in Taiwanese Patients, Ching-Ya Huang, Tsochiang Ma, Liyun Tien, Yow-Wen Hsieh, Shwu-Yi Lee, Hung-Yi Chen, and Gwo-Ping Jong
Volume 2013, Article ID 287696, 6 pages

Association between Polymorphisms of Alpha-Adducin Gene and Essential Hypertension in Chinese Population, Li-na Zhang, Lin-dan Ji, Li-juan Fei, Fang Yuan, Yue-miao Zhang, and Jin Xu
Volume 2013, Article ID 451094, 5 pages

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Volume 2013, Article ID 467682, 3 pages
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Editorial

Cardiometabolic Diseases and Related Complications: Current Status and Future Perspective

Joseph Fomusi Ndisang[1] and Sharad Rastogi[2]

[1] *Department of Physiology, College of Medicine, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK, Canada S7N 5E5*
[2] *Division of Cardiovascular Medicine, Department of Medicine, Henry Ford Heart & Vascular Institute, 2799 West Grand Boulevard, Detroit, MI 48202, USA*

Correspondence should be addressed to Joseph Fomusi Ndisang; joseph.ndisang@usask.ca

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In the past decade, there has been a dramatic increase in chronic diseases like diabetes, hypertension, and obesity not only in industrialized nations but also in developing nations with emerging economies [1, 2]. With the escalation of obesity, diabetes and hypertension, there has been a parallel increase in the incidence and prevalence of cardiometabolic complications [3, 4]. Cardiometabolic complications are multifactorial diseases, and a wide spectrum of different factors including changes in living environments, diets, lifestyles, genetic, and epigenetic factors [1, 2] may be involved. Although significant strides have been made in elucidating the multifaceted mechanisms associated with many cardiometabolic complications [1, 2], much still has to be done. For example studies which unveil novel mechanisms implicated in cardiometabolic diseases may open new therapeutic horizons.

This special issue focuses on original research articles and review papers that address a broad range of mechanisms associated with cardiometabolic diseases, with possible prognostic and therapeutic interventions. It is widely acknowledged that genetic defects are implicated in many cardiometabolic diseases including hypertension [5]. Genetic defects may be caused by altered exonic splicing leading to aberrant gene regulation. In an article featured in this special issue, L. Zhang and coworkers investigated the association between polymorphisms of α-adducin (ADD1) gene and essential hypertension. The authors reported that chromosome rs4963 within the ADD1 gene is associated with the development of essential hypertension in Chinese people, particularly males. Furthermore, the authors reported that the interaction among body mass index, chromosomes rs4963 and rs16843452 constitute an additional detrimental factor that increases the susceptibility to developing essential hypertension. Defective genes are also implicated in impaired lipid metabolism and lipotoxicity [6]. This concept is further elaborated in an article by Z. Song and coworkers that is also featured in this special issue.

Besides defective genes, increased inflammatory episodes are among the pathophysiological driving force in many cardiometabolic complications [7]. Proinflammatory cytokines such as resistin, tumour necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL1β are widely acknowledged as important pathophysiological factors implicated in insulin resistance [7]. In a related study, the interaction between resistin and insulin signaling is elaborated in this special issue in a research article by Z. W. Du et al. The authors showed that human resistin inhibits myogenic differentiation and causes insulin resistance in myocytes. Similarly, P. C. Tsiotra and coworkers reported that the expression of resistin, TNF-α, IL-6, and IL1β in human mononuclear cells was enhanced by hyperinsulinemia and hyperleptinemia in the chronic conditions of obesity, type 2 diabetes, and atherosclerosis. The authors suggested a pathophysiological role of Proinflammatory cytokines in dysfunctional insulin signaling, impaired endothelial function, and dyslipidemia. If these detrimental factors are corrected in a timely manner, their progressive and ultimate transformation into more complicated cardiometabolic diseases may be avoided.

For optimal physiological functions adequate levels of vitamins are necessary. For example, vitamin D is known to enhance insulin sensitivity [8]. However, the effect of vitamin D on insulin sensitivity may be compromised in obese individuals [8]. Accordingly, the article by G. De Pergola et al. showed that in obese individuals, hyperinsulinemia and/or insulin resistance may be responsible for reducing the levels of vitamin D. In another related article featured in this issue, the pathophysiological role of atherogenic lipoproteins such as lipoprotein(a) was examined. Lipoprotein(a) is known to be critical in the development of many cardiovascular pathologies [9]. A detailed analysis of lipoprotein(a) can be found in the review article written by M. Malaguarnera et al. The authors examined the role of lipoprotein(a), in arteriosclerosis, coronary artery disease, and myocardial infarction. Many cardiometabolic complications including hyperlipidemia, dyslipidemia, obesity, type 2 diabetes, hypertension, nephropathy, nonalcoholic fatty liver disease are closely interrelated [7, 10]. Y. Li et al. wrote an article that is included in this special issue that underscores this paradigm.

Besides dyslipidemia, metabolic syndrome and several cardiovascular complications are associated with excessive production of glucocorticoids like cortisol [11]. C. G. Schnackenberg and coworkers expanded on this theme with an article for this issue that elaborates the effects of blocking 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), a key enzyme necessary for the conversion of cortisone to cortisol on metabolic syndrome and cardiometabolic complications. The authors reported that blockade 11β-HSD1 in the liver and adipose tissue leads to reduction of mean arterial pressure, glucose intolerance, insulin resistance, hypertriglyceridemia, and plasma renin activity with no effect on heart rate, body weight gain, or microalbuminuria. It was suggested that 11β-HSD1 may be common mediator of hypertension, hypertriglyceridemia, glucose intolerance, and insulin resistance in metabolic syndrome. Thus, novel pharmaceutical agents capable of lowering lipoprotein(a) and blocking cortisol may retard or suppress the development of many cardiometabolic complications. Accordingly, the study of P. Aramwit and coworkers in this issue proposed a substance which could be explored in the search for novel remedies capable to suppress triglyceride, low-density lipoprotein, and C-reactive protein in patients with dyslipidemia. Similarly, the article by C. Liu and coauthors suggested the possible application of a substance known as the early growth response gene-1 DNA enzyme (EDRz) against cardiovascular complications like intimal hyperplasia and excessive proliferation of vascular smooth muscle cells. Agents that modulate EDRz may have pharmacological application.

Many physiological responses are impaired in patients with cardiometabolic disease and other chronic or debilitating conditions. For example, respiratory insufficiency is common in patients with cardiovascular complications. This notion has been discussed in detail in another article featured in this special issue by M. G. Neto et al. Similarly, response to ischemic insults may also be impaired in cardiovascular diseases. In a related study in this special issue, R. Schier et al. showed that patients with cardiovascular risk and particularly with those hypertension and diabetes mellitus had an aberrant reactive hyperemic response to ischemic insults, and, interestingly, this defect was improved by exercise. Moreover, ischemic insults are implicated in myocardial infarction [7, 12]. To expand on this topic, the article by H. Doods and D. Wu showed that sabiporide, a selective inhibitor of Na^+/H^+ exchanger, reduced ischemic insult in the myocardium and attenuated the severity of ventricular arrhythmias and myocardial infarct size. The underlying mechanism for the cardioprotective effect of sabiporide was attributed in part to the inhibition of ERK1/2 phosphorylation and suppression of inducible nitric oxide.

Diabetes is among the major causes of cardiometabolic complications. A common problem in diabetic patients is the maintenance of glycemic levels within a fairly narrow range, reflecting the recommendations of the Diabetes Control and Complications Trial [13, 14]. Although such tight glycemic control may not be feasible, the study of P. Kopecký et al., in this issue, suggests that a combination approach of two methodologies such as enhanced model predictive control algorithm and continuous glucose monitoring would be more reliable and accurate. Similarly, the usage of two or more antihypertensive drugs is a common practice for the management of hypertension [15]. In this special issue C. Y. Huang et al. discussed polytherapy in the treatment and management of hypertension.

Taken together, the manuscripts in this special issue are based on the recent developments and future perspectives of cardiometabolic diseases. Importantly, these articles also unmask many challenging issues that should be overcome to improve diagnosis, prognosis, treatment and management of cardio-metabolic diseases and related complications.

Joseph Fomusi Ndisang
Sharad Rastogi

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Research Article

A Novel Technique for the Assessment of Preoperative Cardiovascular Risk: Reactive Hyperemic Response to Short-Term Exercise

Robert Schier,[1] Jochen Hinkelbein,[1] Hanke Marcus,[1] Ashley Smallwood,[2] Arlene M. Correa,[3] Reza Mehran,[3] Randa El-Zein,[4] and Bernhard Riedel[5]

[1] *Department of Anaesthesiology and Intensive Care Medicine, University Hospital of Cologne, Kerpener Strasse 62, 50937 Cologne, Germany*
[2] *Department of Anesthesiology and Pain Medicine, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[3] *Department of Thoracic Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[4] *Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[5] *Department of Anaesthesia and Pain Medicine, Peter MacCallum Cancer Centre and The University of Melbourne, Locked Bag 1, A'Beckett Street, Melbourne, VIC 8006, Australia*

Correspondence should be addressed to Robert Schier; robert.schier@gmx.net

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Background. Perioperative vascular function has been widely studied using noninvasive techniques that measure reactive hyperemia as a surrogate marker of vascular function. However, studies are limited to a static setting with patients tested at rest. We hypothesized that exercise would increase reactive hyperemia as measured by digital thermal monitoring (DTM) in association to patients' cardiometabolic risk. *Methods*. Thirty patients (58 ± 9 years) scheduled for noncardiac surgery were studied prospectively. Preoperatively, temperature rebound (TR) following upper arm cuff occlusion was measured before and 10 minutes after exercise. Data are presented as means ± SD. Statistical analysis utilized ANOVA and Fisher's exact test, with P values <0.05 regarded as significant. *Results*. Following exercise, TR-derived parameters increased significantly (absolute: 0.53 ± 0.95 versus 0.04 ± 0.42°C, $P = 0.04$, and % change: 1.78 ± 3.29 versus 0.14 ± 1.27 %, $P = 0.03$). All patients with preoperative cardiac risk factors had a change in TR (after/before exercise, ΔTR) with values falling in the lower two tertiles of the study population (ΔTR < 1.1%). *Conclusion*. Exercise increased the reactive hyperemic response to ischemia. This dynamic response was blunted in patients with cardiac risk factors. The usability of this short-term effect for the preoperative assessment of endothelial function warrants further study.

1. Introduction

The physiological response of peripheral vasodilation during and shortly after exercise is affected by several factors. The vascular endothelium plays a central role in the regulation of vascular tone via nitric oxide, which has a key role in endothelial function, and is involved in exercise-induced vasodilation [1, 2]. Impaired endothelial function is promoted by injury from mechanical forces and processes related to cardiovascular risk factors including ageing [3], hypertension [4], dyslipidaemia [5], impaired fasting glucose [6, 7], insulin resistance [8], hyperhomocysteinemia [9], smoking [10, 11], or acute postprandial hypertriglyceridemia [12]. With an increasing incidence of these risk factors among the patient population presenting preoperatively, noninvasive assessment of endothelial-dependent vascular function in response to exercise might be a diagnostic tool gaining importance in the preoperative risk assessment.

Despite advances in perioperative care, patients undergoing major noncardiac surgery continue to experience a high incidence of postoperative morbidity (15–36%) and mortality (4.8–10.9%, e.g., following pneumonectomy), with increased health care expenditure [13, 14]. Cardiovascular risk factors predispose to perioperative morbidity and mortality, with evidence that patients with microvascular dysfunction undergoing cardiovascular interventions are at increased risk for postoperative complications [15, 16].

Given that the noncardiovascular surgical population increasingly presents with multiple cardiovascular risk factors [17], there is need to explore the role of endothelial dysfunction in this population from a clinical point of view. Recent studies investigating DTM have shown that impaired vascular reactivity correlated with the extent of myocardial perfusion defect and was found in patients with coronary artery disease, metabolic syndrome, and diabetes mellitus [18, 19]. It is increasingly recognized as a diagnostic tool for cardiovascular risk assessment [20–22].

The noninvasive assessment of reactive hyperemia *in response to exercise*, as a surrogate marker of endothelial-dependent vascular function, has not been described in the literature at this point. Therefore, we tested the hypothesis that acute exercise would increase reactive hyperemia regardless of changes in blood pressure, heart rate, and body temperature (primary endpoint). In addition, we tested the hypothesis that a lack of reactive hyperemia increase after exercise would correlate with preoperative cardiovascular risk factors (secondary endpoint).

2. Methods

2.1. Subjects. Following IRB approval (The University of Texas M.D. Anderson Cancer Center, study protocol no. 2003-0434), thirty consecutive patients scheduled for major noncardiac surgery (esophagectomy or major lung surgery, e.g., lobectomy or pneumonectomy) were prospectively enrolled into this observational trial. Exclusion criteria were any condition that deemed a patient unsatisfactory for surgery after the preanesthetic evaluation. Patients were evaluated with standard preoperative risk scores, including the American Society of Anesthesiologists (ASA) Physical Status Classification System and modified Lee Cardiac Risk Index [23, 24].

2.2. Study Endpoints. The primary endpoint of this pilot study investigated whether acute exercise would increase reactive hyperemia, a surrogate marker of vascular function, and that this effect would be blunted in the presence of preoperative cardiovascular risk factors (i.e., coronary artery disease, hypertension, diabetes, and obesity).

2.3. Measurement of Reactive Hyperemia. To ensure consistency, all measurements of reactive hyperemia were performed within one week of scheduled surgery. Measurements were performed before and 10 minutes after exercise in a quiet dimmed room at a controlled ambient temperature (20–25°C) using a VENDYS 5000BC Digital Thermal Monitoring (DTM) system (Endothelix, Inc., Houston, TX, USA). This FDA approved device consists of a computer-based thermometry system (0.006°C thermal resolution), with two special thermocouple fingertip probes designed to minimize the area of skin-probe contact and fingertip pressure. A standard sphygmomanometer cuff and a compressor unit to control cuff inflation and deflation is included to facilitate the occlusion-hyperemia protocol. The test is conducted with the patient at rest for 30 minutes in the supine position, in a quiet, dimmed room with ambient temperature of 24°C to 26°C. VENDYS DTM probes are affixed to the index finger of each hand and after a period of stabilization of basal skin temperature (defined as stabilization within a 0.05°C threshold) the temperature is measured in the index fingers of both hands (of which the right arm only is subjected to occlusion-hyperemia) with an automated, operator-independent protocol. The right upper arm cuff is rapidly inflated to ≥50 mmHg above systolic pressure for 2 minutes and then rapidly deflated to invoke reactive hyperemia distally. Thermal tracings are measured continuously and digitized automatically using a computer-based thermometry system with 0.006°C thermal resolution. Dual channel temperature data is simultaneously acquired at a 1 Hz sample rate. Figure 1 shows a representative example of a temperature-time trace and the primary DTM-derived measures, related to thermal debt and recovery that were recorded and calculated.

3. Cardiopulmonary Exercise Testing (CPET)

Prior to exercise, baseline vitals (heart rate, blood pressure, pulse oximetry, and ECG) and static pulmonary function tests (forced expiratory volume at 1 second, forced vital capacity, and maximal voluntary ventilation) were recorded for all patients. CPET was then performed as a multistage incremental "ramp workload" study using a cycle ergometer and a metabolic cart with standardized exercise software (Medgraphic Cardio-2CP system, Medical Graphics Corporation, St. Paul, MN, USA) for breath-by-breath analysis of gas exchange.

An initial acclimation period consisted of breath-by-breath gas exchange analysis performed in the supine, resting position for five minutes. After acclimation the patient pedaled at 60 rpm with minimal resistance for three minutes (unloaded work). After three minutes, loaded work (increasing pedal resistance, watts per minute) followed a standardized ramp protocol to maximal symptom limited exertion that typically lasted 9–12 minutes. Exercise was terminated by the study patient or by the study investigator if symptoms of cardiovascular, pulmonary distress, and/or fatigue were observed. To ensure consistency, exercise above the anerobic threshold was required for inclusion into the study. Anerobic threshold (AT, mL/kg/min) was defined as the VO_2 at the inflection point as determined by the modified V-slope method of plotting carbon dioxide excretion (VCO_2) against oxygen uptake (VO_2) during increasing exercise intensity, as described by Wasserman et al. [25]. Gas exchange analysis recorded oxygen consumption (VO_2, mL/kg/min) and carbon dioxide production (VCO_2, mL/kg/min) at all phases of exercise.

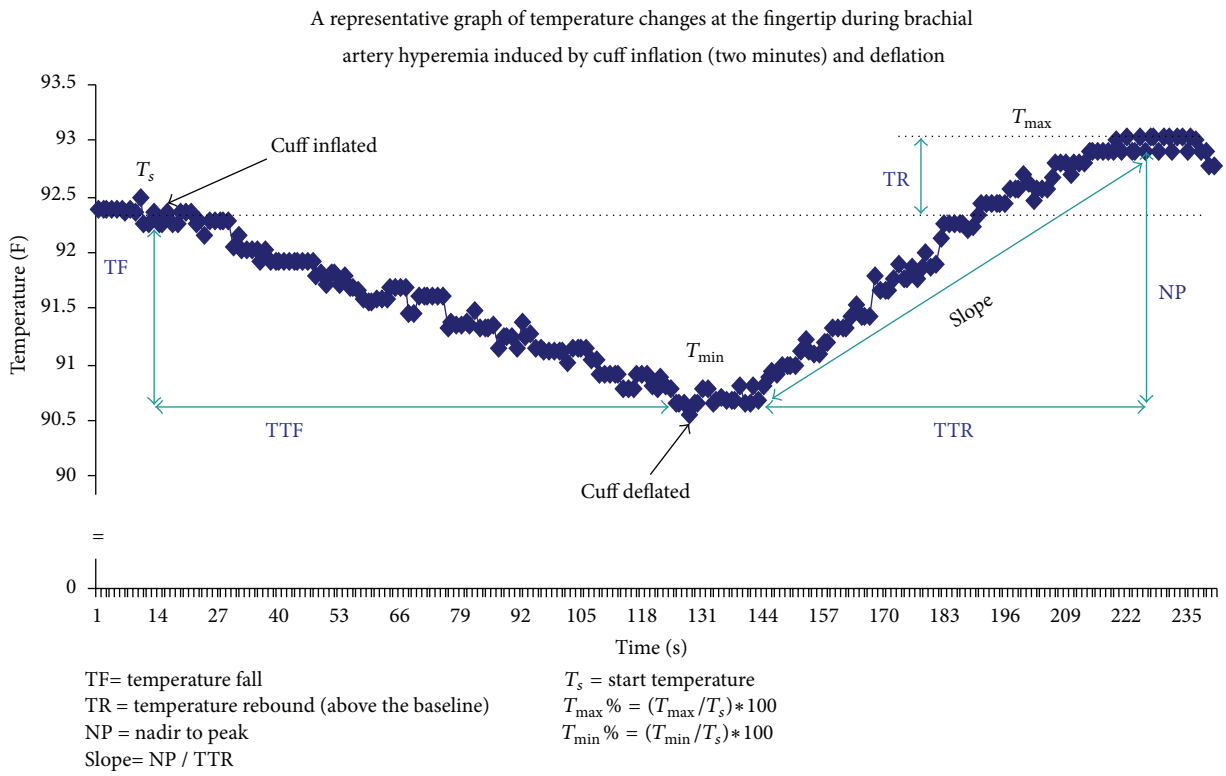

Figure 1: Representative example of a temperature-time trace in response to occlusion-hyperemia.

3.1. Statistical Analysis. The study sample size determination was based on data from a previous study by Harris et al. who enrolled nine patients to detect an increase of reactive hyperemia, as measured by flow-mediated dilation of the brachial artery, immediately after 45 min of exercise on a treadmill at 50% of their VO_2 peak. We calculated that a sample of thirty patients would need to be enrolled to achieve 80% power to detect a log-linear trend in the primary endpoint assuming that the percentage increase of reactive hyperemia after exercise, as measured by TR, was 50 percentage points. Descriptive statistics were used to summarize the patients' demographic, clinical, and TR measures. The relative changes from baseline (before exercise) and after-exercise (10 minutes after peak exercise) were analyzed using repeated measures (ANOVA) and Wilcoxon signed ranks test.

Fisher's exact test was used to analyze for an association of perioperative variables—including patients' comorbidities (i.e., obesity, abdominal obesity, coronary artery disease, and Modified Lee Cardiac Risk Index) with TR measures when tertiles were used as cutoff points. A P value of less than 0.05 was considered to indicate statistical significance. Statistical analyses were carried out using SAS 9.1 (SAS Institute, Cary, NC, USA) and S-Plus (version 8; Insightful Corp., Seattle, WA, USA).

4. Results

4.1. Clinical and Demographic Characteristics of the Study Participants. Thirty patients (18 males and 11 females) with mean age of 58 ± 10 years scheduled for major noncardiac surgery were enrolled in the study. Twenty-eight (93%) patients had an increased perioperative risk with an ASA score >2; thirteen (46%) patients had cardiovascular risk factors, for example, hypertension and dyslipidemia; and twenty-one (70%) patients were current smokers. Table 1 summarizes the demographic and clinical characteristics of the study population.

4.2. Reactive Hyperemia (TR) before and after Exercise. Table 2 summarizes the vital signs (heart rate and blood pressure) and the reactive hyperemia measures before and after exercise. The heart rate was significantly increased 10 min after exercise when compared to baseline (mean: 75 ± 10.58 versus 76±19.88 min^{-1}; $P = 0.021$). There were no differences in blood pressure before and after exercise. The starting temperature at the beginning of the reactive hyperemia measurement did not differ before and after exercise (mean: 32.84 ± 1.78 versus 32.23 ± 2.01°C; $P = 0.147$).

Reactive hyperemia was significantly increased 10 min after exercise with an absolute TR increase of 0.04 ± 0.42 versus 0.53 ± 0.95°C, $P = 0.035$ and a relative TR increase of 0.14 ± 1.27 versus 1.78 ± 3.29%, $P = 0.033$ (Figure 2). Area under the curve (AUC) of the TR slope was significantly lower after exercise with AUC 15 sec: 14.89 ± 4.70 versus 11.92 ± 5.26, $P = 0.019$; AUC 30 sec: 29.01 ± 9.04 versus 23.29 ± 10.23, $P = 0.017$; AUC 45 sec: 41.50 ± 12.86 versus 33.34 ± 14.53, $P = 0.017$; and AUC 60 sec: 52.11 ± 16.15 versus 41.85 ± 18.12, $P = 0.020$.

Table 1: Clinical characteristics.

	n	Mean (±SD)	Median (range)
Age, y	30	58 (±9.93)	59 (45–70)
Height, m	30	1.70 (±0.11)	1.70
Weight, kg	30	83 (±18.62)	81
Waist, cm	28	104 (±38.48)	98
BMI, $(kg/m)^2$	30	28 (±4.76)	28
PreOp hemoglobin, mg/dL	30	13 (±1.16)	13
PreOp fasting glucose, mg/dL	30	103 (±24.63)	100
PreOp creatinine, mg/dL	28	1.03 (±0.24)	1.00
Length of hospital stay, d	30	12 (±13.91)	7
Length of ICU stay, d	30	4 (±13.39)	0
	n (%)		
Sex, female	11 (37)		
Obesity, n (%)	10 (33)		
Abdominal obesity, n (%)	13 (46)		
Smoker, n (%)	21 (70)		
Coronary artery disease**, n (%)	1 (3)		
Hypertension, n (%)	13 (43)		
Diabetes, n (%)	4 (13)		
Dyslipidemia, n (%)	13 (43)		
Statin therapy, n (%)	5 (17)		
β-Blocker therapy, n (%)	6 (20)		
ACE-inhibitor therapy, n (%)	4 (13)		
ASA risk score > 2, n (%)	28 (93)		
Lee Cardiac Risk Index > 2, n (%)	3 (10)		
Chemotherapy, n (%)	13 (43)		
Radiation therapy, n (%)	10 (33)		

**Patient status after myocardial infarction (with or without intervention).

There was no association between clinical characteristics and low TR values (2 lower tertiles) when compared to high TR values (upper tertile) (Table 3).

5. Discussion

The principal finding of the present study is that a single episode of acute exercise above the anerobic threshold enhanced the reactive hyperemia, a surrogate marker of endothelial function. These results imply that a short period of exercise enhances cutaneous perfusion and is associated with an increase in the release and/or bioactivity of endogenous vasodilatative mediators (e.g., NO) in the endothelial cells of skin vasculature. We were able to measure this short-term effect with the use of digital thermal monitoring (DTM) of temperature rebound (TR), which provides a noninvasive assessment of vascular function.

However, the diagnostic value of our findings in terms of preoperative assessment of endothelial dysfunction warrants further research. Although we found that patients with preoperative cardiac risk factors and postoperative complications were within the lower 2 tertiles of the study population ($\Delta TR < 1.1\%$), this observation needs to be validated in a larger patient population.

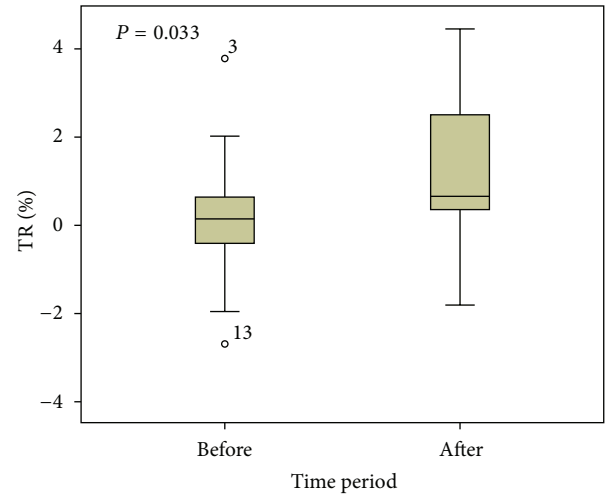

Figure 2: Increase of reactive hyperemia (temperature rebound, TR%) 10 minutes after peak exercise.

In agreement with our findings, previous work has demonstrated that acute exercise increases skin blood flow and cutaneous vascular conductance accompanied by enhanced plasma NO metabolite levels and acetylcholine-induced cutaneous perfusion [26]. These authors suggested that endothelium-dependent dilation in skin vasculature is enhanced by moderate exercise training and reversed to the pretraining state with detraining.

Furthermore, our observations suggest that this effect can be reproduced by a single episode of exercise above the anerobic threshold increasing the aerobic capacity and vascular responsiveness to acute exercise.

In contrast, a previous study investigating on the effect of 6 months of aerobic exercise in patients with type 2 diabetes mellitus was not able to show an improvement of microvascular dysfunction [27]. The authors interpreted their negative results with the hypothesis that micro- and macrocirculation respond differently to the exercise stimulus. We were able to observe a significant increase of reactive hyperemia after a short exercise stimulus. However, it remains unclear how long this effect would have lasted on and we suggest that our observed physiological response to exercise has rather diagnostic than therapeutic value.

Our study has implications for preoperative assessment of endothelial function, as the observed increased reactive hyperemic signal shortly after exercise may serve as a diagnostic tool. Impairment of endothelial function is a precursor for cardiovascular disease and precedes the morphological changes associated with atherosclerosis in the blood vessels [28] and the clinical manifestations of its associated complications (e.g., myocardial infarction, stroke) [29, 30]. Furthermore, any transient inflammatory burden or a systemic inflammatory state also adversely affects endothelium-dependent vascular function with consequent increase in risk

Table 2: Reactive hyperemia (TR) before and after exercise.

	Before exercise			After exercise (10 min after)			P values*
	N	Mean	Std deviation	N	Mean	Std deviation	
Starting temperature (°C)	30	32.84	1.78	30	32.23	2.01	0.147
Temperature rebound (TR°C)	30	0.04	0.42	30	0.53	0.95	**0.035***
Temperature rebound (TR%)	30	0.14	1.27	30	1.78	3.29	**0.033***
Area under curve after 15 sec	30	14.89	4.70	30	11.92	5.26	**0.019***
Area under curve after 30 sec	30	29.01	9.04	30	23.29	10.23	**0.017***
Area under curve after 45 sec	30	41.50	12.86	30	33.34	14.53	**0.017***
Area under curve after 60 sec	30	52.11	16.15	30	41.85	18.12	**0.020***
Heart rate (bpm)	27	75	10.58	28	76	19.88	**0.021**
Systolic blood pressure (mmHg)	27	128	16.94	28	132	16.35	0.216
Diastolic blood pressure (mmHg)	27	76	6.09	28	79	9.06	0.081
Mean blood pressure (mmHg)	27	94	11.59	28	98	2.01	0.094

*Wilcoxon signed ranks test.

Table 3: Clinical characteristics and tertiles of TR % change after exercise (pre-/postexercise difference).

	n	Lower 2 tertiles (<−0.0952 and <1.1162)	Upper tertiles (≥1.1162)	P value*
Age, y	30	57.5 ± 11.3	59.1 ± 6.7	0.685
Sex, n (%) female	11	6 (55)	5 (45)	0.425
Height, m	30	1.7 ± 0.1	1.7 ± 0.1	0.589
Weight, kg	30	84.5 ± 19.3	79.5 ± 17.8	0.501
Waist, cm	28	107.5 ± 46.2	95.9 ± 10.1	0.465
BMI, $(kg/m)^2$	30	28.8 ± 5.4	27.7 ± 3.3	0.567
Obesity, n (%)	10	8 (80)	2 (20)	0.419
Abdominal obesity, n (%)	13	8 (62)	5 (38)	0.505
Smoker, n (%)	21	12 (57)	9 (43)	0.204
Coronary artery disease**, n (%)	1	1 (100)	0 (0)	1.000
Hypertension, n (%)	13	11 (85)	2 (15)	0.119
Diabetes, n (%)	4	4 (100)	0 (0)	0.272
Dyslipidemia, n (%)	13	8 (62)	5 (38)	0.705
Statin therapy, n (%)	5	3 (60)	2 (40)	1.000
β-Blocker therapy, n (%)	6	6 (100)	0 (0)	0.074
Aspirin therapy, n (%)	5	5 (100)	0 (0)	0.140
ACE-inhibitor therapy, n (%)	4	3 (75)	1 (25)	1.000
ASA risk score > 2, n (%)	28	19 (68)	9 (32)	0.615
Lee Cardiac Risk Index > 2, n (%)	3	3 (100)	0 (0)	0.107
Chemotherapy, n (%)	13	7 (54)	6 (46)	0.255
Radiation therapy, n (%)	10	7 (70)	3 (30)	0.101
PreOp echo/EF, %	17	61.4 ± 3.8	62.4 ± 6.9	0.709
PreOp hemoglobin, mg/dL	30	13.3 ± 1.0	13.5 ± 1.4	0.705
PreOp fasting glucose, mg/dL	30	106.7 ± 28.6	95.8 ± 12.0	0.260
PreOp creatinine, mg/dL	28	1.0 ± 0.3	1.0 ± 0.2	0.509
Length of hospital stay, d	30	11.2 ± 11.7	12.6 ± 18.32	0.800
Length of ICU Stay, d	30	2.4 ± 9.0	6.3 ± 19.9	0.462

*Fisher's exact test.
**Patient status after myocardial infarction (with or without intervention).

for cardiovascular complications [31, 32]. In the perioperative context, inflammatory mediator release associated with surgical trauma has been shown to impair vascular function and correlate with both the duration and extent of major surgery [31–35]. This effect may be additive to the underlying endothelial dysfunction that is inherent in certain surgical patients as a result of their preoperative comorbidity burden and thus plays a significant role in certain perioperative complications (e.g., perioperative myocardial infarction, poor wound healing, ALI, and sepsis) [33, 35].

6. Conclusions

Based on our results, we suggest that the preoperative assessment of endothelial function using reactive hyperemia in response to exercise gains clinical importance as a potential risk assessment tool in the prevention of perioperative complications and should be further studied in a larger patient population.

Disclosure

The meeting at which the work has been presented is American Society of Anesthesiologists (ASA) Meeting, October 21, 2008, Orlando, FL, USA.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

A Systematic Review of Effects of Concurrent Strength and Endurance Training on the Health-Related Quality of Life and Cardiopulmonary Status in Patients with HIV/AIDS

Mansueto Gomes Neto,[1,2] Cecília Ogalha,[2] Antônio Marcos Andrade,[2] and Carlos Brites[2]

[1] *Departamento de Biofunção, Curso de Fisioterapia, Universidade Federal da Bahia (UFBA), 40110-160 Salvador, BA, Brazil*
[2] *Programa de Pós-Graduação em Medicina e Saúde da Universidade Federal da Bahia (UFBA), 40110-160 Salvador, BA, Brazil*

Correspondence should be addressed to Mansueto Gomes Neto; netofisio@gmail.com

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Purpose. To determine the effects of concurrent strength and endurance training (concurrent training) on the Health-Related Quality of Life (HRQOL) and cardiopulmonary status among HIV-infected patients, using a systematic search strategy of randomized, controlled trials (RCTs). *Methods*. A systematic review was performed by two independent reviewers using Cochrane Collaboration protocol. The sources used in this review were Cochrane Library, EMBASE, LILACS, MEDLINE, PEDro and Web of Science from 1950 to August 2012. The PEDro score was used to evaluate methodological quality. *Result*. Individual studies suggested that concurrent training contributed to improved HRQOL and cardiovascular status. Concurrent training appears to be safe and may be beneficial for medically stable adults living with HIV. The rates of nonadherence were of 16%. *Conclusion*. Concurrent training improves the HRQOL and cardiopulmonary status. It may be an important intervention in the care and treatment of adults living with HIV. Further research is needed to determine the minimal and optimal duration, frequency, and intensity of exercise needed to produce beneficial changes in the HIV-infected population subgroups.

1. Introduction

The introduction of highly active antiretroviral therapy (HAART) has dramatically reduced mortality and morbidity in HIV-infected patients. On the other hand HIV-infected patients are experiencing an increasing frequency of noninfectious problems, which can significantly impair the benefits of HAART [1, 2].

Exercise training improves and maintains health and reduces the risk of chronic disease in healthy adults [3]. Exercise has been considered an important adjuvant therapy for health promotion of patients with HIV [4, 5]. The proper exercise prescription must take into consideration the choice of exercise's type, in accordance with the objective to be achieved. This includes other important parameters such as intensity, volume, frequency, and duration of exercise [6].

Resistance training has been employed as a therapeutic tool in patients with HIV and is considered safe and effective in improving muscle strength and body composition [7, 8]. Aerobic exercise promotes a significant effect in improving aerobic capacity, measured by maximal oxygen consumption in this population [9, 10].

Recently, the combination of two exercise modalities: concurrent strength and endurance training (concurrent training) has been employed, as recommended by the American College of Sports Medicine [11]. Participation in concurrent training has been recommended for healthy people and adults with chronic medical conditions [12].

The physiological stimuli directed to skeletal muscle as a result of strength training or endurance training are divergent in nature, due to competition in metabolic adaptation to exercise. As a consequence, its effects may be limited when compared to training, in terms of specific parameters [13, 14], but in populations with multiple functional impairments the combination of different modes of exercise is part of rehabilitation programs [15, 16].

Some studies have shown a significant improvement in components of muscle performance and endurance during concurrent training in patients with HIV/AIDS [17–19]. The impact of training on functional capacity and mainly on the HRQOL has not been well documented. In addition, there is no consensus among studies regarding the association of the exercise types, or on what is the best intensity of exercise to be prescribed for this population, with little emphasis on HRQOL. This is an open question and a barrier to a large scale use of such strategies in clinical practice.

The goal of this systematic review was to analyze the impact of concurrent strength and endurance training termed concurrent training on HRQOL and cardiopulmonary status of patients living with HIV/AIDS and discuss their implications for clinical practice.

2. Methods

2.1. Data Sources and Searches. We performed a computer-based search querying Ovid MEDLINE (1950 to August 2012), LILACS (up to August 2012), CINAHL (Cumulative Index to Nursing and Allied Health, 1982 to August 2012), EMBASE (1980 to August 2012), PEDro (Physiotherapy Evidence Database), and the Cochrane Central Register of Controlled Trials for original research articles published in English, Spanish, and Portuguese. We also performed a manual tracking of citations in the selected articles.

The design group included the terms randomized controlled trials, clinical trials, and controlled trials. The HIV group included the terms human immunodeficiency virus, acquired immunodeficiency syndrome, HIV, HIV infections, HIV long-term survivors, AIDS, and HIV/AIDS. The exercise group included the terms exercise, training, physical exercise, fitness, strength training, progressive resistive/resistance aerobic, aerobic training, concurrent strength and endurance training, concurrent training, anaerobic, exercise therapy, or physical training.

The outcome measures group included the terms quality of life, health-related quality of life, life expectancy, and cardiopulmonary status.

2.2. Study Selection

2.2.1. Types of Studies and Participants. We included randomized controlled trials (RCTs) comparing concurrent training with nonconcurrent training or with another exercise modality, performed at least two times per week and lasting at least four weeks. Studies of adults (18 years and older), regardless sexes, at all stages of infection were included.

2.2.2. Types of Interventions. The concurrent training was defined as the application of aerobic and resistance exercise in the same training session, performed at least two times per week for at least four weeks. Resistance training was defined as exercise that requires muscle contraction against resistance. Aerobic exercise was defined as a regimen containing aerobic interventions (walking, treadmill, cycling, rowing and stair stepping). Exercise programs were described with respect to type of exercise, volume, intensity, frequency, and duration.

2.2.3. Types of Outcome Measures. Cardiopulmonary measures considered in this review included but were not limited to maximal/peak oxygen consumption (V02 max/peak) (mL/kg/min), oxygen pulse (02pulse), maximum heart rate (HR_{max}) (beats/min), fatigue (time on exercise), and dyspnea (rate of perceived exertion).

To assess the quality of life related to health we included in the review studies that reported HRQL through standardized and validated scales or questionnaires.

2.2.4. Data Extraction and Quality Assessment. One reviewer made the search and the initial selection of potentially relevant studies that met the inclusion criteria and two independent reviewers selected the articles that fulfill the inclusion criteria, using a standard form adapted from the Cochrane Collaboration [20] model for data extraction, considering (1) aspects of the study population, such as average age and gender, (2) aspects of the intervention performed, (sample size, type of exercise performed presence of supervision, frequency, and duration of each session), (3) followup, (4) loss of followup, (5) outcome measures and (6) results presented.

There are several scales for assessing quality of RCTs. The PEDro scale assesses the methodological quality of a study based on other important criteria, such as concealed allocation, intention-to-treat analysis, and adequacy of followup. These characteristics make the PEDro scale a useful tool to assess the methodological quality of physical therapy and rehabilitation trials [21].

The PEDro scale [22] is based on a Delphi list [23] and consists of 11 items. The first item is related to external validity and is generally not used to calculate the method score, leaving a score range of 0 through 10 [22]. Most trials had already been rated at least twice by trained evaluators of PEDro database (http://www.pedro.fhs.usyd.edu.au/). If a trial was not included in PEDro or had not been previously rated twice, it was rated independently by two investigators. Studies were excluded in subsequent analysis if the cutoff of 4 points was not reached.

3. Results

We identified a total of 98 articles with the search strategy applied to the databases MEDLINE, Scielo, AMED, Lilacs, and PEDro. These 37 items were sent to reviewers for evaluation, selection, and inclusion in the review. Twenty-six were excluded, and 11 papers met entry criterion according to reviewers. Three additional studies were excluded after retrieving the full text. Of these, 2 were RCTs that did not examine outcomes of interest to this review and one study was a duplicate of Mutimura et al. [24].

The remaining eight articles were fully analyzed and approved by both reviewers and had the extraction of data from each RCT (Mutimura et al., 2008 [24]; Hand et al., 2008 [25]; Pérez-Moreno [26]; Dolan et al., 2006 [27] Fillipas et al.,

2006 [28]; Driscoll et al., 2004 [29]; Rojas et al., 2003 [30]. Rigsby et al., 1992 [31]).

Each of the papers was assessed using the PEDro scale methodology by both reviewers, with the pre-defined cutoff [4].

3.1. Characteristics of the Sample. The initial sample size for the selected studies ranged from 35 [30] to 100 [24]. The final sample ranged from 31 [31] to 97 [24], and mean age of participants ranged from 18 to 60 years. The studies included patients of both genders, but there was a predominance of males. All studies analyzed in this review included outpatients diagnosed with HIV, and the majority of these were under antiretroviral therapy.

Participants included adults infected with HIV at various stages of the disease with CD4 counts ranging from <100 to >500 cells/mm^3. Also included were patients with elements of wasting syndrome (either >5% or >10% involuntary weight loss or body weight <90% ideal body weight).

3.2. Outcomes of Included Studies

3.2.1. Cardiopulmonary Status. Stress test was used with a treadmill, stationary bike, and cycle ergometer. Submaximal tests were also used, as the Shuttle test, Kasch Pulse Recovery Test, and six-minute walk test.

3.2.2. Health-Related Quality of Life. WHOQOL-BREF and MOS-HIV health surveys were the tools used to evaluate HRQOL. Table 1 presents summary data from the 8 RCTs eligible for this systematic review.

3.3. Characteristics of Intervention Programs. The exercise intervention characteristics of included studies are provided in Table 2. The parameters used in the application of aerobic and resistance exercise have been reported in most studies, and all described the progressive nature of the training.

The duration of intervention programs with concurrent training ranged from 6 [25] to 24 weeks [24], but in most studies reviewed, the application period ranged from 12 to 16 weeks. Regarding the length of the session, there was a variation from 60 [27, 28] to 120 [29] minutes. The frequency of sessions varied from two to three times a week.

For resistance training only two studies [27, 29] specify the type of muscle contraction performed during training: the exercise was performed with concentric and eccentric contractions lasting 6 to 10 seconds, with use of machines, weight stations, and free weights in six studies, but in two, there was no description of the type of equipment used [24]. The exercise intensity was based on the extent of maximum repetition (MR), ranging from 50 to 80% of MR in most studies. One study did not report the prescribed exercise intensity [24]. The application volume of exercise ranged from 1 to 3 sets of 6–18 repetitions. The volume of exercise was not described in one study [24].

For the application of aerobic exercise, all studies reported the treadmill, bike, walking, or joging. Except for the study of Rigsby et al. [31], all reported the criteria for progression training. In all studies the intensity was adjusted based on heart rate (HR_{max}), ranging from 45 to 80%HR_{max}.

3.4. Effects of Intervention Programs

3.4.1. Cardiopulmonary Status. Seven studies reported significant improvement in the concurrent training group compared to control group. One study did not compare the improvement intergroups, because they used a before and after evaluation [30].

In the study of Mutimura et al. [24], Shuttle's test was used to evaluate the functional capacity to predict maximum oxygen uptake ($VO_{2\,max}$). It was improved from 4.7 ± 3.9 to 0.5 ± 0.3 mL/kg per min in the intervention group compared to control ($P < 0.001$). In the study of Fillipas et al. [28], the Kasch Pulse Recovery test (which evaluates the beats per minute after 3 minutes of stepping) was used to assess the endurance, with a lower HR meaning better conditioning. HR was reduced from 19.6 ± 0.6 to 11.7 ± 2.9 in the exercise group compared to control ($P < 0.001$). In the study of Hand et al. [25], there was an improvement of 21% in VO_2 estimated in the exercise group while there was no improvement in the control group ($P < 0.001$).

Dolan et al. [27] observed an improvement (1.5 ± 0.8 versus −2.5 ± 1.6 mL/kg min^{-1}, $P < 0.001$) in $VO_{2\,max}$ in the training group compared to control. In a study by Driscoll et al., fitness assessment was performed using the time to perform the exercise on a cycle ergometer, with a significant increase in the exercise group compared to control (3 ± 0 0 ± 4 min versus 1.1 min, $P < 0.001$). Rigsby et al. [31] also used maximum time exercise as a parameter for fitness assessment, and he observed a maximum execution time of 1388.46 ± 224.45 versus 965.91 ± 136.14 s in the exercise group and control group, respectively ($P < 0.001$).

In the study by Rojas et al. [30], a significant improvement in $VO_{2\,max}$ after training was observed, compared to baseline. Table 3 provides details of the effects of intervention programs.

3.4.2. Health-Related Quality of Life. Four researches included HRQOL outcome between the endpoints. All reported significant improvement in HRQOL of the concurrent training group compared to control group.

Mutimura et al. [24] assessed HRQOL using a short-form instrument (WHOQOL-BREF) of the WHO Quality of Life HIV (WHOQOL-HIV). The psychological (1.3 ± 0.3 versus 0.5 ± 0.1; $P < 0.0001$), independence (0.6 ± 0.1 versus 0.0 ± 0.0; $P < 0.0001$), social relationships (0.6 ± 0.2 versus 0.0 ± 0.0; $P < 0.0001$), HIV HAART-specific (1.4 ± 0.2 versus −0.1 ± 0.2; $P < 0.0001$), and QoL domains (0.5 ± 0.3 versus 0.0 ± 0.3; $P < 0.05$) significantly improved in the concurrent training compared to control group.

In the Pérez-Moreno et al. study [26], although statistical significance was not reached for the combined effect of group and time ($P = 0.09$), QOL significantly increased ($P < 0.01$) in the training group after the intervention period, whereas no change was observed in controls.

Table 1: Characteristics of the outcomes and results of concurrent training in the trials included in the review.

Study	Patients	Outcomes	Measures		Results	
			Aerobic capacity	HRQOL	Aerobic capacity	HRQOL
Mutimura et al., 2008 [24]	HIV	Aerobic capacity HRQoL	Shuttle test	WHOQOL-BREF	$\uparrow VO_{2peak}$	↑QOL
Hand et al., 2008 [25]	HIV	Aerobic capacity	Graded exercise stress test	NA	$\uparrow VO_{2peak}$	NA
Pérez-Moreno et al., 2007 [26]	HIV	Aerobic capacity HRQoL	Stress test cycle ergometer	QOL	$\uparrow VO_{2peak}$	NS
Dolan et al., 2006 [27]	HIV	Aerobic capacity	Treadmill stress test TCAM6	NA	$\uparrow VO_{2peak}$ ↑TCAM6	NA
Fillipas et al., 2006 [28]	HIV	Aerobic capacity HRQoL	Kasch pulse recovery test	MOS-HIV	↓HR	↑MOS-HIV
Driscoll et al., 2004 [29]	HIV	Aerobic capacity	Submaximal stress test	NA	↑ET	NA
Rojas et al., 2003 [30]	HIV/AIDS	Aerobic capacity HRQoL	Graded exercise stress test	MOS-HIV	$\uparrow VO_{2max}$ $\uparrow O_{2pulse}$	↑MOS-HIV
Rigsby et al., 1992 [31]	HIV	Aerobic capacity	YMCA cicle test protocol	NA	↑ET ↓HR	NA

In the study of Fillipas et al. [28], and Rojas et al. [30], HRQOL was assessed using the Medical Outcomes Study HIV Health Survey (MOS-HIV). In the first study [28] HRQOL showed a between-group difference in only two out of the eleven dimensions. The experimental group improved their overall health while the control group showed slight reduction in this parameter, resulting in a between-group difference of 20.8 points (95% CI 2.0 to 39.7, $P = 0.03$). The experimental group improved their cognitive function while the control group stayed much the same; the between-group difference was 14 points (95% CI 0.7 to 27.3, $P = 0.04$).

In second study [30] six domains were assessed (health status, global quality of life, energy, physical strength, social contact, and emotional well-being); concurrent training group showed better results than controls in five domains. The only unchanged domain was social contact ($P > 0.05$).

3.4.3. Adherence to Exercise Program. Adherence to exercise is the ability to maintain a program for a certain time. In all studies a varied proportion of patients are excluded before the end of program. In this review, from 471 patients that entered the protocol, only 396 (84%) remained on study at closure.

Mutimura et al. [24] showed the lower rate of discontinuation, with only 4% of withdraw. Conversely, the study of Hand el al. [25] presented the greatest loss of patients in the exercise group, starting with 44 and ending with 21 patients, with loss of 53.3%. The proportion of loss to exercise and control groups was 19.1% versus 11.44%, respectively.

4. Discussion

This systematic review demonstrated that there is sufficient evidence to support the inclusion of concurrent training for adults living with HIV/AIDS.

It is evident that the effectiveness of concurrent training improves aerobic capacity in this population. Despite major differences in exercise prescription and duration of different programs, the aerobic capacity was significantly improved. These findings coincide with results of previous studies that found significant improvements in cardiopulmonary fitness [32, 33].

The effect of concurrent training on QOL is less clear. Only one study showed significant improvement in all domains, while two studies showed impact in specific domains, and in one study the statistical significance was not reached for the combined effect of group and time. This discrepancy can be due to intensity, frequency, and duration of the programs in the analyzed studies, which can result in a different impact on such parameters.

Physical therapists can play an important role in diagnosis and management of the physical dysfunction in HIV-infected patients [34, 35]. This systematic review suggests that concurrent exercise may be an important intervention in the care and treatment of adults living with HIV. Performing concurrent exercise for at least 60 minutes, three times per week for at least six weeks, may contribute to improvements in selected outcomes of cardiopulmonary status. These physiological adaptations to concurrent strength and endurance training may decrease functional limitations and reduce physical disability resulting from HIV infection and increase of HRQOL [35].

Intervention strategies should focus on increasing exercise, considering cessation of smoking, dietary counseling, and treatment of arterial blood hypertension and dyslipidemia [36]. Research supports the use of therapeutic exercise as an adjunct therapy in the treatment of symptoms of HIV infection [37].

The number of weekly exercise sessions should be increased until the patient can tolerate three to five sessions weekly. Aerobic exercise should be performed at a moderate intensity: from 11 to 14 on the Borg Rating of Perceived Exertion Scale, at 50% to 85% of peak heart rate, or at 45%

Table 2: Characteristics of the experimental intervention in the trials included in the review.

Study	Type exercise	Intensity/duration (wk)	Volume	Frequency (x per wk)	Time (min)	Length (wk)	Supervision
Mutimura et al., 2008 [24]	Aerobic exercise	45%HR_{max}/3 60% HR_{max}/6 75% HR_{max}/15	15 min warm-up 60 min exercise 15 min cool-down	3	90	24	Yes
	Resistance exercise	NI	NI	3	90	24	Yes
Hand et al., 2008 [25]	Aerobic exercise	50–70% HR_{max}	5 min warm-up 30 min exercise 5 min cool-down	2	40	6	NR
	Resistance exercise	12 RM	1 set—12 reps	2	20	6	NR
Pérez-Moreno et al., 2007 [26]	Aerobic exercise Cycle ergometer	70–80% HR_{max}	10 min warm-up 20 min exercise 10 min cool-down	3	20–40	16	Yes
	Resistance exercise	12–15 RM	1-2 set 12–15 reps	3	50	16	Yes
Fillipas et al., 2006 [28]	Aerobic exercise	60% HR_{max}/3 75% HR_{max}/3	5 min warm-up 20 min exercise 5 min cool-down	2	30	6	Yes
	Resistive exercise	60% MR 80% MR	3 sets 10 reps	2	30	6	Yes
Dolan et al., 2006 [27]	Aerobic exercise	60% HR_{max}/2 75% HR_{max}/14	5 min warm-up 20–30 min exercise	3	35	16	Yes
	Resistive exercise	60–70% MR/2 80% MR/12	3-4 sets 8–10 reps	3	85	16	Yes
Driscoll et al., 2004 [29]	Aerobic exercise Stationary bicycle	60% HR_{max}/2 75% HR_{max}/14	5 min warm-up 20–30 min exercise	3	35	12	Yes
	Resistance exercise	60–70% MR/4 80% MR/12	5 min cool-down 3-4 sets 8–10 reps	3	25	12	Yes
Rojas et al., 2003 [30]	Aerobic exercise	60–80% HR_{max}	10 min warm-up 25 min exercise 10 min cool-down	3	50	12	NR
	Resistive exercise	60–70% MR/4 80% MR/12	2-3 sets 8 reps	3	NI	12	NR
Rigsby et al., 1992 [31]	Aerobic exercise	60–80% HR_{max}	2 min warm-up 30 min exercise 3 min cool-down	3	36	12	NR
	Resistive exercise	NI	1–3 sets 6–18 reps	3	24	12	NR

NR: not reported; maximum heart rate (HR_{max}); MR: maximal repetition; reps: repetitions.

to 85% $VO_{2\,max}$. Resistance training should focus on large muscle groups, such as the chest, biceps brachia, quadriceps, and hamstrings. The intensity should be moderate (set at 60% to 80% of the one MR) and progressively increased. Overload should be selected with the level this patient can comfortably perform, 8 to 12 repetitions [17].

The role of a well-planned exercise program should therefore be emphasized and used as medical treatment among patients and health care professionals. When implementing therapeutic exercise programs for HIV-infected patients, it is recommended that programs be individualized on the basis of the functional capacity and individual symptoms presented by each patient [37, 38].

A patient participating in an exercise intervention should be monitored by a physical therapist qualified health-care provider for potential changes in their health status, especially those in more advanced stages of immunosuppression, to prevent any potential adverse events of exercise [39].

Adherence to exercise is an underresearched area with regards to HIV treatment. Very few studies have been reported on the adherence of HIV patients to exercise in the clinical setting. The strongest motivators of adherence to exercise have been shown to be self-efficacy (the concept that a person is capable of performing a course of action to attain a desired outcome) and outcome expectation (the belief that specific consequences will result from specific personal actions) [40, 41].

Further research into reasons for nonadherence as well as for dropouts would be beneficial. In order to gain the most from the exercise, combined exercise programs including

Table 3: Effects of concurrent training on the cardiopulmonary status.

			Maximal exercise capacity	
	ΔBefore − After	*P* value	Mean difference (CI) for between-group comparison	*P* value
Mutimura et al., 2008 [24]				
Control	0.5 (0.3)	NR		
CT	4.7 (3.9)	NR	4.2 (NE)	$P < .0001$
Hand et al., 2008 [25]				
Control	0 (3.0)	NS		
CT	8.3 (3.1)	$P < .01$	NE	NE
Pérez-Moreno et al., 2007 [26]				
Control	0 (0.0)	NS	10.0 (NE)	
CT	10 (1.0)	$P < .01$		$P < .001$
Dolan et al., 2006 [27]				
Control	−2.5 (1.8)	NR		
CT	1.5 (0.8)	NR	1.0 (NE)	$P < .001$
Fillipas et al., 2006 [28]				
Control	0.6 (2.9)	NR		
CT	−19.6 (11.7)	NR	−20.2 (−25.8 to −14.6)	$P < .001$
CT	3 (0/4)	NR	3.0 (NE)	$P < .05$
Driscoll et al., 2004 [29]				
Control	0 (0/1)	NR		
CT	3 (0/4)	NR	3.0 (NE)	$P < .05$
Rojas et al., 2003 [30]				
Control	NE	NE	(NE)	(NE)
CT	2.99 (0.38)	$P < .003$		
Rigsby et al., 1992 [31]				
Control	18.18 (NR)	NR		$P < .0001$
CT	392.31 (NR)	NR	374.13 (NE)	

CT: concurrent training, NE: not estimated, NR: not reported. Data are reported as mean (SD) or as mean (95% confidence interval (CI)).

targeted psychological support might be the way forward [42].

Future research needs to identify which patient subgroups might benefit the most, the optimal exercise dose needed to lessen disease-related symptoms and maximize clinical benefit, and the effects with different types of programs.

Meta-analyses were not performed due to variability of characteristics of the studies pertaining to the exercise, variation among individual studies in the types of interventions the differences in endpoints, assessment instruments, and variables of exercise prescription.

5. Conclusion

Concurrent training is efficacious in treating disability in outpatient men who are HIV positive and showed to be a safe and beneficial intervention in the treatment. Exercise improves aerobic cardiopulmonary status and HRQOL. It may be an important intervention in the care and treatment of adults with HIV.

Conflict of Interests

The authors have no conflict of interests to disclose.

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Research Article

Chronic Inhibition of 11β-Hydroxysteroid Dehydrogenase Type 1 Activity Decreases Hypertension, Insulin Resistance, and Hypertriglyceridemia in Metabolic Syndrome

Christine G. Schnackenberg, Melissa H. Costell, Daniel J. Krosky, Jianqi Cui, Charlene W. Wu, Victor S. Hong, Mark R. Harpel, Robert N. Willette, and Tian-Li Yue

Heart Failure Discovery Performance Unit, Metabolic Pathways and Cardiovascular Therapeutic Area, GlaxoSmithKline, UW2521, P.O. Box 1539, 709 Swedeland Road, King of Prussia, PA 19406-0939, USA

Correspondence should be addressed to Christine G. Schnackenberg; christine.g.schnackenberg@gsk.com

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Metabolic syndrome is a constellation of risk factors including hypertension, dyslipidemia, insulin resistance, and obesity that promote the development of cardiovascular disease. Metabolic syndrome has been associated with changes in the secretion or metabolism of glucocorticoids, which have important functions in adipose, liver, kidney, and vasculature. Tissue concentrations of the active glucocorticoid cortisol are controlled by the conversion of cortisone to cortisol by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). Because of the various cardiovascular and metabolic activities of glucocorticoids, we tested the hypothesis that 11β-HSD1 is a common mechanism in the hypertension, dyslipidemia, and insulin resistance in metabolic syndrome. In obese and lean SHR/NDmcr-cp (SHR-cp), cardiovascular, metabolic, and renal functions were measured before and during four weeks of administration of vehicle or compound 11 (10 mg/kg/d), a selective inhibitor of 11β-HSD1. Compound 11 significantly decreased 11β-HSD1 activity in adipose tissue and liver of SHR-cp. In obese SHR-cp, compound 11 significantly decreased mean arterial pressure, glucose intolerance, insulin resistance, hypertriglyceridemia, and plasma renin activity with no effect on heart rate, body weight gain, or microalbuminuria. These results suggest that 11β-HSD1 activity in liver and adipose tissue is a common mediator of hypertension, hypertriglyceridemia, glucose intolerance, and insulin resistance in metabolic syndrome.

1. Introduction

Metabolic syndrome is a constellation of interrelated risk factors that promote the development of cardiovascular disease. The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) defined the characteristics of the metabolic syndrome as elevated blood pressure, insulin resistance (with or without glucose intolerance), abdominal obesity, atherogenic dyslipidemia (elevated triglycerides, small LDL particles, and low HDL cholesterol), and prothrombotic and proinflammatory states [1]. Rather than any single factor, the Adult Treatment Panel III specified that combination of three out of five of these factors must be present to establish a diagnosis of metabolic syndrome. These factors include elevated blood pressure, elevated fasting glucose, elevated triglycerides, reduced HDL cholesterol, and abdominal obesity [1, 2]. Using these criteria in population-based studies, investigators reported that the prevalence of metabolic syndrome is increasing and contributes to higher rates of cardiovascular events [3, 4]. However, it remains unclear whether these interrelated risk factors share a common regulatory mechanism.

Glucocorticoids such as cortisol are important mediators in the regulation of cardiovascular and metabolic functions. Through activation of glucocorticoid or mineralocorticoid receptors, glucocorticoids impact vascular, adipose, liver, and kidney functions [5, 6]. Some of the glucocorticoid activities include gluconeogenesis, liposynthesis, insulin resistance,

accumulation of visceral fat, vascular reactivity, vascular remodeling, and sodium reabsorption [5–9]. Prospective, cross-sectional studies on humans have shown that plasma cortisol or 24-hour renal cortisol excretion is correlated with some of the risk factors of metabolic syndrome [10–12]. The pathophysiological importance of glucocorticoid activity in metabolic disorders is exemplified in patients with Cushing's syndrome who have abnormally high plasma cortisol/cortisone ratio, which results from either administration of glucocorticoids or increased adrenal secretion of cortisol, and develop hypertension, obesity, and insulin resistance [13, 14]. Importantly, the ability to therapeutically reverse hypertension and other features of Cushing's syndrome with the antiglucocorticoid agent RU486 [6] suggested that limiting the actions of cortisol may be an important mechanism for controlling the development and maintenance of hypertension and other cardiovascular risk factors in metabolic syndrome. The regulation of cortisol activity is controlled by the local action of the microsomal enzyme 11β-hydroxysteroid dehydrogenase within tissues. There are two isozymes of 11β-hydroxysteroid dehydrogenase: type 1 (11β-HSD1) converts inactive cortisone to active cortisol and type 2 (11β-HSD2) converts active cortisol to inactive cortisone. 11β-HSD1 is most abundantly expressed in liver and adipose tissue [15, 16]. In contrast, 11β-HSD2 is mainly expressed in mineralocorticoid target tissues such as the kidney, colon, salivary, and sweat glands [17] where the enzyme prevents activation of the mineralocorticoid receptor by cortisol.

Inhibition of 11β-HSD2 activity causes hypertension and hypokalemia [18, 19]. However, the contribution of 11β-HSD1 to blood pressure regulation, especially in the context of its role in metabolic syndrome, is less clear. Genetic expression levels of 11β-HSD1 have been associated with blood pressure regulation in preclinical studies [20–22]. For example, mice with genetic overexpression of 11β-HSD1 have high blood pressure but mice with genetic knockout of 11β-HSD1 are normotensive. Clinical studies of an 11β-HSD1 inhibitor have shown mixed blood pressure results [23, 24].

To directly test the hypothesis that 11β-HSD1 is a common mechanism in the hypertension, dyslipidemia, and insulin resistance found in metabolic syndrome, we compared the cardiovascular, renal, and metabolic effects of a pharmacological inhibitor of 11β-HSD1 within the context of a preclinical setting of metabolic syndrome. The leptin receptor deficient spontaneously hypertensive rat (SHR-cp) is a well-established model of metabolic syndrome with hypertension, dyslipidemia, insulin resistance, and obesity [25–27]. Our findings of improved global function by the 11β-HSD1 inhibitor compound 11 [28] in this model support a role of 11β-HSD1 as a coordinated regulator of these diverse processes of metabolic syndrome.

2. Methods

2.1. Animals. Animal procedures were approved by the Institutional Animal Care and Use Committee of GlaxoSmithKline and were in accordance with NIH Guidelines for the Care and Use of Animals. Adult male littermates of obese (cp/cp) and lean (+/+) SHR/NDmcr-cp (SHR-cp, Vassar College) rats aged 4-5 months were used in all studies. Rats were anesthetized, surgically implanted with radiotelemetry catheters (DSI) in the abdominal aorta, and allowed to recover for at least one week before baseline measurements were taken. After cardiovascular, renal, and metabolic functions were determined at baseline, obese and lean SHR-cp were divided into two groups each. Groups were administered vehicle (1% DMSO, 6% Cavitron; $n = 12$ obese, $n = 9$ lean) or compound 11 at 10 mg/kg/d ($n = 13$ obese, $n = 10$ lean) via gavage for 4 weeks. The doses of compound 11 were chosen based on previously published studies [28]. Liver, visceral adipose tissue, and kidney were harvested at the end of the study, rapidly frozen in liquid nitrogen, and stored at −80°C.

Mean arterial pressure and heart rate were measured directly in conscious rats using radiotelemetry before and during vehicle or compound 11 administration. Blood pressure and heart rate were collected every 5 minutes for 22 hours daily and averaged. Urine was collected over 24 hours from rats individually housed in metabolic cages and stored at −80°C until analysis. Plasma was collected at the end of the urine collection for determination of plasma electrolyte, hormone, and creatinine concentrations. Plasma lipids, insulin, and blood glucose concentrations were determined in overnight-fasted rats. Plasma insulin was measured using ELISA (LINCOplex). Whole blood glucose was measured immediately upon sampling using a glucometer (Accu-Chek Advantage). Plasma aldosterone concentration was measured by ^{125}I-radioimmunoassay (Siemens). Plasma renin activity was measured by ^{125}I-radioimmunoassay (Diasorin). Electrolytes, creatinine, microalbumin, cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and nonesterified fatty acids (NEFA) were measured using an Olympus AU640 Clinical Analyzer.

2.2. Oral Glucose Tolerance Test. Oral glucose tolerance testing was conducted before and 4 weeks after vehicle or compound 11 administration in lean and obese SHR-cp. Rats were fasted overnight before challenge with an oral glucose load as previously described [29]. Briefly, blood samples were collected from conscious rats at baseline and 15, 60, and 120 minutes after oral administration of 2 g D-glucose/kg body weight.

2.3. Preparation of Tissue Microsomes. Microsomes were prepared from harvested liver, visceral adipose tissue, and kidney according to the method reported previously [30]. Briefly, the harvested tissues were homogenized using a polytron in a buffer containing 50 mM Tris HCl, 150 mM KCl, and 2 mM EDTA (pH 7.4). The volume of homogenizing buffer was determined by the weight of thawed tissue (1 gram of tissue: 4 mL of buffer). The homogenate was centrifuged at 13,000 g for 20 minutes, and the supernatant was further centrifuged at 109,000 g for 60 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 250 mM sucrose (0.25 mL/g tissue) and stored at −80°C prior to use.

2.4. Measurements of 11β-Hydroxysteroid Dehydrogenase Type 1 and Type 2 Activities. The measurements of 11β-HSD1 activity in liver and adipose tissue and 11β-HSD2 activity in kidney were performed using a scintillation proximity assay (SPA) as reported previously [31, 32]. Briefly, for the 11β-HSD1 assay, 40 μL of [^{3}H]-cortisone diluted in 80 nM in assay buffer (50 mM HEPES, 100 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, pH 7.4) with 1 mM NADPH was dispensed to a 96-well plate. To start the reaction, 10 μL of tissue microsome preparation (adipose 200 μg/mL; liver 10 μg/mL) was added to each well. As a control to determine assay background, assay buffer was added instead of microsomes. The plate was shaken briefly and incubated at 37°C for 2 hours. Meanwhile, a stop solution containing 5 mg/mL protein A-coated YS SPA beads resuspended in Superblock (Pierce), 10 μM 18β-glycyrrhetinic acid, and 1 μg/mL monoclonal cortisol antibody (East Coast Biologics) was prepared and incubated for 2 hours at room temperature to form the SPA bead complex. For each well, 70 μL of the stop solution containing SPA beads was added to terminate the enzyme reaction. The plate was then incubated for another 2 hours. The signal emitted by the SPA/product complex was measured on a TopCount (Packard).

For the 11β-HSD2 assay, 40 μL of [^{3}H]-cortisol diluted in 80 nM in assay buffer with 2 mM NAD was dispensed to a 96-well plate. 10 μL of kidney microsomes (30 μg/mL) was added to the plate. The plate was shaken briefly and incubated at 37°C for 1.5 hour. The stop solution was prepared as described above, and 70 μL of the stop solution containing SPA beads was dispensed to all wells to terminate the enzyme reaction. The plate was then incubated for 1 hour at room temperature while slightly shaking to allow the capture of the remaining substrate, [^{3}H]-cortisol, by the SPA bead complex. The signal emitted by the SPA/product complex was measured on a TopCount (Packard).

2.5. Statistical Analysis. Data are reported as mean ± SEM. Analysis of variance followed by Bonferroni multiple comparison test or Student's t-test was used to evaluate statistical significance. $P < 0.05$ was considered to be statistically significant.

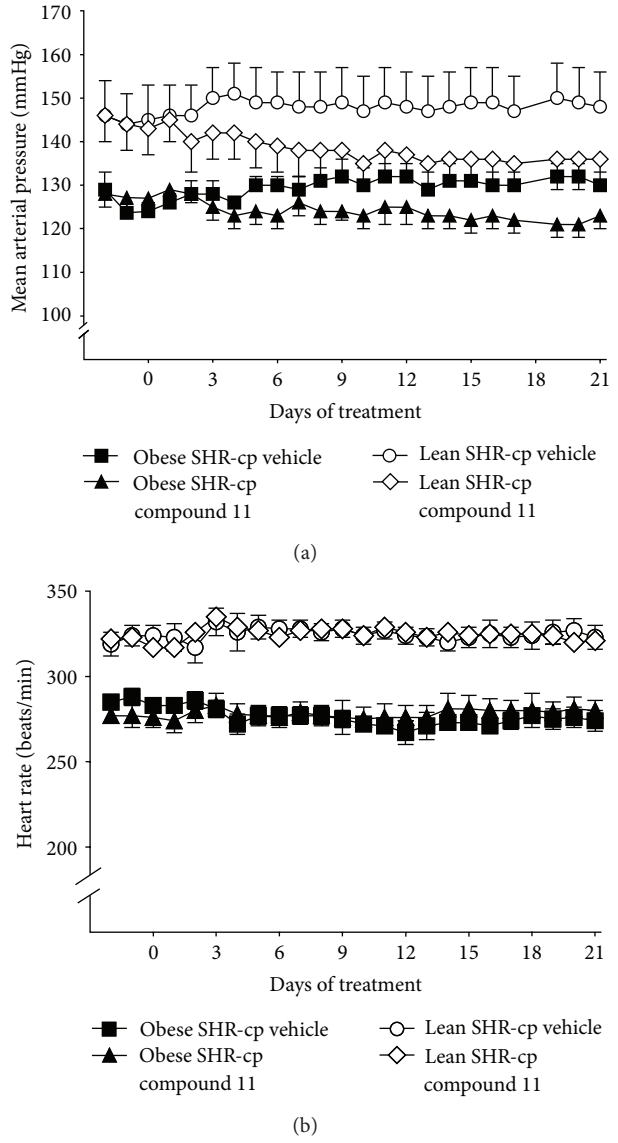

Figure 1: Absolute mean arterial pressure and heart rate in conscious, chronically instrumented obese and lean SHR-cp before and during chronic administration of vehicle or compound 11 (10 mg/kg/d).

3. Results

3.1. Cardiovascular and Renal Function. The blood pressure and heart rate responses to chronic administration of compound 11 or vehicle in obese and lean SHR-cp are illustrated in Figures 1 and 2. At baseline, obese (126 ± 2 mmHg) and lean (144 ± 4 mmHg, $P < 0.05$ versus obese) SHR-cp have significantly higher mean arterial pressure (MAP) than age-matched WKY (105 ± 2 mmHg, data not shown). As shown in Figure 2, compound 11 administration decreased MAP similarly in obese and lean SHR-cp. Three weeks of compound 11 administration significantly decreased MAP in obese SHR-cp by an average of 5.7 ± 0.8 mmHg and in lean SHR-cp by an average of 7.3 ± 1.0 mmHg. In contrast, vehicle administration had no significant effect on MAP in obese (delta 4.5 ± 0.4 mmHg) and lean (delta 2.2 ± 0.2 mmHg) SHR-cp. The circadian rhythm of MAP was not altered by compound 11. Throughout the study, heart rate was significantly lower in obese compared to lean SHR-cp; compound 11 had no effect in either group.

The plasma lipid profiles of lean and obese SHR-cp before and after three weeks of vehicle or compound 11 administration are shown in Table 1. During baseline conditions, obese SHR-cp had significantly higher plasma cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides compared to lean SHR-cp. Chronic administration of compound 11 had a significantly greater effect than vehicle treatment on the plasma lipid profile in lean and obese SHR-cp. In lean SHR-cp, compound 11 significantly decreased triglycerides by 30% and increased HDL by 25%,

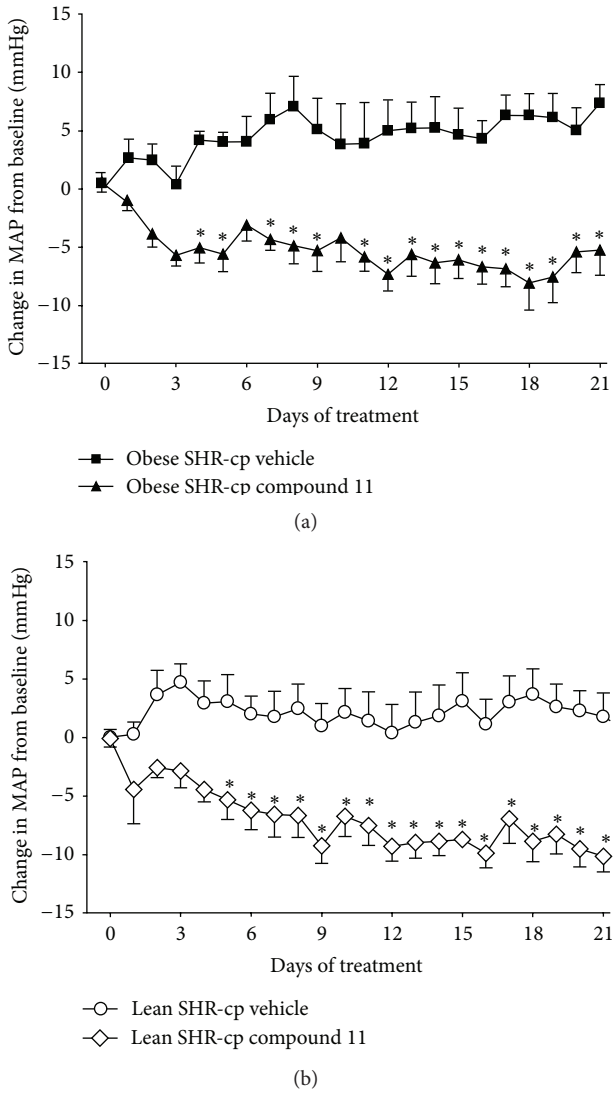

Figure 2: The change in mean arterial pressure (MAP) from baseline in obese (a) and lean (b) SHR-cp during three weeks of vehicle or compound 11 (10 mg/kg/d) administration. $^{*}P < 0.05$ compared to vehicle.

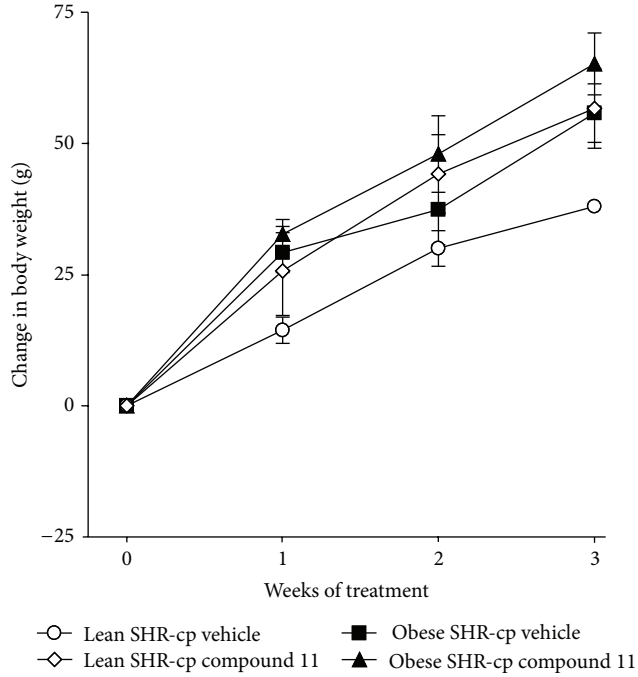

Figure 3: The change in body weight from baseline in obese and lean SHR-cp during three weeks of vehicle or compound 11 administration (10 mg/kg/d).

LDL by 138%, and total cholesterol by 49% from baseline. In obese SHR-cp, compound 11 significantly decreased plasma triglycerides by 59% and increased LDL by 274% and total cholesterol by 94% from baseline, with no change in HDL. Compound 11 significantly decreased triglycerides and increased LDL and subsequently total cholesterol more in obese than in lean SHR-cp.

Table 2 summarizes the renal excretory responses to chronic administration of compound 11 or vehicle in obese and lean SHR-cp. During baseline conditions urine flow, electrolyte excretion, glucose excretion, and microalbumin excretion were significantly higher in obese compared to lean SHR-cp. However, there was no difference in creatinine clearance between groups, suggesting that glomerular filtration rates were similar in obese and lean SHR-cp. In lean SHR-cp, three weeks of compound 11 treatment had no significant effect on renal excretory function. In contrast, compound 11 significantly decreased urine flow by 44%, potassium excretion by 18%, and glucose excretion by 94% in obese SHR-cp. The effects of compound 11 on urine flow and glucose excretion were significantly greater than vehicle in obese SHR-cp. Chronic administration of compound 11 had no effect on sodium excretion, creatinine clearance, or microalbuminuria in obese SR-cp.

Figure 3 depicts the body weight response to compound 11 or vehicle administration in obese and lean SHR-cp. At baseline, obese SHR-cp had significantly higher body weight than lean SHR-cp (525 ± 12 g versus 383 ± 7 g). Three weeks of compound 11 administration at 10 mg/kg/d had no significant effect on body weight gain in obese or lean SHR-cp.

The renin-angiotensin-aldosterone system was assessed by measuring circulating renin and aldosterone concentrations before and after chronic vehicle or compound 11 administration. At baseline, the plasma aldosterone concentration was significantly higher in obese (273 ± 59 pg/mL) compared to lean (130 ± 26 pg/mL) SHR-cp. Compared to vehicle treatment, compound 11 had no significant effect on the plasma aldosterone concentrations in either lean (vehicle: 149 ± 28 pg/mL; compound 11: 186 ± 28 pg/mL) or obese (vehicle: 145 ± 26 pg/mL; compound 11: 184 ± 31 pg/mL) SHR-cp. However, plasma renin activity was significantly reduced in obese SHR-cp treated with compound 11 (vehicle: 10.7 ± 1.6 pg/mL; compound 11: 3.6 ± 0.4 pg/mL).

3.2. Glucose Tolerance and Insulin Resistance. At baseline, obese SHR-cp had impaired glucose tolerance and insulin resistance compared to lean SHR-cp. The fasting blood

Table 1: Plasma lipid profile in lean and obese SHR-corpulent rats before and after three weeks of treatment with vehicle or compound 11.

	Period	Lean SHR-cp vehicle	Lean SHR-cp compound 11	Obese SHR-cp vehicle	Obese SHR-cp compound 11
Total cholesterol (mg/dL)	Baseline	64 ± 2	62 ± 1	122 ± 7$^{\#}$	121 ± 8$^{\#}$
	Week 3	74 ± 3	93 ± 3*	154 ± 6$^{\#}$	233 ± 14$^{\#*}$
LDL cholesterol (mg/dL)	Baseline	8 ± 1	9 ± 1	14 ± 1$^{\#}$	15 ± 2$^{\#}$
	Week 3	16 ± 1	20 ± 1*	34 ± 2$^{\#}$	55 ± 5$^{\#*}$
HDL cholesterol (mg/dL)	Baseline	21 ± 0	21 ± 1	35 ± 2$^{\#}$	34 ± 1$^{\#}$
	Week 3	23 ± 1	25 ± 1*	42 ± 2$^{\#}$	45 ± 2$^{\#}$
Triglycerides (mg/dL)	Baseline	60 ± 4	60 ± 3	516 ± 79$^{\#}$	662 ± 139$^{\#}$
	Week 3	62 ± 8	42 ± 3*	654 ± 91$^{\#}$	253 ± 31$^{\#*}$
NEFA (mmol/L)	Baseline	1.6 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
	Week 3	1.0 ± 0.2	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1

Data are expressed as mean ± SEM. LDL: low-density lipoprotein; HDL: high-density lipoprotein; NEFA: nonesterified fatty acid. $^{*}P < 0.05$ versus vehicle within genotype; $^{\#}P < 0.05$ versus lean SHR-cp.

Table 2: Renal excretory function in lean and obese SHR-corpulent rats before and after three weeks of treatment with vehicle or compound 11.

	Period	Lean SHR-cp vehicle	Lean SHR-cp compound 11	Obese SHR-cp vehicle	Obese SHR-cp compound 11
Urine flow (mL/day)	Baseline	18 ± 1	21 ± 2	57 ± 4$^{\#}$	56 ± 6$^{\#}$
	Week 3	24 ± 2	26 ± 3	46 ± 4$^{\#}$	30 ± 4*
Sodium excretion (mmol/day)	Baseline	2.2 ± 0.1	2.3 ± 0.2	3.5 ± 0.2$^{\#}$	3.1 ± 0.2$^{\#}$
	Week 3	2.4 ± 0.1	2.0 ± 0.2	3.2 ± 0.3$^{\#}$	2.7 ± 0.3$^{\#}$
Potassium excretion (mmol/day)	Baseline	5.0 ± 0.3	5.1 ± 0.3	8.4 ± 0.3$^{\#}$	8.3 ± 0.3$^{\#}$
	Week 3	5.6 ± 0.2	4.8 ± 0.5	8.0 ± 0.6$^{\#}$	6.7 ± 0.4$^{\#*}$
Chloride excretion (mmol/day)	Baseline	3.7 ± 0.2	3.7 ± 0.3	5.8 ± 0.2$^{\#}$	5.3 ± 0.3$^{\#}$
	Week 3	3.7 ± 0.1	3.1 ± 0.3	5.3 ± 0.5$^{\#}$	4.6 ± 0.2$^{\#}$
Glucose excretion (mg/day)	Baseline	7 ± 2	9 ± 1	3568 ± 391$^{\#}$	3638 ± 538$^{\#}$
	Week 3	6 ± 2	4 ± 1	1148 ± 220$^{\#}$	220 ± 156$^{\#*}$
Creatinine clearance (L/day)	Baseline	6.00 ± 0.56	5.86 ± 0.20	5.47 ± 0.35	5.14 ± 0.20
	Week 3	6.28 ± 0.60	5.08 ± 0.77	5.95 ± 1.36	5.00 ± 0.23
Microalbumin excretion (mg/day)	Baseline	2 ± 0	1 ± 0	49 ± 15$^{\#}$	50 ± 22$^{\#}$
	Week 3	5 ± 1	10 ± 8	85 ± 28$^{\#}$	77 ± 28$^{\#}$

Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus obese SHR-cp vehicle; $^{\#}P < 0.05$ versus lean SHR-cp.

glucose concentrations at baseline were similar among obese (vehicle: 93 ± 8 mg/dL; compound 11: 81 ± 8 mg/dL) and lean (vehicle: 79 ± 5 mg/dL; compound 11: 75 ± 4 mg/dL) SHR-cp. However, the fasting blood glucose response to glucose challenge was significantly impaired in obese compared to lean SHR-cp (see Figure 4(a)). The maximum blood glucose concentration that was achieved and the area under the oral glucose tolerance test curve (obese vehicle: 20151 ± 1722 mg/dL/min; obese compound 11: 19676 ± 1482 mg/dL/min; lean vehicle: 4399 ± 856 mg/dL/min; lean compound 11: 3893±67 mg/dL/min) were significantly higher in obese than in lean SHR-cp. At baseline, plasma insulin concentrations (obese vehicle: 6746 ± 508 pg/mL; obese compound 11: 7257 ± 620 pg/mL; lean vehicle: 1419 ± 260 pg/mL; lean compound 11: 1112 ± 88 pg/mL) and the homeostasis model assessment-insulin resistance index (HOMA; obese vehicle: 36 ± 3; obese compound 11: 43 ± 5; lean vehicle: 6 ± 1; lean compound 11: 5 ± 0) were significantly elevated in obese compared to lean SHR-cp (Figures 4(c) and 4(d)).

Four weeks of compound 11 administration significantly improved glucose tolerance and reduced insulin resistance in obese SHR-cp. The maximum blood glucose concentration in response to glucose challenge (Figure 4(b)), the area under the oral glucose tolerance test curve, and HOMA (Figure 4(d)) were significantly lower in obese SHR-cp chronically treated with compound 11 compared to vehicle. In contrast, compound 11 had no effect on glucose tolerance or HOMA in lean SHR-cp.

3.3. 11β-Hydroxysteroid Dehydrogenase Type 1 and Type 2 Activities. Figure 5 illustrates 11β-HSD1 cortisone reductase activities in adipose tissue and liver tissues of lean and obese SHR-cp after chronic treatment with vehicle or compound 11.

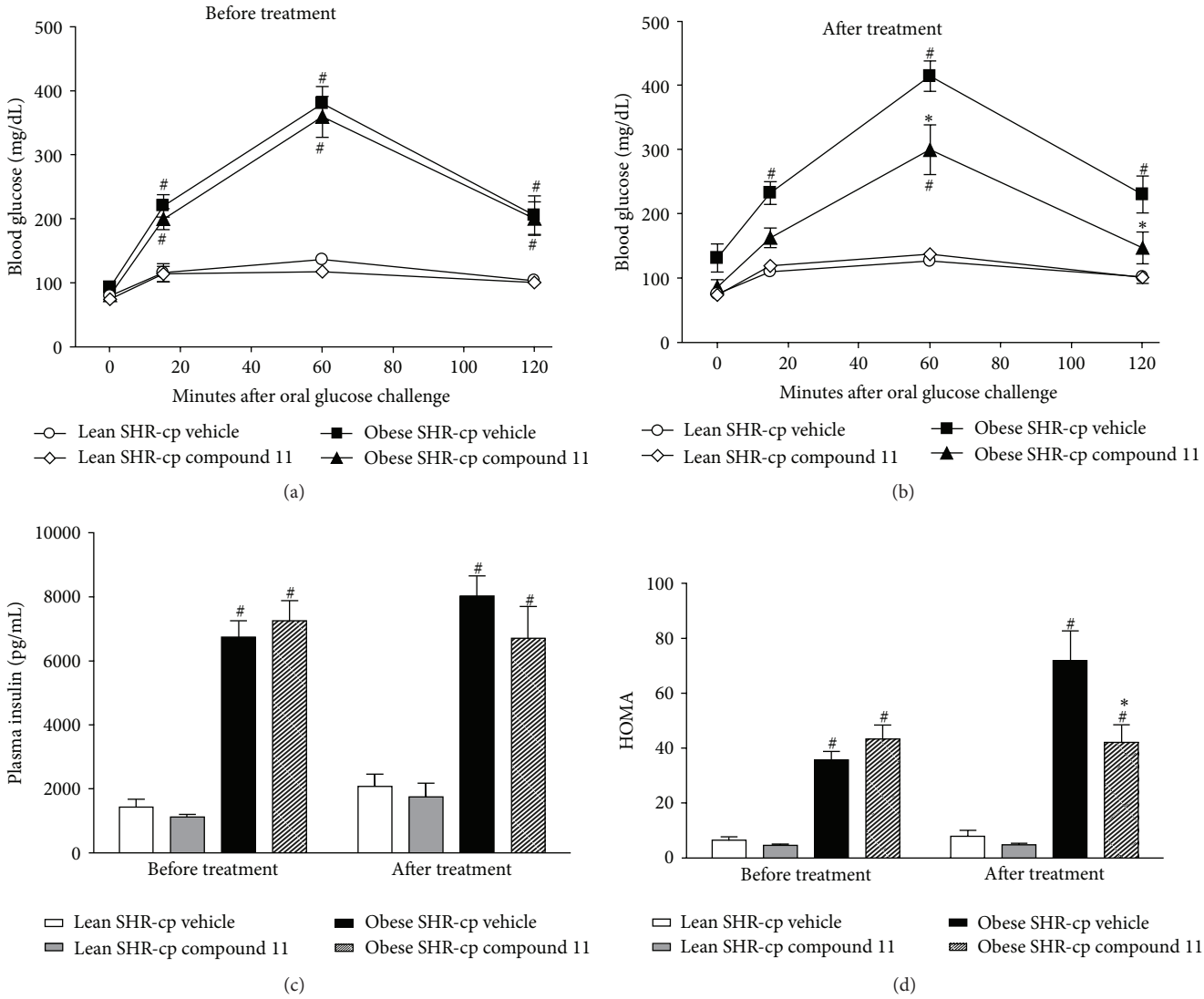

Figure 4: Indices of glucose tolerance and insulin resistance in obese and lean SHR-cp before and after 4 weeks of vehicle or compound 11 administration (10 mg/kg/d). (a) represents the blood glucose concentrations in all rats during oral glucose tolerance testing before vehicle or compound 11 administration. (b) represents the blood glucose concentrations in all rats during oral glucose tolerance testing after chronic vehicle or compound 11 administration. Oral glucose load was administered after fasting blood glucose was measured at $t = 0$ minutes. (c) is the plasma insulin concentration in all groups of rats before and after chronic vehicle or compound 11 treatment. (d) is the homeostasis model assessment index (HOMA) in all groups of rats before and after chronic vehicle or compound 11 treatment. $^{*}P < 0.05$ compared to obese SHR-cp vehicle. $^{\#}P < 0.05$ compared to lean SHR-cp.

After four weeks of treatment with vehicle, 11β-HSD1 activity in obese SHR-cp was significantly higher by 31% in liver and lower by 76% in adipose tissue compared to lean SHR-cp. However, obese SHR-cp had a much greater abundance of visceral fat than lean SHR-cp (authors' observations at tissue harvest), so total adipose 11β-HSD1 activity may have been elevated in obese compared to lean SHR-cp. In lean SHR-cp with compound 11 treatment, 11β-HSD1 activity was significantly lower by 96% in liver and by 92% in adipose tissue compared to vehicle. Similarly, in obese SHR-cp with compound 11 treatment, 11β-HSD1 activity was significantly lower by 90% in liver and by 97% in adipose tissue compared to vehicle. The cortisol dehydrogenase activity of 11β-HSD2 in kidney was similar between lean (4587 ± 98 cpm) and obese (4228 ± 113 cpm) SHR-cp with vehicle treatment, and compound 11 had no effect (lean: 4629 ± 459 cpm; obese: 4762 ± 148 cpm).

4. Discussion

The major objective of this study was to determine the integrated cardiovascular, renal, and metabolic response to 11β-HSD1 inhibition in metabolic syndrome. Previous studies using either genetic models or pharmacological blockade uncovered a role for 11β-HSD1 in one or more of the risk factors in metabolic syndrome. However, no earlier study had

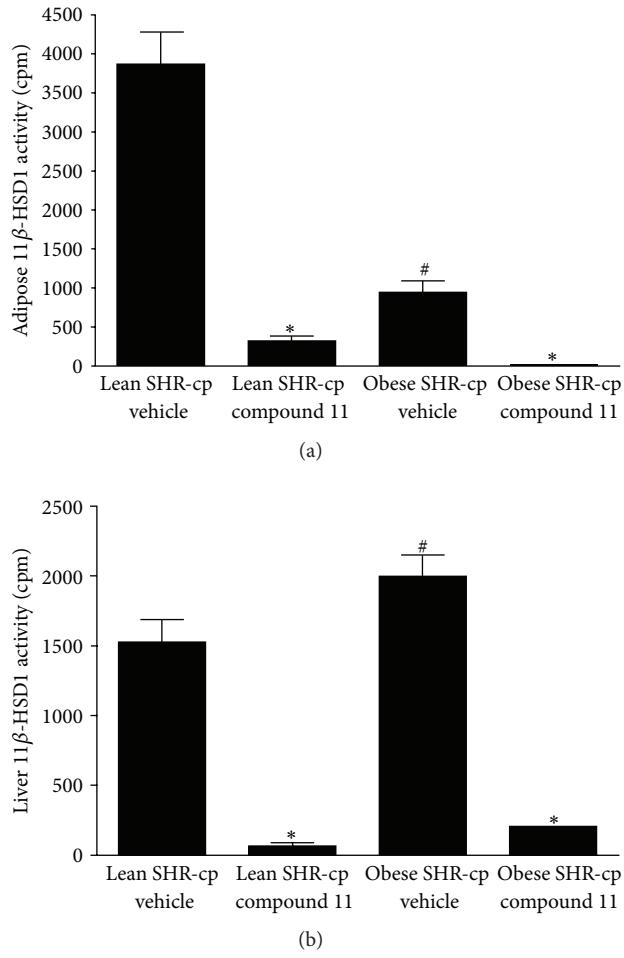

Figure 5: Bar graph depicts 11β-HSD1 activity in (a) adipose tissue and (b) liver of lean and obese SHR-cp after four weeks of vehicle or compound 11 (10 mg/kg/d) administration. $^{*}P < 0.05$ compared to vehicle treatment of same genotype. $^{\#}P < 0.05$ compared to lean SHR-cp vehicle.

investigated the comprehensive response, including blood pressure and renal function, to determine whether 11β-HSD1 is a common mechanism in the multiple risk factors. The results from this study show for the first time that pharmacological inhibition of 11β-HSD1 activity alone in adipose tissue and liver significantly decreases hypertension in a preclinical model of metabolic syndrome. Inhibition of 11β-HSD1 also reduces the glucose intolerance, insulin resistance, and elevated plasma triglycerides in metabolic syndrome. Together, these results expand upon previously published results and indicate that 11β-HSD1 is a common mechanism that contributes to the interrelated risk factors of metabolic syndrome.

Clinical studies previously have shown an association between glucocorticoids and hypertension in the presence or absence of metabolic syndrome. In humans with essential hypertension, the vasoconstrictor sensitivity to glucocorticoids is increased [33] and the ratio of excreted cortisol to cortisone metabolites is increased in some but not all cases [34–36]. Patients with the syndrome of apparent mineralocorticoid excess which is caused by a reduction in the peripheral metabolism of cortisol are hypertensive [18, 19]. Glucocorticoid sensitivity and salivary cortisol concentration are increased in hypertensive humans with insulin resistance and hyperglycemia, as well as in men with a predisposition to high blood pressure [37, 38]. These observations all suggest a primary role for glucocorticoids in the development of hypertension.

Not only there is a strong association between glucocorticoids and hypertension, but also clinical trials have shown a therapeutic benefit of reducing glucocorticoid activity on blood pressure, in some cases on a background of metabolic syndrome. Hypertension associated with Cushing's syndrome was reversed with the antiglucocorticoid agent RU486 [6]. Recently, Feig et al. reported that patients with type 2 diabetes mellitus and metabolic syndrome had a modest but significantly reduced blood pressure after twelve weeks of treatment with the 11β-HSD1 inhibitor MK-0916 [23]. Interestingly, in overweight and obese patients with hypertension, MK-0916 at the same dose had no significant effect on the primary endpoint of sitting blood pressure, but modestly improved other blood pressure endpoints [24]. In association with the small decrease in blood pressure, 11β-HSD1 inhibition also reduced elevated LDL and body weight in these clinical studies using small cohorts [23, 24].

Prior preclinical studies using the genetic manipulation of 11β-HSD1 suggested that the local (nonadrenal) generation of cortisol plays an important role in the regulation of blood pressure in metabolic syndrome (see [39] for review). Paterson et al. reported that liver-selective overexpression of 11β-HSD1 in mice caused a transgene dose-related increase in blood pressure [20]. Furthermore, mice with overexpression of 11β-HSD1 specifically in adipose tissue also exhibited high blood pressure [21]. In contrast to studies that increased the mRNA expression of 11β-HSD1 in selective tissues, Kotelevtsev et al. reported that the mean arterial pressure of 11β-HSD1$^{-/-}$ mice is the same as in wild-type mice [22]. Other mechanisms may be compensating in the 11β-HSD1$^{-/-}$ mice in order to maintain blood pressure. These preclinical studies suggested that increased 11β-HSD1 activity can markedly elevate blood pressure, but is not required for hypertension. We show for the first time that the pharmacological inhibition of 11β-HSD1 activity alone in liver and adipose tissue is sufficient to reduce blood pressure in an animal model of metabolic syndrome.

Glucocorticoids have a wide range of activities within the cardiovascular, renal, and endocrine systems. Previous studies have shown that glucocorticoids can affect insulin resistance, gluconeogenesis, liposynthesis, accumulation of visceral fat, vascular reactivity, vascular remodeling, renal sodium reabsorption, and blood pressure [5–9, 40, 41]. However, the mechanisms whereby glucocorticoids can cause hypertension in humans remain unclear. Most evidence suggested that glucocorticoids increase blood pressure through the modulation of vascular structure and function, renal sodium reabsorption, and metabolic changes [5–9, 40, 41].

In this study, we examined whether pharmacological inhibition of 11β-HSD1 decreases blood pressure through a natriuretic mechanism. The results indicate that inhibition of 11β-HSD1 activity tended to decrease sodium and chloride excretion in obese SHR-cp, an effect opposite to a natriuretic agent. However, the small reduction in salt excretion could have been due to the significant decrease in urine flow, which was secondary to the reduced glucose excretion, since glucose is an osmotically active solute in renal tubules.

An alternative explanation for the reduction in blood pressure may be found in the metabolic changes associated with inhibition of 11β-HSD1 activity. The present data clearly indicate that long-term inhibition of 11β-HSD1 significantly improves glucose tolerance and reduces insulin resistance, which may contribute to the lowering of blood pressure. Previous studies have demonstrated that reducing 11β-HSD1 activity decreased glucose intolerance and hyperinsulinemia in diet-induced obese mice [8, 42] and streptozotocin-induced diabetic mice [8]. However, blood pressure was not reported in those studies, thus rendering impossible any interpretation of the effects on insulin resistance (with or without glucose intolerance) on blood pressure. Whether hyperinsulinemia per se causes chronic elevations in blood pressure is still controversial and may be dependent upon the species studied [43].

Our data suggests that inhibition of 11β-HSD1 decreases blood pressure in hypertensive SHR-cp independent of metabolic changes. Inhibition of 11β-HSD1 in adipose tissue and liver of obese SHR-cp decreases glucose tolerance, insulin resistance, hypertriglyceridemia, and hypertension. Yet in lean SHR-cp, inhibition of 11β-HSD1 similarly decreases hypertension in the absence of changes in glucose tolerance or insulin resistance. With the caveat that the metabolic response to 11β-HSD1 inhibition may simply not be manifested in lean SHR-cp because of their normal metabolic state, our results suggest that 11β-HSD1 is an independent mediator of hypertension in SHR-cp. Regardless, 11β-HSD1 is a common mechanism in multiple risk factors in metabolic syndrome.

Finally, the mechanism for the reduction of high blood pressure likely includes modulation of the renin-angiotensin system. Previous investigators showed that glucocorticoids increased hepatic synthesis of angiotensinogen [44] and angiotensin II receptor subtype 1 in peripheral tissues [45]. Indeed, mice overexpressing 11β-HSD1 activity in adipose tissue had increased plasma angiotensinogen, angiotensin II, and aldosterone concentrations and are hypertensive. The elevated blood pressure was abrogated by blockade of the angiotensin II type 1 receptor [22]. Independent studies of the corpulent SHR showed that blockade of angiotensin II type 1 receptor [46] or mineralocorticoid receptor [25, 47], inhibition of angiotensin converting enzyme [48], or antioxidant therapy [49, 50] all significantly decreased blood pressure indicating that angiotensin II and its downstream effects mediated the hypertension. The decreased plasma renin activity in our study suggests that the antihypertensive response to 11β-HSD1 inhibition is likely due, at least in part, to a reduction in angiotensin II actions.

5. Conclusion

The present study shows that inhibition of 11β-HSD1 activity decreases hypertension, insulin resistance, glucose intolerance, and hypertriglyceridemia in obese SHR-cp. These are prominent features of the metabolic syndrome, and 11β-HSD1 appears to be a common regulatory mechanism among them. Longitudinal clinical studies have confirmed that metabolic syndrome is a risk factor for subsequent development of cardiovascular disease and mortality [51]. The prevalence of metabolic syndrome has increased over decades among adults in the United States [4]. Although adults with hypertension are more likely to be insulin resistant [52, 53] and hypertension tends to cluster with other metabolic risk factors [54], there are currently no guidelines for treating hypertension specifically in individuals with metabolic syndrome. The present study provides preclinical support for the pharmacological inhibition of 11β-HSD1 for the treatment of hypertension and other interrelated risk factors in metabolic syndrome.

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Research Article

Transfected Early Growth Response Gene-1 DNA Enzyme Prevents Stenosis and Occlusion of Autogenous Vein Graft *In Vivo*

Chengwei Liu,[1] Xuesong Zhang,[1] Shi Wang,[1] Mingxun Cheng,[1] Chuanyu Liu,[1] Shuqing Wang,[1] Xinhua Hu,[2] and Qiang Zhang[2]

[1] *First Department of General Surgery, The First Affiliated Hospital of Jiamusi University, Jiamusi 154002, China*
[2] *Department of Vascular Surgery, The First Hospital of China Medical University, Shenyang 110001, China*

Correspondence should be addressed to Shi Wang; shiwangcn@163.com

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The aim of this study was to detect the inhibitory action of the early growth response gene-1 DNA enzyme (EDRz) as a carrying agent by liposomes on vascular smooth muscle cell proliferation and intimal hyperplasia. An autogenous vein graft model was established. EDRz was transfected to the graft vein. The vein graft samples were obtained on each time point after surgery. The expression of the EDRz transfected in the vein graft was detected using a fluorescent microscope. Early growth response gene-1 (Egr-1) mRNA was measured using reverse transcription-PCR and *in situ* hybridization. And the protein expression of Egr-1 was detected by using western blot and immunohistochemistry analyses. EDRz was located at the media of the vein graft from 2 to 24 h, 7 h after grafting. The Egr-1 protein was mainly located in the medial VSMCs, monocytes, and endothelium cells during the early phase of the vein graft. The degree of VSMC proliferation and thickness of intima were obviously relieved compared with the no-gene therapy group. EDRz can reduce Egr-1 expression in autogenous vein grafts, effectively restrain VSMC proliferation and intimal hyperplasia, and prevent vascular stenosis and occlusion after vein graft.

1. Introduction

In 1977, Paterson et al. [1] first inhibited gene transcription using a complementary combination of single-stranded DNA and RNA in a cell-free system. Later, Stephenson and Zamecnik [2] reversely inhibited the replication of the Rous sarcoma virus using a 13 oligodeoxynucleotide and pioneered the direction of gene-based drugs by inhibiting gene expression. A variety of catalytic DNA, called DNA enzymes, was one of the important breakthroughs in life science history since the discovery of catalytic RNA (ribozyme, Rz) [3–7].

In 1994, Breaker and Joyce [8] found that a single-stranded DNA molecule (catalytic DNA) can catalyze the hydrolysis of RNA phosphodiester bonds. This single-stranded DNA molecule was also called DNA enzyme (DRz). The enzyme activity center was the "10–23 motif" [9–15] composed of 15 deoxyribonucleotides (5′-GGC TAG CTA CA A CGA-3′). Its mutation or reverse mutation variants had no activities. Both ends of the active center were substrate-binding regions that can specifically combine with the target RNA through the Watson-Crick base pairing.

Early growth response gene-1 (Egr-1) is a Cys2-His2-type zinc-finger transcription factor. A broad range of extracellular stimuli are capable of activating Egr-1, thus mediating growth, proliferation, differentiation, or apoptosis, therefore, participating in the progression of a variety of diseases such as atherosclerosis [16–19]. Previous studies have demonstrated that Egr-1 can activate the restenosis process and intimal hyperplasia and inhibit vascular smooth muscle cell apoptosis in vein grafts [20]. The DNA enzyme is an oligonucleotide that bound to and interfered with translation of the Egr-1 mRNA and it could inhibit the expression of Egr-1. In the present study, an Egr-1 DNA enzyme (EDRz) was designed for Egr-1 mRNA, used a liposome as a carrying agent, and investigated the inhibitory action of the Egr-1 DNA enzyme on vascular smooth muscle cell (VSMC) proliferation and intimal hyperplasia.

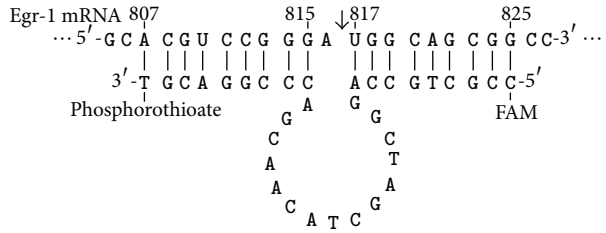


Figure 1: The construction conceptual diagram of Egr-1 DNA enzyme shear and its substrate.

2. Materials and Methods

2.1. Construction of Early Growth Response Gene-1 DNA Enzyme. The primer sequences were as follows: 5′-CC GCT GCC AGG CTA GCT ACA ACG ACC CGG ACG T-3′. The 3′ end was phosphorothioate-modified, the 5′ end was labeled with carboxyl fluorescein (FAM), and a total of 15 OD_{260} (495 μg) of the Egr-1 DNA enzyme was synthesized (Figure 1). Approximately 80 μL of DEPC was added to the solution (1 : 1000), mixed, centrifuged, and then added with 120 μL of the liposome Lipofectamine 2000 (Invitrogen,USA). After 10 min, 32 μL of 1 mmol/L $MgCl_2$ and 568 μL of Pluronic gel 30% F-127 (Sigma, USA) were added to a final volume of 800 μL. The solution was oscillated and homogenized at 4°C and stored until use.

2.2. Establishment of Animal Model and Sample Collection. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Hospital Affiliated to Jiamusi University. Ninety Wistar rats of either sex (200 g to 250 g) were used. The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate solution (300 mg/kg) and underwent a sterile microsurgery under the SXP-1B microscope (10 times). The procedure was as follows: about 5 mm of the rat's right jugular vein was cut; the vein was flushed with heparin saline; and the vein was anastomosed to the infrarenal abdominal aorta using an 11-0 vascular suture line in an end-to-end manner. Up to 8 μL of EDRz was evenly used around the graft vein (including anastomotic) when no signs of active bleeding were found. The retroperitoneum was closed without the use of anticoagulants either before or after the surgery. The animals were randomly divided into 9 groups (10/group), namely, 1, 2, 6, and 24 h and 3, 7, 14, 28, and 42 days after the graft surgery, respectively. The graft vein specimens were cut on each time point. The no-gene therapy group was taken as control group (Figure 2).

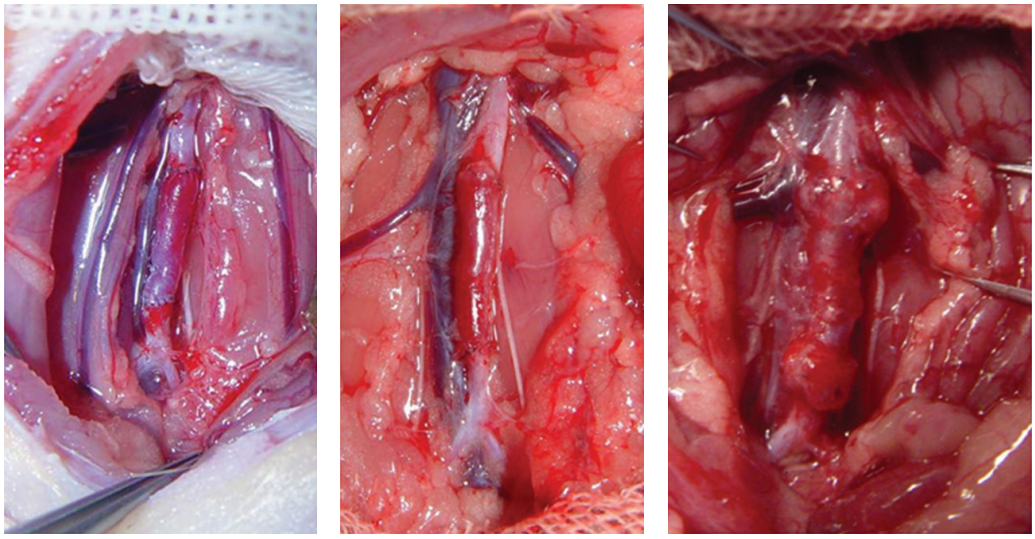
The specimens were fixed with 4% paraformaldehyde in 0.1% diethylpyrocarbonate (DEPC) for 2 h. The gradient sucrose was dehydrated. Then, they were frozen-embedded and cut into 5 μm thick sections. EDRz transfection on the vein graft was observed under a fluorescence microscope. The localization of EDRz was determined by confocal microscopy. The fluorescence gray value was detected using a fluorescence image analyzer and was replicated and verified in multiple samples.

2.3. Histomorphology Staining. Vein grafts were fixed in 10% neutral formalin for 24 h. Conventional dehydration was then performed. The grafts were transparent and wax-dipped. The wax block was embedded and the middle section of the vein graft was cut into 5 μm thick sections. HE staining was conducted and images of the computer image analysis system were collected. Finally, intimal hyperplasia thickness was measured. The data collection and analysis of intimal hyperplasia were performed in a blinded manner.

2.4. In Situ Hybridization. Specimens were processed with fixation. The sucrose gradient was dehydrated, and frozen-embedded, and then a constant cold slicer was used to slice them into 5 μm thick sections. Digoxigenin-labeled oligonucleotides were used as probes, and the *in situ* hybridization was performed according to the manufacturer's instructions (Wuhan Boster Corporation, Wuhan, China). The specimens were dyed with DAB or AEC. The percentage of positive cells in the total cell in eight-unit perspective was randomly counted and performed in a blinded manner.

2.5. Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from cell lines according to instructions of the kit (Wuhan Boster Corporation, Wuhan, China). Primers for Egr-1 were designed using the Jellyfish software according to the sequence in GenBank and synthesized by Shanghai Sangon. For Egr-1, the primers were 5′-CAG TCG TAG TGA CCA CCT TAC CA-3′ (Fwd) and 5′-AGG TTG CTG TCA TGT CTG AAA GAC-3′ (Rev), 448-bp long. For β-actin, the primers were 5′-TTG TAA CCA ACT GGG ACG ATA-3′ (Fwd) and 5′-GAT CTT GAT CTT CAT GGT GCT-3′ (Rev), 668-bp long. The PCR program involved the following procedures: predenaturation for 2 min at 94°C; denaturation for 30 s at 94°C; annealing for 30 s at 58°C; extension for 1 min at 72°C; and final elongation at 72°C for 10 min. Thirty cycles of PCR were performed. PCR products were analyzed by electrofluorescence on 2% agarose gel in a 1x TAE buffer at a voltage of 100 V for 1 h and EB-stained for 20 min. Band intensity was photographed and analyzed on the Gel Imaging System. The gene expression value = Egr-1mRNA/β-actin mRNA.

2.6. Immunohistochemical Staining. Conventional SABC staining was performed according to the kit's instructions (Wuhan Boster Company, Wuhan, China). PBS was used in place of primary antibodies as the negative control. The nucleus or cytoplasm had positive brown-yellow (DAB) or red (AEC) particles at 400 times magnification under the light microscope; it was considered positive regardless of dyeing intensity as long as there was a color display. The percentage of positive cells in the total cell in the eight-unit perspective was randomly counted and was performed in a blinded manner.



(a) (b) (c)

Figure 2: (a) The picture of animal model after graft vein: (b) 28 d after graft in transfection group, the picture of animal model and (c) 28 d after graft in control group, the picture of animal model.

2.7. Western Blot Analysis. The specimens were lysed with a cell lysis solution. The vessel tissues were cut into pieces. The specimens were ultrasound-homogenized. Proteins (100 μg/sample) were separated using 10% SDS-PAGE. The proteins were electrotransferred to nitrocellulose membranes using a semidry system. Then, the membrane was blocked in 5% skimmed milk diluted in TBST for 1 h at room temperature. Thereafter, the membranes were incubated with a primary antibody for 2 h at room temperature. Next, the membranes were further incubated with a horseradish peroxidase-labeled goat anti-mouse IgG antibody at a 1 : 500 dilution. The specimens were washed with TBST three times. Then, 12.5 mg of β-Naphthyl acid phosphate and 12.5 mg of O-Dianisidine tetrazotized (Sigma Corporation) were added to color the specimens. The NC membrane was photographed and analyzed on the Gel Imaging System.

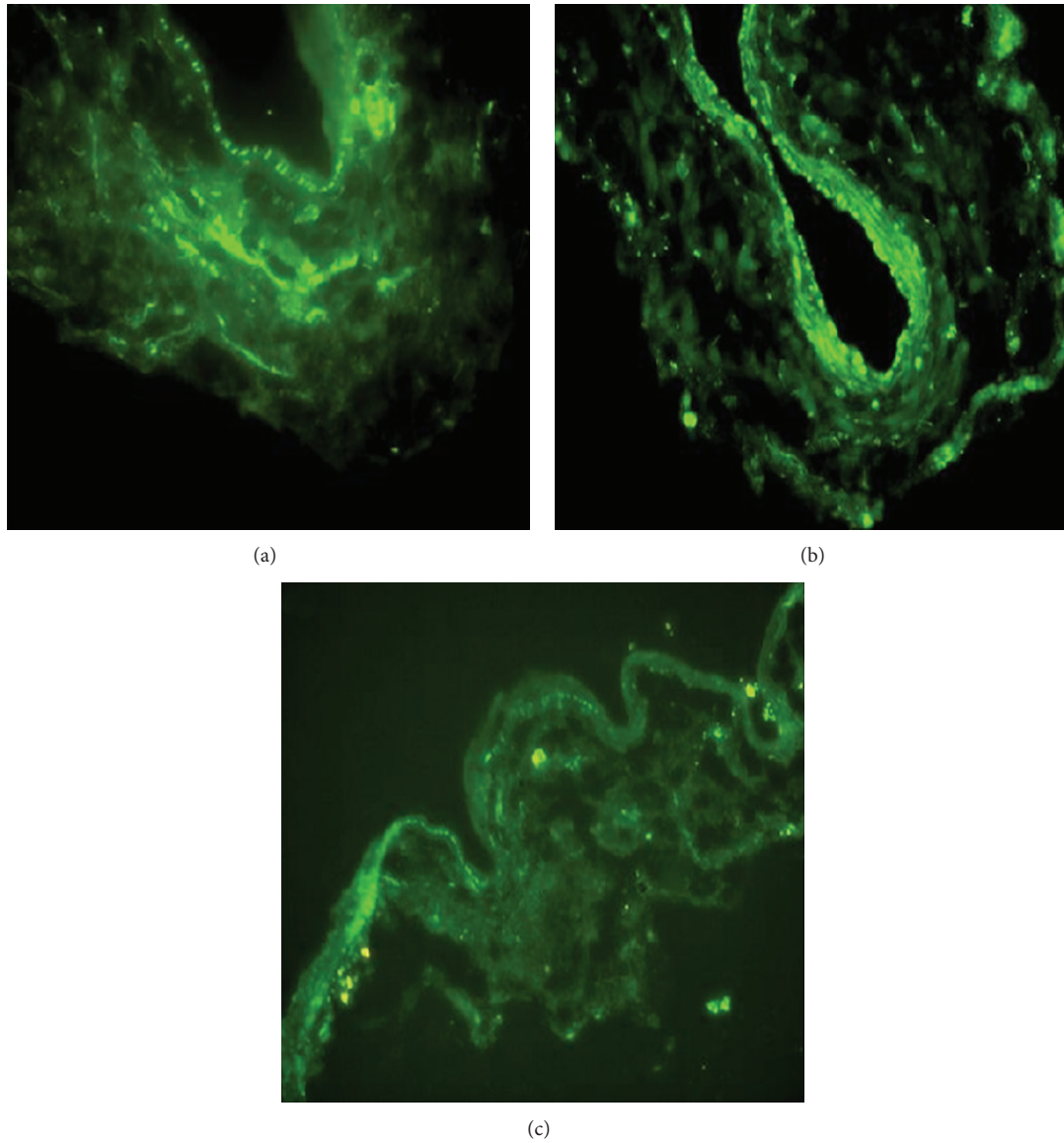
2.8. Statistical Methods. Data were shown as mean ± SD ($\overline{x} \pm s$) and analyzed using the SPSS10 statistical software. The significance of the differences between the group means was determined using ANOVA and post hoc test.

3. Results

3.1. Egr-1 DNA Enzyme (EDRz) Transfection. The early growth response gene-1 DNA enzyme was mainly located in the tunica media, adventitia, and partial endothelial cells of the vein graft 1 h after the grafting in transfection group (fluorescence expression value of 70.3 ± 13.5) (Table 1, Figure 3(a)). The early growth response gene-1 DNA enzyme was located in the tunica media of the vein graft from 2 h to 24 h after-grafting. There was a small amount of EDRz in the tunica media of the vein graft 3 d after the grafting. It was mainly located in the intima of the vein graft 7 d after grafting (Table 1, Figure 3(b)). There were no traces of the early growth response gene-1 DNA enzyme in the vein grafts at 14, 28, and 42 d and control group (Table 1, Figure 3(c)).

3.2. Changes in Histomorphology. There was no expression of PCNA protein in normal vein. There was still a small amount of slightly disordered VSMCs in the media 2 h to 6 h after the vein graft compared with the control group. Slightly positive expression of PCNA at 6 h, positive cell rate of (2.5 ± 0.4)% in transfection group, (5.6 ± 0.4)% in control group. Moreover, VSMCs were also found partly in the thin layer of a thrombus formation in the cavity surface of the intima. The intima was partly damaged at 24 h to 3 d after grafting. The expression of PCNA protein was increased from 24 h to 3 d. In addition, endothelial cells were shed and there was a small amount of thrombosis in the local area. The intima thickened and VSMC proliferation was visible at 7 d. Intimal hyperplasia reached a peak (16.4 ± 4.7 μm) at 14 d. The expression of PCNA protein reached peak at 14 d, (15.3 ± 4.2)% in transfection group, (33.5 ± 6.2)% in control group. The vascellum basically completed endothelialization and disordered VSMC were still visible compared with the control group whose degree of VSMC proliferation and thickness of intima were obviously relieved at the same time. The difference was statistically significant ($F = 3.42$, $P < 0.01$). Intimal hyperplasia thickness decreased at 28 and 42 d compared with 14 d and the expression of PCNA protein was decrease (Tables 1 and 2 and Figure 4).

3.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Egr-1 mRNA expression reached a peak (gene expression value of 1.89 ± 0.63) 1 h after the EDRz was transfected. The expression decreased (0.85 ± 0.42, 0.13 ± 0.03, 0.09 ± 0.04) from 2 h to 24 h after grafting. The expression was weak (0.05 ± 0.01) 3 d after-grafting. There were no more



(a) (b)

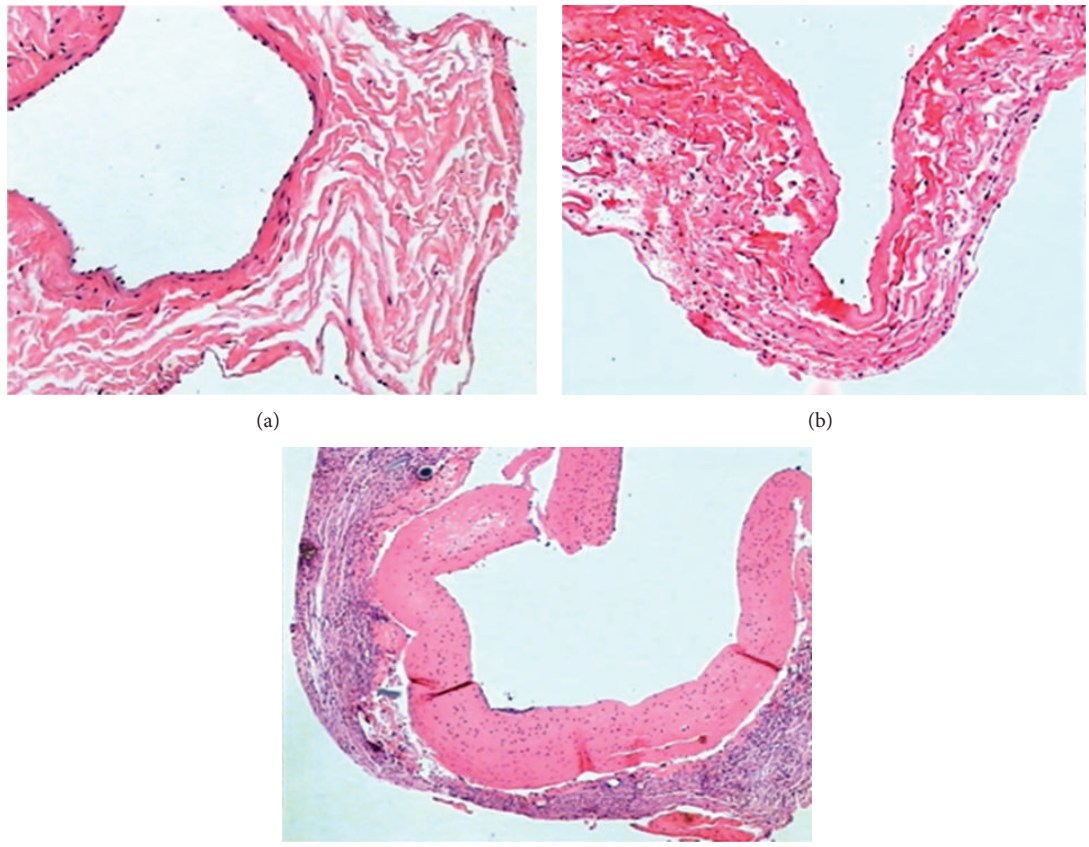
(c)

Figure 3: (a) 1 h after graft in transfection group, EDRz located in adventitia, tunica media, and partial endothelial cells by confocal microscopy (×400). (b) 7 d after graft in transfection group, EDRz located in tunica intima by confocal microscopy (×400). (c) 1 h after graft in control group, there was no EDRz in adventitia, tunica media, and endothelial cells by confocal microscopy (×400).

Egr-1 mRNA expressions at 7, 14, 28, and 42 d after grafting (Figure 5(a)). Egr-1 mRNA expression had biphasic changes in control group. Egr-1 mRNA rapid rise at 1 h after graft, a spontaneous decline at 6 h to 3 d, increase at 7 d after graft operation, a peak at 28 days (Figure 5(b)).

3.4. In Situ Hybridization. Partial VSMC showed an Egr-1 mRNA-positive expression in the media of the vein graft 1 h after EDRz transfection. The strongest positive cell expression was (20.1 ± 6.4)%. The difference was statistically significant ($F = 3.25$, $P < 0.01$) compared with the rest of the time points. Its expression decreased from 2 h to 3 d after grafting. There was no Egr-1 mRNA positive expression of neointimal VSMC 7 d after grafting. The trend of positive cells was consistent with the RT-PCR results (Table 3, Figure 6(a)) in control group, and the positive expression of Egr-1 mRNA was found in the part of VSMCs of the media at 1 h after graft. A peak at 28 d, the positive rate of Egr-1 mRNA was (45.7 ± 6.4)%, Egr-1 mRNA major located in the vascular smooth muscle cells of neointimal (Table 3, Figure 6(b)).

3.5. Western Blot Analysis. Egr-1-positive cells were not detected in the normal vein. Egr-1 protein expression appeared 2 h after EDRz transfection. The optical density value was (26.4 ± 9.2) × 10^3. Its expression decreased from 6 h to 3 d after grafting, with optical density values of (14.5 ± 5.2) × 10^3, (3.4 ± 1.5) × 10^3, and (2.0 ± 0.8) × 10^3. Egr-1 positive cells were no longer present 7 d after grafting



(a) (b) (c)

Figure 4: (a) Normal vein (HE × 100). (b) 14 d after graft in transfection group, intimal hyperplasia reached a peak (HE × 100). (c) 14 d after graft in control group, intimal hyperplasia reached a peak (HE × 100).

Table 1: The result of thickness of intimal hyperplasia in vein graft and EDRz transfection after transfection and no-transfection EDRz ($\overline{x} \pm s$).

	Thickness of intima (μm) in the control group	Thickness of intima (μm) in transfection group	Values of gray scale
Normal vein	2.3 ± 05	2.3 ± 0.5	0
1 h	2.3 ± 0.8	2.3 ± 0.2	70.3 ± 13.5*
2 h	2.3 ± 0.4	2.2 ± 0.3	34.8 ± 9.6
6 h	2.2 ± 0.6	2.3 ± 0.5	25.5 ± 8.7
24 h	2.5 ± 0.3	2.4 ± 0.7	10.1 ± 4.2
3 d	3.2 ± 1.1	3.4 ± 1.2	3.4 ± 1.7
7 d	15.5 ± 2.5	8.9 ± 2.3#	1.6 ± 0.8
14 d	26.4 ± 4.7	16.4 ± 4.7*#	0
28 d	30.2 ± 2.8	14.2 ± 3.5#	0
42 d	22.7 ± 1.9	9.6 ± 2.8#	0

*Compared with other times $P < 0.01$, #compared with control groups $P < 0.01$.

(Figure 7(a)). In control group, we found that Egr-1 protein was expressed at the early phase of 2 h, and continuing to 6 h, the expression of Egr-1 protein was decline from 24 h to 3 d, reincreased at 7 d, and reached peak at 28 d (Figure 7(b)).

3.6. Immunohistochemistry. The Egr-1 protein was mainly located in the medial VSMCs, monocytes, and endothelium cells during the early phase of the vein graft. However, there were no Egr-1 proteins in the medial and neointimal VSMCs after 7 d. The positive expression rates were as follows: positive cell rate of (15.3 ± 4.2)% at 2 h; positive cell rate of (9.7 ± 2.4)% at 6 h; positive cell rate of (6.4 ± 1.8)% at 24 h; and positive cell rate of (2.3 ± 0.2)% at 3 d (Figure 8(a)). In control group, the positive expression of Egr-1 protein reached peak at 28 days (40.7 ± 9.5)% (Figure 8(b)).

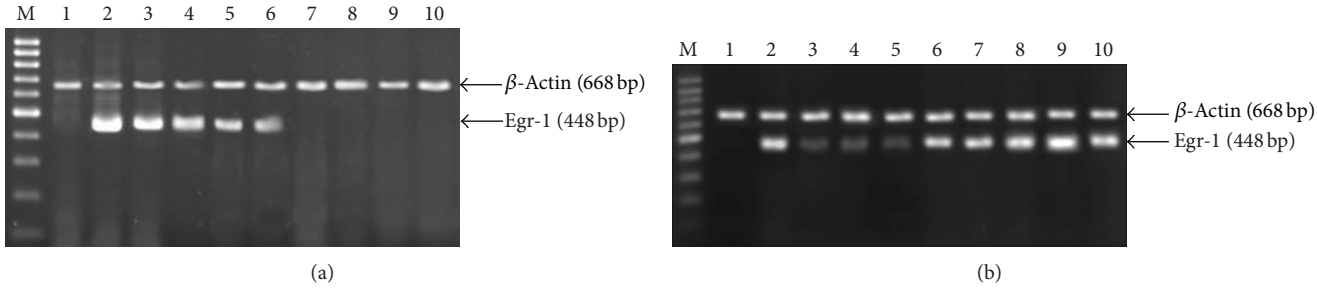


Figure 5: (a) The RT-PCR results of Egr-1 mRNA in transfection group. (b) The RT-PCR results of Egr-1 mRNA in control group. M: Gene Ruler 100 bp DNA Ladder Marker; 1: normal vein; 2–10: transplantation vein at different time after operation, 2: 1 h; 3: 2 h; 4: 6 h; 5: 24 h; 6: 3 d; 7: 7 d; 8: 14 d; 9: 28 d; 10: 42 d.

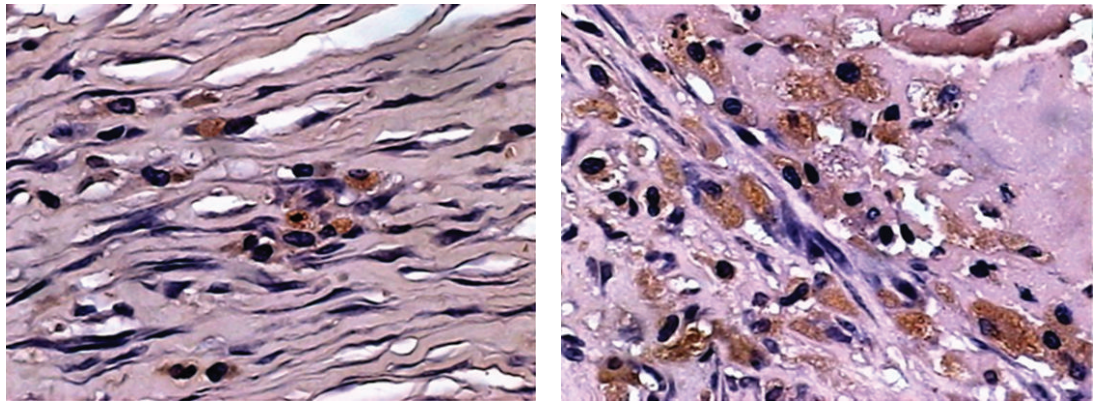


Figure 6: (a) 1 h after graft in transfection group, the positive cells of Egr-1 mRNA were located in cytoplasm of VSMC of neointima by ISH (×400). (b) 1 h after graft in control group, the positive cells of Egr-1 mRNA were located in cytoplasm of VSMC of neointima by ISH (×400).

Table 2: Contrast of PCNA protein by immunohistochemistry ($\overline{x} \pm s$, %).

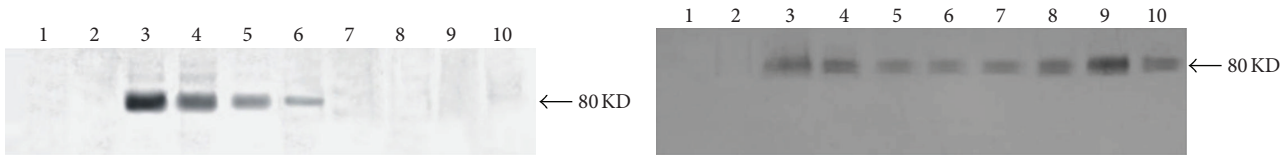
	Control group	Transfection group
Normal vein	0	0
1 h	0	0
2 h	0	0
6 h	5.6 ± 0.4	2.5 ± 0.4
24 h	9.6 ± 1.5	6.2 ± 1.5
3 d	16.7 ± 2.1	8.3 ± 1.6$^{\#}$
7 d	24.6 ± 5.3	10.8 ± 5.7$^{\#}$
14 d	33.5 ± 6.2*	15.3 ± 4.2$^{*\#}$
28 d	18.7 ± 9.1	8.1 ± 3.9$^{\#}$
42 d	9.8 ± 1.7	3.2 ± 0.6$^{\#}$

*Compared with other times $P < 0.01$, $^{\#}$compared with control groups $P < 0.01$.

4. Discussion

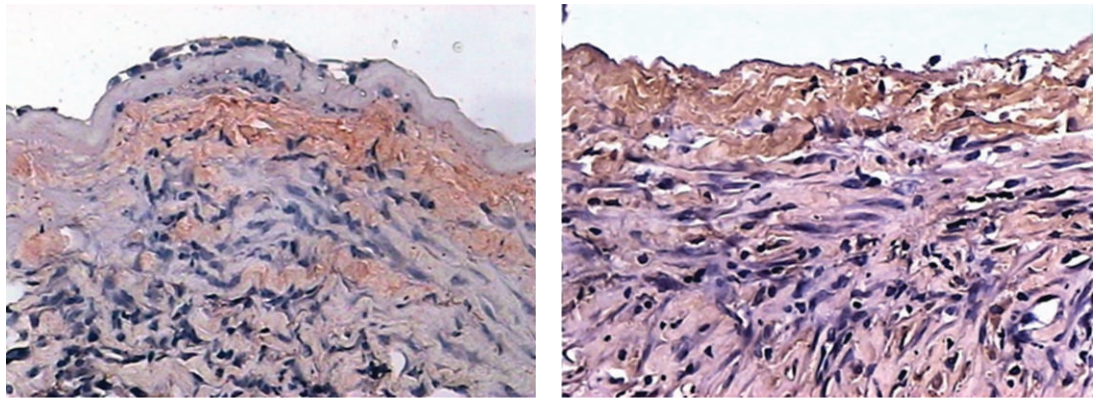
AUG (816 to 818 sequence) is a selected target of the Egr-1 mRNA. The splice site was located between 816 and 817, adding T GCA GGC CC to the 3′ end of DNA enzyme for the 807–815 sequence (A CGU CCG GG) of Egr-1 mRNA and ACC GTC GCC [21–24] to the 5′ end of DNA enzyme for the 817–825 sequence (UGG CAG CGG). A phosphorothioate modification was made in the 3′ end to resist nuclease degradation, and the 5′ end was labeled with carboxy fluorescein (FAM) for detection purposes. The constructed DNA enzyme was called Egr-1 DNA enzyme (EDRz) (Figure 1). The 816 base (A) of the Egr-1 mRNA did not undergo base pairing with EDRz. Meanwhile, the rest of the EDRz sites formed the combination of base pairing with Egr-1 mRNA. Then, the latter underwent conformational changes. The 2′ end at the point of the OH proton was cut with the help of divalent metal cations, such as Mg2. Moreover, a nucleophilic attack occurred on the adjacent phosphate. The Egr-1mRNA molecular structure was dissociated by two transesterification reactions [25–30].

The substrate-binding site can be applied to shear the RNA of a variety of pathogens and mRNAs of disease-related genes after changing its sequence composition in the 10–23 DNA enzyme [31, 32]. In gene therapy, 10–23 DNA enzymes have the advantages of both the ribozyme (Rz) and antisense oligodeoxynucleotide (ASODN) [33, 34]. The 10–23 DNA enzyme has the following features compared with ASODN: it not only has a substrate RNA antisense inhibitory effect by virtue of the two substrate-binding sites, but also kills virus RNA through the "shear" mechanism [35–38]. Furthermore, DNA enzyme molecules can be used repeatedly, which means



(a) (b)

Figure 7: (a) The results of western blot of Egr-1 protein in transfection group. (b) The results of western blot of Egr-1 protein in control group 1: normal vein; 2–10: transplantation vein at different times after operation, 2: 1 h; 3: 2 h; 4: 6 h; 5: 24 h; 6: 3 d; 7: 7 d; 8: 14 d; 9: 28 d; 10: 42 d.



(a) (b)

Figure 8: (a) 2 h after graft in transfection group, the positive cells of Egr-1 protein were located in cell nucleus of VSMC of neointima by immunohistochemistry (×200). (b) 2 h after graft in control group, the positive cells of Egr-1 protein were located in cell nucleus of the VSMC of neointima by immunohistochemistry (×200).

that they can shear a number of RNA molecules. The 10–23 DNA enzyme has the following characteristics compared with a variety of Rz: the identified splice site of 10–23 DNA enzyme is present in a range of RNA molecules, including the RNA translation initiation codon AUG of viruses. It is a good shear target and has more shearing targets to choose from compared with Rz. Its nature is relatively stable. The stability of DNA is about 100,000 times that of RNA in the conditions of physiological pH, temperature, ionic strength, and so on. Its resistance to hydrolysis is about 100 times or more than that of a protein enzyme [39–41]. The sequence of the active center is short. The molecular weight is relatively small with relatively good elasticity. Therefore, it is less affected by the secondary structure of the target sequence. The trend to the substrate is better. Thus, the specificity of the target RNA, combing stability and shear activity, is expressed better than Rz in general [42–44]. It is easier to dissociate the DNA-RNA hybrid molecule than the RNA-RNA hybrid molecule. Therefore, the shear rate of the shear product DRz dissociation process is relatively small [45, 46]. The RNA of the DNA-RNA hybrid molecules can be degraded by the RNA enzyme H. Hence, the DNA enzyme can not only directly kill the target RNA such as Rz, but also cause the hydrolysis of the RNA enzyme H to target RNAs, such as ASODN [47, 48].

The results of this experiment combined with those of previous studies [8, 49, 50] indicated that the early growth response gene-1 DNA enzyme was mainly located in the media and adventitia of the vein graft 1 h after grafting and then gradually shifted to the media. There was a small amount of EDRz in the media of the vein graft 3 d after grafting and was mainly located in the media. It was mainly located in the intima of the vein graft 7 d after grafting. In addition, the Egr-1 DNA enzyme can also be found in some small newborn blood vessels. However, Egr-1 mRNA and protein expressions in the vein graft were not detected 14 d after grafting. There was no EDRz in the vein grafts, suggesting that the EDRz pathway is adventitia → medial → intima and perhaps degraded by a deoxyribonuclease in the end. Egr-1 mRNA and protein expressions decreased at the same time point. Egr-1 mRNA expression decreased obviously 1 h after grafting. This finding indicated that the Egr-1 DNA enzyme rapidly transferred from the adventitia to the media to combine with the Egr-1 mRNA under a short period of time. Hence, the role of the carrier liposome Lipofectamine 2000 was confirmed. Egr-1 proteins were mainly located in the medial VSMCs, monocytes, and endothelium cells during the early phase of the vein graft. However, there were no Egr-1 proteins in medial and neointimal VSMCs 7 d after grafting, indicating that the early growth response gene-1 DNA enzyme can reduce Egr-1 expression in an autogenous vein graft. VSMC proliferation and intimal hyperplasia reached a peak 7 and 14 d after grafting. The degree of VSMC proliferation and thickness of intima were obviously relieved at the same time compared with the no-gene therapy group. Therefore, Egr-1 DNA enzyme transfection of vein grafts with the liposome Lipofectamine 2000 as a carrier can effectively restrain VSMC

Table 3: Contrast of Egr-1 mRNA and protein by *in situ* hybridization and immunohistochemistry ($\overline{x} \pm s$, %).

	mRNA in control group	mRNA in transfection group	Protein in control group	Protein in transfection group
Normal vein	0	0	0	0
1 h	35.4 ± 7.2	20.1 ± 6.4*#	0	0
2 h	17.8 ± 3.1	10.2 ± 2.3#	30.2 ± 5.1	15.3 ± 4.2*#
6 h	8.5 ± 2.2	8.6 ± 1.7	29.4 ± 2.3	9.7 ± 2.4#
24 h	8.9 ± 1.6	5.1 ± 1.2	7.2 ± 3.1	6.4 ± 1.8
3 d	8.7 ± 2.4	3.2 ± 0.8#	7.2 ± 4.5	2.3 ± 0.2#
7 d	15.3 ± 4.5	0	10.8 ± 6.3	0
14 d	25.5 ± 3.6	0	21.6 ± 6.2	0
28 d	45.7 ± 6.8	0	40.8 ± 8.9	0
42 d	28.3 ± 8.4	0	24.1 ± 4.6	0

*Compared with other times $P < 0.01$, #compared with control groups $P < 0.01$.

proliferation and intimal hyperplasia and prevent vascular stenosis and occlusion after vein grafting.

Conflict of Interest

The authors declare that there is no conflict of interests.

Authors' Contribution

C. Liu and X. Zhang contributed equally to this paper.

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Research Article

A Case-Control Study between Gene Polymorphisms of Polyunsaturated Fatty Acid Metabolic Rate-Limiting Enzymes and Acute Coronary Syndrome in Chinese Han Population

Zikai Song, Hongyan Cao, Ling Qin, and Yanfang Jiang

Department of Cardiology, the Second Division of the First Hospital, Jilin University, 3302 JiLin Street, Changchun 130031, China

Correspondence should be addressed to Ling Qin; qinling@medmail.com.cn and Yanfang Jiang; yanfangjiang@hotmail.com

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The purpose of this study is to analyze the relationship between the polymorphisms of fatty acid desaturase 1 (*FADS*1), fatty acid desaturase 2 (*FADS*2), and elongation of very long-chain fatty acids-like 2 (*ELOVL*2) and acute coronary syndrome (ACS) in Chinese Han population. Therefore, we selected three single nucleotide polymorphisms (SNPs) from these candidate genes and genotyped them using PCR-based restriction fragment length polymorphism analysis in 249 ACS patients and 240 non-ACS subjects, as were Han Chinese ancestry. The results showed that rs174556 in the *FADS*1 gene is found to be in allelic association ($P = 0.003$) and genotypic association ($P = 0.036$) with ACS. The frequencies of rs174556 minor allele (T) in case group were obviously higher than in control group. The trans-phase gene-gene interaction analysis showed that the combined genotype of rs174556 (T/T) and rs3756963 (T/T) was associated with ACS ($P = 0.031$). And the results suggest that, for rs174556 C>T, the CT/TT genotypes were more likely to lead in ACS in subjects with hypertension after correction of all risk factors (OR = 4.236, 95% CI, 2.216–7.126). These findings suggest that the polymorphisms of rs174556 in the *FADS*1 gene are very likely to be associated with ACS in Chinese Han population, especially in subjects with hypertension.

1. Introduction

Acute coronary syndrome (ACS) is a common disease, and a major determinant of morbidity and mortality in all races, ethnicities, and cultures [1], which is caused by a combination of genetic background and environmental factors. The epidemic of coronary artery disease (CAD), especially its manifestation as ACS, is a global issue that accounts for more than 80% of the burden of this disease in developing countries [2] and results in approximately 30% of all deaths worldwide each year [3]. The spectrum of ACS ranges from unstable angina pectoris (UAP) to acute myocardial infarction (AMI), including ST-segment elevation myocardial infarction (STEMI) and non-STEMI (NSTEMI) [4]. Atherosclerotic plaque instability is the main feature of ACS pathogenesis.

Blood and tissue contents of polyunsaturated fatty acid (PUFA) and long-chain PUFA (LC-PUFA) are related to numerous health outcomes including cardiovascular health, allergies, mental health, and cognitive development [5]. There are two families of PUFA, and they are classified as omega-3 (n-3) and omega-6 (n-6) based on the location of the last double bond relative to the terminal methyl end of the molecule [6]. Evidence from various research paradigms supports the cardiovascular benefits of a high intake of n-3 polyunsaturated fatty acids (PUFAs), especially the long-chain, marine-derived n-3 PUFA, eicosapentaenoic acids, and docosahexaenoic acids [7, 8]. And n-6 PUFA are well known for their critical role in many physiological functions and seem to reduce risks of CAD [9].

Both families of fatty acids, n-3 and n-6, share and compete for the same enzymes (Δ6-desaturase, Δ5-desaturase, and elongases) in their biosynthesis, and Δ6-desaturase is the rate-limiting step [10–12]. Δ5-desaturase (D5D) and Δ6-desaturase (D6D) are encoded by the genes *FADS1* and *FADS2*, respectively, which form a gene cluster jointly with the gene for fatty acid desaturase 3 (*FADS3*) on the human chromosome 11q12-q13.1 [12, 13]. And D5D and D6D mainly involved in regulating the levels of proinflammatory and

anti-inflammatory eicosanoids derived from PUFAs [14]. Additionally, another essential enzyme, elongase, is involved in the homeostasis of longer chain n-3 fatty acids, which is encoded by elongation of very long-chain fatty acids-like 2 (FEN1/Elo2, SUR4/Elo3, yeast) (*ELOVL2*) gene [15].

Polymorphisms in the genes *FADS1* and *FADS2* are associated with n-3 and n-6 fatty acid levels and especially with arachidonic acid (ARA) amounts in blood and several tissues [16–22]. The presence of a variant T to deletion (T-del) in the promoter of the Δ6-desaturase gene (*FADS2*) leads to reduced timnodonic acid (EPA) concentrations in plasma and adipose tissue [18], suggesting that this variant decreases enzyme activity and therefore conversion from ALA. The presence of the *FADS2* T-del variant is also associated with higher plasma triglyceride concentrations [18]. Additionally, a number of studies have reported significant associations between *FADS* genotypes and the risk of CAD [23, 24]. In CHIANTI study, the genome-wide association study (GWAS) showed that the strongest evidence for association was observed in a region of chromosome 11 that encodes three fatty acid desaturases (*FADS1*, *FADS2*, and *FADS3*) [25]. In 2010, another large GWAS repeated the strongest association between *FADS1* and *ELOVL2* genes and the ratio of product to precursor fatty acids [26]. Several studies, including a recent meta-analysis of genome-wide association (GWA) scans, confirmed that polymorphisms in the *FADS* gene cluster were associated with PUFA concentrations in serum phospholipids and erythrocyte cell membranes in several populations, including Caucasians, East Asians, and African Americans [19, 23, 25, 27–29].

Until now, it is unknown whether or not SNPs in the *FADS1*/*FADS2* gene cluster and *ELOVL2* gene are associated with ACS in Chinese Han population. The aim of this study was to investigate the possible association between the gene SNPs of PUFA metabolic rate-limiting enzymes and risk of ACS in Chinese Han population through the case-control study containing *FADS1*/*FADS2* and *ELOVL2* genes.

2. Subjects and Methods

2.1. Study Subjects. This case-control study included 249 ACS patients and 240 controls in order to undertake a genetic analysis for association between the PUFA rate-limiting enzymes gene polymorphisms and ACS. All patients used for this study were Chinese of Han descent. Patients with ACS were admitted to the First Hospital of Jilin University, Changchun, China, in the period between 2008 and 2010. All subjects gave written informed consent for the study. The study was approved by the ethics committee of Jilin University, Changchun, China.

Diagnosis was carried out independently by at least two well-trained physicians based on the following criteria. All patients (143 males and 106 females) were identified with ACS by coronary computed tomographic angiography (SIEMNS Somatom Definition AS + 128 row spiral CT). ACS was defined by ≥75% stenosis in any major coronary artery. Acute coronary syndrome encompasses three clinical diagnoses: unstable angina, non-ST-segment elevation myocardial infarction, and ST-segment elevation myocardial infarction. Myocardial infarction with cardiac chest pain, serologic evidence of myonecrosis, and persistent (>20 min) ST-segment elevation was confirmed as WHO criteria issued in 1979 [30]; the definition of UAP is when cardiac chest pain was new or worsening without serologic evidence of myonecrosis (i.e., no elevation of serum troponin or creatine kinase MB isoenzyme concentration), or dynamic electrocardiographic (ECG) changes (i.e., ST depression and/or T wave inversion); the definition of NSTEMI is when the patient had cardiac chest pain and serologic evidence of myocardial necrosis in the absence of ST-segment elevation on the ECG [31], and patients with nonatherosclerotic vascular diseases, congenital heart disease, cardiomyopathy, valvular disease, renal or hepatic disease, and cancer were excluded. Based on the principle of epidemiology for setting control group [32], control group contained 240 healthy patients (126 males and 114 females) randomly selected from the same geographical area who were undergoing a routine checkup as part of annual physical examination, which included an ECG, chest X-ray, and serum analysis. The control group was formed on the basis of their unremarkable physical examination, as well as the absence of personal or family history and reasons to suspect ACS.

The presence of cardiovascular risk factors, including diabetes mellitus, blood pressure, and cigarette smoking, was obtained from all participants. Diagnosis of hypertension and diabetes mellitus was performed according to World Health Organization criteria. In this study, hypercholesterolemia was defined as a serum total cholesterol level of 200 mg/dL or more, and a smoking habit was defined as a daily intake of >10 cigarettes [4]. Overnight fasting venous blood samples were collected from all subjects for genomic DNA extraction.

2.2. Genotyping. Three SNPs rs174556 (Mbo I site) in the *FADS1*, rs174617 (Msp I site) in the *FADS2*, and rs3756963 (Hha I site) in the *ELOVL2* were selected as genetic markers. They were all C to T base change present in intron. SNP information was obtained from NCBI dbSNP Build 132 (http://www.Ncbi.nlm.nih.gov/SNP/). The candidates SNPs were restricted to minor allele frequency bigger than 15% in HAPMAP-CHB database (http://www.hapmap.org/). Genomic DNA used for PCR amplification was extracted from the whole blood sample using a DNA extraction kit (Promega, Beijing, China). SNPs were genotyped using standard polymerase chain reaction and restriction fragment length polymorphism (RFLP) analysis. The sequences of primers for amplification are available as follows (Table 1). PCR conditions included predenaturation at 94°C for 5 minutes followed by 35 cycles of 95°C for 35–40 seconds, 63–57°C for 1 minutes, and 72°C for 1 minutes, and a final extension at 72°C for 10 minutes.

2.3. Statistical Analysis. Data were expressed as percentages of total categorical variables, or mean ± SD. The statistical analyses on the characteristics of the subjects were performed with Pearson χ^2 test for the categorical variables such as sex, smokers, and nonnormal distribution variable age was compared by Mann-Whitney rank sum test.

Table 1: The sequences of primers for amplification.

Genes	SNPs	Primers	Sequences
FADS1	rs174556	Forward	5′AAGCAGGGACCTCAAGAC3′
		Reverse	5′AGCCCACCAAGAATGTAA3′
FADS2	rs174617	Forward	5′GAACTGTCAGAGGCAACG3′
		Reverse	5′CTGGGCAATAAAGCAAGA3′
ELOVL2	rs3756963	Forward	5′CCCTTTGTGCGAGAACCT3′
		Reverse	5′ATCCCAAGCGACAGACCC3′

Table 2: General characteristics of patients and controls included in our study.

Subject characteristics	Case ($n = 249$)	Control ($n = 240$)	P value
Age (years)	62.00 ± 17.00	63.00 ± 23.70[a]	0.148
Male, n (%)	143 (57.4)	126 (52.5)	0.273
Diabetes, n (%)	86 (34.5)	30 (14.4)	<0.001
Hypertension, n (%)	154 (61.8)	96 (40.0)	<0.001
Smokers, n (%)	162 (65.1)	86 (35.8)	<0.001

[a] Median ± QR.
Age was compared by using Mann-Whitney rank sum test.
Male, diabetes, hypertension, and smokers were compared by using Pearson's Chi-square test.

The Hardy-Weinberg equilibrium for the genotypic distributions of SNPs was tested using the Chi-square (χ^2) goodness-of-fit test. The Haploview program (version 4.1) was applied to estimate the linkage disequilibrium (LD) measures (D' and r^2) between paired SNPs. Allelic association, genotypic association, and analysis for gene-gene interaction were performed with the UNPHASED program, which is an application for performing genetic association analysis in nuclear families and unrelated subjects [21]. Results are expressed as odds ratio (OR) and 95% confidence intervals (CI). P values < 0.05 were considered significant. With regard to the gene-gene interaction tests, the genotypes with relative frequencies of less than 3% were not considered for analysis. We also applied the permutation test (1000 times) performed with UNPHASED to correct the final P values for the markers used.

3. Results

3.1. Characteristics of Study Subjects. Table 2 lists the demographic and clinical characteristics of the 249 ACS patients and 240 control subjects. Compared with control group, ACS group had more males, more smokers, and more individuals with diabetes. However, there was no significant difference of the mean age and proportion of hypertension between case and control groups.

3.2. Allele and Genotype Analysis. Rs174556 of *FADS1*, rs174617 of *FADS2*, and rs3756963 of *ELOVL2* were genotyped, and they all lie within intron. Rs174556 and rs174617 locate in different LD block on 11q12-q13.1 region ($D' = 0.57$, $r^2 = 0.52$). Rs3756963 locates on 6p24.2. The χ^2 goodness-of-fit test showed that the genotypic distributions of rs174556, rs174617, and rs3756963 were not deviated from Hardy-Weinberg equilibrium in both case and control groups ($P > 0.05$). Tables 3 and 4 present the distributions of alleles and genotypes of 3 SNPs among participants, respectively. For rs174556, C was major allele, frequency was 60.6% and 69.6% in case and control group, respectively, but for rs174617 and rs3756963, T was major allele.

Analysis with the UNPHASED software showed that rs174556 was associated with ACS before ($\chi^2 = 8.592$, $P = 0.003$) and after 1000 permutation test ($P = 0.003996$), and frequency of minor allele T of rs174556 was significantly higher in case than control subjects (Table 3). However, rs174617 and rs3756963 were not associated with ACS. As shown in Table 4, the logistic regression analysis test revealed genotypic association between rs174556 and ACS after being adjusted for confounding factors ($\chi^2 = 6.084$, $P = 0.036$), but not the genotypic association was observed between the other 2 SNPs and ACS.

We further analyzed the associations between the polymorphisms of three SNPs and ACS for subgroups with or without hypertension, DM, and smoking. The results suggest that, for rs174556 C>T, compared with the CC genotype, the CT/TT genotypes were more likely to result in ACS in subjects with hypertension after correction of all risk factors (OR = 4.236, 95% CI, 2.216–7.126) (Table 5). Whereas, another two SNPs, rs174556 and rs3756963, were not associated with ACS after correction of all risk factors (Table 6).

3.3. Trans-Phase Gene-Gene Interaction Analysis. In Table 7, the combined genotypes for rs174556 and rs3756963 with frequency more than 3% are presented. We used the most commonly combined genotype major homozygote as a reference, and the results showed that *ELOVL2* gene had a combined effect with *FADS1* gene ($\chi^2 = 14.112$, df = 6, $P = 0.028$). Rs174556 (C/C)-rs3756963 (T/T) and rs174556 (T/T)-rs3756963 (T/T) were associated with ACS ($\chi^2 = 4.478$, $P = 0.034$, $\chi^2 = 4.656$, $P = 0.031$).

3.4. Logistic Regression Analysis. As shown in Table 8, according to a multivariate logistic regression analysis, the most predictive risk factor for ACS was hypertension, followed by smoking, diabetes, and the T allele in rs174556. The T allele in rs174556 may be a risk factor for ACS (OR = 1.791, 95% CI, 1.088–2.951).

Table 3: Distribution of allele frequencies of SNPs in case and control groups.

Genes	SNPs	Allele	Case (%)	Control (%)	χ^2	P	OR	95% CI
FADS1	rs174556	C	302 (60.6)	334 (69.6)	8.592	0.003[a]	1.485	1.139–1.935
		T	196 (39.4)	146 (30.4)				
FADS2	rs174617	T	386 (77.5)	380 (79.2)	0.395	0.530	1.103	0.813–1.495
		C	112 (22.5)	100 (20.8)				
ELOVL2	rs3756963	T	368 (73.9)	372 (77.5)	1.725	0.189	1.217	0.908–1.631
		C	130 (26.1)	108 (22.5)				

[a] The adjusted P value was 0.003996 from 1000 permutations.

Table 4: Distribution of genotype frequencies of SNPs in case and control groups.

Genes	SNPs	Genotype	Case (%)	Control (%)	χ^2	P	OR	95% CI
FADS1	rs174556	C/C	96 (38.6)	119 (49.6)	6.084	0.036[a]	0.826[a]	0.418–1.459[a]
		C/T	110 (44.2)	96 (40.0)				
		T/T	43 (17.3)	25 (10.4)			0.324[a]	0.126–0.864[a]
FADS2	rs174617	T/T	148 (59.4)	149 (62.1)	0.410	0.815		
		C/T	90 (36.1)	82 (34.2)			1.105	0.759–1.609
		C/C	11 (4.4)	9 (3.8)			1.230	0.495–3.056
ELOVL2	rs3756963	T/T	141 (56.6)	145 (60.4)	2.301	0.317		
		C/T	86 (34.5)	82 (34.2)			1.079	0.737–1.579
		C/C	22 (8.8)	13 (5.4)			1.740	0.844–3.589

[a] Adjustment for age, sex, and the presence of diabetes, hypertension, and smoking by forward logistic regression analysis.

Table 5: Stratified analysis between the rs174556 C>T polymorphisms and risk of ACS by hypertension, DM, and smoking.

	Case (n = 249)		Control (n = 240)		Adjusted OR (95% CI)[a]	
	CC (%)	CT + TT (%)	CC (%)	CT + TT (%)	CC	CT + TT
Rs174556 C>T genotypes						
Hypertension						
No	24 (25.3)	71 (74.7)	43 (29.9)	101 (70.1)	1.00	2.640 (1.410–4.560)
Yes	72 (46.8)	82 (53.2)	76 (79.2)	20 (20.8)	1.00	4.236 (2.216–7.126)
DM						
No	26 (16.0)	137 (84.0)	101 (48.1)	109 (51.9)	1.00	4.120 (2.326–7.824)
Yes	70 (81.4)	16 (18.6)	18 (60.0)	12 (40.0)	1.00	0.424 (0.182–0.926)
Smoking						
No	10 (11.5)	77 (88.5)	49 (31.8)	105 (68.2)	1.00	3.624 (1.842–7.636)
Yes	86 (53.1)	76 (46.9)	70 (81.4)	16 (18.6)	1.00	0.820 (0.326–1.726)
No 3 risk	10 (16.1)	52 (83.9)	36 (32.1)	76 (67.9)	1.00	2.016 (1.046–5.236)

[a] Ajusted for age, sex, and the presence of diabetes, hypertension, and smoking status except the stratified factor at each stratum.

4. Discussion

FADS1, *FADS2*, and *ELOVL2* all encode rate-limiting enzymes in PUFA metabolism. And many studies have confirmed that high levels of PUFA in plasma phospholipids, cell membranes, or whole blood were associated with lower risk of multiple diseases, including metabolic syndrome, CAD, et al. Therefore, we investigate the association between the common variants of the three genes and ACS.

Our results show that the frequency of minor allele T of rs174556 was remarkably higher in case than control group (P = 0.003), as the frequency of TT genotype of rs174556 in case group was also markedly higher than in control group (P = 0.036). And for rs174556 C>T, compared with the CC genotype, the CT/TT genotypes were more likely to lead to ACS in subjects with hypertension after correction of all factors. Rs174617 in the *FADS2* was not associated with ACS. Based on our results, therefore, rs174556 in the *FADS1* has a significant role in the development of ACS, especially in subjects with hypertension. But the locus rs174556 is not a functional SNP, which located in intron of *FADS1* gene. As a result, we infer that the *FADS1* gene may confer susceptibility of ACS through affecting nearby gene. Many studies also have proved that SNPs in *FADS1*/*FADS2* were associated with plasma lipid concentrations in adult populations and children [33, 34]. However, in contrast with our results, Aslibekyan

Table 6: Stratified analysis between the rs174617 and rs3756963 T>C polymorphisms and risk of ACS by hypertension, DM, and smoking.

	Case (n = 249)		Control (n = 240)		Adjusted OR (95% CI)[a]	
	TT (%)	CC + CT (%)	TT (%)	CC + CT (%)	TT	CC + CT
Rs174617 T>C genotypes						
Hypertension						
No	51 (58.0)	37 (42.0)	109 (75.7)	35 (24.3)	1.00	2.326 (1.327–4.120)
Yes	90 (58.4)	64 (41.6)	40 (41.7)	56 (58.3)	1.00	0.492 (0.262–0.812)
DM						
No	88 (54.0)	75 (46.0)	135 (64.3)	75 (35.7)	1.00	1.242 (1.006–2.012)
Yes	60 (69.8)	26 (30.2)	14 (46.7)	16 (53.3)	1.00	0.326 (0.126–0.801)
Smoking						
No	58 (82.8)	29 (17.2)	113 (73.4)	41 (26.6)	1.00	0.524 (0.326–0.884)
Yes	90 (46.9)	72 (53.1)	36 (41.9)	50 (58.1)	1.00	0.628 (0.412–1.021)
No 3 risk	50 (71.4)	20 (28.6)	60 (65.2)	32 (34.8)	1.00	0.782 (0.382–1.410)
Rs3756963 T>C genotypes						
Hypertension						
No	67 (70.5)	28 (29.5)	115 (79.9)	29 (20.1)	1.00	1.542 (0.743–2.732)
Yes	74 (48.1)	80 (51.9)	30 (31.2)	66 (68.8)	1.00	0.524 (0.301–0.980)
DM						
No	89 (16.0)	74 (84.0)	125 (59.5)	85 (40.5)	1.00	1.364 (0.896–1.868)
Yes	52 (60.5)	34 (39.5)	20 (66.7)	10 (33.3)	1.00	0.692 (0.284–1.790)
Smoking						
No	59 (11.5)	28 (88.5)	105 (31.8)	49 (68.2)	1.00	0.423 (0.298–0.792)
Yes	82 (53.1)	80 (46.9)	40 (81.4)	46 (18.6)	1.00	0.920 (0.583–1.492)
No 3 risk	60 (85.7)	10 (14.3)	80 (74.1)	28 (25.9)	1.00	0.524 (0.301–1.162)

[a] Ajusted for age, sex, and the presence of diabetes, hypertension, and smoking status except the stratified factor at each stratum.

Table 7: The trans-phase analysis for genotypic combined effect in case and control groups.

Combined genotype[a]	Case	Control	χ^2	P	OR (95% CI)
rs174556 (C/C)-rs3756963 (T/T)	52	70	4.478	0.034	referent
rs174556 (C/C)-rs3756963 (C/T)	38	46	1.310	0.252	1.112 (0.635–1.946)
rs174556 (C/T)-rs3756963 (C/T)	30	34	0.482	0.487	1.188 (0.806–2.891)
rs174556 (C/T)-rs3756963 (T/T)	70	66	0.023	0.880	1.428 (0.873–2.335)
rs174556 (T/T)-rs3756963 (T/T)	21	9	4.656	0.031	3.141 (1.330–7.418)

Test of overall association: $\chi^2 = 14.112$, df = 6, $P = 0.028$.
[a] Only listed the combined genotypes with frequencies >3%.

Table 8: The logistic regression analysis for the relation between the risk factors and ACS.

Risk	P	OR value	95% CI
Hypertension	0.000	4.114	2.224–7.611
Smoking	0.000	2.444	1.563–3.823
Diabetes	0.035	2.243	1.052–4.798
rs174556 T allele	0.022	1.791	1.088–2.951

Smoking versus nonsmoking, hypertension versus nonhypertension, DM versus non-DM, rs174556 T allele versus rs174556 C allele.

et al. [35] failed to show relationship between rs174556 and MI in the Costa Rica Study. Firstly, this discrepancy may be due to racial differences in two studies. Secondly, the research objects are different. Finally, the difference of sample size also may be another possible reason.

FADS2 is located in chromosome 11 and expressed in almost all human tissues, especially in the liver, heart, and brain [36]. Some studies have report the metabolic effects of polymorphisms in this gene or their effects on the risk of CAD [24]. But the locus rs174617 of *FADS2* is rarely reported. In 2011, our previous study showed that this locus was not associated with CAD [37], which is consistent with our present study. The reason may be that a large proportion of regulatory SNPs, which affect gene expression, are located in the promoter regions [38].

At present, rs174537 near *FADS1* is considered to be the most relevant locus with ARA. GWAS in the In CHIANTI Study showed that minor allele homozygotes rs174537 (TT)

had lower plasma concentrations of ARA compared to major allele homozygotes [25]. And a study in Korea population confirmed that rs174537 T had lower proportions of ARA in serum phospholipids and reduced CAD risk [24]. Many studies suggested the minor allele carriers including rs174556 T may have lower desaturase activity [25, 37]. Therefore, based on above results, a possible causality link between lower desaturase activity and vascular disease has been suggested [39]. However, at present, the further research of gene function is still lacking.

ELOVL2 is a member of the mammalian microsomal *ELOVL* fatty acid enzyme family, involved in the elongation of very long-chain fatty acids including PUFAs required for various cellular functions in mammals [40]. A GWAS study found that an association of EPA with variants in the *FADS1* gene reached genome-wide significance level, and independent follow-up investigation showed associations of a selected *FADS1* variant with erythrocyte membrane levels of EPA, ALA, and DPA and of an *ELOVL2* variant with DPA and DHA [25]. These findings confirm an influence of *FADS1* and *ELOVL2* on selected n-3 PUFAs. In contrast, we do not found the association between rs3756593 of *ELOVL2* gene and ACS. Both allele and genotype analysis all have no statistical significance, but the trans-phase gene-gene interaction test revealed that the *ELOVL2* gene combined with *FADS1* gene had an effect on ACS. Above result implies that the *FADS1* gene-*ELOVL2* gene interaction may affect PUFA concentrations and afford susceptibility to ACS. But the exact mechanisms for the interaction are currently unknown.

In conclusion, this case-control study preliminary indicates that variations in *FADS1* may affect the risk of ACS and provide a genetic basis of molecular biology. We will continue to analyze the gene functional and the serum levels of phospholipids fatty acid in ACS.

Conflict of Interests

All authors have no conflict of interests.

Authors' Contribution

L. Qin and Y.-F. Jiang participated in the design and conduct of the study, data collection and analysis, data interpretation, and paper writing. Z.-K. Song and H.-Y. Cao participated in data collection and analysis. All authors read and approved the final paper. Z. Song contributed to this work.

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Research Article

The Use of Continuous Glucose Monitoring Combined with Computer-Based eMPC Algorithm for Tight Glucose Control in Cardiosurgical ICU

Petr Kopecký,[1] Miloš Mráz,[2] Jan Bláha,[1] Jaroslav Lindner,[3] Štěpán Svačina,[2] Roman Hovorka,[4] and Martin Haluzík[2]

[1] *Department of Anaesthesia, Resuscitation and Intensive Medicine, 1st Faculty of Medicine and General University Hospital, Charles University in Prague, U Nemocnice 2, 128 08 Prague 2, Czech Republic*
[2] *Third Department of Medicine, 1st Faculty of Medicine and General University Hospital, Charles University in Prague, U Nemocnice 1, 128 08 Prague 2, Czech Republic*
[3] *Department of Cardiac Surgery, 1st Faculty of Medicine and General University Hospital, Charles University in Prague, U Nemocnice 2, 128 08 Prague 2, Czech Republic*
[4] *Institute of Metabolic Science, University of Cambridge, Addenbrooke's Hospital, Box 289, Cambridge CB2 0QQ, UK*

Correspondence should be addressed to Martin Haluzík; mhalu@lf1.cuni.cz

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Aim. In postcardiac surgery patients, we assessed the performance of a system for intensive intravenous insulin therapy using continuous glucose monitoring (CGM) and enhanced model predictive control (eMPC) algorithm. *Methods*. Glucose control in eMPC-CGM group ($n = 12$) was compared with a control (C) group ($n = 12$) treated by intravenous insulin infusion adjusted according to eMPC protocol with a variable sampling interval alone. In the eMPC-CGM group glucose measured with a REAL-Time CGM system (Guardian RT) served as input for the eMPC adjusting insulin infusion every 15 minutes. The accuracy of CGM was evaluated hourly using reference arterial glucose and Clarke error-grid analysis (C-EGA). Target glucose range was 4.4–6.1 mmol/L. *Results*. Of the 277 paired CGM-reference glycemic values, 270 (97.5%) were in clinically acceptable zones of C-EGA and only 7 (2.5%) were in unacceptable D zone. Glucose control in eMPC-CGM group was comparable to C group in all measured values (average glycemia, percentage of time above, within, and below target range,). No episode of hypoglycemia (<2.9 mmol) occurred in eMPC-CGM group compared to 2 in C group. *Conclusion*. Our data show that the combination of eMPC algorithm with CGM is reliable and accurate enough to test this approach in a larger study population.

1. Introduction

Stress hyperglycemia (e.g., "diabetes of injury") is a common finding in critical care occurring in up to 90% of patients with critical illness [1, 2]. It is associated with increased morbidity and mortality and poorer prognosis of these patients [1–3]. In 2001, the landmark Leuven study performed in cardiosurgical intensive care unit (ICU) demonstrated that intensive insulin therapy (IIT) aimed at maintaining glycemia between 4.4 and 6.1 mmol/L reduced mortality and decreased frequency of severe organ complications [3]. Several other studies confirmed these findings especially in cardiac surgery patients [4]. However, some of the more recent trials questioned safety, reproducibility, and universality of beneficial effects of tight glycemic control (TGC) in other subgroups of critically ill patients [5–7], leading consequently to a shift towards a less intensive approach to glucose lowering in ICU settings in the last years.

Principally, the need to decrease pathologically elevated glycemia in critically ill subjects has been generally accepted, although the exact target range in various patient subgroups is subject of ongoing discussion [1]. Numerous protocols for IIT of variable effectiveness have been developed [1], with most-recently introduced computer-based predictive algorithms

showing significantly better performance with less adverse effects compared to standard paper-based protocols [8, 9]. The main complication limiting the use of TGC procedures is the occurrence of hypoglycemia, which was associated with increased risk of death and prolonged ICU stay in several studies [5–7]. As the response of ICU patients to hypoglycemia is often blunted [1], frequent glucose monitoring is an essential prerequisite of nearly all IIT algorithms. However, frequent blood sampling increases dramatically the workload of the nursing staff and the intermittent fashion, in which glucose values are obtained, may not always capture significant hypoglycemic events. Continuous glucose monitoring (CGM) could therefore be an ideal tool for catching rapid glycemic excursions (both hypo- and hyperglycemia) and providing the algorithm with (nearly) real-time glycemic data in order to improve its efficacy and safety.

To our knowledge only a minimum number of studies including CGM as input for TGC in the ICU and utilizing predominantly nonpredictive IIT protocols have been performed so far [10–13]. To this end, we performed a single-center randomized open-label trial using a combination of the established computer-based eMPC (enhanced model predictive control) algorithm with a standard system for continuous glucose monitoring Guardian REAL-Time CGMS (MiniMed Medtronic, Northridge, CA, USA). We evaluated the efficacy and safety of the combined system and compared it with the performance of the eMPC algorithm alone.

2. Research Design and Methods

2.1. Study Design and Subjects. The study was designed as a single-center open-label randomized trial. 24 adult patients (16 men and 6 women, aged 46 to 83 years, 5 patients with type 2 diabetes mellitus) undergoing major elective cardiac surgery (aortocoronary bypass or valvular plastic) were enrolled into the study. Twelve patients were randomized for intensive insulin treatment with the eMPC (enhanced model predictive control) protocol combined with continuous glucose measurement (eMPC-CGM group) and 12 were randomized for insulin treatment according to eMPC algorithm alone, which was routinely used at the Department of Cardiovascular Surgery, General University Hospital, Prague. Exclusion criteria were defined as follows: insulin allergy and inability to sign informed consent. Characteristics of both groups are shown in Table 1.

After patients' admission to the ICU the glucose sensor was inserted into the adipose tissue in the abdominal region and continuous glucose monitoring was started after run-in period of 1h 45 min. The insulin infusion in both groups of patients started 1 h 45 minutes (sensor run-in period in eMPC-CGM group) after patients' arrival at the ICU from the operating theater and lasted for 24 hours. No routine protocol was used for perioperative glucose control.

eMPC algorithm and continuous glucose measurement were implemented by the ICU nursing staff with supervision of an ICU physician as required. Protocol training was carried out by the ICU physician and a departmental nurse, usually individually, at bedside.

Table 1: Baseline characteristics of postcardiac surgery patients at the time of admission at ICU. Data are mean ± SEM.

	eMPC	eMPC-CGM
Number of patients (*n*)	12	12
Age (years)	67.5 ± 3.3	68.1 ± 2.2
Female (*n*)	4	6
Ethnicity: Caucasian (%)	100	100
BMI (kg/m^2)	27.8 ± 1.0	29.1 ± 0.8
Type of surgery (*n*):		
CABG	6	8
Valve replacement	4	3
CABG + valve replacement	2	1
History of diabetes (*n*)	4	2
Previous insulin treatment (*n*)	2	0
Arterial hypertension (*n*)	11	11
Dislipidemia (*n*)	2	6

2.2. Informed Consent. A written informed consent was signed by all participants before being enrolled into the study. The study was approved by the Human Ethical Review Committee, General University Hospital, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

2.3. Target Glucose Range. The target glucose range was set to 4.4 to 6.1 mmol/L, a level, which has been shown to reduce mortality and morbidity in cardiosurgical critically ill patients [3].

2.4. Patients' Examination. Clinical parameters and patients' clinical history data including age, sex, race, height, weight, BMI, history of diabetes and other chronic diseases, and type of surgery were collected prospectively.

2.5. Blood Glucose Monitoring, Insulin Treatment Regimens, and Nutrition. Blood glucose (BG) was monitored and insulin was administered according to each protocol rules/suggestions. Undiluted arterial blood for measurement of BG was drawn from an arterial line, inserted for routine monitoring procedures. Whole blood glucose was analyzed by a standard point-of-care testing device (ABL 700, Radiometer Medical, Copenhagen, Denmark).

Insulin (Actrapid HM, Novo Nordisk, Baegsvard, Denmark) was given into a central venous line as a continuous infusion in both groups. A standard concentration of 50 IU of insulin in 50 mL of 0.9% NaCl was used. In all patients, infusion of 10% glucose solution was initiated upon admission to ICU with a glucose dose of 2.5 g/kg of ideal body weight (height in centimeters minus 100) per hour and lasted for 18 hours, when normal oral food intake was started. In ventilated patients, the glucose infusion lasted longer than the monitored 24 hour.

Adverse events, medication, and nutrition were continuously monitored and documented.

2.6. Continuous Glucose Monitoring, eMPC Algorithm, and Their Combination. A real-time continuous glucose monitoring system, Guardian REAL-Time CGMS (MiniMed Medtronic, Northridge, CA, USA), was used for continuous glucose measurement. A subcutaneous glucose sensor was inserted under the skin in the abdominal region immediately after arriving in the ICU. The monitoring started after a run-in period of 1 h 45 min. Glucose was measured every 5 minutes and displayed on the monitoring unit. The system was calibrated using arterial blood glucose concentrations measured by a standard point-of-care testing device (ABL 700, Radiometer Medical A/S, Copenhagen, Denmark).

The enhanced model predictive (eMCP) algorithm used in this study was described in detail elsewhere [9, 14]. Glucose concentration, insulin dosage, and carbohydrate intake were the input variables for the eMPC and the output was the insulin infusion rate. The eMPC was implemented on a laptop computer. Control group was treated by this algorithm alone, while the variable sampling interval for the next blood glucose measurement calculated by the eMPC was respected. Arterial glucose was used as input for the eMPC. For a detailed description of the eMPC algorithm see the Appendix. The main computer interface of eMPC is also shown in Figure 3.

In the eMPC-CGM group data from CGM were entered manually into the eMPC every 15 minutes, while the variable sampling interval was not respected. Each hour glucose value from continuous glucose monitor was compared to reference arterial glucose using the Clarke error-grid analysis (C-EGA) [15] and when clinically unacceptable (zone C, D, or E of C-EGA) reference glycemia was used as input for the eMPC and to recalibrate the Guardian REAL-Time. When no additional calibration was needed, the sensor was calibrated every 12 hours as recommended by the manufacturer. In case of sensor failure (i.e., inability to calibrate) the study was interrupted and TGC was resumed using reference glycemia.

2.7. Outcome Measures. The performance of Guardian REAL-Time CGMS was evaluated using Clarke Error-Grid Analysis (C-EGA), a standard tool for assessing accuracy of glucose meters [15]. The number of additional recalibrations of each sensor and the number of sensor failures were recorded.

Endpoints for effectiveness assessment of the TGC protocols were as follows: entire study average glycemia level; time to the target range of 4.4–6.1 mmol/L (80–110 mg/dL); average blood glucose level after reaching the target range; time within, above and below the target range throughout the whole study period and after reaching the target range; number of hypoglycemic episodes (≤2.9 mmol/L). The percentages of time in the specific ranges were calculated as number of hours in the selected range in each patient/24 ∗ 100.

2.8. Statistical Analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, USA). The results are expressed as mean ± standard error of the mean (SEM). The TGC protocols were compared using Student's t-test or Mann-Whitney Rank Sum test as appropriate. Significance level was set at $P = 0.05$.

3. Results

Baseline characteristics of both study groups are listed in Table 1. The groups did not differ with respect to age, race, BMI, type of surgery, history of diabetes mellitus, and arterial hypertension. Baseline blood glucose was significantly higher in the eMPC-CGM group.

The performance of Guardian REAL-Time CGMS evaluated by C-EGA is shown in Figure 1. Of the 277 paired glucose values (values from the Guardian RT system and reference arterial glucose measured at the same time) obtained during the study, 270 (97.5%) were found in the acceptable A and B ranges of C-EGA (66.4% in A zone and 31.1% in B zone). Only 7 values (2.5%) were in the D zone with none of them being in the C and E zones.

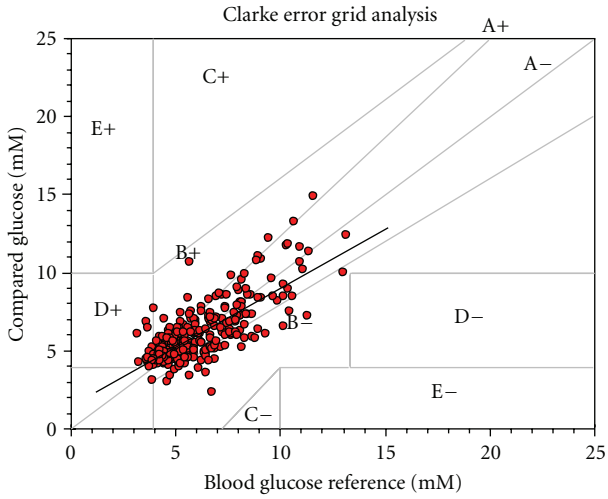
Of the 12 sensors used in the study (1 sensor for each patient), 6 needed no additional calibration except for the 2 obligatory ones (initially and after 12 hours). Of the other 6 sensors, 4 needed 1 extra recalibration, while the remaining 2 sensors had to be calibrated 3 or more times. One sensor failed after 21 hours, while the other 11 completed the designed 24-hour testing time.

Performance of both TGC approaches using blood glucose-based endpoints is summarized in Table 2, while absolute glucose values throughout the whole testing period for both groups are depicted in Figure 2. The eMPC-CGM protocol showed similar glucose control compared to eMPC group as assessed by average blood glucose (6.2 ± 0.1 versus 6.1 ± 0.6 mmol/L, n.s.) and time spent in and above the target range throughout the whole study (46.3 ± 5.5 versus 46.2 ± 6.5 and 40.6 ± 5.9 versus 38.4 ± 5.1% of time, resp., n.s.) and also after reaching the target range. Time below the target range tended to be shorter in the eMPC-CGM group (13.1 ± 2.6 versus 15.4 ± 2.4 and 18.8 ± 3.8 versus 22.2 ± 4.6% of time, resp., n.s.), but without any statistical significance. Two episodes of severe hypoglycemia defined as blood glucose equal or below 2.9 mmol/L were observed in the control group, while no such episode was recorded in patients treated with eMPC-CGM protocol. Both hypoglycemic episodes were classified as "asymptomatic" and were not related to established major risk factors of ICU hypoglycemia such as nutritional interruption, asynchrony of nutrition and insulin administration, delayed glucose measurement, or drug administration. The combination of eMPC and Guardian REAL-Time tended to be more efficient in reaching the target levels of 4.4–6.1 mmol/L (7.6±1.0 versus 8.8 ± 5.4 hours, n.s.).

4. Discussion

In the present study we tested the feasibility of a combination of an established computer-based protocol for tight glucose control (TGC) with a real-time continuous glucose monitoring system (CGM). This combination showed reasonable accuracy and reliability and resulted in similar glucose control as the computer-based algorithm alone.

Compared with diabetic patients, where the precision of various CGM systems has been extensively tested, much less data is available for individuals with critical illness. [16–19].



$y = 0.7546x + 1.3919$

$R^2 = 0.573$

(a)

	EG range	Number	(%)
Clinically acceptable zone	A+	80	28.9%
	A−	104	37.5%
	B+	37	13.4%
	B−	49	17.7%
Clinically unacceptable zone	C+	0	0%
	C−	0	0%
	D+	7	2.5%
	D−	0	0%
	E+	0	0%
	E−	0	0%
	Total	277	100%

(b)

Figure 1: Clarke Error-Grid Analysis of data from Guardian RT during the whole study period. Zone A (accurate), within 20% of reference values, zone B (benign erroneous), outside of 20%, but not leading to inappropriate treatment, zones A and B, clinically acceptable accuracy. Zone C (unnecessary correction), leading to overcorrection of acceptable glucose levels, zone D, potentially dangerous failure to detect hypo- or hyperglycemia, zone E (erroneous treatment), erroneous treatment of hypo- or hyperglycemia (for hypoglycemia in case of hyperglycemia and vice versa), zones C+D+E—clinically unacceptable.

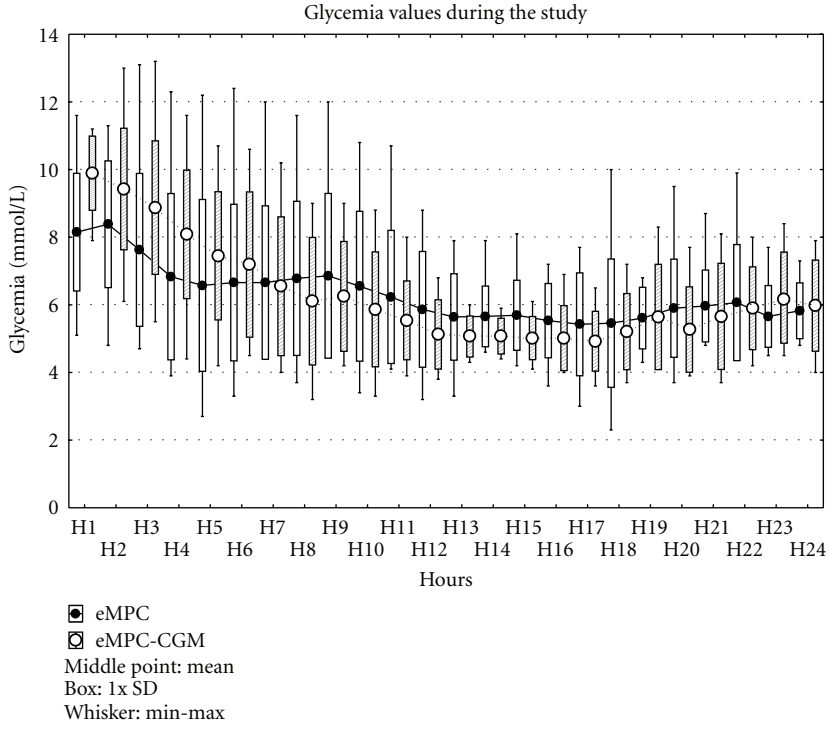


Figure 2: Glucose values in both groups throughout the study period. Values are means ± SD.

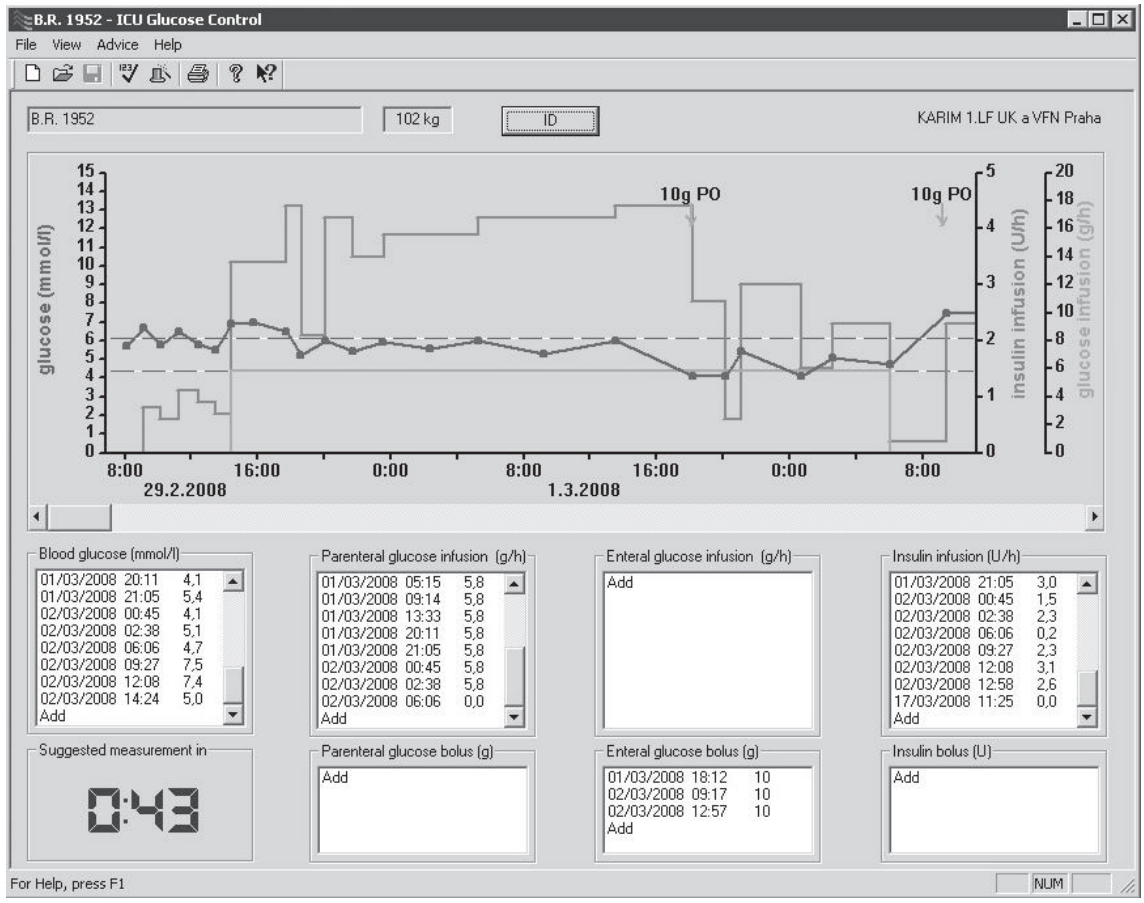


Figure 3

Table 2: The study blood glucose control data. Data are expressed as mean ± SEM. The percentages of time in the specific ranges were calculated as number of hours in the selected range in each patient/24 ∗ 100.

	eMPC	eMPC + Guardian	*P*
Baseline blood glucose	8.1 ± 0.6	9.9 ± 0.4	<0.05
The entire study blood glucose control data			
Average blood glucose (mmol/L)	6.1 ± 0.6	6.2 ± 0.1	n.s.
Time in target range (%)	46.2 ± 6.5	46.3 ± 5.5	n.s.
Time above target range (>6.1 mmol/L; %)	38.4 ± 5.1	40.6 ± 5.9	n.s.
Time below target range (<4.4 mmol/L; %)	15.4 ± 2.4	13.1 ± 2.6	n.s.
Severe hypoglycemia episodes (≤2.9 mmol/L)	2	0	
Blood glucose control data after reaching the target range (4.4–6.1 mmol/L)			
Average blood glucose (mmol/L)	5.2 ± 0.78	5.3 ± 0.1	n.s.
Time to target range (h)	8.8 ± 5.4	7.6 ± 1.0	n.s.
Time in target range (%)	62.8 ± 10.72	63.9 ± 5.4	n.s.
Time above target range (>6.1 mmol/L; %)	15.0 ± 8.6	17.3 ± 6.2	n.s.
Time below target range (<4.4 mmol/L; %)	22.2 ± 4.6	18.8 ± 3.8	n.s.
Severe hypoglycemia episodes (≤2.9 mmol/L)	2	0	

Moreover, most of the studies that evaluated the performance of subcutaneous sensor-based CGM systems in ICU settings yielded conflicting results, with several trials reporting unsatisfactory correlation of continuous and systemic glucose [20, 21], insufficient accuracy of continuous systems [22], or underestimated hypoglycemia [23], whereas in others CGM systems provided clinically reliable measurements and correlated tightly with reference glucose values [24–29]. The accuracy of Guardian REAL-Time CGMS in our study with 97.5% values in the acceptable range of C-EGA was comparable to most of the data collected in other ICU trials and in routine diabetic patients. Nevertheless, it has to be stressed that subjects included into our study were specifically admitted for elective cardiac surgery. Therefore we cannot make general conclusions with respect to sensor performance in different, possibly more severely ill populations. Guardian REAL-Time CGMS sensors showed high reliability with 10 sensors requiring 0 to 1 calibration in addition to the standard 2 calibrations in 24 hours specified by the manufacturer for diabetic patients. Only 1 sensor failed to complete the whole 24-hour testing period. The sensors were well tolerated with no major local complications (significant bleeding, infection, irritation, pain). No serious technical or operational problems were recorded during the study.

The eMPC algorithm proved its effectiveness in maintaining target glycemia in several clinical trials [9, 30–32]. In a study recently conducted in our surgical ICU the performance of eMPC using intermittent glucose values was compared to two other TGC algorithms—the Matias protocol, which uses absolute glucose values, and the Bath algorithm based on relative glucose change. The eMPC protocol demonstrated the highest efficacy in achieving and maintaining glucose in the target range without excessive risk of severe hypoglycemic events [32]. In the present study the performance of the eMPC algorithm corresponded largely to results obtained in previous trials.

Only few studies have tried to combine CGM with TGM algorithms. A system using retrospective CGMS in a real-time manner coupled with a sliding scale algorithm in a closed-loop fashion developed by Chee et al. did not show significantly better performance compared to manual control [10]. In the so far largest trial evaluating real-time CGM in the ICU settings, including 124 mechanically ventilated patients and using a routine Leuven-derived protocol governed either by Guardian REAL-Time or intermittent arterial glycemia, CGM did not improve the allover glycemic control (time spent in target range, time to target range), although it significantly reduced number of hypoglycemic events [12].

In this study the eMPC-CGM combination resulted in similar glucose control compared to the use of eMPC algorithm alone as assessed by no significant differences in average glycemia and percentage of time in or above target range. The combined system required less time to reach the target levels and patients in the eMPC-CGM group tended to spend less time under the target range compared to the control group, but also without statistical significance. However, considering higher baseline blood glucose in the eMPC-CGM study arm, the inclusion of a CGM device seems to at least partially improve the performance of the eMPC algorithm. Moreover, no severe hypoglycemia (≤ 2.9) was observed in the eMPC-CGM group compared to 2 episodes in the eMPC group. These findings are of major importance in the light of recent large multicentric studies aiming at tight glucose control, which were discontinued due to excessive risk of hypoglycemia—the Glucontrol and the VISEP study [5, 7]—and particularly the NICE-SUGAR trial, where intensive insulin treatment targeted at normal glycemic levels was associated with an increased risk of hypoglycemia and overall mortality [6]. A large meta-analysis including all important TGC trials further confirmed a causal relationship between hypoglycemia prevalence and increased mortality. [33]. Therefore, our combination of eMPC and CGM seems to offer promising opportunities to achieve TGC goals in a safer manner, that is, without excessive risk of hypoglycemic episodes.

We are aware of several limitations of our study. As this was a study intended mainly at testing the practical feasibility of the proposed approach, the number of subjects in each study arm was relatively low. The potential of continuous glucose monitoring might not have been completely exploited, as continuous values were inserted into the eMPC in 15-minute intervals, even though they were updated every 5 minutes. Furthermore, the low rate of hypoglycemic events could be attributed to the relatively high constant rate of glucose infusion administered throughout the study. A constant high rate glucose infusion is expected to accelerate glucose turnover and the overall system response [34]. It is still possible that the overall outcome of the study would differ under the condition of a lower parenteral glucose administration and the results thus cannot be generalized. Finally, despite the absence of any severe hypoglycemic episode, the relatively long period of time spent under the target range in the eMPC-CGM group (in spite of being shorter than in the control group) might be of some concern as well.

5. Conclusion

In conclusion, the results of our pilot feasibility trial indicate that a combination of the computer-based enhanced model predictive control algorithm with continuous glucose monitoring by Guardian REAL-Time CGMS in cardiac surgery patients is reliable, accurate, and efficient enough to test this approach in larger populations. This treatment strategy might represent a further step towards a fully automated closed-loop system for insulin delivery in the critically ill, providing a temporary solution until the so-far largely experimental intravenous continuous glucose sensors are generally available.

Appendix

The eMPC Algorithm

The eMPC includes a model of the glucoregulatory system, which adapts itself to the input-output relationship observed during tight glucose control; that is, an incoming glucose measurement is used by the model to update model

parameters such as insulin sensitivity taking into account previously given insulin and parenteral and enteral glucose. Once individualized to a critically ill subject, the eMPC uses the glucoregulatory model to determine the optimum insulin infusion rate which is expected to achieve the target glucose concentration. This is achieved by numerical optimization using simulated experiments with the individualized glucoregulatory model. The output of this optimization is a sequence of insulin infusion rates which are expected, based on model predictions, to result in the target glucose concentration over a period of 4 hours. The first insulin infusion rate is displayed to the user and recommended for the delivery. The determination of the time-to-next glucose sample utilizes prediction accuracy. Through an internal procedure, the eMPC estimates how accurately it is able to predict glucose concentration. The extent of accuracy will differ over time as the unexplained variability in glucose concentration varies due to, for example, temporal variations in insulin sensitivity. The estimated prediction accuracy is used by the eMPC to plot a prediction envelope. This is a funnel-like prediction shape indicating a range of possible glucose concentrations at each time point in the future. Once the prediction funnel crosses a border indicating nonacceptable bounds, this might be a level indicating a risk of hypoglycaemia or unacceptable hyperglycaemia, the eMPC suggests a sample to be taken.

Glucose concentration, insulin dosage, and carbohydrate intake are the input variables for the eMPC. The insulin infusion rate and the time of the next glucose sample are the outputs. The eMPC was implemented on a bedside PC terminal.

Conflict of Interests

P. Kopecký, M. Mráz, J. Bláha, J. Lindner, S. Svačina, and M. Haluzík have no conflict of interests to declare. R. Hovorka has received consultancy fees from BBraun.

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Research Article

Human Resistin Inhibits Myogenic Differentiation and Induces Insulin Resistance in Myocytes

Chun Hua Sheng,[1,2] Zhen Wu Du,[1] Yang Song,[1] Xiao Dong Wu,[1] Yu Cheng Zhang,[1] Mei Wu,[1] Qian Wang,[1] and Gui Zhen Zhang[1]

[1] *Department of Central Research, The Third Clinical College, Jilin University, Changchun, Jilin 130033, China*
[2] *Cell Transplantation Center, The 208th Hospital of Chinese People's Liberation Army, Changchun, Jilin 130021, China*

Correspondence should be addressed to Gui Zhen Zhang; zhangguizhenjlu@yahoo.com

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This study is aimed to investigate the effect of human resistin on myocyte differentiation and insulin resistance. The human resistin eukaryotic expression vector was stable transfected into C2C12 myocyte cells and was transiently transfected into COS7 cells. The effects of human resistin on cell proliferation, cell cycle, and myogenic differentiation of C2C12 cells were examined. Glucose uptake assays was performed on C2C12 myotubes by using [^{3}H] 2-deoxy-D-glucose. The mRNA levels of insulin receptor (IR) and glucose transporter 4 (GLUT4) were evaluated by semiquantitative RT-PCR. Results showed by the C2C12 cells transfected with human resistin gene compared with that without transfecting gene are as follows: (1) cell proliferation was significantly promoted, (2) after inducing differentiation, the myotube's diameters and expression of desmin and myoglobin decreased, and (3) glucose uptake ratio was lowered and expression of IR and GLUT4 decreased. However, there was no significant difference in the glucose uptake ratio between C2C12 myotubes treated with a human resistin conditioned medium of COS7 cells and treated with control medium. These results suggest that maybe human resistin has not a direct role on insulin sensitivity of myocytes. However, maybe it impaired the insulin sensitivity of myocytes through suppressing myogenesis and stimulating proliferation of myoblasts.

1. Introduction

Resistin is known as an adipocyte-specific secretory factor (ADSF) that belongs to a gene family found in the inflammatory zone (FIZZ) or found in the resistin-like molecule (RELM) [1]. Mostly researches reach consensus on the effect of resistin inducing insulin resistance in rodents [1–6]. However, it is controversial in the studies evaluating resistin expression related to type 2 diabetes in humans [7–11]. It is necessary to investigate the effects of human resistin (hR) on the target cells of insulin action. The skeletal muscle is a major tissue to take up and utilize glucose in vivo. Although it is argued that whether resistin is expressed in a skeletal muscle or not [1, 10, 12, 13], it impacts glycometabolism by endocrine, autocrine, and paracrine ways so that it is conceivable that effects of resistin might contribute to the pathogenesis of impaired insulin sensitivity, it is significant to elucidate the effect of human resistin on myocytes. However, some reports considered the activity of recombinant human resistin may be lower than endogenous resistin [14–16], so we constructed a human resistin eukaryotic expression vector. C2C12 myoblast is originated from a skeletal muscle of mouse and forms myotube after inducing differentiation. COS7 cells, as the African green kidney cell line, express SV40 large T antigen and support the replication of plasmid vector which contains SV40 ori (including PcDNA3.1) or some mutant SV40 to obtain high-level expression of exogenous genes. As a high-performance transient expression of eukaryotic systems, it is a commonly used tool to study gene function. In this study, human resistin eukaryotic expression vector were stably transfected into C2C12 myoblast and transiently

transfected into COS7 cells to examine the effect of hR on myocytes.

2. Materials and Methods

2.1. Plasmids' Construction. The pGEM-T human resistin (pGEM-T-hR) clone vector was kindly provided by professor Ying Sun (King's College London, London, UK). Human resistin (hR) gene was amplified by polymerase chain reaction (PCR) from the pGEM-T-hR clone vector. The primer sequences for hR amplification are as follow: forward primer: 5′-TCAGGT ACCATGGCCATGAAAGCTCTCTGTCTCCTC-3′; reverse primer: 5′-TCGGAATTCTCAGGGCTGCACACGACAGC-3′. Both primers include additional leader sequences that corresponded to the KpnI and EcoRI restriction enzymes, respectively, that can direct hR gene cloning into the pcDNA3.1 plasmids (Pc-3.1) (Invitrogen). HR cDNA generated by PCR was confirmed by the gene sequencing and inserted into plasmid Pc-3.1 by gene recombination technology. The hR gene of the construct was named pcDNA-hR (Pc-hR). Pc-3.1 plasmid was used as a control vector.

2.2. Cell Culture and Differentiation Assays. C2C12 and COS7 cells (American Type Culture Collection) were cultured in Dulbecco's minimal essential medium (DMEM) containing 25 mM glucose and 10% fetal bovine serum (FBS) (GIBCO), respectively. C2C12 cells were grown to 100% confluence with 10% FBS serum and then changed to 2% horse serum. By day 10, C2C12 cells were fused into myotubes.

2.3. Stable Transfection in C2C12 Cells. C2C12 cells were transfected with Pc-hR and Pc-3.1 plasmids by using Lipofectamine 2000 reagent (Invitrogen) following the protocol. 72 h after transfection, the medium was changed to the selection DMEM supplemented with 800 μg/mL G418 (GIBCO). Three weeks later, the cell clones were screened and further cultured in DMEM medium containing 400 mg/mL G418.

2.4. Transient Transfection in COS7 Cells. The Pc-3.1 and Pc-hR plasmids were transfected, respectively, into COS7 cells by using Lipofectamine 2000 reagent. 72 h after transfection, the medium of COS7 cells was collected, centrifuged at 500 ×g for 5 min, and stored at 4°C as conditioned medium of hR for less than a week before use.

2.5. Immunocytochemistry and Immunofluorescence Staining. The immunocytochemical detection of hR expression was carried out in C2C12 cells as previously described [17] using mouse anti-human resistin monoclonal antibody (R&D Systems, Inc., 1 : 50 dilution). The cells were visualized using a microscope (Olympus) and the immunoreactivity was identified as brown cytoplasmic staining in cytoplasm. Immunofluorescence staining was performed to identify the expression of desmin and myoglobin in C2C12 myotubes using monoclonal mouse antibody to desmin (Sigma-Aldrich, 1 : 50 dilution) and myoglobin (R&D Systems, Inc., 1 : 50 dilution) and goat anti-mouse Cy3-conjugated secondary antibody (R&D Systems, Inc., 1 : 200 dilution). The cells were visualized under a fluorescence confocal microscopy (OLYMPUS confocal microscope FV500). The grayscale value of desmin and myoglobin expression and the myotubes diameters were measured from randomly selected microscope fields from five different wells of Pc-3.1-transfected and Pc-hR-transfected C2C12 myotubes, respectively, and analyzed by image analytical system HPIAS-1000. At least five fields were selected and the diameter of 150 myotubes was measured per well.

2.6. Methyl Thiazolyl Tetrazolium (MTT) Assays to Determine Cell Growth. C2C12 cells transfected with Pc-3.1 and Pc-hR were seeded in 96-well tissue culture plates (2×10^3 cells/well) respectively. At the indicated time points, the assays were performed as the manufacturer's instruction.

2.7. Cell Cycle Analyses. C2C12 cells were cultured in culture flasks. After reaching 30% confluence, the cells were treated with DMEM without FBS for 24 h for synchronization. At the next day, cells were cultured with DMEM with 10% FBS. Forty-eight hours later, cells were trypsinized, fixed in 70% ethanol, and were analyzed by flow cytometry using a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

2.8. Glucose Uptake Assays. The measurements for glucose uptake were performed as described by Nakamori et al. [18]. Briefly, C2C12 myotubes (10 days after differentiation) transfected with pc-hR and pc-3.1 were grown in serum-free DMEM for 4 h and then incubated in the presence of insulin for 30 minutes at 37°C. Transport was started by adding 1 μCi [^{3}H]2-Deoxy-D-glucose (Amersham Pharmacia Biotech) in 1 mL of the Krebs-Ringer phosphate buffer for 10 min at 37°C and stopped by rapidly washing for three times with ice-cold phosphate-buffered solution (PBS). Cells were lysed in 0.4 mL PBS containing 0.1% Triton X-100 for 45 min. Aliquots of the cell lysates were used for liquid scintillation counting and determination of protein content by the Bradford method. Nonspecific transport was assayed in the presence of 10 μmol/L cytochalasin B (Sigma-Aldrich). In addition, C2C12 myotubes (10 days after differentiation) were incubated in conditioned medium of COS7 cells transfected with pc-3.1 and pc-hR, respectively, for 24 h. The cells were then changed to serum-free DMEM for 4 h and performed glucose uptake assays as above.

2.9. Semi Quantitative RT-PCR Analysis of mRNA Expression. Total RNA was extracted from cells with the use of TRIzol reagent (GIBCO). RNA (1 μg) was reverse transcribed using RevertAid H Minus M-MuL V reverse transcriptase (Helena Biosciences, Europe, Sunderland, UK) and random hexamers in 20 μL reaction volume, according to the manufacturer's instructions. The products (1 μL of cDNA) were subjected to PCR with Ex Taq and primers.

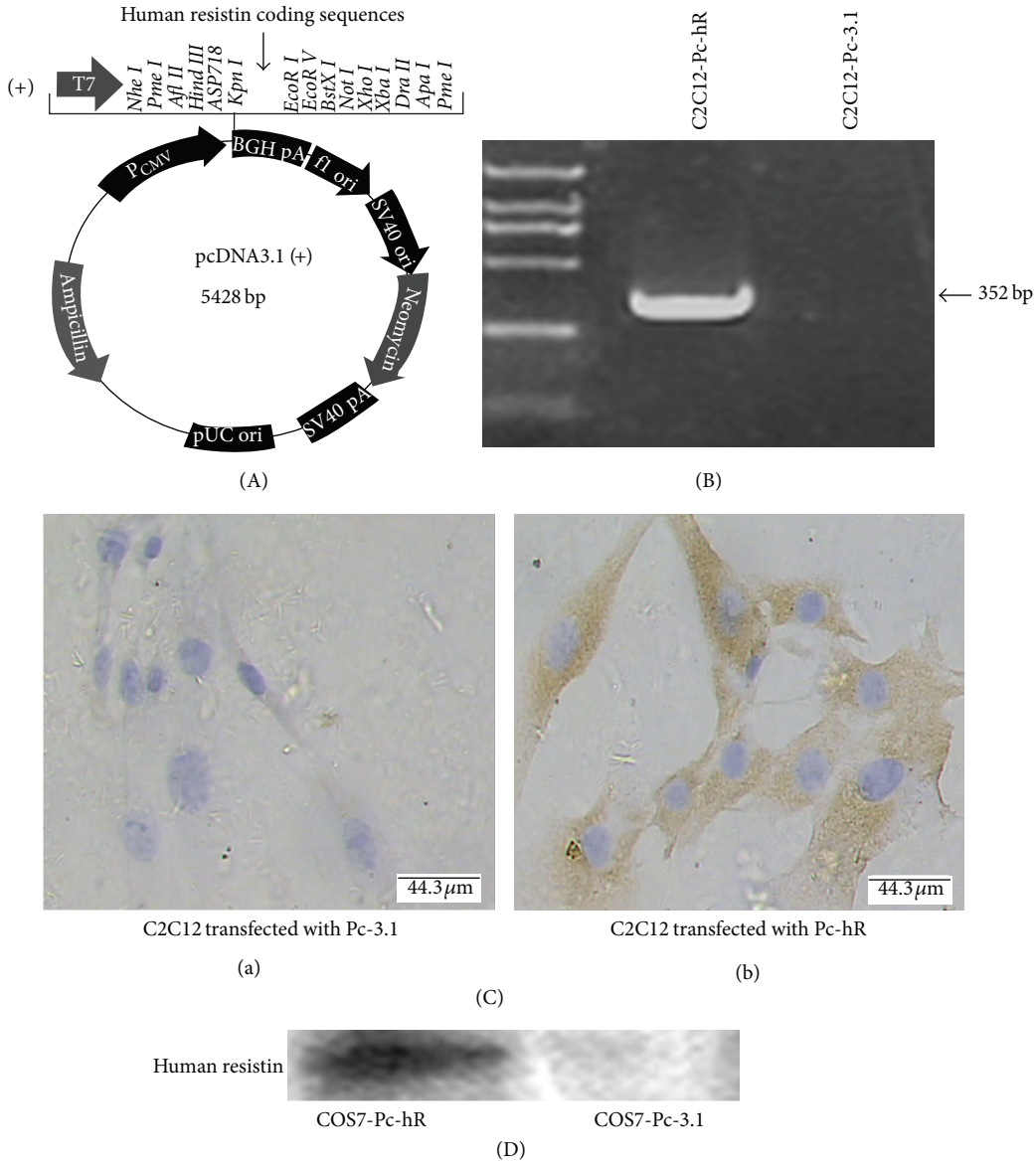

Figure 1: Construction of recombinant hR expression vector and transfection in cell lines. (A) A full length of hR coding sequence was inserted into PcDNA-3.1 (Pc-3.1). (B) The expression of hR mRNA was identified in C2C12 cells transfected with PcDNA-hR (Pc-hR). (C) Immunocytochemistry analysis showed hR immunoreactive protein in C2C12 cells transfected with Pc-hR. (D) Western blot analysis showed expression of hR in COS7 cells transfected with Pc-hR. C2C12-Pc-3.1: C2C12 cells transfected with Pc-3.1 plasmids; C2C12-Pc-hR: C2C12 cells transfected with Pc-hR plasmids.

Primer sequences are as follow: IR: forward primer: 5′-ATGGACATCCGGAACAACCT-3′; reverse primer: 5′-TTGATGACAGTGGCAGGACA-3′ (the product was 493 bp), GLUT4: forward primer: 5′-CAACGTGGCTGGGTAGGCA-3′; reverse primer: 5′-ACACATCAGCCCAGCCGGT-3′ (the product was 587 bp), β-actin 1: forward primer: GATGGTGGGTATGGGTCAGAAGGA; reverse primer: GCTCATTGCCGATAGTGATGACCT (the product was 632 bp), and β-actin 2: forward primer: 5′-GGGACCTGACAGACTACCT-3′; reverse primer: 5′-CAGGATTCCATACCCAAG-3′ (the product was 268 bp). The PCR products were separated by electrophoresis on agarose gel, visualized by ethidium bromide staining, and quantitated with Gel Image Systems. The abundance of each specific mRNA was normalized on the basis of that of β-actin mRNA.

2.10. Western Blot Analysis. Cell supernatants of Cos7 cells transfected with Pc-3.1 and Pc-hR plasmids were collected and treated by western blot 72 h after transfection as described [19]. In brief, the proteins were subjected to SDS polyacrylamide gel electrophoresis, electroblotted onto Polyvinylidene fluoride (PVDF) membrane (Millipore), and immunodetected using mouse anti-human resistin monoclonal antibody and goat anti-mouse IgG horseradish peroxidase conjugate.

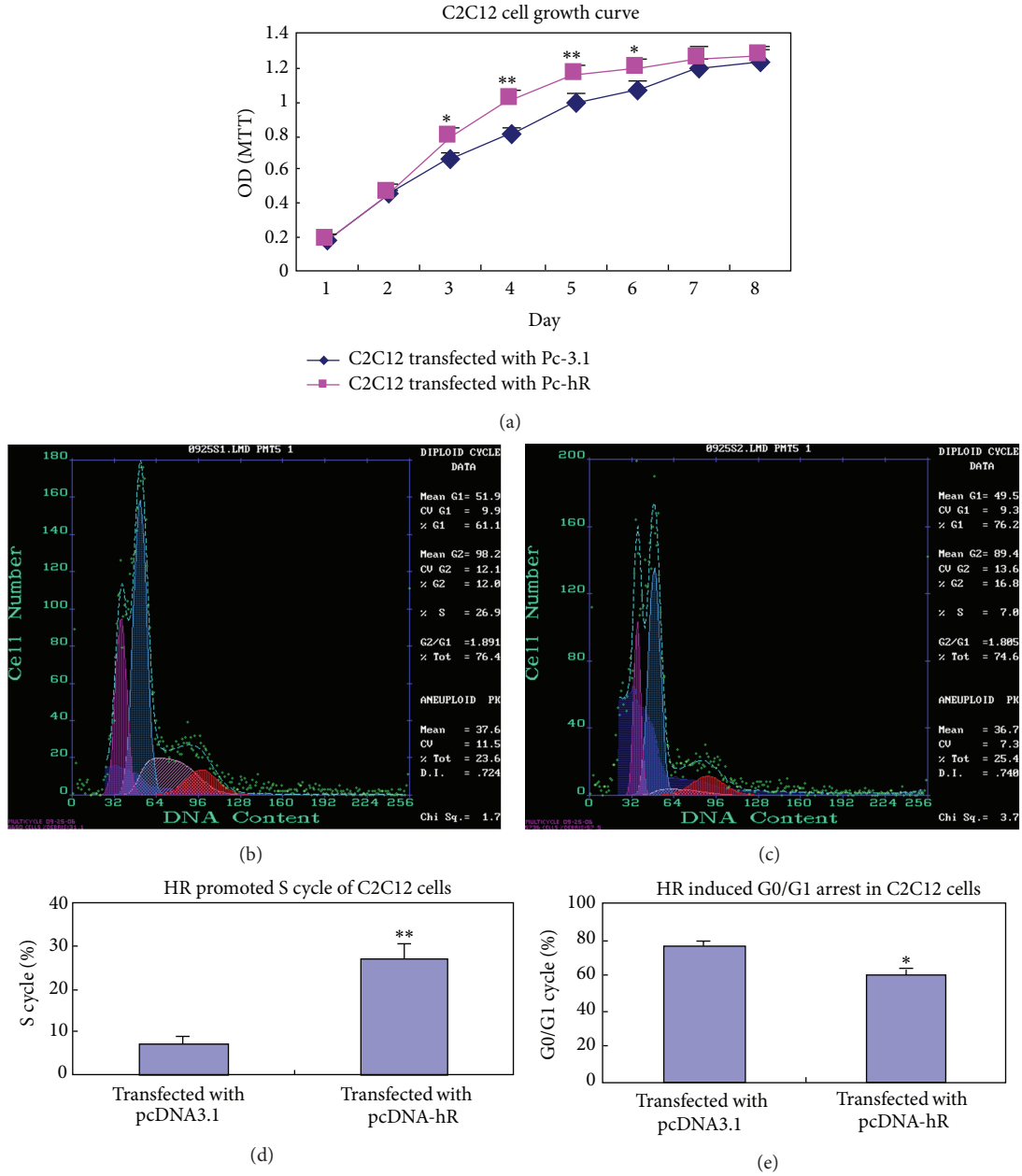

FIGURE 2: Effects of hR on proliferation and cell cycle of the C2C12 cells. (a) MTT showing significant increased absorbance of C2C12 cells transfected with Pc-hR compared with the control cells transfected with Pc-3.1. (b) Flow cytometry showing the cell cycle of C2C12 cells transfected with Pc-hR. (c) Flow cytometry showing cell cycle of C2C12 cells transfected with Pc-3.1. (d) Analysis of the percentages of S cycle. (e) Analysis of the percentages of G0/G1 cycle. Results are expressed as the means ± SEM of six measurements. $^{*}P < 0.05$; $^{**}P < 0.01$.

2.11. Statistical Analysis. Statistical analyses were carried out using SPSS (SPSS Inc. 12.0, Woking, UK) software. All qualitative data are representative of at least three independent experiments, with at least four wells per group per experiment. Quantitative data are presented as means ± SEM and were compared with Student's t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Construction of Recombinant Human Resistin (hR) Expression Vector and Transfection into Cell Lines. The full length of cDNA encoding hR coding sequences (327 bp) was cloned into PcDNA3.1 (Pc-3.1) vectors (Figure 1(A)). The recombinant plasmid PcDNA-hR (Pc-hR) and the control plasmid Pc-3.1 were stably transfected into C2C12 cells and transiently transfected into COS7 cells. The expression of recombinant hR at mRNA and protein levels in C2C12 cells were identified by RT-PCR and immunocytochemistry respectively. Expression of hR mRNA and protein was observed in C2C12 cells transfected with Pc-hR plasmids (Figures 1(B) and 1(C-b)), but was not observed in C2C12 cells transfected with Pc-3.1 plasmids (Figures 1(B) and 1(C-a)). The expression of hR in COS7 cells was identified by Western blot (Figure 1(D)). HR

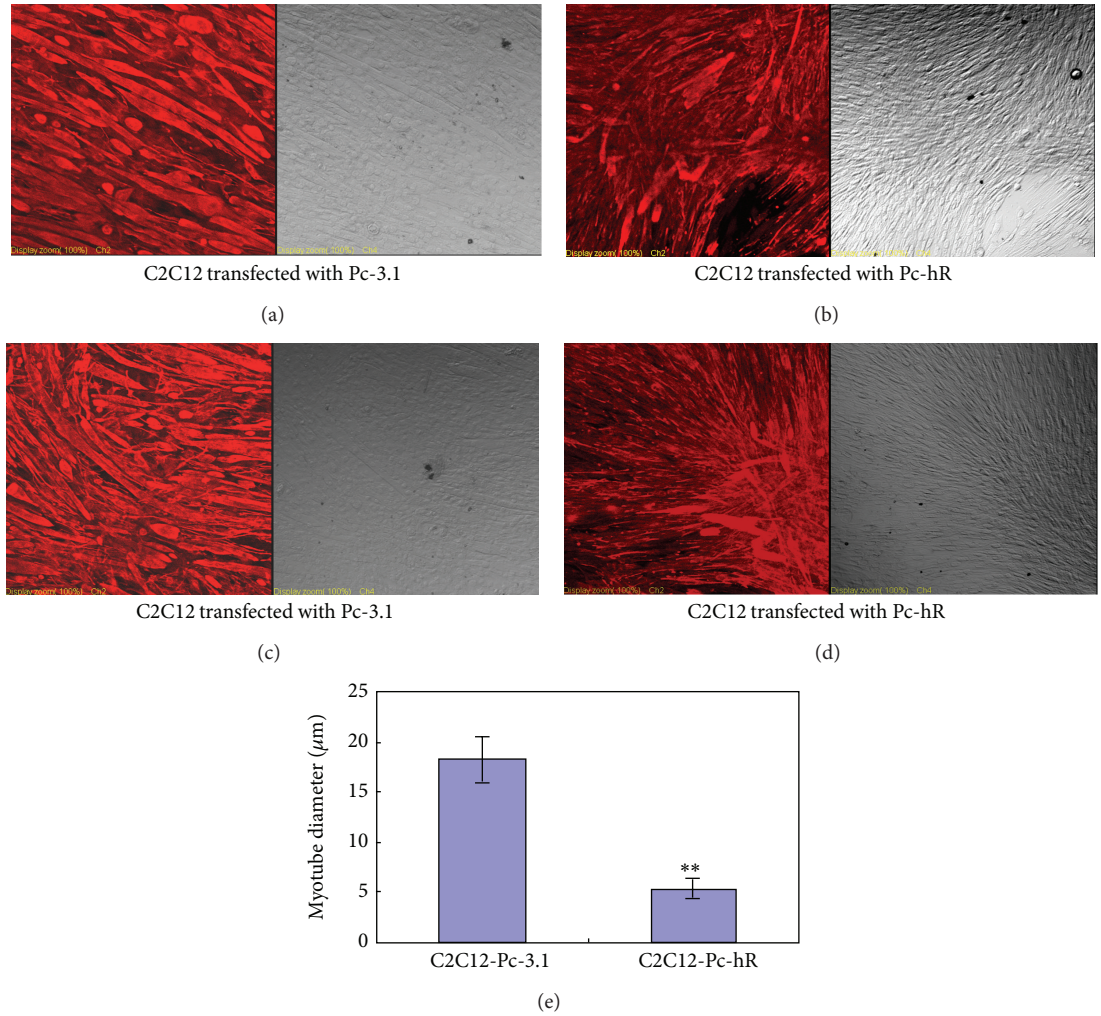

Figure 3: Effects of hR on myogenic differentiation of C2C12 cells (immunofluorescence staining). ((a) and (b)) The expression of desmin in C2C12 myotubes transfected with Pc-3.1 and transfected with Pc-hR vectors (magnification 200x). ((c) and (d)) The expression of myoglobin in C2C12 myotubes transfected with Pc-3.1 and transfected with Pc-hR vectors (magnification 200x). (e) Analysis of myotubes diameter. $^{**}P < 0.01$.

expressed significantly in COS7 cells transfected with Pc-hR whereas not expressed in control cells.

3.2. Effects of hR on Proliferation and Cell Cycle of C2C12 Cells. MTT assays were performed to determine cell proliferation. Compared with the control, proliferation of C2C12 cells transfected with Pc-hR was significantly promoted (Figure 2(a)). Overexpression of hR also significantly induced the S cycle accumulation in C2C12 cells. There were 26.9% of the S phase cells in C2C12 cells transfected with Pc-hR versus 7.0% in the control groups ($P < 0.01$) (Figures 2(b), 2(c), and 2(d)). At the same time, overexpression of hR significantly reduced the ratio of G0/G1 cycle in C2C12 cells transfected with Pc-hR (61.1% versus 76.2% in the control) ($P < 0.01$) (Figures 2(b), 2(c), and 2(e)).

3.3. Effects of hR on Myogenic Differentiation of C2C12 Cells. Desmin is a muscle-specific intermediate filament protein which is expressed in both smooth and striated muscles. It and myoglobin are important markers of myogenic differentiation. Immunofluorescence staining was performed to detect the expression of desmin and myoglobin. Compared with the control cells transfected with Pc-3.1, expression of desmin and myoglobin decreased significantly in Pc-hR-transfected C2C12 myotubes (Figures 3(a), 3(b), 3(c), and 3(d)). HR also decreased the diameters of myotubes ($P < 0.01$) (Figure 3(e)). These data suggest that hR inhibited myogenic differentiation of C2C12 myoblasts.

3.4. Effects of hR on Glucose Uptake and Expression of Relevant Genes in C2C12 Myotubes. Data showed that insulin-stimulated glucose uptake in C2C12 myotubes transfected with Pc-hR was significantly decreased as compared with controls (Figure 4(a)). To further investigate the mechanisms, we examined the mRNA expression of IR and GLUT4 of C2C12 myotubes transfected with Pc-3.1 and Pc-hR plasmids.

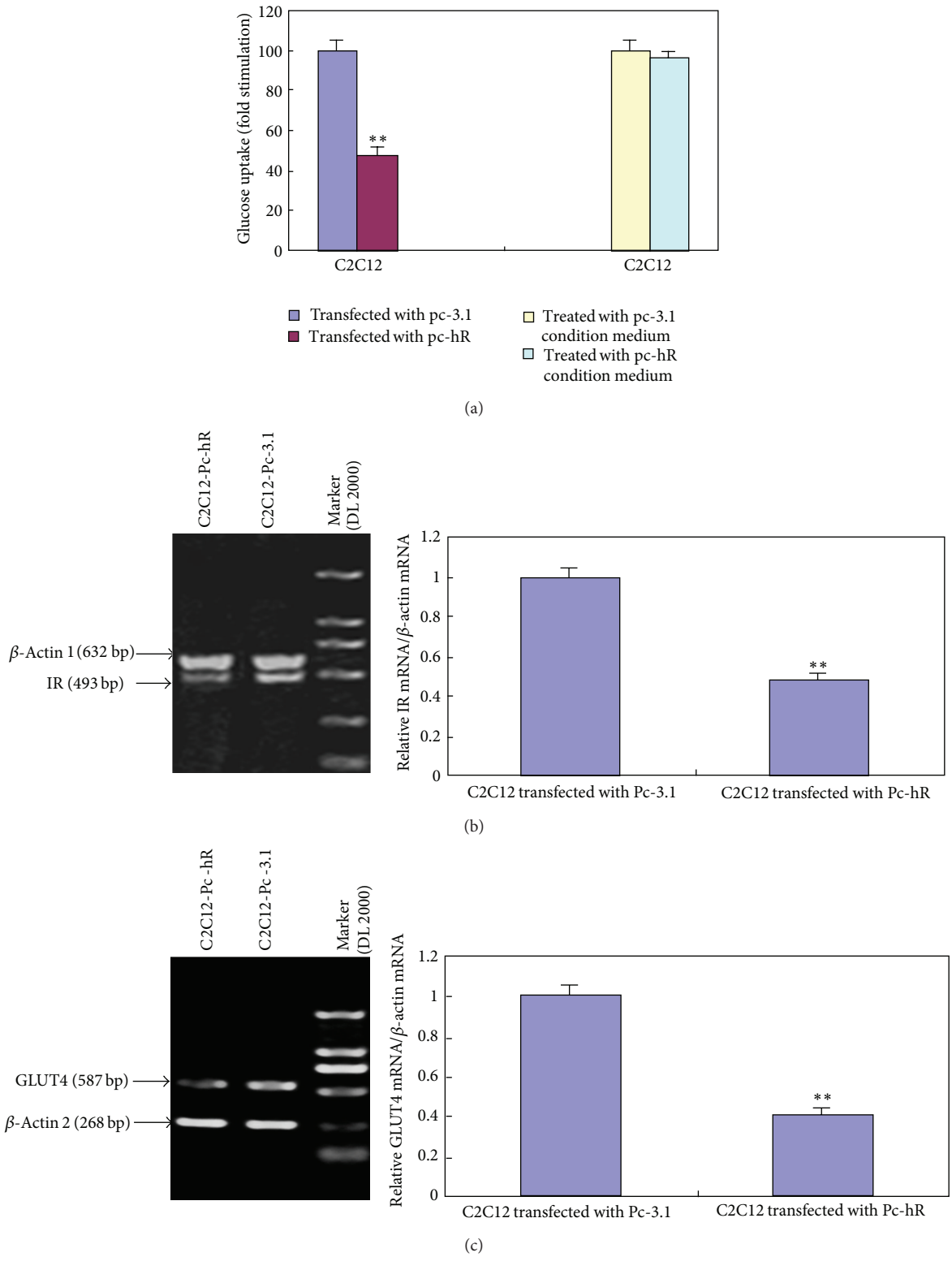

FIGURE 4: Effects of hR on glucose uptake and expression of relevant genes in C2C12 myotubes. (a) The effects of hR on glucose uptake in C2C12 myotubes. (b) Expression of insulin receptor (IR). (c) Expression of glucose transporter 4 (GLUT4). The amount of each target mRNA was normalized by the amount of β-actin mRNA and was expressed relative to the abundance of the target mRNA in cells transfected with Pc-3.1. $^{**}P < 0.01$.

Semiquantitative RT-PCR analysis showed that expression of IR and GLUT4 mRNA decreased significantly in C2C12 myotubes transfected with Pc-hR as compared with the controls ($P < 0.01$) (Figures 4(b) and 4(c)).

To observe whether hR has a direct effect on insulin sensitivity of myocytes, condition mediums of COS7 cells transfected with Pc-3.1 and Pc-hR were used to culture C2C12 myotubes. The result showed that there was no significant difference in glucose uptake between the myotubes treated with Pc-hR condition medium and the myotubes treated with the Pc-3.1 condition medium (Figure 4(a)).

4. Discussion

Since the discovery of resistin in 2001, there has been controversy on the role of resistin on glucose metabolism of skeletal muscles. It was reported not only that the resistin had no effect on insulin sensitivity of skeletal muscles [2, 20] but also that the resistin impaired insulin sensitivity of myocytes [3, 21, 22]. However, in the studies where resistin induced insulin resistance in myocytes, its mechanism was different or even contradictory. Fan et al. [3] and Palanivel et al. [21] thought that resistin impaired insulin sensitivity of rat skeletal muscle cells (differentiated from L6 myoblasts) by the inhibition of PI-3K insulin signal transduction pathway. Moon et al. [22] and his colleagues transfected recombined mouse resistin vector in L6 cells and the glucose uptake decreased significantly whereas PI-3K insulin signal transduction pathway was not affected. So they presumed that resistin degraded intrinsic activity of GLUTs. However, it was not mentioned whether resistin affects proliferation and differentiation of L6 myoblasts in these studies.

In the current study, we chose C2C12 myotubes as a cell culture model to test whether resistin can induce insulin resistance in muscle cells. C2C12 cells are immortalized cell lines from the C3H mouse skeletal muscle satellite cells, often used as the model of the study of the development and differentiation of muscle lineage [23], and insulin resistance is readily induced in this cell model with palmitate [24]. In order to observe the effect of resistin on muscle cell differentiation for a long time, resistin gene was expressed in C2C12 cells by the stable transfection technology so that it can offer the continuing effect of resistin on C2C12 cells. As well as to observe the direct effect of resistin on C2C12 myotubes, resistin gene was expressed in COS7 cells by the transient transfection technology so that it can offer the resistin protein direct effect on C2C12 myotubes.

That resistin inhibits adipogenic differentiation has been reported [5, 12, 14]. However, to our knowledge, its effect on myogenic differentiation has not been reported, but it was reported that Fizz1, which also belongs to Fizz family inhibited myogenesis [25]. Our data showed that hR dramatically stimulated the proliferation and increase of S phase cells whereas decreased G0/G1 phase cells. It also decreased diameter of myotubes and suppressed the expression of myogenic markers including desmin and myoglobin. These results indicate that resistin inhibits myogenesis and promotes proliferation of immature myocytes. Compared with the control cells, the glucose uptake and expression of IR and GLUT4 were dramatically decreased in C2C12 myotubes. However, compared with the controls, hR condition medium did not affect glucose uptake of C2C12 myotubes; nevertheless, it inhibits myogenesis and promotes proliferation of immature myocytes so that the population of mature myocytes and expression of IR and GLUT4 cutdown, which led to less glucose uptake.

In a word, our results demonstrated for the first time that human resistin acted on cell cycle of myoblasts, inhibited myogenic differentiation, and promoted proliferation of myoblasts, which may be relevant to the glucose metabolic disorder.

Author's Contribution

Dr Zhen Wu Du and Chun Hua Sheng contributed equally to this work that Dr Zhen Wu Du and Chun Hua Sheng is owned first author.

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Research Article

Mulberry Leaf Reduces Oxidation and C-Reactive Protein Level in Patients with Mild Dyslipidemia

Pornanong Aramwit,[1] Ouppatham Supasyndh,[2] Tippawan Siritienthong,[1] and Nipaporn Bang[1]

[1] *Bioactive Resources for Innovative Clinical Applications Research Unit, and Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, PhayaThai Road, Phatumwan, Bangkok 10330, Thailand*
[2] *Division of Nephrology, Department of Medicine, Phramongkutklao Hospital and College of Medicine, Bangkok 10400, Thailand*

Correspondence should be addressed to Pornanong Aramwit; aramwit@gmail.com

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C-reactive protein (CRP) is the inflammatory marker that could represent the inflammation in blood vessels resulted from dyslipidemia. The objective of this study was to evaluate the antioxidative activity of mulberry leaf powder using DPPH assay and the effect of mulberry leaf powder on lipid profile, CRP level, and antioxidative parameters in mild dyslipidemia patients. A within-subjects design was conducted and patients received three tablets of 280 mg mulberry leaf powder three times a day before meals for 12 weeks. Total of 25 patients were enrolled but one subject was excluded. After three months of mulberry leaf consumption, serum triglyceride and low-density lipoprotein (LDL) level were significantly reduced and more than half of all patients' CRP levels decreased every month as well as the mean CRP level but no statistically significant difference was found. The average erythrocyte glutathione peroxidase activity of patients was increased but not at significant level; however, the mean serum 8-isoprostane level was significantly lower after mulberry treatment for 12 weeks. It can be concluded that mulberry leaf powder exhibited antioxidant activity and mulberry leaf powder has potential to decrease serum triglyceride, LDL, and CRP levels in mild dyslipidemia patients without causing severe adverse reactions.

1. Introduction

Mulberries (*Morus alba* L., Moraceae) have been widely used in traditional Oriental medicine for several applications including prevention of diabetes [1]. It contains various nutritional components such as flavonoids and polyphenols, especially 1-deoxynojirimycin (DNJ), a potent glucosidase inhibitor, which shows hypoglycemic [2], hypolipidemic [3], and antiatherogenic effects [4, 5] in certain animal models. Shibata et al. [6] reported that mulberry leaf-derived aqueous fractions inhibit tumor necrosis factor-α-induced nuclear factor κB activation and lectin-like oxidized low-density lipoprotein receptor-1 expression in vascular endothelial cells. Harauma et al. [5] also found that mulberry leaf powder can prevent atherosclerosis in apolipoprotein E-deficient mice. Our earlier research also found that mulberry leaf powder was effective in reducing lipid profile in mild hyperlipidemia patients [3]. Even though mulberry leaf powder seems to have several advantages in cardiovascular diseases, very few studies have been investigated in human subjects.

Dyslipidemia with high serum cholesterol, both total cholesterol and low-density lipoprotein (LDL), and low high-density lipoprotein (HDL), is a critical cardiovascular risk factor [7, 8]. It is now established that oxidation of LDL constitutes a key event in inflammation and atherogenesis [9]. Mechanisms of LDL oxidation involve concerted modification by oxidants produced by arterial wall cells, such as reactive nitrogen species, hydroxyl radicals, and lipid-soluble free radicals [10]. Because most mechanisms involve the oxidation process, antioxidants may be useful in preventing endothelium blood vessel related to atherosclerosis.

There is extensive evidence that link hypercholesterolemia with increased lipid peroxidation and increased oxidative stress [11, 12]. The oxidative modification of lipoproteins, particularly LDL, has emerged as a fundamental process

in the development of atherosclerosis [13]. Oxidative stress due to increased reactive oxygen species (ROS) generation and unbalanced oxidative/antioxidative equilibrium are also implicated in the development of coronary arteriosclerotic cardiovascular disease especially in patients with hyperlipidemia [14]. Glutathione peroxidase (Gpx) is considered as one of the primary defense systems which eliminate excess ROS and maintain equilibrium between oxidative and antioxidative activity under normal physiological conditions [15] while 8-isoprostane, the final esterified product of oxidized arachidonic acid, seems to be a good marker of oxidative injury and is considered a gold standard for measuring oxidative stress *in vivo*.

C-reactive protein (CRP), generated from the liver, is a protein in the kind of acute phase reactant. It will respond immediately after the rising of nonspecific inflammation in the blood vessel [16, 17]. CRP has been identified in human atherosclerotic lesions and has been hypothesized to mediate endothelial dysfunction through induction of endothelial adhesion molecules, tissue factor, and proinflammatory cytokine synthesis [18]. Accordingly, CRP in blood can reflect the process involved with inflammation and it is a strong independent predictor of future peripheral arterial disease [19], myocardial infarction, and stroke among apparently healthy men and women [20]. As a marker of inflammation, CRP measurement has been recommended in cardiovascular risk stratification and clinical treatment guidelines, additional to traditional cardiovascular risk assessment [21, 22]. When combined with lipid screening, CRP improves global risk prediction in patients who would not be identified for primary prevention of cardiovascular events by lipid assessment alone [23].

The purposes of this study are to evaluate the antioxidant property of mulberry leaf-derived aqueous fraction *in vitro*; the effect of mulberry leaf on the serum lipid profile, erythrocyte glutathione peroxidase activity, and 8-isoprostane, and the effect of mulberry leaf in reducing the CRP levels in patients with mild dyslipidemia.

2. Materials and Methods

2.1. Materials. Mulberry leaf powder and tablets were derived from Kitayamakit Co. Ltd. (Kyoto, Japan). They were produced from chemical-free mulberry leaves. Each tablet weighed 280 mg and contained 254.8 mg mulberry leaf powder, which had 0.367 mg of the active ingredient DNJ.

2.2. Methods

2.2.1. Analysis of Antioxidant Activity. Antioxidant activity of mulberry leaf was determined by free radical scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [24]. Mulberry leaf powder (0.3 g) was extracted in 3 mL of hot water (95°C) with continuous shaking for 30 minutes. After getting cool at room temperature, it was centrifuged at 5,000 rpm for 20 minutes then filtered through 0.45 μm filter. The supernatant obtained was considered as undiluted mulberry leaf extract (100%). Serial dilution of undiluted extract was performed in order to obtain extract at 50, 25, 12.5, and 6.25%, respectively. Then 100 μL of different concentrations of mulberry leaf extracts were mixed with 150 μL of 0.1 mM DPPH methanolic solution. The samples were shaken vigorously and allowed to stand for 20 minutes at room temperature. A control was prepared without any sample and purified water was used for baseline correction. The decrease in the absorbance of the formed blue to violet reagent was determined after 20 minutes at 550 nm and the percentage inhibition activity was calculated from the following equation:

$$\left(\frac{(A_0 - A_1)}{A_0}\right) \times 100, \quad (1)$$

where A_0 = Absorbance of the control and A_1 = Absorbance of the extract/standard.

Experiments were carried out 5 times. Data were expressed as the half maximal inhibitory concentration (IC_{50}), which is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed.

2.2.2. Subjects and Study Design. A within-subjects research design was conducted at the outpatient internal medicine clinic, Phramongkutklao Hospital, Thailand. All patients were screened at the beginning of the study. Eligibility criteria for entry into the study included the following: (1) age between 20 and 60, (2) met the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) criteria for dyslipidemia [9, 25], (3) must have serum LDL level in the range of ≥ 140 and < 190 mg/dL and fasting plasma glucose < 126 mg/dL, (4) should not have more than one major cardiovascular disease (CVD) risk factor according to NCEP ATP III guidelines, (5) must not be receiving lipid lowering drugs except for diet control, only diuretics were allowed for patients with hypertension and their blood pressure had to be controlled at level < 140/90 mmHg, and (6) should have liver enzymes (alanine aminotransferase and aspartate aminotransferase) < 40 U/L and blood urea nitrogen < 20 mg/dL, with serum creatinine in the range of 0.6–1.2 mg/dL. The patients with the following criteria were excluded from the study: (1) patients needing to receive lipid lowering drugs according to NCEP ATP III guidelines and if they had a high risk factor for CVD or equivalent, (2) had a Framingham risk score greater than 20%, (3) had abnormal liver or renal function tests, (4) had severe complications or had been admitted to the hospital for cardiovascular events in the three months prior to enrollment in this study, and (5) patients with cancer and those who were pregnant or breastfeeding. Written informed consent was obtained from all study participants after a thorough discussion of the protocol, its rationale, and potential risks. The protocol was approved by the Ethics Committee of the Institutional Review Board of Phramongkutklao Hospital.

Prior to the enrollment, all subjects were asked about underlying diseases, current medications, and personal profile. On the first visit of the screening period, blood samples

were collected from all patients and a dietician advised them during the four-week period of diet control. This advice included the diet consumed, food exchange, and a diet that was appropriate for each patient, and they were carefully instructed on how to record their total oral intake using household measures. Each patient was requested to make a three-day food record by recording all of the food and beverages that they consumed over two working days and one day of the weekend. Blood samples were collected to determine the lipid profile after four weeks of diet control. Patients who reached the target lipid profile after diet control were withdrawn from the study while patients who could not reach the target according to NCEP ATP III guidelines continued in this study and the lipid profile before receiving mulberry leaf tablet therapy was examined. All included subjects were assigned to receive three tablets of 280 mg mulberry leaf powder three times a day before meals, the dose which has been proven to reduce the LDL in mild dyslipidemia patients [3], for 12 weeks. CRP measured by high-sensitivity methods was used in order to measure low levels of CRP more accurately. Briefly, blood samples were completely coagulated and centrifuged as serum and then stored frozen at −80°C. The CRP levels were determined by particle-enhanced immunonephelometry. The serum sample of 200 μL was diluted and reacted with CRP reagent (suspension of polystyrene particles coated with mouse monoclonal antibodies to CRP) to form an immune complex. A beam of light at the wavelength of 840 nm was then passed through the sample; the amount of light scattering was measured by photodetector. The intensity of the scattered light was proportional to the concentration of the relevant protein in the samples. The result was evaluated by comparing with a standard of known concentration. Routine blood analyses including lipid parameters, CRP, fasting plasma glucose, and liver function tests of all subjects were performed every four weeks. Clinical evaluation for side effects and pill counts to determine compliance were also performed every four weeks at the follow-up visits. If total pill count indicated more than 20% of mulberry leaf tablets untaken, subjects were excluded from the study.

Glutathione peroxidases (Gpx) was measured by the modified method of Paglia and Valentine [26] as described by Jacobson et al. [27]. The rate of glutathione oxidation was measured by monitoring the disappearance of $NADPH+H^+$ in the reaction medium (decrease of absorbance at 340 nm), since $NADPH+H^+$ was consumed for the reduction of oxidized glutathione by glutathione reductase. 8-isoprostane concentrations were measured in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA). The detection limit was 5 pg/mL and the intraassay and interassay variabilities were 6 and 7%, respectively.

2.2.3. Statistical Analysis. Results were expressed as mean and standard deviations. The *t*-test was used to evaluate the difference between pre- and posttreatment. Repeat-measures one-way ANOVA by Bonferroni was used to test the differences in CRP levels in each period. A $P < 0.05$ was considered statistically significant.

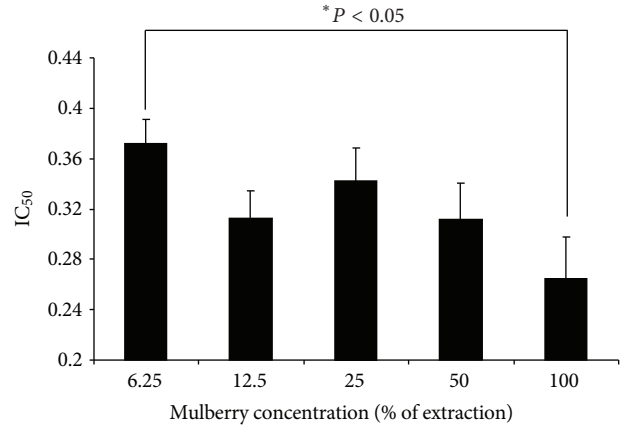

Figure 1: IC_{50} of mulberry leaf extracts at room temperature.

Table 1: Patient demographic characteristics and baseline laboratory data ($n = 25$).

Characteristics	
Gender (M : F)	5 : 20
Age (years)	35.88 ± 10.87
Body mass index (kg/m^2)	23.18 ± 3.12
Fasting plasma glucose (mg/dL)	92.14 ± 6.72
Alanine aminotransferase (U/L)	18.42 ± 3.25
Aspartate aminotransferase (U/L)	17.44 ± 3.62
Serum creatinine (mg/dL)	0.62 ± 0.04
Blood urea nitrogen (mg/dL)	11.79 ± 2.73
CVD risk factor (none : 1 risk)	21 : 5

3. Results

Free radical scavenging activities at room temperature from mulberry leaf extracts, indicated as IC_{50}, are shown in Figure 1. The results showed that mulberry leaf extracted by hot water exhibits good antioxidant activity. Undiluted mulberry leaf extracts exhibit the highest free radical scavenging activities (the lowest IC_{50} value) while the most diluted mulberry leaf extracts show the lowest free radical scavenging activities. The concentration of mulberry leaf extracts correlates well with their antioxidant activities. There is a significant difference in IC_{50} of 100% and 6.25% mulberry leaf extract.

Twenty-five subjects have enrolled in the study. Table 1 summarizes the demographic characteristics and baseline laboratory data of all participants in this study. None of the participants had underlying disease or current medications. The mean age was 35.88 years with the majority being female. Generally, it can be concluded that the subjects with mild dyslipidemia and having normal body weight were at low risk of coronary heart disease.

Most subjects complied with their medication regimens. The percentage of compliance ranged from 89.63 to 98.52% with the mean of 95.00%. One subject was eliminated from the study since she discontinued her lifestyle modification by consuming a high fat diet routinely after starting the

Table 2: Serum lipid profile in patients with mild dyslipidemia after 12 weeks of treatment ($n = 24$).

	Mulberry leaf tablet therapy		
	Baseline	Week 12	Change (%)
Total cholesterol (mg/dL)	216.3 ± 6.1	211.5 ± 4.3	−1.8
Triglyceride (mg/dL)	114.2 ± 8.7	86.2 ± 7.4	−10.6*
Low-density lipoprotein (mg/dL)	163.7 ± 5.1	151.2 ± 3.8	−8.2*
High-density lipoprotein (mg/dL)	41.5 ± 3.4	45.3 ± 1.9	+6.3
TC : HDL	5.6 ± 0.2	3.1 ± 0.3	−6.4*

TC: total cholesterol, HDL: high-density lipoprotein.
*$P < 0.05$ compared with the baseline.

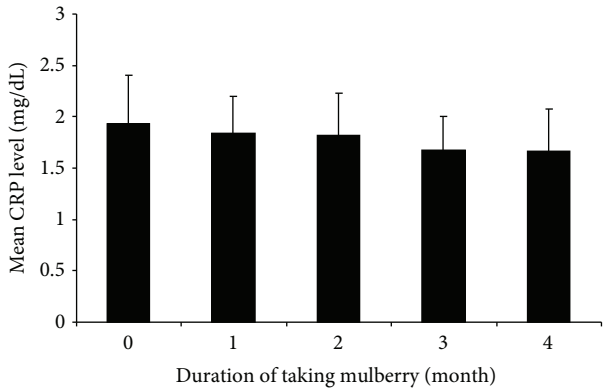

Figure 2: Mean CRP level of subjects in each month ($n = 24$).

mulberry leaf tablet regimen. Thus, her lipid level could increase and affect the CRP level to be higher than usual.

Table 2 represents the serum lipid profile in patients with mild dyslipidemia after 12 weeks of treatment with mulberry leaf. After 12 weeks of treatment, serum triglyceride and LDL level were significantly reduced by approximately 10.6% and 8.2%, respectively ($P < 0.05$), from baseline, while diet control did not improve lipid profile. Moreover, the HDL was increased by 6.3% even though no significant difference was found.

According to the monthly CRP blood level, more than half of all patients' CRP levels decreased every month compared to the CRP level before the intake of mulberry leaf tablets as indicated in Table 3. The mean CRP level of subjects also decreased every month as shown in Figure 2. After three months of mulberry leaf tablet consumption, 16 patients (66.67%) had lower CRP levels compared to baseline after lifestyle modification. However, there is no statistically significant difference of CRP level between each month.

The average erythrocyte glutathione peroxidase activity of patients at baseline (after diet control) was 12.16 ± 3.54 U/g Hb while this parameter increased to 14.22 ± 2.86 U/g Hb after mulberry treatment for 12 weeks but no significant difference was found. The level of 8-isoprostane showed the same trend; however, the significance difference between

Table 3: Mean difference of CRP level and number of patients whose CRP level decreased comparing to baseline (month 0, after diet therapy) ($n = 24$).

Duration	Mean difference of CRP level (mg/L) ± SD	Number of patients with decreased CRP (%)
Month 1–month 0	−0.0175 ± 0.898	14 (58.33)
Month 2–month 0	−0.1606 ± 1.201	14 (58.33)
Month 3–month 0	−0.1753 ± 1.180	16 (66.67)

Table 4: Incidence of adverse reactions from the mulberry leaf powder treatment ($n = 25$).

Adverse reactions	Number of patients (%)
Gastrointestinal	
Flatulence	1 (4)
Diarrhea	6 (24)
Constipation	1 (4)
Miscellaneous	
Increase appetite	2 (8)
Dizziness	2 (8)

baseline and after mulberry treatment was found. At baseline the average 8-isoprostane level was 563.12 ± 14.16 pg/mL and the value decreased to 244.68 ± 22.17 pg/mL after mulberry treatment.

Twelve adverse reactions from mulberry leaf tablet consumption were observed. Details are shown in Table 4. Most common adverse reaction was diarrhea, which could occur on the first day of the mulberry consumption. However, the diarrhea, as well as other symptoms, was considered as minor and the patients were able to well tolerate mulberry leaf consumption after one week. All conditions disappeared after the patients followed the advice by taking mulberry leaf tablets immediately after meals. No severe adverse reaction was found in this study.

4. Discussion

Our present study indicated that mulberry leaf tablet therapy is more effective than diet control alone for controlling lipid profile in mild dyslipidemia patients as shown by a significant fall in serum triglycerides and LDL as well as total cholesterol/HDL ratio. It also showed a rise in HDL in all patients and it is well known that improving the lipid profile potentially reduces the risk of major cardiovascular events.

C-reactive protein can be used as a marker of acute inflammation and it also has been widely used for monitoring disease activity in cardiovascular disease and diabetes [28–30], which emphasizes the likely role of chronic inflammation in the aetiology. C-reactive protein also rises with vascular insufficiency and damage of most types, which includes acute myocardial injury or infarction, stroke, and peripheral vascular compromise. Elevation of the CRP level has predictive value for an increased risk of an acute coronary event compared to very low CRP levels [31]. Regarding

to gender, women normally have significantly higher CRP levels than men [32] and stronger correlation was found in women between the association of obesity and CRP level [33]. Our results indicated that mulberry powder extracted exhibited strong antioxidant property, which is similar to results found by others [5, 34]. Even though earlier report showed that mulberry leaf extracted by water had very little effect on cell cycle progression and had less 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities compared to mulberry leaf extracted by methanol or butanol [35], it still shows strong free radical scavenging activity *in vitro*. From our result, it could be stated that lifestyle modification can prevent more inflammation in blood vessel that occurs from dyslipidemia resulting in the decreased CRP levels especially when the intake of the mulberry leaf tablet was included. We found that CRP mean level in subjects receiving mulberry leaf tablets had a tendency to decrease every month, especially after 12 weeks of mulberry leaf consumption. It could be stated that mulberry leaf tablet consumption can prevent more inflammation of blood vessels that occurs from dyslipidemia. However, the difference of CRP levels in each month was not statistically significant which may be due to a small number of the participants and the initial CRP levels in all subjects were rather low. Even though most of subjects in this study were female who has high tendency of elevated CRP, most subjects were considered as normal (healthy weight) according to their body mass index resulting in small-elevated CRP. Despite its high sensitivity, it has only 19-hour half-life which might have caused deviation [36]. However, according to patient interviews, there was no illness, inflammation or infection, injury, or medication use during the study period, which could relate to their particular CRP levels.

Maximum CRP levels > 3 mg/dL had positive predictive values > 20% for proven or probable early-onset infections or inflammation [37]. Only 5 out of 25 patients in our study had CRP levels higher than 3 mg/dL at initial stage with no acute phase reaction of inflammation or infection reported in their patient profiles and their CRP levels after 3 months of mulberry consuming reduced significantly in all patients and the levels are within normal range (data not shown). In month 3, the average CRP levels decreased only slightly, possibly due to the fact that most subjects had mild dyslipidemia with low risk of coronary heart disease and their initial CRP levels were rather low. The intake of mulberry leaf tablet could, thereby, reduce CRP levels to a certain extent.

Taking other factors into consideration, one patient who took oral contraceptives during the research participation exhibited a high initial CRP level. This might be due to the estrogen/progestogen hormone use. After changing her lifestyle and consuming mulberry leaf tablets, her CRP level continued to decrease and reduced by 77% in the third month (data not shown). This might, therefore, be an indication that the decrease in CRP level will be obvious when the patient's initial CRP level was high.

Glutathione peroxidase activity is a key antioxidant enzyme within most cells including endothelial cells. Due to its important role in the prevention of oxidative stress, it is considered to be an antiatherogenic enzyme [38]. Guo et al. [39] showed that reduced expression of Gpx resulted in an increase of cell-mediated oxidation of LDL in mice. In human, the lack of Gpx activity in atherosclerotic lesions appeared to be associated with the development of more severe lesions [40]. Our results indicate that mulberry leaf enhances the antioxidant activity in human by increasing Gpx activity even though no significant difference between before and after mulberry consumption was found which may possibly due to; again, most subjects had only mild dyslipidemia with no severe life-threatening condition.

The isoprostanes are a unique series of prostaglandin-like compounds formed *in vivo* via a nonenzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid [41]. 8-isoprostane has been focused due to its stability, specificity for lipid peroxidation, and relative abundance in biological fluid [42]. In this present study, the mean serum 8-isoprostane level was significant lower after mulberry treatment for 12 weeks despite that its initial serum level at the baseline was much higher. This observation may verify the notion that mulberry leaves possess antioxidative properties in clinical application.

During this study, there was no severe adverse reaction found. A minor diarrhea occurred, however, possibly due to the fact that mulberry leaf tablets are rich in dietary fiber, which could stimulate defecation. Accordingly, constipation is also possible when there is insufficient water in the bowels' contents. An active ingredient in mulberry leaf, DNJ, also acts as alpha-glucosidase inhibitor, which prevents disaccharide digestion, thereby causing gastrointestinal side effects such as flatulence or abdominal distention. Moreover, mulberry-induced reduction in blood glucose might increase appetite or cause dizziness.

5. Conclusions

Extraction of mulberry leaf powder by hot water exhibited strong antioxidative activity. This research also reveals the tendency of mulberry leaf powder in reducing serum LDL and triglyceride as well as blood vessel inflammation stemmed from dyslipidemia, by the measurement of decreased CRP levels. Moreover, mulberry leaf powder can increase the erythrocyte glutathione peroxidase activity and decrease 8-isoprostane in serum. No severe adverse reaction was found and minor side effects can be relieved by taking mulberry leaf tablets immediately after meals.

Conflict of Interests

All authors have indicated that they have no financial/commercial conflict of interests regarding the content of this paper.

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Research Article

Possible Role of Hyperinsulinemia and Insulin Resistance in Lower Vitamin D Levels in Overweight and Obese Patients

Giovanni De Pergola,[1] Alessandro Nitti,[1] Nicola Bartolomeo,[2] Antonella Gesuita,[1] Vito Angelo Giagulli,[3] Vincenzo Triggiani,[4] Edoardo Guastamacchia,[4] and Franco Silvestris[1]

[1] *Clinical Nutrition Unit, Department of Biomedical Sciences and Human Oncology, Section of Clinical Oncology, School of Medicine, University of Bari, Policlinico, Piazza Giulio Cesare 11, 70124 Bari, Italy*
[2] *Department of Biomedical Sciences and Human Oncology, School of Medicine, University of Bari, Policlinico, Piazza Giulio Cesare, 70124 Bari, Italy*
[3] *Outpatient Clinic for Metabolic Diseases and Endocrinology, Conversano Hospital, ASL Bari, 70014 Conversano, Italy*
[4] *Section of Endocrinology and Metabolic Diseases, Department of Emergency and Organ Transplantation, School of Medicine, University of Bari, Policlinico, Piazza Giulio Cesare, 70124 Bari, Italy*

Correspondence should be addressed to Giovanni De Pergola; gdepergola@libero.it

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A cohort of 66 healthy overweight and obese patients, 53 women and 13 men were examined. Waist circumference and fasting 25(OH)D, insulin, glucose, lipid (cholesterol, HDL cholesterol, and triglyceride), C-reactive protein (CRP), and complement 3 (C_3), and 4 (C_4) serum concentrations were measured. Insulin resistance was assessed by the homeostasis model assessment ($HOMA_{IR}$). *Results.* 25(OH)D levels showed a significant negative correlation with BMI ($P < 0.01$), waist circumference ($P < 0.05$), fasting insulin ($P < 0.01$), $HOMA_{IR}$ ($P < 0.01$), triglycerides ($P < 0.01$), CRP ($P < 0.01$), C_3 ($P < 0.05$), and C_4 ($P < 0.05$). Multiple regression analyses were performed with 25(OH)D as the dependent variable and BMI (or waist circumferences), fasting insulin (or $HOMA_{IR}$), triglycerides, and CRP (or C_3 or C_4) as independent variables. Only insulin or $HOMA_{IR}$ maintained a significant independent association with 25(OH)D levels, whereas vitamin D did not maintain a significant independent association with CRP or C_3 or C_4 concentrations. *Conclusions.* The present study, performed in overweight and obese subjects, shows that 25(OH)D levels are negatively associated with inflammatory parameters such as CRP and C_3 and C_4 levels, but not independently of BMI, body fat distribution, insulin levels, or insulin resistance. Our results suggest that hyperinsulinemia and/or insulin resistance are directly responsible for decrease of 25(OH)D levels in obesity.

1. Introduction

Vitamin D is well known to be involved in the calcium and bone metabolism, and most observational and randomized placebo-controlled trials concerning this vitamin have focused on the skeletal effects and have linked low vitamin D levels to fractures [1–3]. However, interest is growing in the nonskeletal effects of vitamin D [4] and in the relationship between vitamin D deficiency and diseases such as obesity, diabetes mellitus, and metabolic syndrome.

Serum 25-hydroxyvitamin D3 (25(OH)D) level reflects total vitamin D from dietary intake and sunlight exposure, as well as the conversion of vitamin D from adipose stores in the liver. Therefore, it is the best indicator of overall vitamin D status [5].

Several studies have demonstrated that serum 25(OH)D concentrations are inversely correlated with measures of obesity such as body mass index (BMI), waist circumference, and subcutaneous and visceral fat [6–10].

Insulin resistance and hyperinsulinemia are typical features of abdominal obesity, and, interestingly, inverse associations between 25(OH)D levels and fasting insulin concentrations [11–13] or insulin resistance (measured by $HOMA_{IR}$ index) [11–13] or insulin sensitivity index [14, 15] have been

reported in most of the studies, even independently of BMI or obesity [13].

Some cross-sectional studies indicate that low vitamin D is also associated with higher serum levels of inflammatory biomarkers, such as C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) in either healthy [16–19] or obese subjects [20]. However, the relationship between vitamin D and inflammation is not fully clear. In fact, other studies have not confirmed an inverse relationship between vitamin D and inflammation parameters in normal weight [21, 22] and obese patients [23]. Moreover, when healthy overweight subjects participating in a weight-reduction program were supplemented with vitamin D (3332 IU cholecalciferol/day for twelve months), they did not show a decrease in serum CRP and IL-6 and concentration [24]. Lastly, the National Health and Nutrition Examination Survey (2001 to 2006) observed a significant independent inverse relation between 25(OH)D and CRP levels only when 25(OH)D concentrations were lower than 21 ng/mL [25].

Obesity is characterized by a condition of low-grade chronic inflammation, and it is well known that adipocytes express TNF-α and may produce 30%–40% of circulating levels of IL-6 [26], the main regulator of CRP production in the liver. It is noteworthy that low-grade chronic inflammation is responsible for the activation of the complement system, a situation that could contribute to the metabolic complications observed in obesity. No study has investigated the relationship between 25(OH)D and complement protein circulating levels.

The complement is a system of proteins functionally interacting with each other in order to provide many of the effector functions of humoral immunity and inflammation. The central component of the complement system is the C_3 fraction, as all the pathways for the activation of the system converge there. Interestingly, adipocytes are an important source of C_3 production, in addition to that produced in the liver and in activated macrophages. Adipose tissue also produces all the factors of the alternative pathway for the complement activation [27], and human adipocytes express the genes that code the proteins activating the complement system [28]. A positive correlation was found between baseline insulin levels and C_3 levels [29], and serum C_3 has been considered as an inflammatory marker of insulin resistance [30]. Moreover, we recently found a direct association between waist circumference and C_3 serum concentrations in overweight and obese patients, independently of insulin resistance [31].

To the best of our knowledge, no study has simultaneously evaluated vitamin D, insulin, insulin resistance, CRP, and C_3 and C_4 serum levels in a population of healthy obese subjects. Therefore, the present study was addressed to examine whether CRP and C_3 and C_4 serum levels are associated with vitamin D concentrations in overweight and obese patients, independently of BMI, body fat distribution, and insulin levels, all parameters well known to influence vitamin D concentrations. To this aim, a cohort of 66 overweight and obese patients, aged 18–55 years, were investigated.

2. Methods

2.1. Subject Population. The patients were consecutively enrolled at the Outpatient Clinic of Clinical Nutrition, Section of Clinical Oncology, Department of Internal Medicine and Clinical Oncology, University of Bari, School of Medicine.

Concerning the inclusion criteria, subjects were recruited at the first medical examination whether they showed a BMI higher than 25.0 and were not using any drug (including oral contraceptives for premenopausal women and hormone replacement therapy for postmenopausal women). They came to the Outpatient Clinic with the aim of losing weight and/or to have dietetic and lifestyle suggestions. Subjects were defined overweight whether they had a BMI between 25.0 and 29.9 kg/m^2 and obese whether they had a BMI $\geq$ 30 kg/m^2.

Patients with the following disorders were excluded: endocrinological diseases, diabetes mellitus, chronic inflammatory diseases, stable hypertension, angina pectoris, heart infarction, congenital heart disease, stroke, and transient ischemic attack. Thus, the study enrolled 66 patients, including 53 women and 13 men, aged 18–55 years.

All subjects gave their informed consent for the study, which was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

All patients had fasting blood glucose levels lower than 126 mg/dL, and they were judged in good health on the basis of physical examination, medical history, routine blood work, urinalysis, and electrocardiogram. None of the patients was on hypocaloric diet or had been involved into intensive or competitive physical activity prior to the enrollment. During the testing period, all subjects were asked to keep their normal mixed diet and not to perform any sporting activity. The day before the measurement, they were abstained from both caffeinated and alcoholic drinks but maintained their normal diet.

2.1.1. Anthropometric Measurements and General Data. Body weight was measured to the nearest kg. Height was determined to the nearest cm. BMI was calculated as the weight (kg) divided by the square of height (m). Waist circumference was measured at the narrowest part of the abdomen, that is, at the natural indentation between the 10th rib and the iliac crest (minimum waist).

2.1.2. Hormone and Metabolic Parameters. Blood samples were drawn between 08:00 h and 09:00 h after an overnight fast. Serum insulin concentrations were measured by radioimmunoassay (Behring, Scoppito, Italy), and intra- and interassay coefficients of variation were 3.7% and 7.5%, respectively. Plasma 25(OH)D was quantified by a chemiluminescence method (Diasorin Inc., Stillwater, USA), and all samples were analyzed in duplicate. The assay had intraclass CV of 5.3%–6.7% and interclass CV of 4.6%–8.7%.

The C_3 and C_4 fractions of the complement were determined by immunonephelometry (Behring Nephelometer II (BNII); Dade-Behring, Marburg, Germany). Intra- and interassay coefficients of variation were lower than 6%. CRP was measured by a particle-enhanced turbidimetric

immunoassay (PETIA) technique (Siemens Healthcare Diagnostic Inc., Newark, DE, USA). Intra- and interassay coefficients of variation were lower than 8%. It is noteworthy that this assay meets the recommendations of the American Heart Association and the Centers for Disease Control (2003) for determining patients at high risk for cardiovascular disease [32].

Plasma glucose levels were determined by the glucose-oxidase method (Sclavo, Siena, Italy). Plasma lipids (triglycerides, total cholesterol, and HDL cholesterol) were determined by an automatic colorimetric method (Hitachi; Boehringer Mannheim, Mannheim, Germany).

Insulin resistance was assessed by using the homeostasis model assessment ($HOMA_{IR}$) [33].

2.2. Statistics. The sample characteristics were expressed by mean and standard deviation of the measured parameters. Variables with a skewed distribution were transformed before any statistical analyses, to improve the approximation to a Gaussian distribution: insulin, $HOMA_{IR}$, BMI, triglycerides, CRP, and C_4 (positively skewed) were transformed into their logarithms, while C_3 (negatively skewed) was elevated to the square. Several univariate linear regression analyses were performed to test the joint effect of different variables on plasma 25(OH)D levels, and, for each model, the assumptions of linearity, the normality of the residuals (Shapiro-Wilk test), the homoskedasticity (Breusch-Pagan test), and the absence of serial correlation (Durbin-Watson value) were verified; the measures of relationships were also evaluated by Pearson's correlation coefficient. The variables having a P value <0.05 in univariate t-test were selected for inclusion in multivariate linear regression model analyses. Concerning the power analysis, based on Type III F test of one predictor adjusting for the other three predictors (excluding the intercept) in a regression model with a significance level of 0.05, assuming an unconditional model with normal predictors, a sample size of 66 (i.e., the number of patients examined in the present study) has a power of 0.797. Statistical and power analyses were performed using SAS software Version 9.3 for PC.

3. Results

Table 1 shows the general, anthropometric, hormone, and metabolic parameters in the study population.

Table 2 shows the relationship between 25(OH)D levels and general, anthropometric, hormone, and metabolic parameters. Variables significantly predictive of 25(OH)D levels in univariate linear regression models were BMI, waist circumference, fasting insulin, $HOMA_{IR}$, triglycerides, CRP, C_3 and C_4.

Concerning multiple regression analyses, a first analysis was performed by considering 25(OH)D as the dependent variable and BMI, fasting CRP, insulin (or $HOMA_{IR}$), and triglycerides as independent variables (fitted model: $F = 5.81$, $P < 0.001$, adjusted $R^2 = 0.237$), and 25(OH)D did not maintain an independent association with CRP ($\beta = -1.685$, $P = 0.136$). A second multiple regression analysis was performed by considering 25(OH)D as the dependent variable and fasting C_3, BMI, insulin (or $HOMA_{IR}$), and triglycerides as independent variables (fitted model: $F = 5.73$, $P < 0.001$, adjusted $R^2 = 0.240$), and 25(OH)D did not maintain an independent association with C_3 ($\beta = -2.702$, $P = 0.158$). A third multiple regression analysis was performed by considering 25(OH)D as the dependent variable and fasting C_4, BMI, insulin (or $HOMA_{IR}$), and triglycerides as independent variables (fitted model: $F = 5.06$, $P = 0.001$, adjusted $R^2 = 0.213$), and 25(OH)D did not maintain an independent association with C_4 ($\beta = -1.108$, $P = 0.755$). 25(OH)D maintained a significant independent association only with insulin ($P < 0.05$) or $HOMA_{IR}$ ($P < 0.05$), but not with BMI and triglycerides, in all of these multiple regressions.

Table 1: General, anthropometric, hormone, and metabolic parameters in subjects under study ($n = 66$).

Age (years)	36.3 ± 11.7
Body mass index (kg·m^{-2})	31.7 ± 5.0
Waist circumference (cm)	103.7 ± 12.6
Systolic blood pressure (mmHg)	128.3 ± 15.1
Diastolic blood pressure (mmHg)	86.2 ± 10.4
25(OH)D (ng/mL)	18.2 ± 7.7
Fasting blood glucose (mg/dL)	90.3 ± 8.4
Fasting insulin (μUI/mL)	18.7 ± 8.1
$HOMA_{IR}$	4.20 ± 1.98
Triglycerides (mg/mL)	90.1 ± 51.3
HDL cholesterol (mg/mL)	50.8 ± 13.0
Total cholesterol (mg/mL)	187.0 ± 42.5
CRP (mg/dL)	0.48 ± 0.46
C_3 (g/L)	1.18 ± 0.22
C_4 (g/L)	0.27 ± 0.08

$HOMA_{IR}$: homeostasis model assessment.

Table 2: Pearson correlation coefficients of 25(OH)D levels with general, anthropometric, hormone, and metabolic parameters in subjects under study ($n = 66$).

	25(OH)D (ng/mL)
Age (years)	−0.118
Body mass index (kg·m^{-2})	−0.346**
Waist circumference (cm)	−0.296*
Systolic blood pressure (mmHg)	−0.120
Diastolic blood pressure (mmHg)	−0.137
Fasting blood glucose (mg/dL)	−0.149
Fasting insulin (μUI/mL)	−0.350**
$HOMA_{IR}$	−0.344**
Triglycerides (mg/mL)	−0.343**
HDL cholesterol (mg/mL)	−0.016
Total cholesterol (mg/mL)	−0.159
CRP (mg/dL)	−0.320**
C_3 (g/L)	−0.258*
C_4 (g/L)	−0.302*

$HOMA_{IR}$: homeostasis model assessment.
$^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

At the best of our knowledge, this is the first study examining the relationship between complement factors and vitamin D levels in apparently healthy overweight and obese subjects.

We show that serum vitamin D levels are negatively related to C_3 and C_4 concentrations, but these correlations were not maintained after adjustment for BMI, insulin levels, and insulin resistance. Seemingly, as previously shown in severely obese patients [20], vitamin D and CRP levels were inversely associated, but also this correlation was not maintained after adjustment for BMI, insulin, and insulin resistance, suggesting that the negative association between vitamin D and investigated inflammation parameters (CRP and C_3 and C_4) is only indirectly mediated by factors associated with obesity.

According to previous studies [4–10], we found an inverse correlation between vitamin D levels and BMI (or waist circumference). As far as the mechanism why obese individuals have lower serum 25(OH)D concentrations, 25(OH)D has a high lipid-solubility, and it may well be that this vitamin is under sequestration when adipose tissue is in excess, resulting in reduced 25(OH)D bioavailability [34]. In line with this hypothesis, weight loss of 10%, obtained by a 20-week low-calorie diet, increased 25(OH)D levels in obese women [35]. Another study confirmed that serum vitamin D levels were higher after weight loss induced by a 2-year dietary intervention [36]. An alternate explanation is that higher leptin and IL-6 circulating levels, mostly secreted by adipose tissue, may have inhibitory effects on 25(OH)D synthesis via their receptors [37].

According to previous studies [11–16], we also found a negative correlation between vitamin D levels and insulin levels and insulin resistance (measured by $HOMA_{IR}$). It is noteworthy that insulin or $HOMA_{IR}$ was the only parameter maintaining an inverse relationship with vitamin D levels independently of other variables significantly correlated with vitamin D in this study (BMI, waist circumference, triglycerides, CRP, and C_3 and C_4).

These results suggest that hyperinsulinemia and insulin resistance drive the negative association between serum vitamin D and inflammation parameters (CRP, and C_3 and C_4) in obesity.

This is the first study raising the possibility that higher insulin levels and/or insulin resistance are directly responsible for lower vitamin D levels, but we do not have our own data to explain this result, and this is the limitation of this study. How hyperinsulinemia can cause a decrease in plasma vitamin D concentration needs to be addressed further, and this may be the direction of future studies in the obesity research area. However, it may well be that hyperinsulinemia *per se* or nonalcoholic fatty liver disease (NAFLD) associated with insulin resistance inhibits the activity of liver 25-hydroxylase, thus explaining lower production and circulating levels of 25(OH)D in obesity.

Interestingly, since low vitamin D is a risk factor for diabetes mellitus [38], it may well be that the effect of hyperinsulinemia on vitamin D levels may be one of the mechanisms that through insulin resistance enhances the risk of diabetes.

How low plasma vitamin D levels can change insulin concentration and sensitivity has been shown. In fact, vitamin D increases both insulin secretion [39, 40] and insulin sensitivity. Concerning insulin secretion, vitamin D receptors are expressed in both pancreatic β-cells and skeletal muscle cells, and vitamin D supplementation results in increased insulin release and insulin-mediated glucose transport [41]. Concerning the possible influence of vitamin D on insulin effect, two randomized controlled trials of high-dose vitamin D supplementation showed a significant increase in insulin sensitivity in insulin-resistant Indian men or South-Asian women, either after 6 weeks or 6 months of treatment, respectively [42, 43]. Interestingly, in vitro studies have even shown that vitamin D may influence insulin sensitivity via a direct stimulatory effect on insulin receptor expression [44].

In conclusion, the present study shows for the first time that vitamin D is negatively related to complement factor (C_3 and C_4) levels. These correlations were not maintained after adjustment for BMI (or waist circumference), insulin (or $HOMA_{IR}$), and triglyceride levels, and, in particular, only insulin and $HOMA_{IR}$ maintained an inverse relationship with vitamin D levels independently of other variables. Therefore, our study strongly suggests that hyperinsulinemia and insulin resistance are the main factors responsible for decrease of vitamin D concentrations in obese patients.

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Research Article

Serum Fetuin-A Levels Related with Microalbuminuria in Diet-Induced Obese Rats

Yanyan Li, Xiaodong Sun, and Yerong Yu

Department of Endocrinology and Metabolism, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

Correspondence should be addressed to Yerong Yu; yerongyu@scu.edu.cn

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The aim of the study was to investigate the association between elevated serum fetuin-A and increased urine albumin excretion in obese rats, and whether increased urine albumin excretion was modified by improving hepatic steatosis and lipid metabolism disorder. Male Wistar rats 4 weeks in age were randomly divided into three groups and fed with normal chow (control group), high-fat chow (obesity group), or high-fat chow plus fenofibrate (fenofibrate group). After 24 weeks, both body weight and visceral fat/body weight ratio in obese rats were higher than in controls. A difference in serology markers and pathology associated with hepatic steatosis was also found among the three groups. Serum fetuin-A and the expression of NF-κB in the liver were increased, while serum adiponectin was decreased in obese rats in comparison to controls ($P < 0.01$). Urinary albumin/creatinine ratio (ACR) was increased in the obesity group compared to controls ($P < 0.01$). The fenofibrate intervention reduced serum fetuin-A and NF-κB expression in the liver and increased serum adiponectin compared to obese rats and was accompanied by decrease in ACR. A positive correlation was found between ACR and fetuin-A ($r = 0.602$, $P < 0.01$), and a negative correlation was found between ACR and adiponectin ($r = -0.635$, $P < 0.01$). We conclude that elevated fetuin-A levels are associated with microalbuminuria in obese rats, and abnormal albuminuria is reversible by improving hepatic steatosis.

1. Introduction

In recent decades, changes in lifestyle and diet have led to an increased frequency in overweight and obese individuals. Obesity has become one of the most urgent public health problems and causes a major threat to human health worldwide. Obesity is associated with chronic diseases such as nonalcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD), among others. Growing recognition of the importance of obesity-related NAFLD and obesity-related CKD has provoked interest in the potential link between NAFLD and CKD. There is increasing evidence that NAFLD is associated with an increased prevalence and incidence of CKD. This association seems to be independent of obesity, hypertension, diabetes, and other possibly confounding factors [1, 2]. Moreover, prediabetic and newly diagnosed diabetic patients with NAFLD had higher prevalence rates of microalbuminuria than those without NAFLD [3]. However, the mechanistic relationship between NAFLD and CKD remains unknown.

Fetuin-A is an abundant serum protein, that is, predominantly synthesized and secreted by the liver. Higher serum fetuin-A levels have been found in diet-induced obesity, NAFLD and metabolic syndrome (MetS) [4–6]. Stefan and Haring reported that fatty liver might play an important role in the pathogenesis of the metabolic diseases type-2 diabetes mellitus (T2DM) and cardiovascular disease, and fetuin-A is directly involved in the pathogenesis of these diseases. However, recent novel findings revealed that under certain conditions fatty liver is not associated with metabolic disorders; the terms "metabolically benign" has been used to describe this state [7]. Adiponectin, a plasma protein primarily secreted by adipose tissue, was found to be decreased in NAFLD [8]. Both human fetuin-A and adiponectin genes reside on chromosome 3q27, which has been mapped as a T2DM and MetS susceptibility locus [9, 10]. Hennige and colleagues demonstrated that the liver-derived fetuin-A induced low-grade inflammation and repressed adiponectin production in animals and humans [11]. Moreover, adiponectin is a key regulator of albuminuria and is inversely related to

albuminuria [12]. Low-grade inflammation, one of the characteristics of chronic kidney disease (CKD), is also associated with albuminuria. From this information, we hypothesized that high serum fetuin-A levels may be associated with increased urine albumin excretion. One study reported that serum fetuin-A level was elevated in subjects with fat accumulation in the liver, and a decrease in liver fat was accompanied by a decrease in serum fetuin-A concentration [13]. In addition, elevated fetuin-A level in obesity is significantly reduced during exercise- and diet-induced weight loss [4]. However, it is not known whether improving lipid metabolism disorder is associated with ameliorating elevated fetuin-A level or reversing increased urine albumin excretion.

Therefore, this study investigated whether high circulating fetuin-A is associated with increased urine albumin excretion in diet-induced obese rats and examined the potential mechanism associating fetuin-A with microalbuminuria. We also studied whether the increased urine albumin excretion induced by higher serum fetuin-A levels was modified by improving hepatic steatosis and lipid metabolism disorder.

2. Methods

2.1. Animal Studies. 4-week-old male Wistar rats were purchased from the experimental animal center of west China, Sichuan University, China. The animal protocol was approved by the local ethics committee for animal studies and animals were handled according to "Principles of laboratory animal care". All rats were housed under controlled temperature (20 ± 2°C), humidity (50%), and light conditions (12-h light-dark cycle), and water was freely available. The rats were randomly divided into three groups: the control group (n = 8) was fed with normal chow (320 kcal/100 g/day), the obesity group (n = 8) was fed with high fat chow (586 kcal/100 g/day) and the fenofibrate group (n = 8) was fed with high fat chow plus fenofibrate (100 mg/kg/d). At the end of 24 weeks the rats were sacrificed and the liver was harvested. Liver lobes were weighed and processed for liver triglycerides (LTG), protein, and histological analysis. The blood was collected and serum samples were stored and kept frozen at −80°C for analyzing the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), free fatty acids (FFAs), fetuin-A, and adiponectin. Abdominal visceral fat (including perirenal fat, epididymal fat, and mesenteric fat) was collected and weighted.

2.2. Blood Measurements. Plasma glucose was measured by the glucose oxidase method. Serum insulin was assayed by radioimmunoassay (Linco Research, Inc., St Louis, MI, USA). Serum ALT and AST levels were measured by automatic biochemical analysis meter (Beckman, USA). Serum FFA concentrations were assayed by the colorimetric method with serum FFAs kit (E1001, Applygen Technologies Inc., Beijing, China). Serum fetuin-A and adiponectin levels were measured by an ELISA technique using the rat fetuin-A and adiponectin kits (E90178Ra and E90605Ra, resp., Uscn Life Science Inc., China), according to the manufacturer-supplied instructions.

2.3. Analysis of Liver Triglycerides (LTG). Frozen liver tissues were used for analyzing LTG using the tissue TG kit (E1013, Applygen Technologies Inc., Beijing, China). The liver was weighted and put into a manual glass homogenizer with lysis buffer (20 uL/mg), which was heated and then centrifuged to obtain supernatant for future enzymatic assays, according to the manufacturer-supplied instructions.

2.4. Liver and Kidney Histological Examination. Hepatic and renal cortex tissues were excised from each rat, fixed in 10% formalin, and embedded in paraffin wax. Paraffin-embedded tissues were sectioned and stained with hematoxylin-eosin (H&E).

2.5. Liver NF-κB p65 Immunohistochemical Staining. Sections of formalin-fixed, paraffin-embedded tissue were stained by means of an anti-rabbit SP kit (SP-9001, Zhong Shan Inc., Beijing, China). The specimens were incubated with rabbit anti-rat polyclonal NF-κB (p65) antibody (1 : 200 dilution; sc-101748, Santa Cruz Biotechnology, Inc.) overnight at 4°C. The procedure was performed essentially as described [14]. Semiquantitative analysis of NF-κB p65 expression in the liver used image analysis techniques (Image-Pro Plus software 6.0) to determine the gray scale value.

2.6. Measurement of Urine Albumin Excretion. Urine was collected in a metabolic cage at the end of 24 weeks. Urinary albumin was measured using a radioimmunoassay kit (China Institute of Atomic Energy, Beijing, China). Urinary creatinine concentration was measured by the Jaffe method. Urine albumin excretion was defined as urine albumin-to-creatinine ratio (ACR).

2.7. Statistical Analysis. Statistical analysis was performed by the statistical software package SPSS 17.0 for Microsoft windows (SPSS Inc). Data were expressed as means ± standard deviation. For three group comparisons, data were analyzed by ANOVA. Statistical comparison among two groups was performed with the student's t-test. Pearson correlation coefficient "r" was used to measure the relationship between two variables. P values less than 0.05 were considered statistically significant.

3. Results

Body weight increased 32.8% in obese rats in comparison to controls, and visceral fat increased 3.67 times in the obesity group compared to the control group. Compared with controls, obese rats had significantly higher visceral fat and weight ($P < 0.01$). In the fenofibrate group, body weight was duplicated (with some slight differences) compared to the obesity group, but visceral fat decreased 25.7% in the fenofibrate group when compared to the obesity group; visceral fat and weight were significantly decreased in the fenofibrate group in comparison to the obesity group (Table 1).

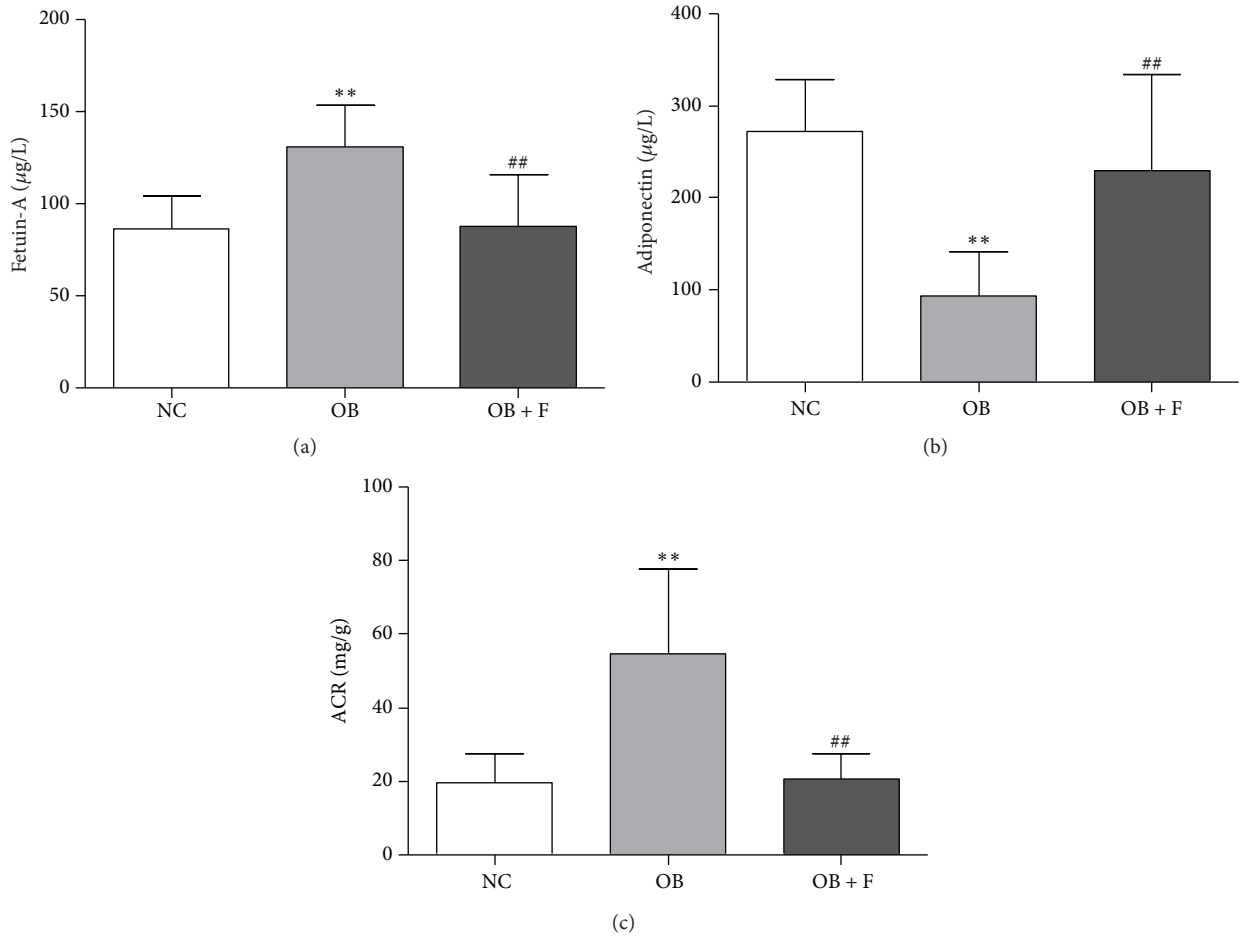

Figure 1: Representative parameters of rats in three groups: levels of fetuin-A (a), adiponectin (b), and ACR (c) in each group. Data was represented by means ± SD ($n = 8$). $^{**}P < 0.01$ versus control group; $^{\#\#}P < 0.01$ versus obesity group; (NC = control group, OB = obesity group, OB + F = fenofribate group).

Table 1: Biometric parameters of rats in the studied groups ($n = 8$).

Group	Body weight (g)	Visceral fat (g)	Visceral fat/weight (10^{-3})
Control group	521.3 ± 35.2	14.93 ± 1.68	28.58 ± 1.86
Obesity group	692.3 ± 93.9**	54.90 ± 14.18**	73.85 ± 18.76**
Fenofibrate group	657.4 ± 56.9**	40.78 ± 5.31$^{\#\#**}$	55.71 ± 8.47$^{\#\#**}$

Data are shown as mean ± SD. $^{**}P < 0.01$ versus control group. $^{\#\#}P < 0.01$ versus obesity group.

There was a significantly increased level of serum insulin, free fatty acids (FFAs), ALT, AST, and LTG in obese rats compared to controls ($P < 0.05$, Table 2). A significant decrease in most measurements was detected when fenofibrate was compared to the obesity group ($P < 0.05$), except for LTG content (Table 2). Although the LTG content was apparently decreased in the fenofibrate group, no significant difference was found between the fenofibrate and obesity groups. The data show that the degree of variability was high in obesity group, which might cause the above phenomenon.

There was a significant increase in serum fetuin-A levels in obese rats compared to controls (130.88 ± 22.41 versus 86.24 ± 17.97 μg/L, $P = 0.001$). A significant decrease was detected in the fenofibrate group when compared to the obesity group (87.94 ± 27.7 versus 130.88 ± 22.41 μg/L, $P < 0.01$). Serum adiponectin levels were significantly reduced in obese rats in comparison to controls (93.69 ± 47.45 versus 272.27 ± 56.71 μg/L, $P < 0.001$). A significant increase was found when the fenofibrate group was compared to the obesity group (228.70 ± 104.4 versus 93.69 ± 47.45 μg/L, $P < 0.01$). Urinary albumin/creatinine ratio (ACR) was significantly increased in obese rats accompanied by higher fetuin-A levels and lower adiponectin levels compared to controls (54.47 ± 23.26 versus 19.62 ± 7.81 mg/g, $P < 0.01$). The decrease was significant when comparing the fenofibrate group to the obesity group (20.68 ± 6.67 versus 54.47 ± 23.26 mg/g, $P < 0.01$). Data are shown as mean ± SD (Figure 1).

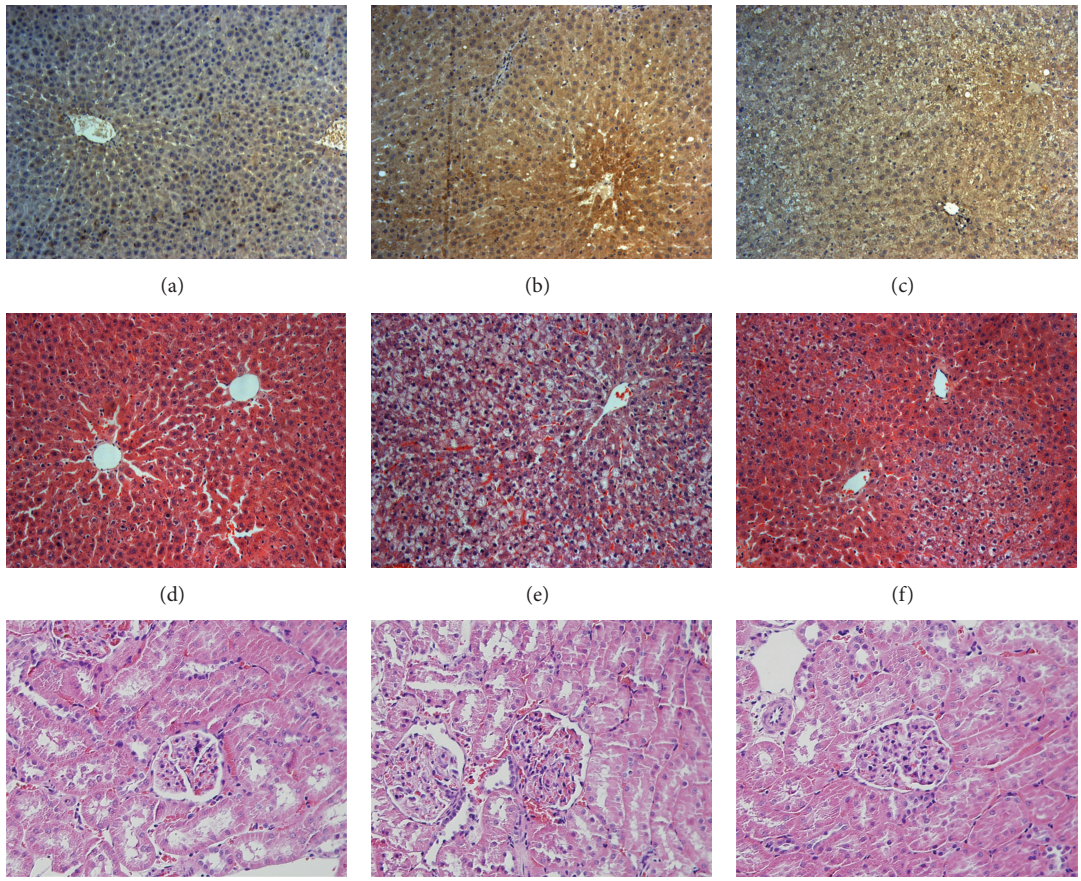

Figure 2: Immunohistochemical staining for NF-κB p65 in the liver in control group (a), obesity group (b), and fenofibrate group (c), respectively. Liver H&E stain from control group (d), obesity group (e), and fenofribate group (f), respectively. Kidney H&E stain from control group (g), obesity group (h), and fenofibrate group (i), respectively. Original magnification ×200; (NC = control group, OB = obesity group, and OB + F = fenofribate group).

Table 2: Blood parameters of rats in three groups ($n = 8$).

Group	FBG (mmol/L)	FINS (mIU/L)	ALT (IU/L)	AST (IU/L)	FFAs (mmol/L)	LTG (μmol/g)
Control group	5.7 ± 0.4	0.52 ± 0.19	48.5 ± 13.7	124 ± 32.4	0.37 ± 0.12	110.9 ± 49.46
Obesity group	6.0 ± 0.4	1.62 ± 0.24*	83.6 ± 39.5*	222 ± 24.3*	1.45 ± 0.33*	258.8 ± 127.6*
Fenofibrate group	5.9 ± 0.5	0.89 ± 0.24$^{\#*}$	51.5 ± 12.6$^{\#}$	108 ± 13.8$^{\#}$	0.78 ± 0.11$^{\#*}$	192.4 ± 77.9*

Data are shown as mean ± SD. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus obesity group. FBG: fasting blood glucose, FINS: fasting insulin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, FFAs: free fatty acid, LTG: liver triglyceride.

Positive NF-κB staining was located in the cytoplasm or nucleus of the liver cells. The NF-κB expression in the liver was apparently increased in the obese rats (Figure 2(b)) while little NF-κB expression in the liver was observed in normal rats (Figure 2(a)). After fenofibrate intervention, the increase in NF-κB expression observed in the liver of obese rats was suppressed (Figure 2(c)). Semiquantitative analysis of NF-κB expression in the liver by image analysis techniques showed that there was a 26.3% increase in obese rats compared to controls (0.385 ± 0.031 versus 0.306 ± 0.028, $P = 0.001$) and an 18.9% decrease in the fenofibrate group compared to the obesity group (0.313 ± 0.045 versus 0.385 ± 0.031, $P < 0.05$). HE-stained liver from diet-induced obese rats revealed a significantly increased steatosis when compared to controls, which was significantly improved after fenofibrate intervention (Figures 2(d)–2(f)). HE-stained kidney revealed marked glomerular enlargement and increased interstitial inflammatory cells (Figures 2(g)–2(i)). However, lowering circulating and renal FFA levels by fenofibrate intervention reduced the glomerular hypertrophy.

Serum FFA concentration and LTG levels were increased in diet-induced obese rats and accompanied by higher serum fetuin-A levels and higher ACR. However, after fenofibrate intervention, lipid metabolism was improved, and simultaneously serum fetuin-A levels were significantly decreased and urine albumin secretion was significantly reduced.

A significant positive correlation was found between serum fetuin-A levels and ACR ($r = 0.602$, $P = 0.002$). Furthermore, a significant positive correlation was detected between ACR and each of these proteins: serum FFAs, serum ALT, serum AST, and LTG ($r = 0.839$, $P < 0.001$; $r = 0.727$, $P < 0.001$; $r = 0.742$, $P < 0.001$; $r = 0.715$, $P < 0.001$, resp.). In addition, there was a significant negative correlation between serum adiponectin level and ACR ($r = -0.653$, $P = 0.001$; Figure 3).

In addition, there was a significant positive correlation between serum fetuin-A and each of these proteins: serum ALT, AST levels, serum FFAs, and LTG content ($r = 0.509$, $P < 0.05$; $r = 0.637$, $P < 0.01$; $r = 0.590$, $P < 0.01$; $r = 0.428$, $P < 0.05$, resp.). A strong inverse correlation was found between serum fetuin-A and serum adiponectin ($r = -0.537$, $P < 0.01$; Figure 4).

We divided all parameters into tertiles based on ACR: lower, middle, and upper tertiles. Increasing tertiles of ACR were associated with increasing levels of fetuin-A, adiponectin, and NF-κB (Table 3). In addition, there was an obvious increase in serum ALT, AST, and FFAs with increasing tertiles of ACR (Table 3). Although there was no significant difference in LTG content, an apparent increase was found accompanied by increasing tertiles of ACR.

4. Discussion

Chronic kidney disease (CKD) is defined by the presence of a marker of kidney damage, such as proteinuria or a decreased glomerular filtration rate for three or more months [2]. CKD has emerged as a growing public health problem worldwide. Microalbuminuria is an early marker of chronic kidney injury, and several cross-sectional studies have noted an association between metabolic syndrome and microalbuminuria in individuals with and without diabetes [15–17]. Ix et al. has documented that higher fetuin-A human concentrations are strongly associated with MetS and an atherogenic lipid profile [6]. Increasing quartiles of fetuin-A are linearly associated with the number of MetS components and increased serum triglyceride concentrations [6]. Additionally, fetuin-A levels strongly, and independently from other important parameters were found to be associated with hyperglycemia [18]. Fetuin-A has been studied as an important circulating inhibitor of ectopic calcium deposition in the renal field, and fetuin-A deficiency in humans was associated with vascular calcification and mortality in patients on hemodialysis [19]. Fetuin-A might, however, have other functions; fetuin-A is an important promoter of insulin resistance and exerts proinflammation effects. This led us to investigate whether higher serum fetuin-A is associated with abnormal albuminuria.

In the current study, serum fetuin-A levels and microalbuminuria were measured in rats of three different intervention groups to clarify their potential relationship. We are able to show that there was a strong, positive correlation between serum fetuin-A levels and abnormal albuminuria, as reported previously by Li et al. [20]. The results showed a significant increase in serum fetuin-A levels in obese rats compared to controls. Our study proved that there is a significant positive correlation between serum fetuin-A level and serum ALT, AST level, serum FFA concentration, and LTG content. These results are in accordance with the previous studies that serum fetuin-A levels are elevated in NAFLD [4, 5, 21]. Yilmaz et al. found that serum fetuin-A levels are significantly higher in patients with biopsy-proven NAFLD and may serve as a biochemical marker of fibrosis in NAFLD patients [21]. However, after diet- and exercise- or surgery-induced weight loss, the homeostatic model assessment (HOMA) index and fetuin-A decreased, and a significant decrease in the prevalence of NAFLD was found [4, 22]. In addition, a cross-sectional and longitudinal study reported that serum fetuin-A levels were elevated in subjected with fat accumulation in the liver, and a decrease in liver fat was accompanied by a decrease in serum fetuin-A concentrations [13]. These findings raise the possibility that fetuin-A may be a new promising link between obesity and its comorbidities. Simultaneously, a significant increase was detected in ACR in obese rats compared to controls. This increase reflects renal impairment and suggests the presence of CKD in obese rats with higher serum fetuin-A levels. A number of studies have shown a positive correlation between obesity and microalbuminuria [23, 24], and that obesity is an independent risk factor for the development of microalbuminuria [25], which is also associated with the MetS and its different components. The prevalence of NAFLD has been estimated to be approximate 90% in obesity [26]; both NAFLD and dyslipidemia contribute to the rate of progression of renal disease [1, 24]. Therefore, higher serum fetuin-A is a potential risk factor in diet-induced obese rats and contributes to the prevalence of abnormal albuminuria.

The previous studies have reported that a negative correlation was found between adiponectin and microalbuminuria in obese patients [12, 23]. Fetuin-A is deemed to repress the production of adiponectin [11]. Based on these findings, we further investigated the association of fetuin-A with adiponectin to clarify the potential mechanism that fetuin-A induced the increased urine albumin excretion. We found that serum adiponectin levels were significantly decreased in obese rats in comparison to controls, and our results revealed a strong inverse correlation between serum fetuin-A and serum adiponectin, and a significant inverse correlation between ACR and serum adiponectin. Adiponectin, an adipocyte-derived hormone, has anti-inflammatory properties by modulating multiple signaling pathways and exerts largely beneficial effects to improve insulin sensitivity. The decreased level of serum adiponectin represents an independent risk factor for NAFLD [27]. It is well known that chronic low-grade inflammation is a common feature of progression of NAFLD. The results of the present study are in accordance with previous studies [28, 29], showing that there was a higher levels of an inflammatory marker, as evidenced by the significant increase in liver NF-κB expression, in obese rats compared to controls. These results are in accordance with a report that FFAs enhanced fetuin-A secretion commensurate with over-expression and activity of NF-κB [30]. Another study demonstrated that fetuin-A acted as an endogenous ligand of TLR4, played a key role in FFA-induced

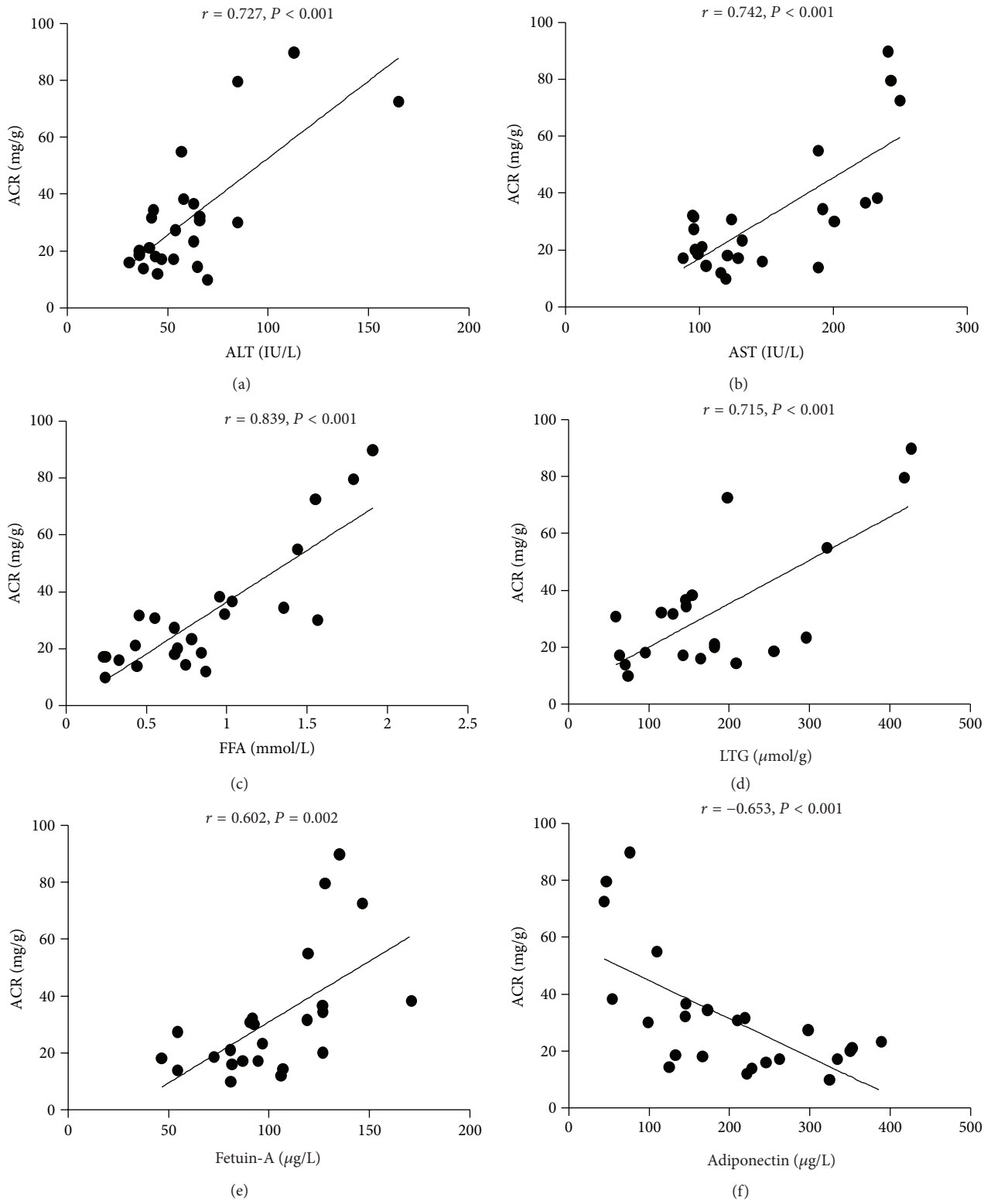

Figure 3: Correlation of serum ALT and AST, serum FFAs, LTG, serum fetuin-A, and adiponectin with the level of ACR; data are from three groups.

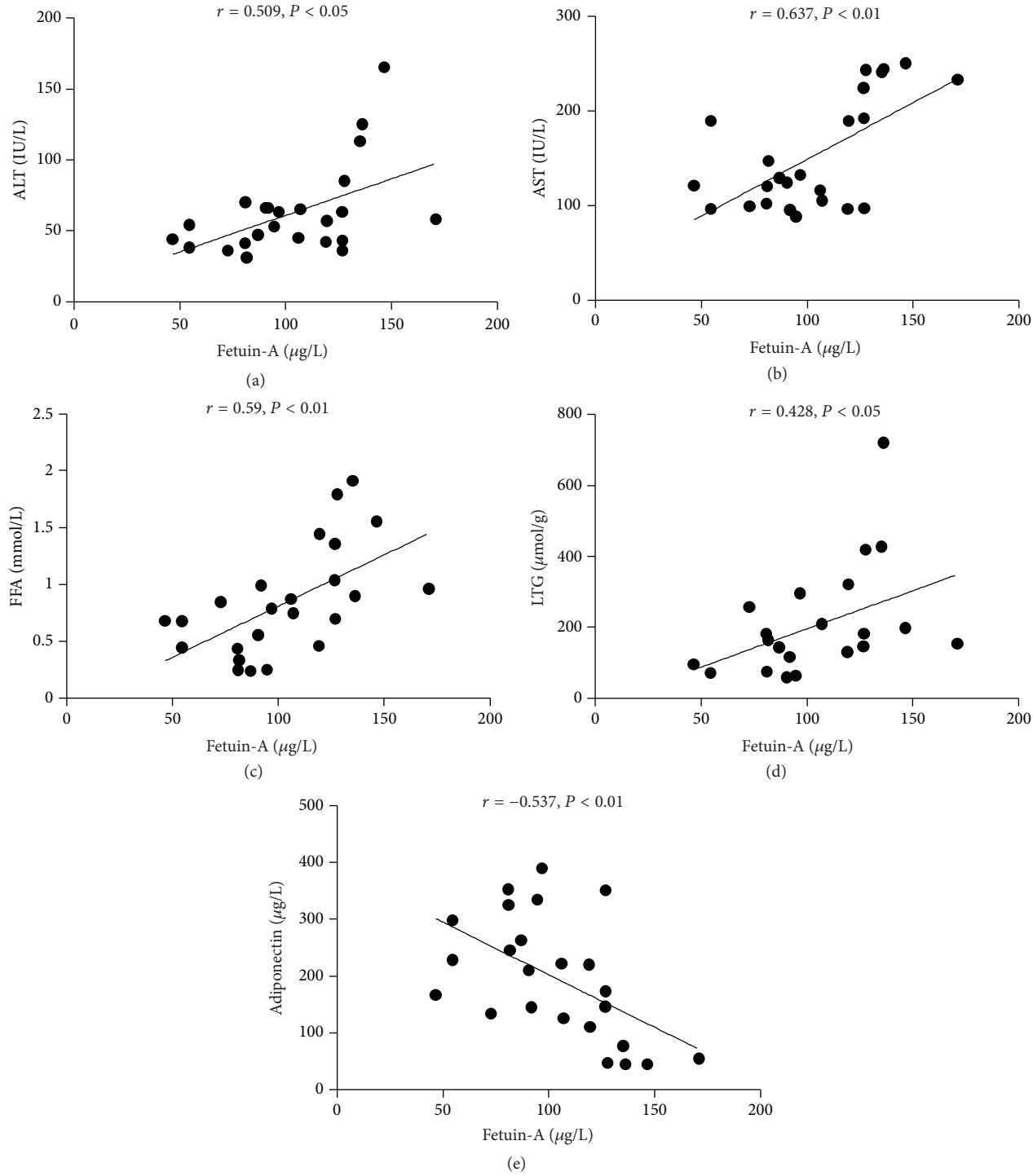

Figure 4: Correlation of serum ALT and AST, serum FFAs, LTG, and serum adiponectin with the level of serum fetuin-A; data are from three groups.

proinflammatory cytokine expression, and promoted lipid-induced insulin resistance [31]. Moreover, the fatty liver itself can exacerbate insulin resistance, thus increasing the risk of renal impairment [32], and several proinflammatory factors from steatosis and inflamed liver can further exacerbate kidney dysfunction [33]. These findings are in accordance with the current study showing that increasing tertiles of ACR were related with a gradual increase in liver NF-κB expression. These findings provide evidence that higher fetuin-A levels induce increased urine albumin excretion by a mechanism related with repressing adiponectin and triggering inflammation.

Several studies have demonstrated that lifestyle intervention or surgery-induced weight loss leads to a significant

Table 3: Parameters of rats by ACR tertiles.

	Lower tertile 14.7 ± 2.9 (mg/g)	Middle tertile 25.3 ± 5.2 (mg/g)	Upper tertile 54.7 ± 22.9 (mg/g)	*P*
Fetuin-A (μg/L)	82.4 ± 22.1	91.9 ± 23.6	130.8 ± 22.6	0.001
ALT (IU/L)	49.1 ± 13.1	52.9 ± 17.5	81.3 ± 40.0	<0.05
AST (IU/L)	126.9 ± 30.4	118.4 ± 36.2	208.4 ± 51.2	<0.001
FFA (mmol/L)	0.47 ± 0.25	0.75 ± 0.36	1.38 ± 0.37	<0.001
LTG (μmol/g)	117.3 ± 55.7	184.1 ± 85.1	240.9 ± 128.5	0.075
Adiponectin (μg/L)	238.7 ± 71.4	256.6 ± 107.6	99.4 ± 50.8	0.001
NF-κB	0.303 ± 0.046	0.314 ± 0.026	0.385 ± 0.031	<0.05

Data are shown as mean ± SD. Data was analyzed by ANOVA. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, FFA: free fatty acid, LTG: liver triglyceride.

decrease of fetuin-A [4, 22]. In a longitudinal study, a large decrease in liver fat induced by lifestyle intervention was paralleled by a decrease in serum fetuin-A levels [13]. Moreover, Ix et al. found a particularly strong association of human fetuin-A with an atherogenic lipid profile among the components of MetS. However, whether reduced FFA levels could reverse the elevated serum fetuin-A remain unclear. It is known that higher serum FFAs contribute to lipid ectopic deposition in liver, and our findings show that there was a significant increase in FFA concentration and LTG content in obese rats compared to controls. In addition, our study demonstrated that fenofibrate alleviated hepatic steatosis by regulating lipid metabolism (reduced serum FFAs and LTG content); the results also revealed a significant decrease in serum fetuin-A levels, which extends previous findings of decreased fetuin-A levels with improvement of NAFLD after a short-term lifestyle intervention [13]. These findings reflect the reversibility of the increased fetuin-A concentrations. However, in our present study we found that there was no significant difference of LTG levels between the obesity and fenofibrate groups, and that not every LTG level was significantly elevated in the obesity group. This condition indicated that in diet-induced obese rats, fatty liver stayed at a certain stage, as reported by the previous review [7], which is associated with a metabolically benign state. Interestingl, a significant increase was detected in serum adiponectin levels with fenofibrate treatment in comparison to the obesity group, which is in accordance with the previous study that adiponectin was increased after short-term fenofibrate therapy [13, 34]. Moreover, fenofibrate alleviated hepatic steatosis and decreased serum fetuin-A levels accompanied by improvement in hepatic chronic low-grade inflammation. As the results revealed, a significant decrease was detected in liver NF-κB expression with fenofibrate compared to the obesity group. The steatotic and inflamed liver was alleviated after the fenofibrate intervention in the wake of a reduction in ACR. Therefore, the increased urine albumin excretion is reversible by improving hepatic steatosis.

5. Conclusions

The results of the present study indicates that in diet-induced obese rats higher serum fetuin-A levels represent a novel risk factor for the presence of microalbuminuria, and the mechanism may be related with repressing adiponectin production and triggering of chronic low-grade inflammation. Abnormal albuminuria induced by increased fetuin-A levels is reversible by improving hepatic steatosis. Therefore, attention should be paid to obesity or NAFLD with early kidney lesions, and the use of therapeutic agents or lifestyle intervention could be used to prevent NAFLD patients from developing microalbuminuria, thus delaying further renal impairment.

Abbreviations

ACR: Urinary albumin/creatinine ratio
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
CKD: Chronic kidney disease
FFAs: Free fatty acid
IR: Insulin resistance
LTG: Liver triglyceride
MAU: Microalbuminuria
MetS: Metabolic syndrome
NAFLD: Non-alcoholic fatty liver disease
T2DM: Type 2 diabetes mellitus.

Conflict of Interests

All authors declare that there are no known conflict of interests related with this publication.

Authors' Contribution

Y. Li wrote the paper and conducted the research; X. Sun conducted the research; Y. Yu reviewed the paper and contributed to the paper.

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Review Article

Lipoprotein(a) in Cardiovascular Diseases

Michele Malaguarnera,[1] Marco Vacante,[2] Cristina Russo,[2] Giulia Malaguarnera,[1] Tijana Antic,[2] Lucia Malaguarnera,[3] Rita Bella,[4] Giovanni Pennisi,[4] Fabio Galvano,[5] and Alessandro Frigiola[6]

[1] *International Ph. D. Program in Neuropharmacology, University of Catania, 95123 Catania, Italy*
[2] *Department of Senescence, Urological, and Neurological Sciences, University of Catania, 95126 Catania, Italy*
[3] *Department of Biomedical Sciences, University of Catania, 95124 Catania, Italy*
[4] *Department of Neurosciences, University of Catania, 95123 Catania, Italy*
[5] *Department of Biological Chemistry, Medical Chemistry, and Molecular Biology, University of Catania, 95123 Catania, Italy*
[6] *Pediatric Cardiology and Cardiac Surgery Department, Guch Unit, IRCCS Policlinico San Donato, 20097 Milan, Italy*

Correspondence should be addressed to Michele Malaguarnera; m.malaguarnera@email.it

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Lipoprotein(a) (Lp(a)) is an LDL-like molecule consisting of an apolipoprotein B-100 (apo(B-100)) particle attached by a disulphide bridge to apo(a). Many observations have pointed out that Lp(a) levels may be a risk factor for cardiovascular diseases. Lp(a) inhibits the activation of transforming growth factor (TGF) and contributes to the growth of arterial atherosclerotic lesions by promoting the proliferation of vascular smooth muscle cells and the migration of smooth muscle cells to endothelial cells. Moreover Lp(a) inhibits plasminogen binding to the surfaces of endothelial cells and decreases the activity of fibrin-dependent tissue-type plasminogen activator. Lp(a) may act as a proinflammatory mediator that augments the lesion formation in atherosclerotic plaques. Elevated serum Lp(a) is an independent predictor of coronary artery disease and myocardial infarction. Furthermore, Lp(a) levels should be a marker of restenosis after percutaneous transluminal coronary angioplasty, saphenous vein bypass graft atherosclerosis, and accelerated coronary atherosclerosis of cardiac transplantation. Finally, the possibility that Lp(a) may be a risk factor for ischemic stroke has been assessed in several studies. Recent findings suggest that Lp(a)-lowering therapy might be beneficial in patients with high Lp(a) levels. A future therapeutic approach could include apheresis in high-risk patients in order to reduce major coronary events.

1. Introduction

Cardiovascular diseases cause 3% of all deaths in North America being the most common cause of death in European men under 65 years of age and the second most common cause in women. These facts suggested us to consider new strategies for prediction, prevention, and treatment of cardiovascular disease [1]. Inflammatory mechanisms play a central role in the pathogenesis of atherosclerosis and its complications [2]. It has been demonstrated that atherogenic lipoproteins such as apo(B-100), oxidized low-density lipoprotein (LDL), remnant lipoprotein (beta-VLDL), and lipoprotein(a) play a critical role in the proinflammatory reaction. High-density lipoprotein (HDL) is antiatherogenic lipoproteins that exert anti-inflammatory functions [3–5]. Plasma LDL cholesterol is a well-established predictor of coronary artery disease (CAD), and many observations have pointed out that Lp(a) and apolipoprotein(a) (apo(a)) levels may be risk factors for cardiovascular diseases (CVD) [6–8].

2. Native Lp(a)

Lp(a) is an LDL-like molecule consisting of an apolipoprotein B-100 (apo(B-100)) particle attached by a disulphide bridge to apo(a). Lp(a) plasma concentrations are controlled by the apo(a) gene located on chromosome 6q26-27 [9]. The unique character of Lp(a) is based on the apo(a) highly glycosylated protein structurally homologous to plasminogen [10].

Several published data indicated the existence of unbound forms of apo(a) in blood and urinary excretion of apo(a) fragments [11, 12]. Animal experiments showed that apo(a) serves as a distinctive marker of Lp(a) and represents an atherogenic component of Lp(a) [13]. Furthermore, apo(a) has also been reported to be correlated to coronary artery disease as well as renal disease [14–16]. Dissociation of apo(a) may lead to the exposure of an additional lysine-binding site, increasing the affinity of free apo(a) for plasmin modified fibrin, thus impeding fibrinolysis [17]. Apo(a) is a member of a family of "kringle" containing proteins, such as plasminogen, tissue plasminogen activator (tPA), prothrombin, factor XII, and macrophage stimulating factor (MSF). Lp(a) shares a high degree of sequence identity with plasminogen. These similarities could explain the role of Lp(a) in thrombogenesis and as a proinflammatory factor [18]. Native Lp(a) has been shown to enhance the expression of adhesion molecules [13, 19–23]. Because of the structural homology with plasminogen, Lp(a) might have important antithrombolytic properties, which could contribute to the pathogenesis of atherothrombotic disease. For example, Lp(a) binding to immobilised fibrinogen and fibrin results in the inhibition of plasminogen binding to these substrates [24, 25]. In addition, Lp(a) competes with plasminogen for its receptors on endothelial cells, leading to diminished plasmin formation, thereby delaying clot lysis and favouring thrombosis. The high affinity of Lp(a) for fibrin provides a mechanistic basis for their frequent colocalization in atherosclerotic plaques [26, 27]. Moreover Lp(a) induces the monocyte chemoattractant (CC chemokine I-309), which leads to the recruitment of mononuclear phagocytes to the vascular wall [28, 29].

3. Oxidized Lp(a)

Lp(a) particles can suffer oxidative modification and scavenger receptor uptake, with cholesterol accumulation and foam cell formation [30], leading to atherogenesis. Oxidation of LDL and Lp(a) affects the catabolism of the lipoproteins, including changes in receptor recognition, catabolic rate, retention in the vessel wall, and propensity to accelerate atherosclerosis. Oxidative modification of apo(a) may have an influence on Lp(a) recognition by scavenger receptors of macrophages. Some studies showed that Lp(a) particles are prone to oxidation and that the increased risk of coronary artery disease associated with elevated Lp(a) levels may be related in part to their oxidative modification and uptake by macrophages, resulting in the formation of macrophage-derived foam cells [31]. The oxidative form of Lp(a) (ox-Lp(a)) might attenuate fibrinolytic activity through the reduction of plasminogen activation, might enhance PAI-1 production in vascular endothelial cells, and might impair endothelium-dependent vasodilation. Particularly, the role of ox-Lp(a) is linked to macrophages that take up ox-Lp(a) via scavenger receptor as well as oxidized LDL. Lp(a) particles are susceptible to oxidative modification and scavenger receptor uptake, leading to intracellular cholesterol accumulation and foam cell formation, which contributes further to atherogenesis [25, 32]. Morishita et al. demonstrated increased values of ox-Lp(a) in patients with coronary artery disease [33]. A study of autopsy findings demonstrated a deposition of ox-Lp(a) in the vessel margin inside the calcified areas [34]. Probably it was related to the promotion of an antifibrinolytic environment, foam cell formation, generation of a fatty streak, and an increase in smooth muscle cells. Moreover ox-Lp(a) is a potent stimulus of monocyte adhesion to endothelial cells, thus contributing to atherogenic changes in human blood vessels. Komai et al. compared the effects of oxidized lipoproteins and no oxidized lipoprotein on the progression of atherosclerosis. It was investigated the mitogenic actions of Lp(a) and ox-Lp(a) on human vascular smooth muscle cells (VSMC). The results were that Lp(a) significantly stimulated the growth of human VSMC in a dose-dependent manner, whereas ox-Lp(a) showed a stronger stimulatory action on VSMC growth than native Lp(a). This study demonstrated that ox-Lp(a) has a more potent effect than native Lp(a) in developing atherosclerosis diseases [35].

4. Glycated Lp(a)

Nonenzymatic glycation of lipoprotein may contribute to the premature atherogenesis in patients with diabetes mellitus by diverting lipoprotein catabolism from nonatherogenic to atherogenic pathways. It has been observed that the proportion of apo (B-100) in glycated form was significantly higher in diabetic patients than in nondiabetic controls, and equally that plasma Lp(a) levels might be increased in diabetic patients [36]. Anyway, glycation does not appear to significantly enhance the atherogenic potential of unmodified Lp(a) [37]. The kringle of apo(a) is homologous to the kringle IV of plasminogen, and each of these kringles contains a potential site of N-linked glycosylation. The carbohydrate content of apo(a) has been determined and represents approximately 28% by weight of the protein. Peripheral levels of Lp(a) have been examined in a number of studies involving diabetic patients because Lp(a) concentration is associated with a high-risk of coronary heart disease, and diabetic patients are prone to develop coronary heart disease. It has been demonstrated that glycation enhances the production of PAI-1 and attenuates the synthesis of t-PA induced by Lp(a) in arterial and venous endothelial cells (EC). The formation of advanced glycation end-products (AGEs) and EC-mediated oxidative modification may contribute to the alterations of the generation of PAI-1 and t-PA induced by glycated Lp(a) [13]. The combination of hyperglyaemia and hyperlipoprotein(a) may reduce EC-derived fibrinolytic activity, which may promote the development of thrombosis and atherosclerosis in subjects with diabetes [36].

5. Atherogenic and Proinflammatory Mechanisms of Lp(a)

5.1. Lp(a) and Endothelial Dysfunction. As the atherosclerotic plaque progresses, growth factors and cytokines secreted

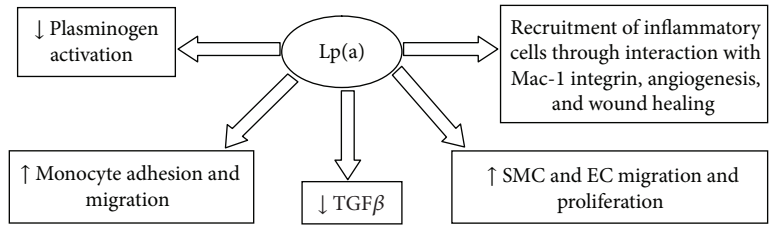

Figure 1: Mechanisms underlying the Lp(a)-induced cardiovascular disease. Lp(a) inhibits the activation of TGF and promotes the proliferation and migration of smooth muscle cells to endothelial cells. Moreover Lp(a) inhibits plasminogen activation and decreases the activity of fibrin-dependent tissue-type plasminogen activator.

by macrophages and foam cells in the plaque stimulate vascular smooth muscle cell growth and interstitial collagen synthesis [37]. Moreover, the apo(a) component of Lp(a) has been shown to enhance the expression of ICAM-1 [13]. Thus, these effects on endothelial cell function may provide mechanisms by which Lp(a) contributes to the development of atherosclerotic lesions. Reduction in nitric oxide (NO) availability also initiates the activation of matrix metalloproteinases MMP-2 and MMP-9 [38, 39], and further it reduces inhibition of platelet aggregation [40]. Thus, endothelial dysfunction with reduced NO bioavailability, increased oxidant excess, and expression of adhesion molecules contributes not only to initiation but also to progression of atherosclerotic plaque formation and triggering of cardiovascular events. In vitro studies indicated that Lp(a) enhances the synthesis of PAI-1 by endothelial cells. PAI-1 is the main inhibitor of the fibrinolytic system [41]. Another potentially important action of Lp(a) is the reduction of activation of latent transforming growth factor-β (TGF-β) by displacing plasminogen from the surfaces of macrophages in atherosclerotic plaques. In the absence of activated TGF-β, cytokines might induce smooth muscle cell proliferation and the transformation of these cells to a more atherogenic cellular phenotype [42, 43]. Furthermore, studies on cultured human umbilical vein or coronary artery endothelial cells revealed a novel effect of Lp(a) that was mediated by its apo(a) component: impairment of the barrier function of endothelial cells through cell contraction occurring as a consequence of a rearrangement of the actin cytoskeleton [44]. In 1992, Cohn et al. studied the abnormalities of vascular compliance in hypertension and demonstrated that vasodilation is inhibited by ox-Lp(a). Also, they showed that elevation of ox-Lp(a) may explain the endothelial dysfunction observed in hypertensive patients because ox-Lp(a) enhanced Lp(a)-induced PAI-1 production in vascular endothelial cells [45]. However, the exact role of ox-Lp(a) is still largely unknown, and at the moment a stronger involvement of the ox-Lp(a) in atherosclerotic development and worse evolution in stroke and heart failure are just supposed. The matter of fact is that ox-Lp(a) might cause more pronounced stimulation of superoxide production, whereas native Lp(a) itself caused a moderate, dose-dependent stimulation of superoxide production. Accumulation of native Lp(a) may enhance the stimulation of ox-Lp(a), a more potent atherogenic lipoprotein, in the vessel wall [46].

5.2. Inflammation, Atherosclerosis, and Lp(a). Lp(a) may act as a proinflammatory mediator that augments the lesion formation in atherosclerotic plaques [47]. Lp(a) may lead to an inflammatory process by inducing the expression of adhesion molecules on endothelial cells, the chemotaxis of monocytes, and the proliferation of smooth muscle cells [48]. Moreover Lp(a) can augment the production of cytokines by vascular cells, and through the autocrine and paracrine mechanisms, the inflammatory reaction may lead to a vicious cycle resulting in lesion progression [49]. Lp(a) acts on the fibrinolytic system in several ways which include the inhibition of plasminogen binding and activation, thereby impairing fibrinolytic activity and the dissolution of thrombi. High concentrations of Lp(a) might increase the risk of thrombus formation by impeding fibrinolytic mechanisms in the region of the plaque. Some mechanisms are involved into the development of atherosclerosis: Lp(a) inhibits the activation of transforming growth factor (TGF) and contributes to the growth of arterial atherosclerotic lesions by promoting the proliferation of vascular smooth muscle cells and the migration of smooth muscle cells to endothelial cells [50, 51]. Moreover Lp(a) inhibits plasminogen binding to the surfaces of endothelial cells and decreases the activity of fibrin-dependent tissue-type plasminogen activator. Furthermore Lp(a) increases plasminogen activator inhibitor activity in endothelial cells and promotes atherothrombosis [52]. Other functions have been related to recruitment of inflammatory cells through interaction with Mac-1 integrin, angiogenesis, and wound healing (Figure 1). However, individuals without Lp(a) or with very low Lp(a) levels seem to be healthy. Thus plasma Lp(a) is certainly not vital, at least under normal environmental conditions [53–55].

6. Lp(a) and Cardiovascular Diseases

The relationship between Lp(a) levels and the severity of coronary atherosclerosis in patients with unstable angina or acute myocardial infarction (MI) has been analyzed in several studies with controversial results [77–79]. The potential value of small apo(a) isoforms in predicting severe angiographically demonstrable atherosclerosis remains unclear. Elevated serum Lp(a) is an independent predictor of coronary artery disease (CAD) and myocardial infarction [64, 68, 80]. Motta et al. studied the transient increased serum levels of this lipoprotein during acute myocardial infarction (AMI). The positive correlation between mean Lp(a) values on day

1 and 7 and the size of the necrotic area suggested an atherogenic and prothrombotic role of Lp(a). Moreover, elevated Lp(a) values were related to greater tissue damage. This study suggested that periodical determination of Lp(a) values in subjects with coronary disease may be useful in order to predict further acute vascular events [81]. Lp(a) levels should be a marker of restenosis after percutaneous transluminal coronary angioplasty [82], saphenous vein bypass graft atherosclerosis [83], and accelerated coronary atherosclerosis of cardiac transplantation [84]. Some studies have shown that Lp(a) is not associated with atherosclerosis, and others have demonstrated that a high serum Lp(a) level is a major risk factor for atherosclerosis and progression of coronary artery disease [85–87]. A high serum Lp(a) level may be a high-risk factor for CCSP (clinical coronary stenosis progression) and restenosis after PCI (percutaneous coronary intervention). An elevated Lp(a) concentration is a significant predictor of long-term adverse outcome in AMI patients treated by primary percutaneous transluminal coronary angioplasty [88]. Serum Lp(a) levels $\geq$ 25 mg/dL are noted in 67% of patients with rapid progression of coronary artery disease but in only 33% of patients without progression of coronary artery disease [89]. Tamura et al. studied the association between serum Lp(a) level and angiographically assessed coronary artery disease progression without new myocardial infarction, reporting a significant association [90]. In patients with serum Lp(a) levels $\geq$ 30 mg/dL, coronary stenosis progression occurred, and revascularizations for target restenotic lesions or new lesions were performed approximately 7 months after the first myocardial infarction; CCSP occurred in a relatively short period after the first AMI in the high-Lp(a) patients [91]. A meta-analysis demonstrated that Lp(a) levels can be considered as a risk factor for cardiovascular disease [92, 93]. The first study of the association between Lp(a) and a range of cardiovascular endpoints including cognitive and disability indices in the elderly was conducted by Gaw et al. The main finding was that Lp(a) level, although influenced by a number of baseline characteristics, is not a significant predictor of cognitive function or levels of disability but is a predictor of combined cardiovascular events over an average 3.2-year followup [94]. Sandholzer et al. reported that in patients with premature coronary heart disease (CHD), alleles at the apo(a) locus determine risk for CHD through their effect on plasma Lp(a) level. This study suggested that Lp(a) can be considered a primary genetic risk factor for CHD [95]. Several epidemiologic studies have assessed the association between Lp(a) and atherosclerotic disease (Table 1). Many population-based prospective studies had reported a controversial association between Lp(a) levels and CHD risk. Few studies, however, have adequately examined important aspects of the association, such as the size of relative risks in clinically relevant subgroups (such as in men and women or at different levels of established risk factors) [56–63, 65–67, 69, 70, 73, 74, 96–98]. A task force for emerging risk factor assessed the relationship between Lp(a) concentration and risk of major vascular and nonvascular outcomes. In this long-term prospective study, Lp(a) plasma levels and subsequent major vascular morbidity and/or cause-specific mortality were recorded. Lp(a) was weakly correlated with several conventional vascular risk factors, and it was highly consistent within individuals over several years. The risk ratio for CHD, adjusted for age and sex only, was 1.16 per 3.5-fold higher usual Lp(a) concentration (i.e., per 1 SD), and it was 1.13 following further adjustment for lipids and other conventional risk factors. The corresponding adjusted risk ratios were 1.10 for ischemic stroke, 1.01 for the aggregate of nonvascular mortality, 1.00 for cancer deaths, and 1.00 for nonvascular deaths other than cancer. The results showed that there are continuous, independent, and modest associations of Lp(a) concentration with risk of CHD and stroke that appear exclusive to vascular outcomes [99]. The Copenhagen City Heart Study (CCHS) found that extreme Lp(a) levels > 95th percentile predict a 3- to 4-fold increase in risk of myocardial infarction (MI) and absolute 10-year risks of 20% and 35% in high-risk women and men [100]. In this study it was observed larger risk estimate for Lp(a) than most previous studies, most likely because the authors focused on extreme levels, measured levels shortly after sampling, corrected for regression dilution bias, and considered MI rather than ischemic heart disease (IHD). For the first time CCHS provided absolute 10-year risk estimates in the general population for MI and IHD as a function of Lp(a) levels stratified for other risk factors, allowing clinicians to use extreme Lp(a) levels in risk assessment of individual patients [101]. In conclusion, most but not all prospective studies on Lp(a) and risk of CHD have found positive associations, and levels of Lp(a) have also been related to severity of disease. Lp(a) levels differ between ethnic groups, and thus results from one study may not be applicable to other ethnic groups. Recent recommendations stated that Lp(a) screening is not warranted for primary prevention and assessment of cardiovascular risk at present, but that Lp(a) measurements can be useful in patients with a strong family history of cardiovascular disease or if risk of cardiovascular disease is considered intermediate on the basis of conventional risk factors [102]. The European Atherosclerosis Society Consensus Panel [103] have suggested screening for elevated Lp(a) in those at intermediate or high CVD/CHD risk, a desirable level < 50 mg/dL as a function of global cardiovascular risk, and use of niacin for Lp(a) and CVD risk reduction.

7. Conclusions

The clinical interest in Lp(a) is largely derived from its role as a cardiovascular risk factor. Although not considered an established risk factor, Lp(a) levels have been associated with cardiovascular disease in numerous studies [72, 104, 105]. Recently Lp(a) serum levels were found to be associated with the severity of aortic atherosclerosis, especially in abdominal aorta, as well as coronary atherosclerosis [106]. Moreover a study by Momiyama et al. [107] demonstrated that elevated Lp(a) has incremental prognostic value in symptomatic patients with coronary artery revascularization [108]. Lp(a) is involved in the development of atherothrombosis and activation of acute inflammation exerting a proatherogenic and hypofibrinolytic effect. Lp(a) plays a critical role in the proinflammatory reaction and can be considered as a

Table 1: Lp(a) values in patients that developed atherosclerotic disease.

	Cases		Controls		Years of follow up	Year of the study
	n	Lp(a) (mg/dL)	*n*	Lp(a) (mg/dL)		
Alfthan et al. [56]						
Males	97	73	148	108	8	1994
Females	97	113	121	91		
Assmann et al. [57]	33	90	828	50	8	1996
Coleman et al. [58]	49	402	192	288	1–9	1992
Cremer et al. [59]	107	180	5124	90	5	1994
Jauhiainen et al. [60]	138	131	130	111	6-7	1991
Klausen et al. [61]	74	124	190	94	8 or 15	1997
Ridker et al. [62]	296	103	296	102.5	5.02	1995
Rosengren et al. [63]	26	277.7	109	172.7	6	1990
Schaefer et al. [64]	233	237	390	195	7–10	1994
Sigurdsson et al. [65]	104	230	1228	170	8.6	1992
Wald et al. [66]	229	85.8	1145	55.6	5–12	1994
Wild et al. [67]						
Males	90	125.1	90	63.5	13	1997
Females	44	97.3	44	72.7		
Bostom et al. [68]	305	>30	3103		12	1994
Bostom et al. [69]	129		2191		15.4	1996
Cantin et al. [70]	116	>30	2156		5	1998
Cressman et al. [71]		38.4	129	16.9	4	1992
Stubbs et al. [71]		>30	266 197		3	1998
Kronenberg and Utermann [72]		32.8	826	8.8	5	1999
Bennet et al. [72]	2047	43.8	3921	40.4	12	2008
Dahlén et al. [73]	62	250.2	124	134.7	11	1998
Cantin et al. [70]	116 with IHD	41	2040	32.7	5	1998
Nguyen et al [74]	9936	32.8	826	8.8	14	1997
Ariyo et al. [72]	3972	4.2			7.4	2003
Hoogeveen et al. [75]	57	12.65	46	9.15		2001
Dirisamer et al. [76]	103	20	103	15		2002

common joint among different metabolic systems. Other actions of Lp(a) can be resumed as follows: inhibition of the activation of plasminogen; inhibition of the activation of TGF-β; activation of acute inflammation; induction of the expression of adhesion molecules; elevation of the production of cytokines. Moreover Lp(a) is implicated in the activation of endothelial uptake, oxidative modification, and foam cell formation, suggesting that these processes could play an important role in atherosclerosis. Recent findings suggest that Lp(a)-lowering therapy might be beneficial, at least in some subgroups of patients with high Lp(a) levels. A possible future therapeutic approach could include apheresis in high-risk patients with already maximally reduced LDL cholesterol levels in order to reduce major coronary events [72]. However, further studies are needed to define such subgroups with regard to Lp(a) levels, apo(a) size, and the presence of other risk factors.

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Research Article

Sabiporide Reduces Ischemia-Induced Arrhythmias and Myocardial Infarction and Attenuates ERK Phosphorylation and iNOS Induction in Rats

Henri Doods[1] and Dongmei Wu[2,3]

[1] *CNS Diseases Research, Boehringer Ingelheim Pharma KG, D-88397 Biberach, Germany*
[2] *Department of Research, Mount Sinai Medical Center, Miami Beach, FL 33140, USA*
[3] *WCU Program, Department of BIN Fusion Technology, Chonbuk National University, Jeonju 561-756, Republic of Korea*

Correspondence should be addressed to Dongmei Wu; dongmeiwu@bellsouth.net

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The aim of the present study was to investigate the effects of sabiporide, a potent and selective NHE1 inhibitor, on myocardial ischemia-induced arrhythmias and myocardial infarction and the possible pathways related to the cardioprotection afforded by sabiporide treatment. Anesthetized rats were subjected to myocardial ischemia via left main coronary artery occlusion for 30 minutes, followed by 2 hours of reperfusion. Administration of sabiporide (0.01–3.0 mg/kg) prior to coronary artery occlusion dose-dependently reduced ischemia-induced arrhythmias and infarct size with an ED50 value of 0.14 mg/kg. Administration of sabiporide (1.0 mg/kg) prior to reperfusion also reduced infarct size by 38.6%. The reduction in infarct size was accompanied by a decrease in circulating levels of creatine phosphokinase and troponin I. In addition, sabiporide (1.0 mg/kg) given prior to coronary artery occlusion or immediately before reperfusion significantly reduced phosphorylation of the extracellular signal-regulated kinase (ERK1/2) and the expression of the inducible nitric oxide synthase (iNOS) following myocardial ischemia-reperfusion. This study demonstrates that sabiporide is a potent and effective cardioprotective agent during myocardial ischemia and reperfusion, by reducing serious ventricular arrhythmias and myocardial infarct size. The cardioprotection afforded by sabiporide is attributed in part to inhibition of ERK1/2 phosphorylation and suppression of iNOS expression.

1. Introduction

Na^+/H^+ exchangers (NHEs) are membrane proteins that regulate ion fluxes. Physiologically, they extrude one intracellular proton in exchange for one extracellular sodium, thereby regulating intracellular pH. NHE1, the housekeeping isoform present in all mammalian cells, is the most predominant isoform in cardiomyocytes [1, 2]. It is implicated in heart hypertrophy and heart failure and mediates myocardial damage that occurs after ischemia-reperfusion injury. For this reason, regulation of NHE1 has been proposed as a therapeutic target for cardioprotection [1–3].

Alterations in energy metabolism during acute ischemia and reperfusion cause disturbances in the ion homeostasis of myocardial cells. Intracellular acidosis is the major stimulus that regulates NHE1 activity. The reduction in intracellular pH during ischemia due to anaerobic metabolism and ATP hydrolysis stimulates Na^+/H^+ exchange leading to increased sodium influx and elevation in intracellular calcium concentration through increased Na^+/Ca^{2+} exchange, resulting in cellular injury [1–3]. In addition to activation of NHE1 during ischemia-reperfusion as a result of proton-dependent processes, a variety of endogenous mediators and oxidant stress produced by ischemia-reperfusion act to stimulate phosphorylation of the NHE1 cytosolic domain. These agents shift the set point of the antiporter such that it remains active in a more alkaline pH range [4, 5]. Inhibition of NHE1 has been shown to provide marked cardioprotection in a number of *in vitro* and *in vivo* models [1, 2].

Sabiporide, a benzoguanidine, is a potent selective inhibitor of NHE1. Considering its ability to inhibit initial rates

of $^{22}Na^{+}$ uptake, sabiporide is considered as one of the best NHE-1 inhibitors (Ki of $5 \pm 1.2\ 10^{-8}$ M). Furthermore, it discriminated efficiently between the NHE1, 2, and 3 isoforms (Ki for NHE2: $3 \pm 0.9\ 10^{-6}$ M and Ki > 1 mM for NHE3), and rinse-out kinetics showed that inhibition with sabiporide is extremely persistent as compared to amiloride and cariporide (half time of 7 hours for sabiporide and of 1 and 2.5 minutes for amiloride and cariporide, resp.) [6–8]. Our recent study showed that postresuscitation pharmacological conditioning with sabiporide afforded protection from whole body ischemia-reperfusion injury by improving cardiac function, enhancing blood flows to vital organs and attenuating systemic proinflammatory response in an experimental model of asphyxia-induced cardiac arrest in piglets [9]. However, the efficacy of sabiporide has not been examined in an animal model of regional ischemia-reperfusion injury. The present study investigated the dose-response effects of sabiporide on myocardial ischemia-induced arrhythmias and myocardial infarction in anesthetized rats and examined the potential signaling pathways underlying this protective response.

2. Methods

The Wistar rats (Chbb: Thom 350–380 g) used in this study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Performance of this project was granted approval by the local IACUC Review Board. The NHE1 selective inhibitor, sabiporide ((N-(aminoiminomethyl)-4-[4-(1H-pyrrol-2-ylcarbonyl)-1-piperazinyl]-3-(trifluoromethyl)-benzamide) was synthesized by Boehringer Ingelheim Pharma KG, Ingelheim, Germany [6].

2.1. Ischemia-Reperfusion Protocol. Rats were anesthetized with sodium pentobarbitone: initial induction at the 60 mg/kg dose (intraperitoneally [i.p.]) followed by continuous infusion at the dose of 30 mg/kg/h (subcutaneously [s.c.] in the abdominal skin through a 23G needle using a solution of 10 mg/mL) throughout the experiment. The animals were maintained in a deep surgical plane of anesthesia throughout the experiment by continuous blood pressure monitor and using the pin-prick of the hind leg method to determine the degree of the anesthesia. The trachea was cannulated, and the animals were artificially ventilated (80 strokes/min) with room air supplemented with oxygen. The body temperature was maintained at 37°C with a heating pad. The right carotid artery and left jugular vein were cannulated for continuous measurement of arterial blood pressure and intravenous administration of test agents (or vehicle: saline), respectively. Heart rate was derived from the blood pressure signal.

A left-sided thoracotomy was performed at the level of the fifth intercostal space. A 5-0 silk suture was placed around the left main coronary artery approximately 1-2 mm from its origin. Four pieces of number 16 sewing cotton were coligated along with the coronary artery to facilitate reperfusion. The coronary artery was occluded for 30 minutes followed by 2 hours of reperfusion. Reperfusion was instituted by removing the ligature. Blood pressure and heart rate were measured continuously throughout the experiment.

A lead II electrocardiogram (ECG) was recorded on a computer by Chart V3.5 program. Arrhythmias were evaluated according to the guidelines of the Lambeth conventions [10]. Ventricular premature beats (VPBs) were defined as discrete and identifiable premature QRS complexes (premature in relation to the P wave). All the VPBs occurring in the ischemic period (30 minutes) were counted. Ventricular tachycardia (VT) was defined as a run of four or more consecutive VPBs. The duration of VT, in seconds, was measured. Ventricular fibrillation (VF) was defined as a signal for which individual QRS deflections could no longer be distinguished from one another. The incidence of VF was quantitated in all rats.

At the end of the reperfusion period, the coronary artery was reoccluded. Evan's blue dye (1 mg/mL) was infused to the right ventricle to define the area of myocardium at risk. After this procedure, the heart was removed. Both atria and the roots of the great vessels were removed. The entire ventricle was cut from the apex to base into four transverse slices and incubated in 2,3,5-triphenyltetrazolium chloride (TTC) (10 mg/mL in phosphate buffer) for a period of 10 min at 37°C to visualize the infarct area. Each section was scanned by a color image scanner, and infarct size on the surface of each slice was determined by Photoshop 6.0 program. The infarct area were traced manually on the digital images and automatically measured. The infarct size was expressed as percentage of area at risk (AAR). The area at risk was similar between all animals, with an average of 62 ± 4% of LV. In some hearts (those which were not subjected to TTC staining), the free walls of the left ventricles (ischemia region or sham) were separated and snap-frozen for immunoanalysis.

Saline or sabiporide (0.01, 0.1, 0.3, 1.0, and 3.0 mg/kg) was given 10 min before coronary artery occlusion; or sabiporide (1.0 mg/kg) was administered immediately before the start of reperfusion.

2.2. Measurement of Creatine Phosphokinase (CPK) Activity and Troponin I. Blood samples were removed from carotid artery before drug treatment, before reperfusion, and at the end of experiment. They were promptly centrifuged at 12,500 rpm, 4°C, for 15 min, the plasma removed, and stored at −80°C until assayed. Creatine phosphokinase levels were determined using a CPK kit (Sigma, Steinheim, Germany). Plasma troponin I levels were measured using a cardiac troponin I enzyme immunoassay kit (Life Diagnostics, Inc., West Cheater, PA, USA).

2.3. Western Blot Analysis. Rat left ventricle tissue lysate was prepared by homogenization in ice cold RIPA buffer. Tissue and cell debris were removed by centrifugation. Protein concentration was determined by the Bio-Rad protein assay. The protein extracts were boiled for 5 min in SDS loading buffer and loaded onto NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). After electrophoresis, the separated proteins were transferred onto nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). The blots were

Table 1: Hemodynamics in control and sabiporide-treated rats.

	Baseline	After 5 min drug treatment	Occlusion 30 min	Reperfusion	
				1 h	2 h
MBP (mmHg)					
Control	119.1 ± 6.5	114.2 ± 8.5	65.9 ± 3.9#	69.8 ± 4.5#	67.6 ± 5.1#
Sabiporide (0.01 mg/kg Pre-Isch)	115.6 ± 8.1	122.3 ± 7.6	70.1 ± 4.5#	67.3 ± 5.2#	70.4 ± 6.4#
Sabiporide (0.1 mg/kg Pre-Isch)	120.8 ± 9.3	118.9 ± 10.6	68.4 ± 5.3#	65.3 ± 5.3#	66.9 ± 5.6#
Sabiporide (1 mg/kg Pre-Isch)	113.4 ± 8.7	117.8 ± 7.9	68.5 ± 4.9#	69.2 ± 7.1#	67.3 ± 5.1#
Sabiporide (3 mg/kg Pre-Isch)	121.8 ± 7.9	120.3 ± 8.4	72.4 ± 6.1#	67.2 ± 5.8#	71.2 ± 6.6#
Sabiporide (1 mg/kg Pre-Rep)	114.5 ± 6.7	115.4 ± 6.8	66.1 ± 3.5#	64.5 ± 5.5#	71.3 ± 5.3#
HR (bpm)					
Control	428.1 ± 18.5	423.7 ± 25.1	323.0 ± 22.4#	337.3 ± 20.2#	330.1 ± 23.1#
Sabiporide (0.01 mg/kg Pre-Isch)	416.7 ± 20.5	423.6 ± 22.7	315.6 ± 20.3#	323.4 ± 19.7#	330.5 ± 23.5#
Sabiporide (0.1 mg/kg Pre-Isch)	423.7 ± 23.7	416.4 ± 19.9	324.9 ± 18.6#	321.7 ± 23.4#	319.7 ± 19.5#
Sabiporide (1 mg/kg Pre-Isch)	430.3 ± 21.8	425.7 ± 21.6	310.5 ± 18.4#	332.9 ± 21.7#	324.6 ± 17.8#
Sabiporide (3 mg/kg Pre-Isch)	411.7 ± 19.2	419.3 ± 22.5	304.7 ± 17.9#	326.8 ± 19.7#	321.8 ± 21.9#
Sabiporide (1 mg/kg Pre-Rep)	431.2 ± 20.7	420.2 ± 17.7	326.6 ± 24.3#	312.5 ± 18.5#	323.7 ± 21.2#

All values are the mean ± SEM, $n = 6$. # $P < 0.05$ versus the baseline values.

incubated in 5% nonfat dry milk in Tris-buffered saline for 2 h at room temperature and incubated overnight at 4°C with primary antibody: extracellular signal regulated protein kinase (ERK1/2) (1: 500) (Abcam Inc., Cambridge, MA, USA), phospho-ERK1/2 (1: 2000); inducible nitric oxide synthase (iNOS) (1: 250); and GAPDH (1: 3000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were rinsed with Tris-buffered saline and incubated with HRP-conjugated donkey anti-rabbit or goat anti-mouse IgG secondary antibody for 2 hours. Immunoreactivity was detected using electrochemiluminescence autoradiography (ECL kit; Amersham, Piscataway, NJ, USA). The levels of the signals were detected by a color image scanner and quantified by densitometry with the use of the NIH Image J software. All bands were normalized against GAPDH. The intensity of the protein bands in the MI groups were expressed as percentage of the negative control tissue samples (from the sham animals involving an identical surgical procedure without MI). All the relative ratio values obtained from 6 rats/groups were pooled and presented as mean ± SEM.

2.4. Statistical Analysis. Data were analyzed for significance using GraphPad InStat software. Differences between groups in hemodynamic and infarct sizes were compared using ANOVA (with Bonferroni posttest) for multiple comparisons. Comparisons for the number of VPBs and duration of VT during ischemia were analyzed with Mann-Whitney U test. Comparisons for the incidence of VF were analyzed with Fischer's exact probability test. The results are presented as means ± SEM. P values <0.05 were considered to be significant.

3. Results

3.1. Hemodynamic and Arrhythmia Data. Consistent with previous findings on the activities of NHE1 inhibitors, sabiporide has no direct effect on blood pressure and heart rate (Table 1). All control rats exhibited VPB; 15 of 17 rats showed VT, while 13 of 17 showed VF. In addition, 5 of 17 control rats died by VF-induced cardiac arrest during the 30-minute coronary artery occlusion period. However, administration of sabiporide prior to coronary artery occlusion dose-dependently reduced the number of VPB, the duration of VT, and the incidence of VF (Figures 1(a), 1(b), and 1(c)). VF and death was completely prevented by sabiporide at the doses ranging from 0.1 to 3.0 mg/kg. One out of 7 rats died at the doses of 0.01 and 0.03 mg/kg, respectively.

3.2. Myocardial Infarction. Occlusion of the left main coronary artery for 30 minutes followed by 2 hours of reperfusion resulted in substantial injury to the myocardium. In the control group, occlusion and reperfusion produced an infarction of 49.8% (IF/AAR) (Figure 2). Meanwhile, treatment of sabiporide prior to coronary artery occlusion dose-dependently reduced the infarct size (Figure 2). The ED50 value was found to be 0.14 mg/kg. Administration of sabiporide (1.0 mg/kg) prior to reperfusion also reduced infarct size by 39% (Figure 2).

3.3. The Release of CPK and Troponin I. As shown in Figure 3, plasma levels of CPK and troponin I were significantly elevated after ischemia-reperfusion. Treatment with sabiporide reduced the plasma levels of CPK and troponin I by 63% and 55%, respectively. Sabiporide (1.0 mg/kg) given prior to reperfusion also reduced the plasma levels of CPK and troponin I by 42% and 40%, respectively.

3.4. Western Blot Analysis. Activation of ERK1/2 and induction of iNOS are known to be involved in myocardial ischemia-reperfusion injury [11, 12]. To further identify the possible pathways underlying sabiporide-induced cardioprotective effects, we measured the ERK1/2 expression and phosphorylation and iNOS expression in myocardium at the end of reperfusion by western blotting (Figures 4(a), 4(b), 4(c), and 4(d)). No difference in total ERK1/2 expression was observed among different treatment groups.

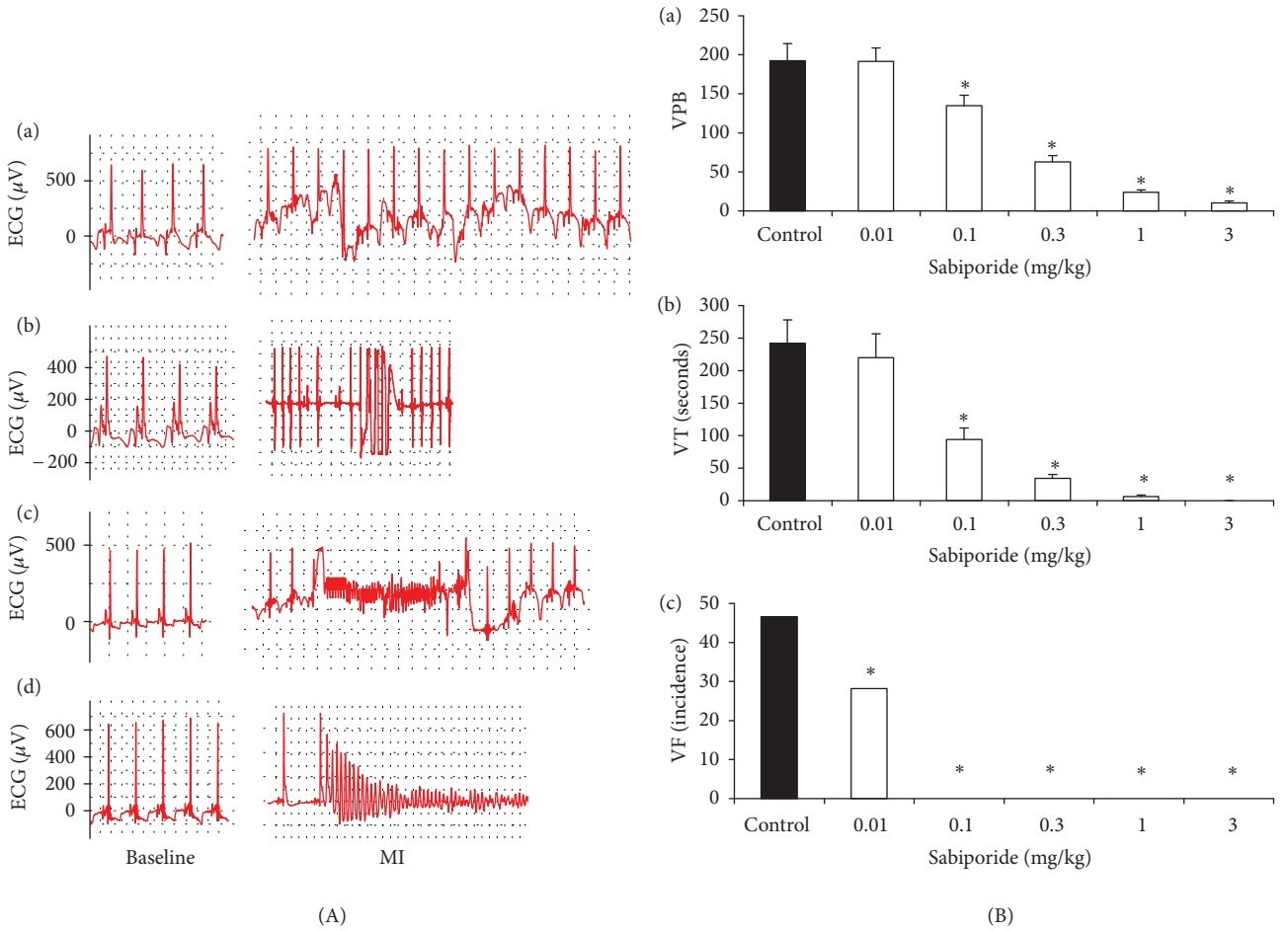

FIGURE 1: (A) Sample tracing EKG data from rats at baseline and myocardial ischemia (MI). (a) ventricular premature beats; (b) Ventricular tachycardia (VT); (c) ventricular fibrillation (VF) (reversible, animal survived); (d) ventricular fibrillation (irreversible, animal died). (B) Dose-dependent effects of sabiporide on (a) ventricular premature beats, (b) ventricular tachycardia duration, and (c) ventricular fibrillation incidence in anesthetized rats. All values are the mean ± SEM, $n = 6$. $^{*}P < 0.05$ versus the vehicle control group.

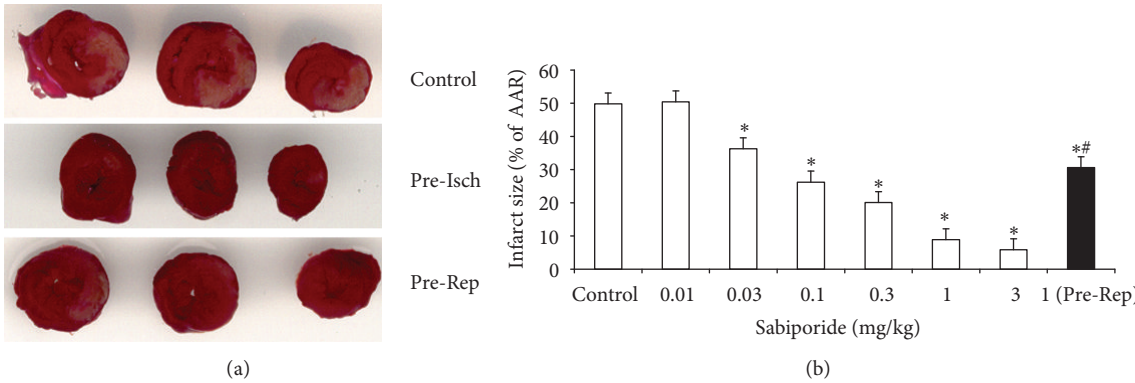

FIGURE 2: (a) Scanning graphs of the infarct area in control rats and those that received sabiporide (1 mg/kg) prior to ischemia (Pre-Isch) or reperfusion (Pre-Rep). (b) Dose-dependent effects of sabiporide in myocardial infarct size. Open column: sabiporide (0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg) or saline was administered intravenously 10 minutes before occlusion. Black column: sabiporide (1.0 mg/kg) was administered intravenously at the start of reperfusion. All values are the mean ± SEM, $n = 6$. $^{*}P < 0.05$ versus the control group. $^{\#}P < 0.05$ versus sabiporide (1.0 mg/kg) prereperfusion group.

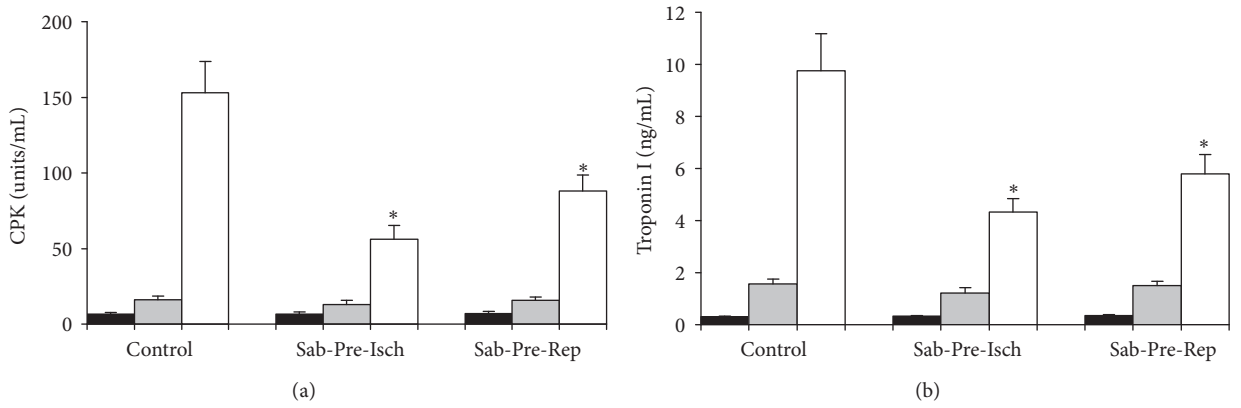

FIGURE 3: Effect of sabiporide (1 mg/kg) given prior to ischemia or prior to reperfusion on plasma levels of CPK (a) and troponin I (b) following myocardial ischemia-reperfusion on anesthetized rats. All values are the mean ± SEM, $n = 6$. $^{*}P < 0.05$ versus the control group. Black column: baseline; grey column: before reperfusion; white column: at the end of experiment.

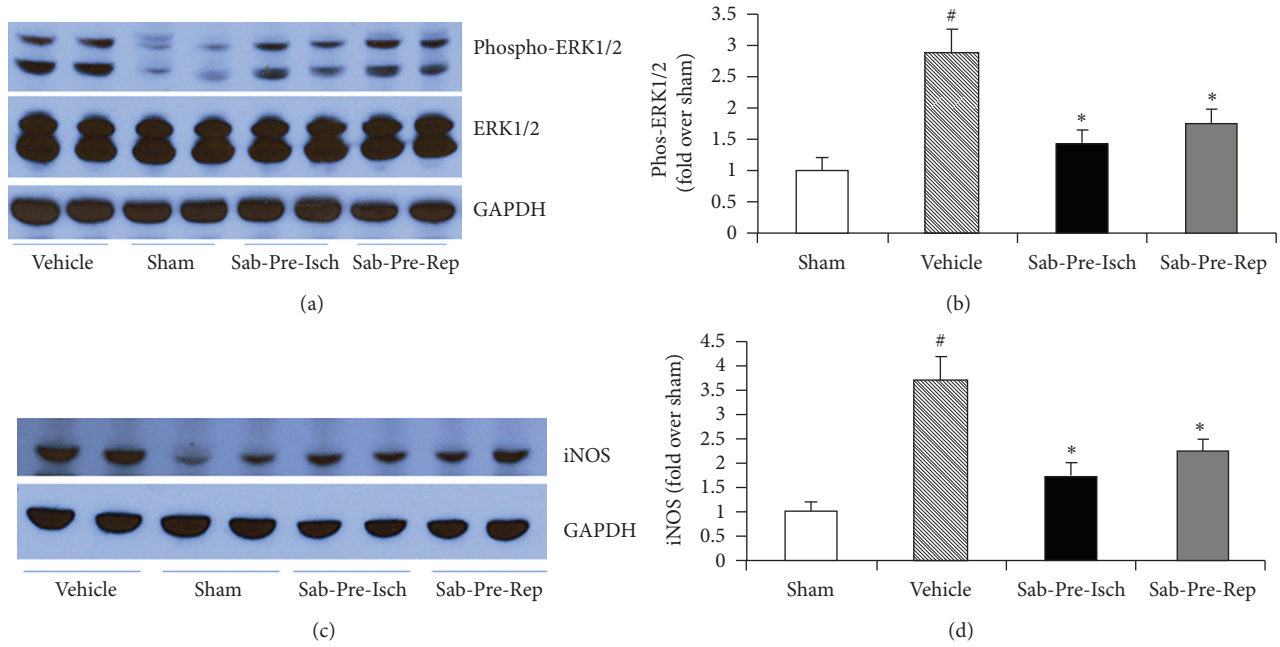

FIGURE 4: ERK1/2 expression and phosphorylation and iNOS expression in heart tissues from rats subjected to coronary ligation and reperfusion. Sabiporide (1.0 mg/kg) was given 10 min prior to coronary occlusion or immediately before reperfusion. (a) Representative blots of phosphorylated ERK1/2 and total ERK1/2 in heart tissues. (b) Statistical data obtained from quantitative densitometry of phos-ERK1/2 in heart tissues. (c) Representative blots of iNOS expression. (d) Statistical data obtained from quantitative densitometry of iNOS in heart tissues. Data are the mean ± SEM, $n = 6$. $^{\#}P < 0.05$ versus sham. $^{*}P < 0.05$ versus the vehicle control group.

However, ischemia-reperfusion resulted in a significant increase in ERK1/2 phosphorylation and sabiporide (1.0 mg/kg) given prior to coronary artery occlusion or prior to reperfusion significantly reduced ERK1/2 phosphorylation following ischemia-reperfusion by 51% and 38%, respectively. Ischemia-reperfusion also resulted in significantly increased iNOS expression. Treatment of sabiporide (1.0 mg/kg) prior to coronary artery occlusion or prior to reperfusion significantly reduced iNOS expression in heart tissue following ischemia-reperfusion by 53% and 40%, respectively.

4. Discussion

The effects of sabiporide on ischemia arrhythmias, myocardial infarction, ERK1/2 phosphorylation, and iNOS expression were studied in a rat model of myocardial ischemia-reperfusion injury. Administration of sabiporide prior to ischemia resulted in a dose-dependent reduction of ischemia-induced arrhythmias, including ventricular tachycardia, and ventricular fibrillation as well as reduction of myocardial infarct size. In addition, treatment with sabiporide prior to coronary artery occlusion or prior to reperfusion significantly

reduced ERK1/2 phosphorylation and cardiac iNOS expression following ischemia-reperfusion.

Coronary artery disease still remains the leading cause of death in the industrialized world despite considerable progress in its management. In patients with severe left ventricular dysfunction, more than 60% of deaths are attributed to the development of ventricular arrhythmias during periods of myocardial ischemia or infarction [13, 14]. The mechanisms of ventricular arrhythmias in acute myocardial ischemia and infarction have been mainly studied using animal models. A number of *in vivo* studies in rats have shown that acute myocardial ischemia with coronary artery occlusion results in severe ventricular arrhythmias [14–16]. Consistent with the findings of previous reports, the present study found that left main coronary artery occlusion resulted in serious ventricular arrhythmias, including ventricular premature beats, ventricular tachycardia and fibrillation, resulting in early death in rats.

Acute myocardial ischemia is associated with significant intracellular and extracellular ionic and metabolic alterations of the myocardium. Some of extracellular changes include elevated potassium, increased lactate and carbon dioxide production, acidosis, and catecholamine release, which concomitantly produce intracellular acidosis, elevated concentrations of calcium, magnesium, and sodium ions [14, 17]. These biochemical and metabolic changes alter inward and outward transmembrane ionic current fluxes, causing profound alterations of the resting membrane and action of potential characteristics of the myocyte. Changes such as depolarization of the resting membrane potential, diminished upstroke velocity, slowed conduction, decreased excitability, shortening of the action potential duration, altered refractoriness, dispersion of repolarisation, and abnormal automaticity, can all occur [14]. The resultant biochemical and electrical changes do not all occur at once; however, intracellular Na^+ and Ca^{2+} overload may be the final common pathway leading to cardiac arrhythmias following myocardial ischemia by activating the transient inward current or causing electrical uncoupling of cardiac myocytes [18, 19]. Thus, altered intracellular Na^+ and Ca^{2+} resulting from pH-regulated NHE1 activation (see reviews [4, 5]) may be an important contributor to cardiac arrhythmias following cardiac ischemia and that blocking NHE1 could be effective in attenuating ischemia-induced cardiac arrhythmias. Consistent with the observation that NHE1 inhibition can reduce ventricular arrhythmias [20, 21], the present study found that NHE1 inhibition with sabiporide dose-dependently reduced ventricular premature beats and ventricular tachycardia and prevented ventricular fibrillation and early death. This indicates that sabiporide is a potent and effective agent to attenuate cardiac arrhythmias.

Reducing myocardial infarct size is the primary goal in patients with coronary heart disease. Early reperfusion of an occluded coronary artery is a well-known and effective strategy to reduce ischemia-induced myocardial damage [22, 23]. However, reperfusion itself has been shown to cause significant cardiac injury through several complex and unresolved mechanisms [24, 25]. It is well established that NHE1 activation during ischemia-reperfusion through pH-regulatory pathway and other pathways mediated by endogenous ischemia metabolites results in increased intracellular Na^+ and Ca^{2+}, leading to myocardium damage [1–3]. NHE1 inhibitors have been shown to protect the myocardium against ischemia-reperfusion damage [14, 15, 21]. In this study, administration of sabiporide prior to ischemia dose-dependently reduced the infarct size. Administration of 1.0 mg/kg sabiporide prior to reperfusion also reduced infarct size by 38.6%. The reduction in infarct size was accompanied by a reduction in CPK and troponin I release, indicating that ischemia-reperfusion- induced myocardium damage can be attenuated by NHE1 inhibition with sabiporide.

Cardiac ischemia-reperfusion is associated with activation of various signaling pathways (e.g., extracellular signal regulated protein kinase (ERK1/2) pathway and inducible nitric oxide synthase (iNOS) mediated cellular injury pathway) that play an important pathophysiological role in the progression of ischemia-reperfusion injury and myocardial dysfunction [11, 12]. ERK1/2 participates in cellular signal transduction cascades and is activated by a diverse range of stimuli including oxidative stress, ischemia-reperfusion and vasoactive agents [11, 26–28]. Recent studies indicate a link between NHE1 activation and activation of ERK1/2 signaling pathways [29, 30]. In cardiac myocytes, sustained intracellular acidosis activates NHE1 and the ERK1/2 pathway [31]. Activation of ERK1/2 has been shown to stimulate NHE1 phosphorylation during acidosis and myocardial ischemia-reperfusion in neuron cultures, cardiac myocytes, and isolated hearts [29, 31]. However, the link between NHE1 and the ERK1/2 pathway in animal models of myocardial ischemia-reperfusion has not yet been identified. In the present study, we showed that pretreatment with sabiporide significantly reduced ERK1/2 phosphorylation following ischemia and reperfusion. This finding indicates that NHE1 activation may have directly triggered ERK1/2 phosphorylation, or indirectly by causing cellular injury which triggered oxidative stress and the release of vasoactive agents that induced ERK1/2 activation. Physical stress (ischemia-reperfusion) and some vasoactive agents (phenylephrine, endothelin-1, and angiotensin II) have been known to trigger activation of both NHE1 and ERK1/2 [1, 2, 29–31]. Therefore, further studies are needed to determine whether activation of NHE1 can directly or indirectly trigger ERK1/2 phosphorylation.

Increased iNOS expression is a component of the immune response and has been demonstrated in cardiomyocytes in ischemia-reperfusion, septic shock, myocarditis, transplant rejection, dilated cardiomyopathy, and heart failure [32–34]. Studies indicate that nitric oxide produced by iNOS is cardiotoxic in that it suppresses myocardial contractility and increases myocyte apoptosis and mortality [33, 35, 36]. Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death, suggesting a pathological role of iNOS induction in heart diseases [37]. Induction of iNOS expression is mediated through cytokine-inducible transcription factors, such as IFN regulatory factor-1 and nuclear factor kappa B (NF-κB), to elements within the iNOS promoter, as well

as through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species (ROS) formation [38–41]. In a rabbit model of pacing-induced heart failure, Aker et al. [42] have shown that sabiporide significantly reduced myocardial apoptosis, fibrosis, myocyte cross-sectional area, p38MAPK phosphorylation, and iNOS protein expression. They have also shown that the progression of heart failure in rabbits is attributed in part to p38 MAP kinase activation and ROS formation [43]. We have previously reported that inhibition of NHE1 attenuates NF-κB activation and reduces cytokines production in traumatic hemorrhage shock in pigs [44]. In the present study, treatment with sabiporide attenuated the induction of iNOS following ischemia-reperfusion. Thus, the findings from the present study suggest that in addition to blunting cellular ionic derangement through inhibition of pH-regulatory activation of NHE1 pathway, the salutary cardioprotection afforded by sabiporide may also in part be attributed to the inhibition of ERK1/2 phosphorylation and iNOS expression following ischemia-reperfusion.

Collectively, the present study demonstrates that sabiporide is a very potent and effective agent for cardioprotection during myocardial ischemia and reperfusion by reducing serious ventricular arrhythmias and myocardial infarct size. The cardiac protection afforded by sabiporide is possibly in part attributed to inhibition of ERK1/2 phosphorylation and suppression of iNOS induction.

In addition to the detrimental role of NHE1 activation in acute cardiac injury, studies have also demonstrated that NHE1 activation contributes to chronic maladaptive myocardial responses to injury such as postinfarction myocardial remodeling, and likely contributes to the development heart failure [45]. Furthermore, NHE1 is ubiquitously expressed in all mammalian cells. Recent studies have also shown that NHE1 inhibition protects from multiorgan injury in conditions of whole body ischemic-reperfusion injury and global metabolic acidosis, including cardiac arrest and resuscitation, traumatic hemorrhagic shock, and sepsis [9, 46, 47]. Thus, NHE1 inhibitors offer substantial promise for clinical development for the treatment of acute myocardial injury and heart failure and could also have potential implications for whole body protection from systemic metabolic acidosis.

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Research Article

High Insulin and Leptin Increase Resistin and Inflammatory Cytokine Production from Human Mononuclear Cells

Panayoula C. Tsiotra,[1] Eleni Boutati,[2] George Dimitriadis,[2] and Sotirios A. Raptis[1,2]

[1] *Hellenic National Center for the Research, Prevention and Treatment of Diabetes Mellitus and its Complications (H.N.D.C), 3 Ploutarchou, 10675 Athens, Greece*
[2] *2nd Department of Internal Medicine, Research Institute and Diabetes Center, Athens University Medical School, University General Hospital "Attikon", 1 Rimini, Athens, 12462 Haidari, Greece*

Correspondence should be addressed to Panayoula C. Tsiotra; ytsiotra@hndc.gr

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Resistin and the proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, produced by adipocytes, and macrophages, are considered to be important modulators of chronic inflammation contributing to the development of obesity and atherosclerosis. Human monocyte-enriched mononuclear cells, from ten healthy individuals, were exposed to high concentrations of insulin, leptin, and glucose (alone or in combination) for 24 hours *in vitro*. Resistin, TNF-α, IL-6, and IL-1β production was examined and compared to that in untreated cells. High insulin and leptin concentrations significantly upregulated resistin and the cytokines. The subsequent addition of high glucose significantly upregulated resistin and TNF-α mRNA and protein secretion, while it did not have any effect on IL-6 or IL-1β production. By comparison, exposure to dexamethasone reduced TNF-α, IL-6, and IL-1β production, while at this time point it increased resistin protein secretion. These data suggest that the expression of resistin, TNF-α, IL-6, and IL-1β from human mononuclear cells, might be enhanced by the hyperinsulinemia and hyperleptinemia and possibly by the hyperglycemia in metabolic diseases as obesity, type 2 diabetes, and atherosclerosis. Therefore, the above increased production may contribute to detrimental effects of their increased adipocyte-derived circulating levels on systemic inflammation, insulin sensitivity, and endothelial function of these patients.

1. Introduction

Adipose tissue secretes a number of bioactive molecules, such as resistin, TNF-α, and IL-6 collectively called adipokines, which affect peripheral insulin sensitivity and may be important players in the development of type 2 diabetes and atherosclerosis [1, 2]. On the other hand, these molecules expressed by the human macrophages can modulate chronic inflammation and have an impact in cardiometabolic complications such as atherosclerosis. Increased production of these adipokines occurs with expanding obesity, particularly visceral obesity, by both the adipocytes and the nonfat cells, mostly macrophages that infiltrate the adipose tissue [3].

The adipokine, resistin, was proposed originally to be a link between obesity and type 2 diabetes [4]. Administration of recombinant resistin in rodents impaired hepatic insulin sensitivity and glucose metabolism [5, 6], and treatment with anti-resistin anti-sense oligonucleotides reversed hepatic insulin resistance [7]. Although in humans there were studies demonstrating increased serum resistin levels in individuals with obesity and/or type 2 diabetes [8–10], resistin's biological role as an insulin resistance molecule was debated by others, which failed to confirm a causal relationship of circulating resistin with insulin sensitivity or other metabolic parameters [11, 12]. Notably, in mice resistin is produced solely by the adipose tissue, whereas in humans resistin is barely detectable in adipocytes, and its mRNA levels are much higher in monocytes and macrophages [13, 14]. Along with this, resistin's proinflammatory nature in man was suggested by the finding that human resistin could stimulate the expression of the proinflammatory cytokines TNF-α and IL-6 in both human and murine macrophages via

the NF-kB-dependent pathway [15, 16], while intravenous administration of endotoxin and activation of this inflammatory cascade could result in hyperresistinemia in humans [17], indicating the importance of this signalling pathway in the resistin-mediated inflammation [15]. In agreement with these experimental data, serum resistin concentrations are significantly elevated in patients with severe inflammatory disease [18] and correlate with markers of inflammation in their inflamed joints [16]. Resistin has also been shown to have direct effects on endothelial cell activation by inducing the expression of endothelin-1 (ET-1) and the cell adhesion molecules VCAM-1 and ICAM-1 [19, 20], and resistin's secretion by the atherosclerotic macrophages could promote atherosclerosis in humans [21]. Indeed, circulating resistin is significantly increased in patients with previous myocardial infarction [22], while it is positively correlated with markers of inflammation in patients with coronary artery disease (CAD) [23] and the risk of heart failure in the Framingham Offspring study [24].

Proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, circulating and locally produced in the endothelium, by tissue macrophages are thought to be involved in the atherogenic process. They have been shown to induce the expression of cellular adhesion molecules in the endothelium, thus, facilitating the entrapment of leukocytes and monocytes and the initiation of the atherogenic process [25]. Proinflammatory cytokines also increase fibrinogen production and enhance thrombosis, thus increasing the risk of vascular occlusion and cardiovascular events. Thus, elevated TNF-α plasma levels are associated with abdominal obesity and an increased risk of myocardial infraction in men [26, 27], while IL-6 circulating levels increase with obesity and are associated with increased risk for myocardial infarction and new onset type 2 diabetes [28, 29].

In the present study, we examined the *in vitro* effects of 24 h exposure to high concentrations of insulin, leptin, and glucose, commonly seen in obesity and type 2 diabetes, on the mRNA expression and protein secretion of resistin, TNF-α, IL-6, and IL-1β from human peripheral blood monocyte-enriched mononuclear cells. We also compared these effects to those of dexamethasone, a potent anti-inflammatory agent and correlated them to measures of obesity and insulin resistance.

2. Methods

2.1. Subjects. Ten healthy volunteers, aged 31 to 41 years, (BMI: 25.7 ± 1.2), participated in our study. The study was approved by the Hospital Ethics Committee in accordance with the Declaration of Helsinki, and all volunteers gave written informed consent. All subjects had no history of recent infection, myocardial infection, or CAD, and were taking no medication. Body Mass Index (BMI) was calculated as the ratio of weight (Kg) per height in the square (m^2). Insulin resistance was calculated by the homeostasis model assessment index (HOMA-IR) as (fasting insulin (IU/L) × fasting glucose (mmol/L))/22.5. Fasting glucose (YSI 2300 STAT Plus Glucose & Lactate Analyser, YSI Incorporated, Ohaio, USA), insulin levels (INSI-CTK irma, DiaSorin, Saluggia, Italy), and free fatty acid (FFA) levels (Falcor 300 Chemical Analyser, Menarini Diagnostics, Italy) were measured in the plasma from all individuals. High sensitivity C-reactive protein (CRP) was measured using the BN Prospec Nephelometer (Dade Behring, Newark, DE, USA) according to manufacturer's instructions.

2.2. Mononuclear Cell Isolation and Incubation Protocol. Peripheral blood was obtained from all subjects after an overnight fast and the mononuclear cells were separated from heparinized blood samples immediately after collection, using a Ficoll-Paque (Amersham, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient [30], while the plasma from the same samples was separated, aliquoted, immediately frozen, and kept at −80°C until assayed.

The isolated mononuclear cells were left selectively to adhere into a 100 mm-petri dish for 1 hour for the isolation of the primary monocyte-derived macrophages, as it has been described previously [31]. The adhered monocyte-enriched mononuclear cells were collected, counted, and plated at a density of 0.8×10^6 cells/well in 24-well plates in RPMI 1640 supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, and penicillin/streptomycin under an atmosphere of 5% CO_2 at 37°C. Cells were left to incubate with either of the following agents: 138.9 nM insulin (Humulin Regular, Lilly France, Fegersheim, France), 100 ng/mL human leptin (R&D Systems, Oxon, UK), 33 mM glucose (GIBCOBRL, Invitrogen Corp., Carlsbad, USA), 10^{-7} M dexamethasone (G. A. Pharmaceuticals, Athens, Greece), or combination of the former three agents for 24 hrs. After the incubation period, the medium was collected for measurement of the resistin, TNF-α, IL-6, and IL-1β secreted proteins, while at the same time total RNA was extracted from the cell pellet. Cell viability and cell counting were done using the trypan blue exclusion.

2.3. RNA Extraction and cDNA Synthesis. Total RNA was isolated from the human peripheral monocyte-enriched mononuclear cells using the Trizol Reagent (Invitrogen Corp., Carlsbad, USA). The integrity of RNA samples was determined on agarose gels (1.2%) and spectrophotometrically, using the absorption ratio at 260/280 nm. Before the reverse transcription reaction, removal of any residual DNA contamination was done by incubating all RNA samples with RQ1-RNase free DNase I (Promega, Madison, WI, USA), at 37°C for 25 minutes, as suggested by the manufacturer.

cDNA synthesis was performed in all samples, using oligo random hexanucleotides and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Promega Corp. Madison, USA), as previously described [30]. The reaction volume was precipitated with NaAcetate and ethanol at −20°C overnight. The cDNAs were diluted in nanopure water and were kept as a stock at −80°C, before the real-time PCR analysis.

2.4. Quantitative Real-Time PCR. A relative quantitative real-time PCR was performed in a spectrofluorometric thermal cycler (LightCycler, ROCHE, Manheim, Germany)

using the LightCycler Fast Start DNA Master HybProbe kit (ROCHE, Manheim, Germany), primers and fluorescently labeled hybridization probes specifically designed by TIM-MOLBIOL (Berlin, Germany) to detect the genes of interest. The specific reaction conditions for each set of genes (primer concentration, annealing temperature, magnesium chloride) were optimized first using the SYBR Green fluorescent dye (LightCycler FastStart DNA Master SYBR Green I, Roche Diagnostics). Hybridization primers and probes for all target and reference genes were as shown in Tables 3 and 4.

Relative quantification RT-PCR is the method that determines the changes in steady-state mRNA levels of a gene across various samples and expresses them relative to the levels of an internal reference control gene, usually a housekeeping gene. In order to quantify resistin, TNF-α, IL-6, and IL-1β relative mRNA levels, we used the calibrator normalized assay with an efficiency correction and the LightCycler Relative Quantification 1.01 Software (Roche Diagnostics) as it has been described before [31]. Briefly, using this quantification method, results are expressed as the target/reference ratio of the sample divided by the target/reference ration of the calibrator, and it does not require a standard curve in each run. The human housekeeping genes of β-actin and porphobilinogen deaminase (PBGD) were used as standards for normalization. Selection of either β-actin or PBGD as housekeeping genes was necessary, because the copy number of the housekeeping gene should be in a similar range with that of the target gene to make comparative quantification possible [8]. The RNA-DNase I treated samples, in which no M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase was added, together with the water containing samples (no DNA template), were used as negative controls in the RT-PCR reaction.

Relative quantification results were expressed in arbitrary units (AUs). Results from some PCR runs were run in ethidium bromide stained agarose gels and photographed in an Image Analyser VDS System (Amersham Biotech-Pharmacia, Sweden) for visualization purposes.

2.5. Immunoassays. Resistin (BioVendor, Czech Republic), TNF-α, IL-6, and IL-1β (Quantikine, R&D Systems, Oxon, UK) secreted proteins were measured in the supernatant from the human cultured monocyte-enriched mononuclear cells, by the use of standard commercial ELISAs according to the manufacturer recommended protocols. Circulating resistin (BioVendor, Czech Republic), TNF-α (R&D Systems, Oxon, UK), adiponectin, IL-6, and IL-1β proteins (Human Serum Adipokine Panel A and Panel B LINCOplex kits, Linco-Millipore Corp., USA) were measured in the plasma of all volunteers using either standard commercial sandwich ELISA kits or the multiplex assay (xMAP technology) and the fluorescently labeled microsphere beads in a LUMINEX 200 instrument (Luminex Corp., USA). Due to the low circulating IL-6 and IL-1β protein levels in the human plasma, we could not detect IL-1β in the plasma of the healthy individuals, while IL-6 could be detected in only 4 out of 10 individuals making the analysis of circulating IL-1β and IL-6 problematic.

The sensitivities of the assays were: 0.2 ng/mL (resistin), <4.4 pg/mL (TNF-α-Elisa assay), <0.70 pg/mL (IL-6-Elisa assay), <1 pg/mL (IL-1β-Elisa assay), 0.1 pg/mL (IL-1β multiplex assay), 145.4 pg/mL (adiponectin), and 1.6 pg/mL (IL-6-multiplex assay). The intra-and interassay coefficients of variation were, respectively, 3.6% and 6.7% (resistin), 4.9% and 5.8% (TNF-α-Elisa assay), 2.6% and 4.5% (IL-6, Elisa assay), 4.8% and 5.6% (IL-1β-Elisa assay), 1.4–7.9% and <21% (adiponectin, IL-6, and IL-1β-multiplex assays). Plasma samples were measured in duplicate in a single experiment.

2.6. Statistical Analysis. Statistical analysis was performed using the SPSS 14.0.1 software (SPSS Corp, Chicago, IL, USA). Data are expressed as mean ± SEM of n independent expreriments. Because the investigated variables showed a nonnormal distribution, nonparametric statistical analyses were applied (Spearmann's correlation test, Wilcoxon signed rank Test). Multivariate regression analysis was also performed if any adjustments were necessary. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Patients' Characteristics. The clinical and biochemical characteristics of the volunteers studied, as well as their plasma resistin, TNF-α, IL-6, and adiponectin levels are shown in Table 1. All individuals had normal glucose tolerance, a mean BMI of 25.7 ± 1.2 Kg/m^2, and their CRP levels were within physiological range (0–1.4 mg/L). Circulating adiponectin levels were higher in women compared to men (30.72±6.32 μg/mL versus 11.26±3.49 μg/mL, $P = 0.016$), but no other differences were observed with regard to age, BMI, fasting insulin and glucose plasma levels, insulin resistance as assessed by HOMA-IR index, or plasma resistin, TNF-α and IL-6 proteins between men and women (Table 1).

Overall, circulating adiponectin was negatively associated with HOMA-IR index ($r = -0.636$, $P = 0.048$), while circulating resistin was positively associated and plasma TNF-α negatively associated with FFA plasma levels ($r = 0.773$, $P = 0.015$ and $r = -0.835$, $P = 0.005$, resp.). The former significance was retained in a stepwise multivariate analysis independent of age, gender, BMI, and insulin resistance (HOMA-IR index) as independent variables (B ± SE: 10.74 ± 3.18, $P = 0.043$) ($R^2 = 0.92$), while the latter significance was lost in the multivariate analysis. Furthermore, circulating plasma resistin was also negatively associated with circulating plasma TNF-α levels ($r = -0.678$, $P = 0.045$).

3.2. Regulation of Resistin mRNA and Protein Expression by Insulin, Leptin, and Glucose in Human Mononuclear Cells. Leptin by itself significantly upregulated resistin protein secretion ($P = 0.049$) and tended to induce resistin mRNA levels ($P = 0.059$) from the monocyte-enriched mononuclear cells, after the 24-h incubation period, and its combination with insulin further increased by 52% resistin protein secretion ($P = 0.024$), but it did not alter basal resistin mRNA levels (Figures 1(a) and 1(b)). Insulin alone did not

Table 1: Anthropometic and biochemical characteristics and plasma resistin, TNF-α, IL-6, and adiponectin levels of the 10 healthy volunteers as a whole, and further divided in women and men.

	Total subjects ($n = 10$)	Women ($n = 5$)	Men ($n = 5$)	P value
Age (years)	34.7 ± 1.17	32.8 ± 1.31	36.6 ± 1.6	0.056
BMI (Kg/m^2)	25.7 ± 1.19	24.48 ± 2.28	26.83 ± 0.69	0.151
Fasting insulin (μU/mL)	10.8 ± 1.3	10.38 ± 1.81	11.23 ± 2.07	0.841
Fasting glucose (mg/dL)	79.4 ± 3.67	81.96 ± 7.3	76.84 ± 1.97	0.222
HOMA-IR index	2.16 ± 0.32	2.17 ± 0.54	2.15 ± 0.41	0.690
FFA (mM)	0.22 ± 0.05	0.28 ± 0.09	0.14 ± 0.04	0.190
CRP (mg/L)	0.38 ± 0.15	0.31 ± 0.16	0.46 ± 0.31	0.905
Resistin (ng/mL)	6.27 ± 0.49	6.85 ± 0.77	5.55 ± 0.36	0.190
TN-α (pg/mL)	1.46 ± 0.37	1.19 ± 0.29	1.73 ± 0.69	0.841
IL-6 (pg/mL)	7.3 ± 3.90	1.69 ± 0.19	12.90 ± 5.32	0.333
Adiponectin (μg/mL)	20.99 ± 4.7	30.72 ± 6.32	11.26 ± 3.49	**0.016**

BMI: Body Mass Index; HOMA-IR: homeostasis model assessment; FFA: free fatty acids; CRP: C-reactive protein. Data were expressed as means ± SEM. P value gives the statistical difference between women and men.

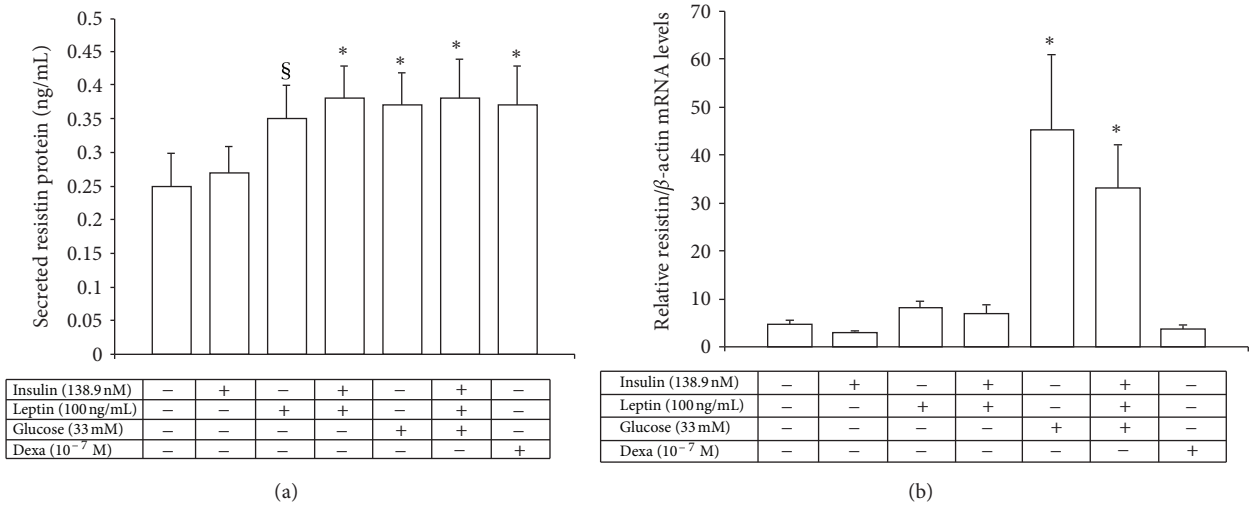

Figure 1: Secreted resistin protein levels (a) and relative resistin/β-actin mRNA levels (b) from human monocyte-enriched mononuclear cells after *in vitro* exposure for 24 h to high concentrations of insulin, leptin, glucose, and dexamethasone. $^*P < 0.03$ versus control cells, $^xP = 0.049$ versus control cells.

have any effect on resistin protein or mRNA levels. In contrast, high concentrations of glucose powerfully and significantly increased the relative resistin mRNA and protein levels ($P = 0.004$ for both), an effect that remained significant when glucose was combined with insulin and leptin (Figures 1(a) and 1(b)). Finally, dexamethasone (10^{-7} M) increased significantly resistin protein production ($P = 0.006$, Figure 1(a)), but failed to change resistin basal mRNA levels (Figure 1(b)).

3.3. Regulation of TNF-α mRNA and Protein Expression by Insulin, Leptin, and Glucose in Human Mononuclear Cells. High concentrations of insulin and leptin either alone or combined together significantly increased TNF-α protein secretion ($P < 0.02$) from human peripheral monocyte-enriched mononuclear cells *in vitro* (Figure 2(a)). Furthermore, glucose alone significantly increased TNF-α protein ($P = 0.013$) and its combination with insulin and leptin upregulated further TNF-α protein secretion levels ($P < 0.001$, Figure 2(a)).

With regard to TNF-α mRNA production glucose and leptin, either alone or combined with insulin, significantly increased relative TNF-α mRNA levels (2.0-fold, 2.1-fold, and 5.2-fold, resp., $P < 0.05$, $P < 0.02$ and $P < 0.002$, resp.), while dexamethasone significantly suppressed by 50% both TNF-α mRNA levels and protein secretion *in vitro* ($P < 0.02$ and $P < 0.001$, resp.) (Figures 2(a) and 2(b)).

3.4. Regulation of IL-6 and IL-1β mRNA and Protein Expression by Insulin, Leptin, and Glucose from the Human Mononuclear Cells. Insulin and leptin either alone or both combined together increased significantly IL-6 protein levels ($P = 0.005$, $P = 0.004$, and $P = 0.016$, resp.) from human peripheral monocyte-enriched mononuclear cells (Figure 3(a)), while they did not change basal IL-6 mRNA levels (Figure 3(b)). Dexamethasone, however, powerfully suppressed up

Table 2: Bivariate correlations of secreted resistin, TNF-α, IL-6, and IL-1β protein with their corresponding basal mRNA levels and the studied metabolic parameters.

	Secreted resistin (ng/mL)	Secreted TNF-α (pg/mL)	Secreted IL-6 (pg/mL)	Secreted IL-1β (pg/mL)
Secreted TNF-α (pg/mL)	−0.167			
Secreted IL-6 (pg/mL)	−0.600	0.491		
Secreted IL-1β (pg/mL)	0.048	**0.733***	0.017	
Relative resistin mRNA levels	0.083	−0.224	−0.321	0.167
Relative TNF-α mRNA levels	0.143	**0.667****	0.033	**0.833***
Relative IL-6 mRNA levels	−0.071	−0.357	0.286	0.690
Relative IL-1β mRNA levels	0.071	0.381	0.429	0.036
BMI (Kg/m^2)	−0.017	−0.127	0.042	−0.433
Fasting insulin (μU/mL)	−0.317	−0.127	0.176	−0.417
Fasting glucose (mg/dL)	0.450	−0.552	−0.018	−0.517
FFA (mM)	−0.108	−0.017	0.487	−0.143
CRP (mg/L)	−0.514	−0.479	0.297	**−0.730***

BMI: Body Mass Index; FFA: free fatty acids. CRP: C-reactive protein. Numbers represent r values. $^{*}P < 0.025$, $^{**}P < 0.05$.

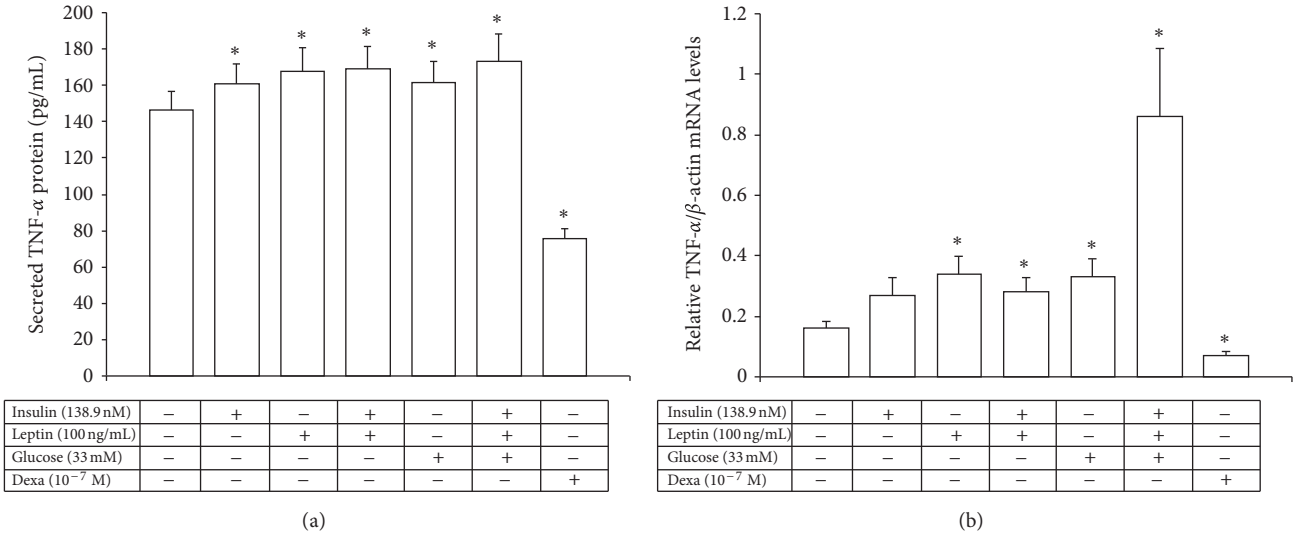

Figure 2: Secreted TNF-α protein levels (a) and relative TNF-α/β-actin mRNA levels (b) from the human monocyte-enriched mononuclear cells after *in vitro* exposure for 24 h to high concentrations of insulin, leptin, glucose, and dexamethasone. $^{*}P < 0.045$ versus control cells.

to 6-fold IL-6 protein and mRNA levels ($P < 0.001$ and, $P < 0.05$ resp.) (Figures 3(a) and 3(b)). Glucose on the other hand, either alone or combined together with insulin and leptin had no significant effect on IL-6 mRNA or protein secretion levels, although there was a nonsignificant 3-fold increase of IL-6 mRNA levels under these conditions (Figures 3(a) and 3(b)).

Only leptin or its combination with insulin had a significant increased effect on IL-1β protein secretion ($P < 0.05$ and $P < 0.02$, resp.) from human peripheral monocyte-enriched mononuclear cells *in vitro* (Figure 4(a)). Glucose either alone or combined together with insulin and leptin had no effect on IL-1β protein levels. No effect on IL-1β mRNA levels was observed upon incubation of the cells with glucose, leptin, insulin, or their combination (Figure 4(b)). Only dexamathasone reduced IL-1β mRNA ($P = 0.013$) and tended to reduce IL-1β protein expression ($P = 0.061$) from the human cells *in vitro* (Figure 4).

Additionally, secreted TNF-α protein levels correlated positively with secreted IL-1β protein levels ($r = 0.733$, $P = 0.025$) and TNF-α mRNA levels ($r = 0.667$, $P = 0.05$), while secreted IL-1β protein correlated negatively with CRP ($r = -0.730$, $P = 0.025$) and with TNF-α mRNA levels ($r = 0.833$, $P = 0.010$) (Table 2). Moreover, IL-6 basal mRNA levels correlated positively with fasting glucose plasma levels ($r = 0.786$, $P = 0.021$).

4. Discussion

We have demonstrated that the combined exposure of human peripheral monocyte-enriched mononuclear cells to high concentrations of insulin and leptin for 24 hours *in vitro* clearly stimulated resistin, TNF-α, IL-6, and IL-1β protein expression. The further addition of glucose increased significantly resistin and TNF-α protein and mRNA levels but failed to induce similar changes in IL-6 and IL-1β protein produc-

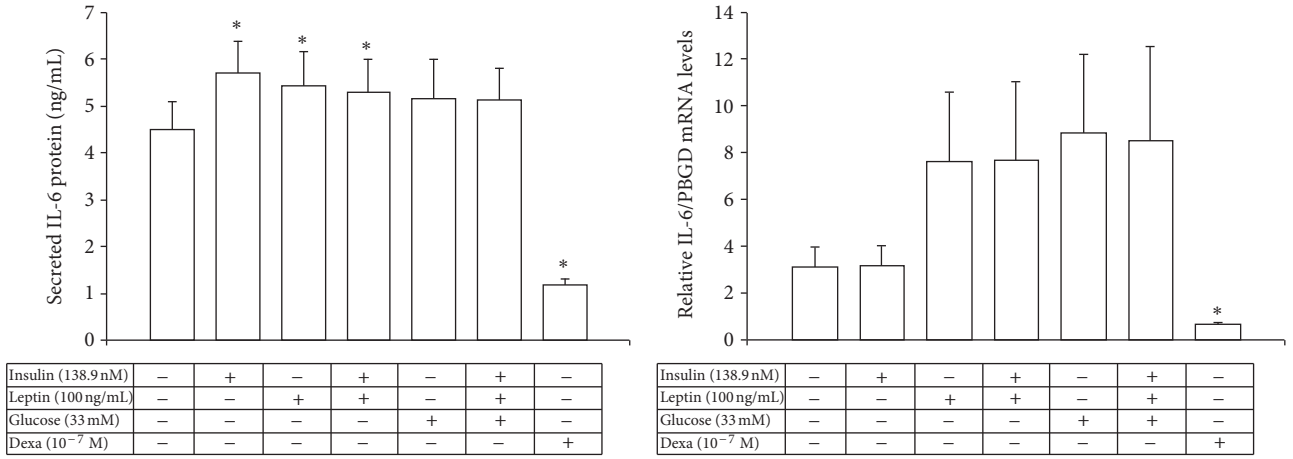

Figure 3: Secreted IL-6 protein levels (a) and relative IL-6/PBGD mRNA levels (b) from the human monocyte-enriched mononuclear cells after exposure to high concentrations of insulin, leptin, glucose, and dexamethasone. $^{*}P < 0.05$ versus control cells.

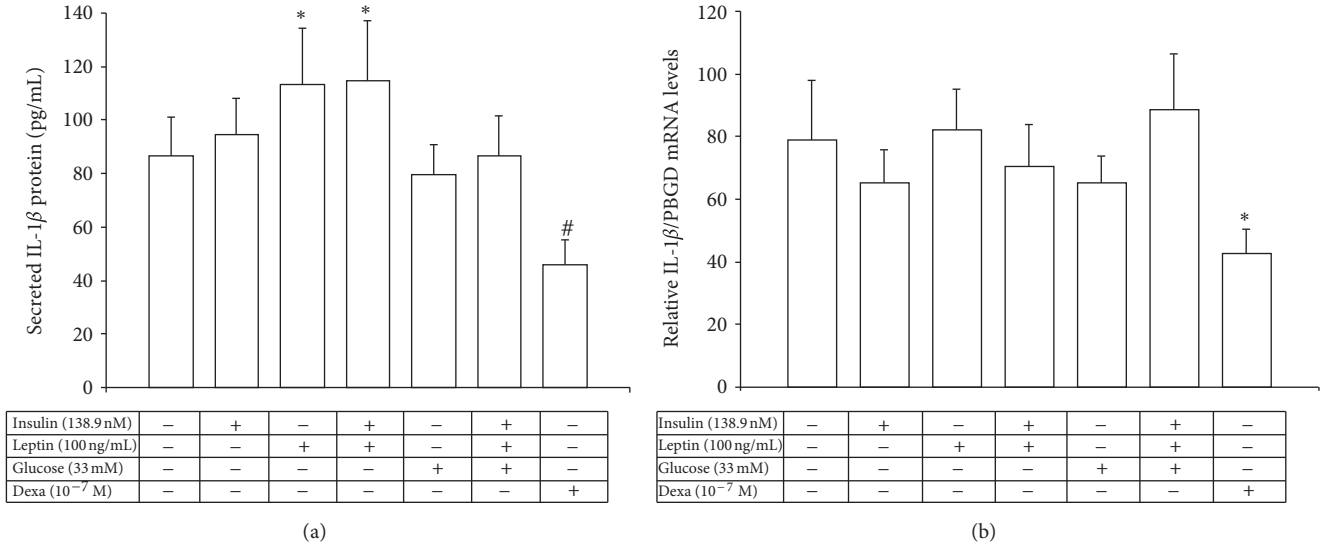

Figure 4: Secreted IL-1β protein levels (a) and relative IL-1β/PBGD mRNA levels (b) from the human monocyte-enriched mononuclear cells after exposure to high concentrations of insulin, leptin, glucose, and dexamethasone. $^{*}P < 0.05$ versus control cells, $^{\#}P = 0.061$ versus control cells.

tion. Moreover, as expected, exposure to dexamethasone decreased powerfully TNF-α, IL-6, and IL-1β production while it increased resistin protein secretion.

The above findings are in agreement with previous results where dexamethasone or high glucose concentrations increased the expression of both resistin mRNA and protein in 3T3-L1 murine adipocytes [32]. High glucose concentrations also upregulated resistin gene expression and protein levels in the human U937 monocytic cell line, an effect mediated by MAPK and NF-kB-dependent mechanisms [33]. In contrast, insulin diminished resistin expression by 30–37% in 3T3-L1 mouse adipocytes acting possibly through the Phosphoinositide (PI)-3, ERK, or p38-MAP- kinases pathways [32, 34]. Insulin in diabetic mice caused a marked increase in adipose tissue resistin mRNA levels [35] and induced resistin protein secretion but not mRNA production in human differentiated adipocytes [36]. However, in humans, there are a number of studies that failed to identify any correlation of resistin with various markers of insulin resistance [11, 12]. In agreement with this, resistin plasma levels and gene expression in mononuclear cells and in macrophages did not differ between women with polycystic ovary syndrome (PCOS) and controls [37]. Furthermore leptin treatment either did not alter resistin mRNA expression in human peripheral blood mononuclear cells [38] or decreased adipose tissue resistin gene expression and improved insulin sensitivity in the ob/ob mice [39]. These discrepancies are probably due to the fact that resistin's

Table 3

	5′-sense primer-3′	5′-a-sense primer-3′
Resistin	GGGCTGTTGGTGTCTAGCAAG	GTCTCGGCGCGCACAT
TNF-α	ACAAGCCTGTAGCCATGTT	AAAGTAGACCTGCCCAGACT
IL-6	CCAATCTGGATTCAATGAGGAGACT	GAGCCCTCAGGCTGGACTG
IL-1β	CAGGGACAGGATATGGAGCAA	GCAGACTCAAATTCCAGCTTGTTA
β-actin	CTTCTACAATGAGCTGCGTGTG	GTGAGGATCTTCATGAGGTAGTCAGTC
PBGD	GGCTGCAACGGCGGAA	CCTGTGGTGGACATAGCAATGATT

Table 4

	5′-FL probe-3′	5′-LC Red640 probe-3′
Resistin	GCGACCTCCTGGATCCTCTCATTGA	GCTTCTTCCATGGAGCACAGGGTC
TNF-α	GCATTGGCCCGGCGGTTC	CCACTGGAGCTGCCCCTCAGCT
IL-6	AGATGCAATAACCACCCCTGACCCAA	CACAAATGCCAGCCTGCTGACGAA
IL-1β	GCTTATCATCTTTCAACACGCAGGACA	GTACAGATTCTTTTCCTTGAGGCCCA
β-actin	GGTATGCCCTCCCCCATGCC	TCCTGCGTCTGGACCTGGCTG
PBGD	CATACAGACGGACAGTGTGGTGGCAAC	TGAAAGCCTCGTACCCTGGCCTG

expression is regulated differently between tissues as well as between mouse and human species. It is of interest that although the human resistin gene appears to be the true ortholog of the murine resistin gene, the derived proteins not only have a very low degree of amino acid conservation, but also exhibit a different tissue distribution profile [40, 41]. These findings, along with the evidence that only two RELM genes have been discovered in humans compared to three in mice, imply that the two genes possibly serve different biological functions in the two species. In our study, however, exposure of the human monocyte-enriched mononuclear cells to either dexamethasone or leptin *in vitro* induced resistin protein secretion while no change was observed in resistin mRNA levels.

We have also demonstrated that high concentrations of either insulin or leptin could enhance cytokine (TNF-α, IL-6, IL-1β) production while the addition of high glucose induced further TNF-α mRNA and protein expression, a finding also supported by previous reports in both humans and mice [42, 43]. Interestingly, we have previously demonstrated that monocyte-enriched mononuclear cells from individuals with type 2 diabetes show increased levels of visfatin, resistin, TNF-α, IL-6, and IL-1β mRNA expression indicating that hyperinsulinemia and hyperglycemia could enhance cytokine production [8, 31]. Surprisingly, in the present *in vitro* study we could not detect an increase in IL-6 or IL-1β production from the monocyte-enriched mononuclear cells isolated from healthy individuals after glucose, insulin, and leptin treatment. Notably IL-6 mRNA levels were increased around 3-fold, but not significantly, under these conditions. Finally, the inhibitory effect of dexamethasone on IL-6, TNF-α, and IL-1β production demonstrated in our study (a finding that had also been reported previously by Bessler et al. [44]), confirms that cytokine production by the isolated peripheral monocyte-enriched mononuclear cells under the *in vitro* conditions we used is subject to regulation. This serves also as a control for the changes we observed after exposure to glucose, insulin, and leptin.

Hyperinsulinemia, hyperglycemia, and hyperleptinemia are commonly seen in obesity-related cardiometabolic disorders, like type 2 diabetes and its complications such as dyslipidemia, hypertension, and atherosclerosis. Low grade systemic inflammation seems to be the common soil hypothesis for these metabolic disorders and recently it was demonstrated that inflammasome components are key players in the induction of obesity and insulin resistance in mice [45]. Circulating monocytes as well as macrophages infiltrating the arterial wall and the adipose tissue are an important source of inflammatory cytokines, and adipokines like resistin, participating in the process of atherogenesis and contributing to systemic inflammation in the adipose tissue and the atheromas, could aggravate this process under hyperglycemic, hyperinsulinemic and possibly hyperleptinemic conditions [3, 46]. Indeed, resistin was found to be more highly expressed in atherosclerotic aneurysms than in normal arteries, while at the same time induces the proliferation and migration of the human vascular smooth muscle cells [21] leading to progression of atherosclerosis in rabbit carotid artery [47]. Plasma resistin levels were significantly higher in patients with coronary artery disease (CAD) and in individuals with acute inflammatory disease than controls, and correlated with markers of inflammation predicting coronary atherosclerosis in humans [18, 22, 23, 48]. Resistin may also induce endothelial dysfunction [19, 20, 49] and treatment of macrophages with resistin could induce lipid accumulation, supporting further resistin's role in atherosclerosis [50]. It is intriguing that in our study dexamethasone, a known anti-inflammatory agent, increased resistin protein levels in human mononuclear cells while at the same time it decreased powerfully TNF-α, IL-6, and IL-1β production. Moreover, monocytes from DM2 patients compared to controls express higher resistin mRNA levels

with a similar increase in their corresponding plasma levels [8]. Resistin's proinflammatory nature is characterized by its induction by various proinflammatory molecules, while at the same time it can stimulate the expression of cytokines such as TNF-a and IL-12 [15–17]. Moroever, in inflammasome, deficient animals which are protected against the development of high-fat diet (HFD), induced obesity and insulin resistance, the production of protein resistin is significantly reduced [45]. Taken all together the aforementioned, suggest that resistin may have a major role in inflammation-associated cardiometabolic disorders [17, 51].

5. Conclusions

In the present study, we have demonstrated for the first time that insulin and leptin and possibly glucose, at concentrations commonly seen in obesity and type 2 diabetes powerfully stimulate resistin and cytokine proinflammatory expression in cultured human monocytes which in turn could aggravate the already increased inflammatory load. It may be suggested that with expanding obesity the increase of resistin and TNF-α and secondarily of IL-6 and IL-1b expression from monocytes/macrophages that infiltrate the stroma of the adipose tissue and/or the vascular endothelium could enhance the effects of their adipocyte-derived levels, and thus may contribute to the risk of atherosclerosis that accompanies obesity and type 2 diabetes.

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Research Article

A Retrospective Longitudinal Cohort Study of Antihypertensive Drug Use and New-Onset Diabetes in Taiwanese Patients

Ching-Ya Huang,[1,2] Tsochiang Ma,[3] Liyun Tien,[4] Yow-Wen Hsieh,[1,2] Shwu-Yi Lee,[5] Hung-Yi Chen,[1,2] and Gwo-Ping Jong[6]

[1] *Department of Pharmacy, China Medical University Hospital, Beikang Campus, Taichung 40402, Taiwan*
[2] *Institute of Pharmacy, China Medical University, Taichung 40402, Taiwan*
[3] *Department of Health Services Administration, China Medical University, Taichung 40402, Taiwan*
[4] *Central Region Branch, Bureau of National Health Insurance, Taichung 40709, Taiwan*
[5] *Yuan Rung Hospital, Changhua 51043, Taiwan*
[6] *Division of Internal Cardiology, Armed Forces Taichung General Hospital, and Basic Science, Central Taiwan University of Science and Technology, Taichung 41168, Taiwan*

Correspondence should be addressed to Gwo-Ping Jong; cgp8009@yahoo.com.tw

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Antihypertensive drugs have been linked to new-onset diabetes (NOD); however, data on the effect of these drugs on the development of NOD in hypertensive patients has not been well determined in a clinical setting. The aim was to investigate the association between antihypertensive drugs and NOD in Taiwan. We conducted a retrospective study of hypertensive Taiwanese patients receiving antihypertensive drugs treatment between January 2006 and December 2011. Clinical information and laboratory parameters were collected by reviewing the medical records. We estimated the odds ratios (ORs) of NOD associated with antihypertensive drug use; nondiabetic subjects served as the reference group. A total of 120 NOD cases were identified in 1001 hypertensive patients during the study period. The risk of NOD after adjusting sex, age, baseline characteristics, and lipid profiles was higher among users of thiazide diuretics (OR, 1.65; 95% confidence interval (CI), 1.12–2.45) and nondihydropyridine (non-DHP) calcium channel blockers (CCBs) (OR, 1.96; 95% CI, 1.01–3.75) than among nonusers. Other antihypertensive drug classes were not associated with risk of NOD. Our results show that patients with hypertension who take thiazide diuretics and non-DHP CCBs are at higher risk of developing NOD than those who take other classes of antihypertensive drugs in Taiwan.

1. Introduction

Diabetes mellitus is a major global public health problem, and it is associated with an estimated annual cost of US$174 billion in the USA alone [1, 2]. Concerns regarding new-onset diabetes (NOD) have been raised because of the economic burden it poses in various countries [3]. Recently, some multiple prospective trials of treatments for hypertension initiated a debate about the clinical impact of NOD in hypertensive patients [4–8]. It seems obvious that cardiovascular risk is increased when diabetes and hypertension coexist than when the two conditions stand alone; however, data from these studies are limited due to clinical trials [5, 6] or head-to-head comparisons of drugs [9, 10]. In particular, it is not completely clear whether certain antihypertensive drug classes are associated with higher risk of NOD. Our previous article [11] provided an estimate of the effects of antihypertensive drugs on the development of NOD from the data of the Bureau of National Health Insurance in Taiwan from January 2002 to December 2007. This data suggests that while hypertensive patients who took angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), or alpha-blockers were at a lower risk of NOD, diuretics, beta-blockers, and calcium-blockers were associated with a significant

increased risk of NOD. However, many reports have provided conflicting results about the effects of antihypertensive drugs on NOD under various conditions [12, 13]. Therefore, we conducted another retrospective cohort study to explore the relationship between antihypertensive drugs and NOD in a clinical setting.

The aim of this paper is to determine the effect of antihypertensive drugs [thiazide diuretics, beta-blockers, dihydropyridine (DHP) calcium channel blockers (CCBs), nondihydropyridine (non-DHP) CCBs, alpha-blockers, vasodilators, ACE inhibitors, and ARBs] on NOD in a clinical setting.

2. Materials and Methods

2.1. Subjects. Our data were taken from medical records provided to the China Medical University Hospital from January 2006 to December 2011. By medical record (electronic chart) review method, selected patients were further clarified to see if they fulfilled the inclusion and exclusion criteria. Electronic chart review contains information regarding patient identification numbers, sex, age, diagnostic codes, current smoking, familial history of diabetes mellitus (DM), body mass index (BMI), blood pressure, total cholesterol, triglyceride, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, fasting blood glucose, serum creatinine, and drugs prescription's information. The LDL cholesterol level was obtained by calculation from Friedewald equation, LDL cholesterol = total cholesterol – HDL cholesterol – (triglyceride/5). Due to the influence of Taiwan BNHI policy, the majority of LDL levels were obtained by calculation instead of direct measurement. The prescription table contains the quantity and expenditure for all drugs, operations, and treatments. Patients were included in the study if they had hypertension only without diabetes at baseline (January 1, 2006). We summarized the medical records of each patient into one record.

2.2. Study Procedure. We used the International Classification of Diseases, Ninth Revision (ICD-9) Clinical Modification code to define hypertension (ICD-9 codes 401–405) and diabetes (ICD-9 codes 250). Any patient with a diabetes diagnosis or prescription for antidiabetic drugs during 2 years prior to their antihypertensive prescription on January 1, 2004 was excluded. The primary endpoint was NOD, which was the first time that a diabetes code or antidiabetic prescription appeared in the medical records. We identified all prescriptions for antihypertensive drugs administered to patients with and without NOD within a 6-year period before the date NOD was diagnosed. Patients who had used only one type of antihypertensive drug in the 180 days before the date NOD was diagnosed were categorized according to the antihypertensive drug class that they took: thiazide diuretics, alpha-blockers, beta-blockers, DHP CCBs, non-DHP CCBs, vasodilators, ACE inhibitors, and ARBs. Patients using more than one type of antihypertensive drug in the 180 days before the date NOD was diagnosed were categorized as combined users. Patients who had used antihypertensive drug within the previous 6 years, but not within 180 days before the date NOD was diagnosed were excluded from the analyses. Finally, we excluded 17 patients who were lost to follow-up or died. A total of 1,001 patients with hypertension only were selected for this study. This study was approved by ethics committee of China Medical University Hospital.

2.3. Drug Classes. The antihypertensive drugs were categorized into 8 drug classes (thiazide diuretics, alpha-blockers, beta-blockers, DHP CCBs, non-DHP CCBs, vasodilators, ACE inhibitors, and ARBs). There are 6 drugs in the alpha-blocker class, 5 drugs in the ACE inhibitor class, 5 drugs in the ARB class, 10 drugs in the BB class, 6 drugs in the DHP CCB class, 7 drugs in the non-DHP CCB class, 5 drugs in the thiazide diuretic class, and 10 drugs in the vasodilator class in the China Medical University Hospital.

2.4. Statistical Analysis. Continuous variables are presented as mean ± SD. They were compared by the Welch t-test. Categorical and discrete variables are presented as frequencies and percentages. When appropriate, they were compared by either the Fisher's exact test or the chi-square test. This study aimed to find out what drug classes might increase or decrease the incidence probability of developing NOD. The 8 drug classes are the main effects adjusted by total drug days (tdays). Logistic regression analysis was applied. The odds ratio was used to measure the incidence probability of NOD. The Wald confidence interval for odds ratio (θ) was used to define the significant difference under $\alpha = 0.05$. If the confidence interval for θ contains 1.0, it is plausible that the true odds of developing NOD are equal among drug classes. If it is greater than 1, the probability of developing NOD among patients who took this drug class is higher than that among patients who did not take that class of drug. An odds ratio less than 1 indicates that the drug class has a low probability of being associated with the development of NOD. Finally, multiple logistic regression models including sex, age, current smoking, familial history of DM, BMI, total cholesterol, triglyceride, LDL cholesterol, HDL cholesterol, and fasting blood sugar were implemented. All analyses were performed using Statistical Analysis software, version 9.1 (SAS). A two-tail P value < 0.05 was interpreted as significant.

3. Results

3.1. Baseline Data of All Patients. Of the 1,001 eligible patients from January 2006 to December 2011, 120 (12.0%) patients developed NOD. Table 1 describes the baseline characteristics of study participants of the two groups. Patients ranged in age from 31 to 81 years; the mean age for NOD patients was 59.8 years and that of non-NOD patients was 56.4 years. There were significant differences in age between these two groups of patients ($P = 0.003$). Men comprised less than half (447, 45%) of the sample population.

There were no significant differences in current smoking, blood pressure, and familial history of DM between these two groups of patients ($P > 0.05$). BMI, total cholesterol, Triglyceride, LDL cholesterol, and fasting blood glucose of the NOD group were significantly higher than the non-NOD

Table 1: Baseline characteristics of all patients.

	NOD (n = 120)	Non-NOD (n = 881)	Total (n = 1001)	P^* value
Age (year-old)	59.8 ± 11.4	56.4 ± 13.6	56.8 ± 13.4	0.003
Male (%)	50 (42)	397 (45)	447 (45)	0.537
Current smoking (%)	19 (16)	122 (14)	141 (14)	0.496
Familial history of DM (%)	18 (15)	105 (12)	123 (12)	0.338
BMI ≥ 27 (%)	43 (36)	202 (23)	245 (24)	0.002
Systolic blood pressure (mm Hg)	148.7 ± 10.2	149.0 ± 9.8	149.0 ± 9.9	0.824
Diastolic blood pressure (mm Hg)	77.8 ± 6.2	77.8 ± 6.0	77.8 ± 6.0	0.892
TC (mg/dL)	227 ± 28	219 ± 35	220 ± 34	0.005
TG (mg/dL)	152 ± 52	130 ± 45	133 ± 51	<0.001
LDL-C (mg/dL)	138 ± 30	131 ± 36	132 ± 35	0.019
HDL-C (mg/dL)	58 ± 13	60 ± 17	60 ± 16	0.114
FBG (mg/dL)	101 ± 10	85 ± 12	87 ± 12	<0.001
Serum creatinine (mg/dL)	1.16 ± 0.32	1.13 ± 0.33	1.13 ± 0.33	0.351
Number of prescription (%)				0.040
1	29 (24)	205 (23)	234 (23)	
2	42 (35)	308 (35)	350 (35)	
3	40 (33)	302 (34)	342 (34)	
4	8 (7)	56 (6)	64 (6)	
5	1 (1)	10 (1)	11 (1)	
Drug class				
Thiazide diuretics (%)	38 (32)	215 (24)	253 (25)	0.054
Beta-blockers (%)	76 (63)	518 (59)	594 (59)	0.399
DHP CCBs (%)	68 (57)	474 (54)	542 (54)	0.528
Non-DHP CCBs (%)	13 (11)	63 (7)	76 (8)	0.102
Alpha-blockers (%)	15 (13)	148 (17)	163 (16)	0.233
ACE inhibitors (%)	17 (14)	132 (15)	149 (14)	0.771
ARBs (%)	37 (31)	289 (33)	326 (33)	0.634
Vasodilators (%)	16 (13)	107 (12)	123 (12)	0.591

* P value between NOD and non-NOD.
DM: diabetes mellitus. BMI: body mass index. TC: total cholesterol. TG: triglyceride. FBG: fasting blood glucose. DHP-CCB: dihydropyridine calcium channel blockers. Non-DHP CCB: Nondihydropyridine calcium channel blockers. ACE: angiotensin converting enzyme. ARB: angiotensin receptor blocker.

Table 2: Incidence of odds ratios (ORs) with 95% confidence intervals (CIs) for NOD according to prescriptions for antihypertensive drugs compared with nonuser subjects.

Drugs	Adjusted OR*	Adjusted 95% CI	P^{**} value
Thiazide diuretics	1.65	1.12–2.45	0.012
Beta-blockers	1.39	0.94–2.06	0.099
DHP CCB	1.24	0.84–1.82	0.276
Non-DHP CCB	1.96	1.01–3.75	0.044
Alpha-blockers	0.71	0.31–1.68	0.445
ACE inhibitors	1.53	0.90–2.64	0.117
ARBs	1.16	0.77–1.75	0.470
Vasodilators	0.92	0.53–1.60	0.777

*ORs were adjusted for age, sex, current smoking, familial history of DM, BMI, total cholesterol, Triglyceride, LDL cholesterol, HDL cholesterol, and fasting blood sugar.
** P value between users and nonusers.
DHP-CCB: dihydropyridine calcium channel blockers.
Non-DHP CCB: nondihydropyridine calcium channel blockers.
ACE: angiotensin converting enzyme.
ARB: angiotensin receptor blocker.

group ($P < 0.05$). Only there was no significant difference in serum creatinine and HDL cholesterol of lipid profile between these two groups of patients ($P > 0.05$).

Approximately 23% (234) of the patients took only one drug class, 35% (350) took two drug classes, 34% (342) took three drug classes, and 7% (2,353) of patients took from four to five drug classes (Table 1). Over half of the patients took DHP CCBs (59%) beta-blockers (54%). Only 8% (76) of the patients took non-DHP CCBs. The distributions of prescription thiazide diuretics, alpha-blockers, ACE inhibitors, ARBs, and vasodilators are shown in Table 1.

3.2. Multiple Logistic Regression Results after Adjusting Sex, Age, Current Smoking, Familial History of DM, BMI, Total Cholesterol, Triglyceride, LDL Cholesterol, HDL Cholesterol, and Fasting Blood Glucose. The risk estimate of NOD after adjusting sex, sex, current smoking, familial history of DM, BMI, total cholesterol, Triglyceride, LDL cholesterol, HDL cholesterol, and fasting blood glucose for users of thiazide diuretics (OR, 1.65; 95% confidence interval [CI], 1.12–2.45) and non-DHP CCBs (OR, 1.96; 95% CI, 1.01–3.75) was significantly higher ($P < 0.05$) than for nonusers. Beta-blockers, DHP CCBs, alpha-blockers, ACE inhibitors, ARBs, and vasodilators were not associated with increased risk of NOD ($P > 0.05$) (Table 2).

4. Discussion

The present study demonstrates that thiazide diuretics and non-DHP CCBs were independently associated with an increased risk of NOD in a clinical setting. The use of alpha-blockers, beta-blockers, DHP CCBs, ACE inhibitors, ARBs, or vasodilators was not associated with NOD.

The present results differ from those of our previous report on beta-blockers, ACE inhibitors, ARBs, and alpha-blockers [11]. However, these differences may be due to the relatively younger age (53 versus 68 years) of participants, different study periods, relatively smaller sample size (1001 versus 24688), and different population of patients taking these classes of antihypertensive drugs in this study. Nonetheless, both studies demonstrate that vasodilators are not associated with a risk of NOD.

Two differences between the results of our previous study and those in this study need to be emphasized. Firstly, in this study, we only collected thiazide diuretics that have been reported to accelerate NOD in patients with hypertension [14, 15]. Our results are comparable with those reported by Taylor et al. who studied the risk of NOD in three cohorts of 74,186 patients taking different classes of antihypertensive drugs [16]. They found that the relative risk of NOD in individuals taking thiazide diuretics was 1.20 (95% CI, 1.08–1.33) in the cohort of older women, 1.45 (95% CI, 1.17–1.79) in younger women, and 1.36 (95% CI, 1.17–1.58) in men. Many meta-analyses have also confirmed that diuretics increase the incidence of NOD compared with other antihypertensive drug classes [17, 18]. This could be a dose-related effect as higher doses are more likely to be associated with NOD [19]. A change in serum potassium levels could be a possible mechanism for thiazide-induced NOD. In one study, a large potassium infusion increased insulin release by two to three times above the basal levels [20]. This is an important mechanism of potassium disposal because insulin can induce increased cellular uptake of potassium. Secondly, we further determined the effect of DHP and non-DHP calcium channel blockers on NOD in a clinical setting. Calcium channel blockers are generally considered to have mild or no impact on the risk of NOD [16]. Many meta-analyses have indicated that CCBs are associated with a greater increase in NOD than ACE inhibitors and ARBs but a lower increase in NOD than beta-blockers and thiazide diuretics. However, no data has effectively demonstrated the effect of DHP and non-DHP CCBs on the development of NOD. To the best of our knowledge, our study is the first to show that non-DHP CCBs are associated with an increased risk of NOD, while DHP CCBs are not. Two recent meta-analyses showed that CCB therapy is not associated with an increased risk of NOD compared with placebo [15, 18]. The mechanism underlying the increase in NOD in patients with non-DHP CCB therapy, observed in the current study, has not been identified [18]. There is only the report suggesting that the DHP CCB azelnidipine may influence inflammation and oxidative stress indirectly and have a beneficial effect on glucose intolerance and insulin sensitivity in nondiabetic patients with essential hypertension [21]. From our study, it showed significant difference of BMI, total cholesterol, Triglyceride, LDL cholesterol, and fasting blood glucose between these two groups of patients. Probably, non-DHP CCBs therapy have related to metabolic disturbance.

Padwal et al. evaluated 76,176 patients with hypertension and reported that the use of beta-blockers was not associated with NOD [22]. However, experts have commented that the study had a mean follow-up time period of less than one year. Furthermore, theirs was an observational community study, and therefore, the results may have lacked the statistical power necessary to demonstrate an association between that class of antihypertensive drugs and NOD [23]. Our study excludes those factors, and the results further demonstrated the same findings as that report.

Recent studies have indicated that ACE inhibitors and ARBs reduce the risk of developing NOD when compared with the results for other classes of antihypertensive agents [24, 25]. In the current study, both ACE inhibitors and ARBs were found to be unassociated with NOD in patients with hypertension. Our result is the same as that from the Diabetes Reduction Assessment with Ramipril and Rosglitazone Medication (DREAM) trial [26], which failed to show a statistically significant reduction in NOD with the ACE inhibitor ramipril versus placebo in patients with IFG. Another study, Telmisartan Randomized Assessment Study in ACE Intolerant Subjects with Cardiovascular Disease (TRANSCEND) [27] had findings similar to our result that the ARB telmisartan cannot reduce the incidence of diabetes.

In the current study, alpha-blockers and vasodilators were found to be unassociated with NOD. Numerous studies have consistently demonstrated that alpha-blocker classes of antihypertensive medications have differential effects on carbohydrate and lipid metabolism in humans [28]. Our

result is different from that of our previous report on the alpha-blocker classes [11]. However, the difference could have been due to the relatively younger age (53 versus 68 years), different study period, and different population of the patients taking this class of antihypertensives in our more recent study.

Some limitations in this study need to be emphasized. First, this was a retrospective and descriptive study in CMUH over a period of six years. Also, we performed analyses that excluded participants with untreated, ongoing hypertension, so caution must be exercised in interpreting our data. Second, all cases in this study were collected from medical records, and diagnoses were based on physician reporting only in CMUH; therefore, it is not clear how our findings can be generalized to patients in different areas. Third, this is a descriptive study and no data regarding time of administering the antihypertensive drugs; therefore, it is not the most effective for determining the relationship between antihypertensive drugs and NOD. Furthermore, the process of insulin resistance in this study of patients who developed NOD must have started many years before the diagnosis, and insulin resistance might have coexisted with the hypertensive condition for which the antihypertensive drug was used. In this situation, the temporality and subsequently the causality of the antihypertensive drugs cannot be determined.

5. Conclusion

In conclusion, our findings provide some support for the hypothesis that there are differences in the risk of developing NOD among the different classes of antihypertensive drugs. Our results show that patients with hypertension who take thiazide diuretics and non-DHP CCBs are at higher risk of developing NOD than those who take other classes of antihypertensive drugs in a clinical setting. We suggest that doctors do not use non-DHP CCBs for stage 1 hypertension alone.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Pharmacist C.-Y. Huang and Professor T. Ma share equal contribution. Dr. H.-Y. Chen and Dr. G.-P. Jong share equal contribution.

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Research Article

Association between Polymorphisms of Alpha-Adducin Gene and Essential Hypertension in Chinese Population

Li-na Zhang,[1] Lin-dan Ji,[2,3] Li-juan Fei,[1] Fang Yuan,[1] Yue-miao Zhang,[1] and Jin Xu[1,3]

[1] *Department of Preventive Medicine, School of Medicine, Ningbo University, Ningbo 315211, China*
[2] *Department of Biochemistry, School of Medicine, Ningbo University, Ningbo 315211, China*
[3] *State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China*

Correspondence should be addressed to Jin Xu; xujin1@nbu.edu.cn

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The association between polymorphisms of α-adducin (*ADD1*) gene and essential hypertension is still unclear. Thus, we carried out a case-control study and an interaction analysis to test whether *ADD1* is a common candidate gene for hypertension in the Chinese population. Blood samples and information including body mass index (BMI), smoking habit, and alcohol abuse were collected. Meanwhile, total cholesterol, high density lipoprotein, triglyceride were measured by automatic biochemistry analyzer. All 6 tag single nucleotide polymorphisms (tagSNPs) within *ADD1* gene were genotyped by SNPstream genotyping system. Multifactor dimensionality reduction (MDR) was used to identify the interactions among the SNPs and the non-genetic factors. Results showed that plasma triglyceride, total cholesterol, and BMI were significantly higher in the hypertensive group than in the control group. Result from genotyping indicated that rs4963 was significantly associated with essential hypertension. After stratification by gender, rs4963 was associated with essential hypertension only in males. MDR analysis indicated that interaction among BMI, rs4963, and rs16843452 were involved in susceptibility of hypertension. The present study indicated that rs4963 within *ADD1* gene was associated with essential hypertension in Chinese population, which might be related to altered exonic splicing and disrupted gene regulation.

1. Introduction

Adducin (ADD) is a heterodimeric cytoskeleton protein consisting of an α-subunit with either a β- or a γ-subunit [1], and they are encoded by three different genes *ADD1*, *ADD2*, and *ADD3*, respectively. The α-subunit is known to increase renal sodium reabsorption and may be involved in the pathophysiology of essential hypertension [2]. Therefore, it is considered as a major candidate gene for essential hypertension. Not surprisingly, a number of studies have investigated the association of α-adducin gene (*ADD1*) polymorphisms with essential hypertension in the past two decades.

Human *ADD1* gene is located on chromosome 4p16.3 and comprises 16 exons. One well-studied polymorphism in *ADD1* gene is a substitution of Gly for Trp at amino acid residue 460 (G460W, rs4961), which was first described by Cusi et al. [3]. *ADD1* W460W was found to be associated with a form of low renin salt-sensitive hypertension in Caucasians, with reduced response to pressure natriuretic function and with increased proximal sodium reabsorption [4, 5]. Therefore, although a great number of similar studies have been conducted, the results are often inconsistent.

To convince the association of this polymorphism with essential hypertension, several meta-analyses were recently published [6–10]. Most of these analyses failed to provide evidence for the genetic association of *ADD1* G460W polymorphism with essential hypertension in global population; but it is suggested that the W carrier might increase the risk of essential hypertension in Han Chinese population [7]. However, most studies focus on *ADD1* G460W polymorphism, and whether other single nucleotide polymorphisms (SNPs) in *ADD1* gene were associated with essential hypertension is not clear. Therefore, we carried out a case-control study

and an interaction analysis to verify whether *ADD1* gene is associated with hypertension in the Chinese population.

2. Materials and Methods

2.1. Population for Case-Control Study. The participants were chosen from our established community-based epidemiology study of common diseases. We have collected more than 10,000 health records. Subsequently, participants who fulfilled the following criteria were put into our database: 30 to 75 years old, Han Chinese, living in Ningbo City (East Coast of China) for at least three generations and do not have a migration history. The hypertensive subjects and control subjects were matched for age and sex. In addition, only subjects who do not have other cardiovascular disease or major chronic illnesses according to their health records were included in the control group. Lastly, 905 essential hypertension cases and 905 healthy controls, including 392 males and 513 females in each group, were involved in the following studies.

2.2. Blood Pressure and Clinical Parameters. Blood pressure measurements were conducted in the morning after the participant had been in the sitting position for 10 minutes. Three BP measurements were obtained at 5-minute intervals using standard mercury sphygmomanometer, and the average of last two measurements was taken as the BP for that participant. Hypertension in this study is defined as a sitting systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg, or self-reported use of antihypertension medication (also confirmed by health records). Patients with secondary hypertension were excluded. Normal blood pressure is defined with SBP ≤ 120 mmHg and DBP ≤ 80 mmHg.

Blood samples were collected with informed consent. Subsequently, total cholesterol (TC), high density lipoprotein (HDL), and triglyceride (TG) were measured from these blood samples by Hitachi automatic biochemistry analyzer 7100. Clinical information including body mass index (BMI), and weekly alcohol and cigarettes consumption were also obtained. In this study, who drank ≥70 g alcohol per week for more than 1 year were defined as individuals with alcohol abuse. Moreover, who smoked ≥70 cigarettes per week for more than 1 year were defined as individuals with smoking habit. The protocol of this study was reviewed and approved by the Ethics Committees of Ningbo University.

2.3. SNP Genotyping. All six tagSNPs were retrieved from HapMap (http://hapmap.ncbi.nlm.nih.gov/), with tagger pairwise method in CHB: *R* Square cut off = 0.8 and MAF cut off = 0.1. Genomic DNA was obtained from the whole blood using standard phenol/chloroform extraction. Genotyping was performed using the GenomeLab SNPstream Genotyping System (Beckman Coulter Inc.) according to the manufacturer's protocol [11].

2.4. Statistical Analysis. Continuous variables are presented as the mean ± SD and analyzed by *t*-test between two groups. Statistical analyses of allele and genotype frequencies between hypertensive cases and healthy controls and between different sex groups were performed by chi-squared test (SPSS 16.0, SPSS Inc.). Hardy-Weinberg equilibrium (HWE) was tested by the software PEDSTATS V0.6.8 (http://www.sph.umich.edu/csg/abecasis/). Linkage equilibrium (LD) blocks were defined based on the confidence intervals method [12] and the most likely haplotypes within each block for individuals were inferred using the Haploview software [13]. Multifactor dimensionality reduction (MDR) was used to identify and characterize interactions among the SNPs and the nongenetic factors, including BMI, serum HDL, TC, and TG level, as well as percentage of smoking and alcohol abuse [14]. The software used for MDR is distributed in a JAVA platform with a graphical user interface and is freely available (http://www.epistasis.org/mdr.html).

All tests were two sided, and *P* values less than 0.05 were considered statistically significant.

Table 1: Baseline characters of the investigated participants.

Variables	Hypertensive	Control	*P* value
Number	905	905	N/A
Sex (male) (%)	43.3	43.3	N/A
Age (y)	56.91 ± 7.37	56.60 ± 7.51	$P = 0.38$
TG (mM)	2.02 ± 1.68	1.63 ± 1.12	$P < 0.01$
HDL (mM)	1.41 ± 0.35	1.41 ± 0.32	$P = 0.72$
TC (mM)	5.34 ± 1.00	5.17 ± 0.93	$P < 0.01$
BMI (Kg/m^2)	24.65 ± 3.24	23.21 ± 2.86	$P < 0.01$
Regular smoking	173	147	$P = 0.11$
Regular alcohol drinking	152	148	$P = 0.80$

TG: triglyceride; HDL: high density lipoprotein; TC: total cholesterol; BMI: body mass index.

3. Results

The baseline characteristics of our study population are summarized in Table 1. Age and sex distribution, HDL, smoking percentage, and percentage of alcohol abuse showed no difference between hypertensive and control groups. However, TG, TC, and BMI were significantly higher in the hypertensive group than in the control group ($P < 0.05$).

Genotype distributions of six tagSNPs within *ADD1* gene were shown in Table 2. None of them deviated from the HWE ($P > 0.05$). Only rs4963 was significantly associated with essential hypertension ($P = 0.02$, odds ratio (OR) = 0.85, 95% confident interval (CI) = 0.75–0.97). After stratification by gender, rs4963 was associated with essential hypertension only in males, and the other 5 SNPs were still negative (Table 3). We further performed haplotype analysis within LD blocks of *ADD1*. Only one LD block and three common haplotypes within the LD block (including rs16843452 and rs12503220) were identified (Figure 1). Of these three haplotypes, no haplotype was significantly associated with hypertension (Table 4).

Finally, MDR was used to analyze the interaction among different SNPs and nongenetic risk factors for hypertension.

Table 2: Association analysis of 6 tagSNPs.

SNP	Group	Genotype			*P* value	OR	95% CI
rs16843452	Hypertensive	243 (CC)	462 (CT)	195 (TT)	0.89	1.01	0.89~1.15
	Control	243 (CC)	461 (CT)	199 (TT)			
rs12503220	Hypertensive	11 (AA)	253 (AG)	636 (GG)	0.26	0.90	0.75~1.08
	Control	22 (AA)	257 (AG)	624 (GG)			
rs4963	Hypertensive	192 (CC)	432 (CG)	278 (GG)	0.02	0.85	0.75~0.97
	Control	224 (CC)	441 (CG)	239 (GG)			
rs3755885	Hypertensive	62 (CC)	379 (CG)	461 (GG)	0.24	0.92	0.79~1.06
	Control	67 (CC)	402 (CG)	435 (GG)			
rs2071694	Hypertensive	447 (CC)	374 (CG)	82 (GG)	0.81	0.98	0.85~1.13
	Control	455 (CC)	366 (CG)	83 (GG)			
rs3775067	Hypertensive	318 (AA)	435 (AG)	148 (GG)	0.27	1.08	0.94~1.23
	Control	287 (AA)	469 (AG)	149 (GG)			

OR: odds ratio; CI: confidence interval.

Table 3: Association analysis of 6 tagSNPs in men and women.

SNP	Group	*P* value	OR	95% CI
rs16843452	Male	0.51	0.94	0.77~1.14
	Female	0.45	1.07	0.90~1.27
rs12503220	Male	0.31	0.87	0.66~1.14
	Female	0.53	0.93	0.73~1.17
rs4963	Male	0.02	0.79	0.65~0.97
	Female	0.25	0.90	0.76~1.07
rs3755885	Male	0.80	1.03	0.83~1.28
	Female	0.08	0.84	0.69~1.02
rs2071694	Male	0.28	0.89	0.72~1.10
	Female	0.53	1.06	0.88~1.29
rs3775067	Male	0.21	1.14	0.93~1.40
	Female	0.71	1.03	0.87~1.23

OR: odds ratio; CI: confidence interval.

Table 4: Association analysis of haplotypes with hypertension.

Haplotype sequence	Haplotype frequency	Case/control ratio	*P* value
TG	0.472	0.473, 0.470	0.840
CG	0.368	0.360, 0.377	0.274
CA	0.157	0.165, 0.149	0.208

After input the genotypes of 6 SNPs together with information about TG, TC, HDL, BMI, smoking, and alcohol abuse, the software outputs the best model for "BMI, rs4963, rs16843452" with 10/10 crossvalidation consistency (Table 5).

4. Discussion

Among the three adducin genes, *ADD1* has received more attention than the other two due to its association with several diseases, such as hyperlipidemia [15], renal disease [16], and coronary heart disease [17]. Series of studies in humans reveal that mutation of *ADD1* gene may lead to the stimulation of the sodium and potassium-adenosine triphosphatase (ATPase) activity in renal tubular cells, increased renal sodium reabsorption, and subsequently hypertension [18, 19].

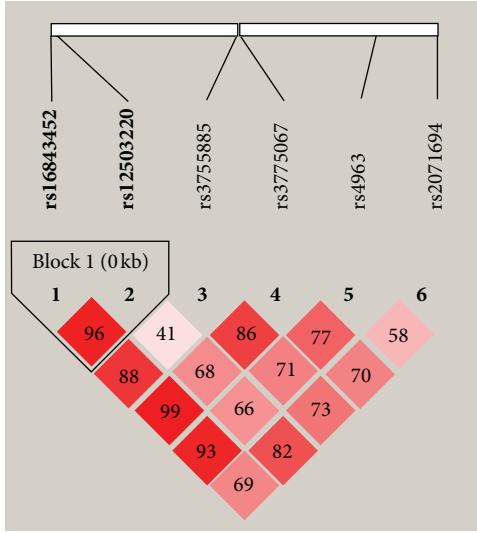

Figure 1: The LD block of 6 tagSNPs within *ADD1* gene. Pairwise linkage disequilibrium (LD) coefficients $D' \times 100$ are shown in each cell. Only one LD block (including rs16843452 and rs12503220) is identified by confidence intervals method.

Since hypertension is considered polygenic, resulting from the interaction of several genes and together with environmental factors, a single SNP has weak effects on the phenotype. A family study from van Rijin et al. [20] showed that *ADD1* G460W polymorphism was able to explain only a very minute proportion of the heritability of BP traits. Therefore, it is necessary to explore whether there are any other SNPs within *ADD1* gene that are also associated with essential hypertension. In order to answer this question, we carried out a case-control study in the Chinese population. The result showed that rs4963 was associated with essential hypertension, especially in males. One possible reason for

Table 5: MDR analysis of gene-environment interaction.

Best model	Testing accuracy	Testing sensitivity	Testing odds ratio	Testing χ^2	Crossvalidation consistency
BMI	0.59	0.43	2.26 (95% CI: 1.20–4.27)	6.50 ($P = 0.010$)	10/10
BMI, rs4963, rs16843452	0.61	0.54	2.42 (95% CI: 1.33–4.44)	8.40 ($P = 0.004$)	10/10
BMI, TG	0.58	0.57	1.98 (95% CI: 1.10–3.58)	5.18 ($P = 0.023$)	7/10

this result might be that men have higher TG (1.93 versus 1.69 mM) and TC (5.39 versus 5.08 mM) level than women.

Out of 1,113 SNPs associated with *ADD1* gene [21], 9 are identified to be nonsynonymous by functional single-nucleotide polymorphism (F-SNP) database [22]. rs4963 is one of them, and the amino acid change found is from Serine to Cysteine. The G allele of rs4963 has deleterious effect by disrupting the activity of exonic splicing and thus disrupt proper gene regulation [23]. In current study, G allele of rs4963 increases the susceptibility of hypertension, which might be associated with altered protein structure and function.

Moreover, environmental factors and individual's biological characteristics, including excess dietary sodium intake, alcohol abuse, smoking, obesity, stress, BMI, TC, and TG, cannot be neglected [24, 25]. We also found interesting interactions between genetic factors and nongenetic risk factors. High BMI and serum TG level were already confirmed to be risk factors for hypertension [26]. The MDR analysis in this study demonstrated that these important risk factors interacted with the genetic factor. Thus, the present interaction analysis gave a little more information than the single genetic study.

In conclusion, the present study indicated that rs4963 within *ADD1* gene was associated with essential hypertension in Chinese population, which might be related to altered exonic splicing and disrupted gene regulation. Regulation of sodium transport is such a complex process that at least 189 genes are involved according to gene ontology (GO: 0002028). *ADD1* is only a common member of this system, and polymorphisms within this gene may play a tiny role. Other genes involved in sodium transport regulation system need to be studied. In addition, in our interaction analysis, a significant interaction was found between genetic and nongenetic factors, demonstrating that genetic study alone is not a sufficient indicator for hypertension. To decipher causal factors leading to the development and the pathogenesis of hypertension, future work will require analysis of gene-environmental interaction.

Authors' Contribution

These authors contributed equally to this work.

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