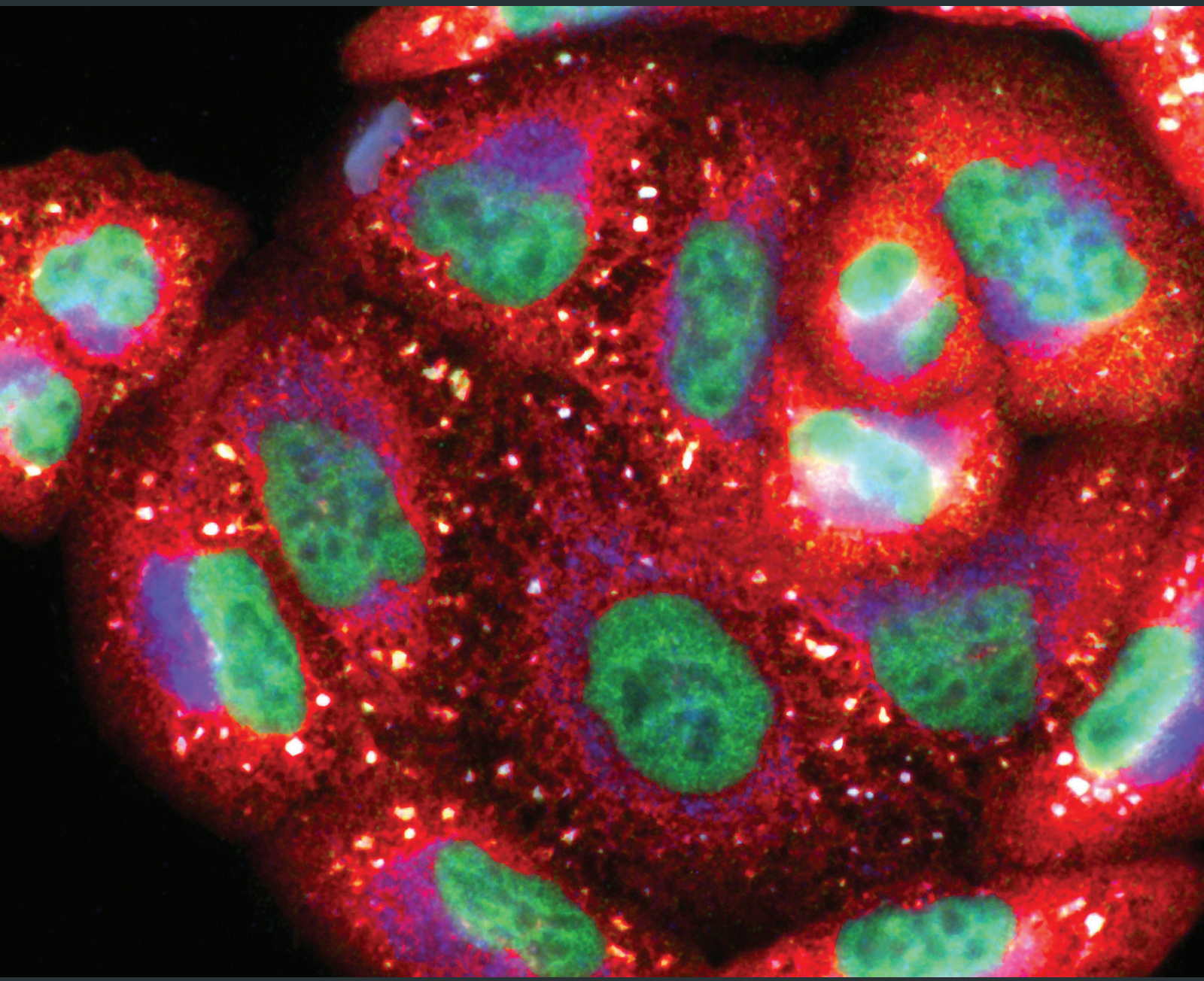
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Metabolic Disorders: Pathogenesis, Prevention, and Therapeutics

Guest Editors: Umesh C. S. Yadav, Vibha Rani, Gagan Deep, Rakesh K. Singh, and Komaraiah Palle



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Editorial Board

Contents

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Editorial

Oxidative Stress in Metabolic Disorders: Pathogenesis, Prevention, and Therapeutics

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Metabolic disorder is characterized by dyslipidemia, insulin refractoriness, defective insulin secretion, glucose intolerance, and chronic inflammation which contribute to dysfunctional cellular physiology and cellular redox imbalance. The increased oxidative environment contributes to a chronic inflammatory condition in the body and creates primary risk factor for the development of several diseases such as diabetes, arthritis, cancer, and cardiovascular complications. The increased oxidative stress in obesity and metabolic disorder can in turn cause progression and sustenance of inflammatory condition by upregulating redox signaling pathways and altered gene expression of inflammatory markers creating a vicious cycle.

Many evidences implicate oxidative stress in the metabolic perturbations in obesity, diabetes, and cardiovascular pathogenesis [1, 2]. The oxidative damage and inflammation originate from both our environmental milieu such as chemicals, toxicants, and nutrients and body's own metabolism as metabolic byproducts and intermediate. The understanding of their roles and effects on cellular physiology has led to the development of novel biomarkers and molecular targets which could be useful in devising innovative approaches in the prevention, diagnosis, and treatment of inflammatory and metabolic diseases. Additionally, the continued intensive research in this area is yielding novel information which is vital towards further advancing our understanding in this complex field.

Till recently, the major focus and efforts have been towards employing antioxidant as preventive/therapeutic agent against metabolic disorders, considering the central role of oxidative stress therein. However, several studies including clinical ones have clearly shown that this strategy has not yielded expected outcomes [3, 4]. Alternatively, several studies have suggested that it would be better to target the pathways involved in reactive oxygen species (ROS) generation rather than ROS neutralization by antioxidants [1, 2]. Several studies have also shown that cells own machinery gets overinvolved in the production of ROS and resultant molecular signaling leads to activation of inflammatory signaling and incessant inflammation. Mitochondrial dysfunction led ROS generation, weakening of cellular antioxidant machinery, aberrant activation of NADPH oxidase system, and lipid peroxidation mechanisms are few examples of oxidative stress sources. Several new molecular targets that are affected by oxidative stress and in turn distort the cellular physiology resulting in pathogenesis are reported in this special issue. These targets include NOTCH-1, Pin-1, galectin-3, paraoxonase-1, HMGB-1, and mitochondrial respiratory complexes.

In the obese diabetic patients, excessive uric acid has been shown to induce cardiovascular disease (CVD) through the

generation of ROS and subsequent endothelial dysfunction [5]. The epidemiological studies suggest that uric acid is an independent risk factor for developing CVDs, especially in people with diabetes, hypertension, and heart failure [6]. However, the clear molecular targets of uric acids have not yet been identified. In this issue, H. Xie et al. presented that uric acid induces ROS generation and increased the expression of several inflammatory molecules such as IL-6, TNF-alpha, and MCP-1 through Notch-1 pathway. Silencing of Notch-1 reversed these changes, and so did (−)-epigallocatechin-3-gallate (EGCG) treatment via inhibiting Notch-1. Authors concluded that downregulation of Notch-1 by EGCG could be an effective approach to decrease inflammation and oxidative stress induced by uric acid.

In their study, K. Gawlik et al. explored the antioxidant defense biomarkers present in the blood of type-2 diabetes patients. They reported that ferric reducing antioxidant power (FRAP) and uric acid levels were significantly elevated in obese diabetic patients; however, no significant difference was observed in the biomarkers of antioxidant defense system between patients with or without chronic diabetes. They concluded that in the presence of excessive ROS production the antioxidant system tends to crumble as reflected by decreased level of erythrocytes glutathione.

Y. Wang et al. discussed the role of peptidyl-prolyl isomerase (Pin-1), a key protein involved in cell division and many other cellular functions both in yeast and humans, in protection against high-dose alcohol-induced apoptosis in mouse cardiomyocytes. They reported that, on the one hand, high-dose alcohol-induced Pin-1 promotes mitochondrial dysfunction leading to ROS increase, while, on the other hand, Pin-1 suppresses endothelial nitric oxide synthase (eNOS) expression leading to apoptosis of cardiomyocytes. The authors concluded that due to these critical activities in alcohol-stimulated cardiomyocytes Pin-1 could be a potential therapeutic target in alcohol-induced cardiomyopathy.

In a review article, I. Petyaev elaborated the important role of lycopene, a member of tetraterpene carotenoids, in scavenging the lipid radicals, ROS, and nitric oxide affording protective effects against prooxidant species. However, with ageing and increased accumulation of oxidative species in the body, lycopene effectiveness might be depleted leading to type-2 diabetes and increased risk for CVDs. The limited bioavailability of lycopene due to decreased intestinal absorption with aging and its enzymatic and oxidative degradation could be addressed by designing novel nutraceuticals.

Galectin-3 (Gal-3), a member of lectin family and important regulator of cellular metabolism, has been implicated in obesity, type-2 diabetes, heart failure, and cancer [7–11]. S. Menini et al. in their review article discussed the controversial role of Gal-3 in this field. On the one hand, Gal-3 has been found elevated in obese and diabetic patients, while, on the other hand, Gal-3 knockout mice showed increased adiposity and systemic inflammation related with altered glucose homeostasis suggesting negative modulation of nutrition-induced immune responses. On the contrary, a few studies showed decrease in fat mass and body weight in high fat diet-fed Gal-3 knockout mice. Although Gal-3 has emerged as an important prognostic marker for heart failure, fibrotic diseases, and inflammatory pathways, the authors concluded that more investigations are warranted to clarify its role in high fat diet-induced obesity and diabetes.

While presenting association between oxidative stress marker plasma 8-isoprostane and the activity of paraoxonase-1 with coronary artery disease (CAD), A. Kuchta et al. suggested that 8-isoprostane could be an important biomarker of lipid peroxidation. They reported a correlation of decreased activity of paraoxonase-1 with decreased protection against lipid oxidation which could be linked with CAD.

High Mobility Group Box-1 (HMGB-1) is a nuclear protein that regulates the transcription of many genes and also involved in the immune response and inflammation. H. Wu et al. investigated how HMGB-1 protein is associated with diabetes-induced oxidative stress that critically regulates endothelial progenitor cell (EPC) dysfunction. In their study, they showed that advanced glycation end products enhanced HMGB-1 levels in EPCs which was downregulated by antioxidant N-acetylcysteine (NAC), suggesting that HMGB-1 could be an important regulator in diabetes-induced oxidative stress and resultant EPC dysfunction.

A. Keller et al. discussed the critical role of mitochondrial perturbation in vascular smooth muscle cells caused by nutritional stress during diabetes. They explored the premise that nutritional stress in diabetes may impair adaptive mitochondrial plasticity via NOS-mediated pathway. They reported that in Goto-Kakizaki diabetes mouse model high glucose enhanced nitric oxide, ROS, and respiratory control ratio while at the same time decreasing phospho-eNOS, uncoupled respiration and mitochondrial respiratory complex expression. With these findings, the authors suggested that eNOS and mitochondria could be potential drug targets in nutritional stress-induced vascular pathogenesis.

Overall, several new findings have been presented in this special issue which have further advanced our knowledge in this exciting area. Also, these studies suggest that oxidative stress is a critical component of metabolic disorders and sustained research efforts are necessary to unravel the complexity associated with oxidative stress in metabolic disorders and related pathologies.

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Research Article

Differential Mitochondrial Adaptation in Primary Vascular Smooth Muscle Cells from a Diabetic Rat Model

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Diabetes affects more than 330 million people worldwide and causes elevated cardiovascular disease risk. Mitochondria are critical for vascular function, generate cellular reactive oxygen species (ROS), and are perturbed by diabetes, representing a novel target for therapeutics. We hypothesized that adaptive mitochondrial plasticity in response to nutrient stress would be impaired in diabetes cellular physiology via a nitric oxide synthase- (NOS-) mediated decrease in mitochondrial function. Primary smooth muscle cells (SMCs) from aorta of the nonobese, insulin resistant rat diabetes model Goto-Kakizaki (GK) and the Wistar control rat were exposed to high glucose (25 mM). At baseline, significantly greater nitric oxide evolution, ROS production, and respiratory control ratio (RCR) were observed in GK SMCs. Upon exposure to high glucose, expression of phosphorylated eNOS, uncoupled respiration, and expression of mitochondrial complexes I, II, III, and V were significantly decreased in GK SMCs ($p < 0.05$). Mitochondrial superoxide increased with high glucose in Wistar SMCs ($p < 0.05$) with no change in the GK beyond elevated baseline concentrations. Baseline comparisons show persistent metabolic perturbations in a diabetes phenotype. Overall, nutrient stress in GK SMCs caused a persistent decline in eNOS and mitochondrial function and disrupted mitochondrial plasticity, illustrating eNOS and mitochondria as potential therapeutic targets.

1. Introduction

Diabetes imparts staggering social and economic costs worldwide. It is known that people with diabetes have a 3–5-fold higher risk of cardiovascular disease (CVD) than the nondiabetic population [1]. Vascular remodeling, characterized by endothelial dysfunction and vascular stiffness and seen in the context of diabetes, hyperglycemia, and elevated oxidative stress, heralds CVD onset [2, 3]. Elucidating early cellular mechanistic pathology is critical to understanding disease progression.

Mitochondria have recently emerged as therapeutic targets in chronic diseases. They are critical signaling hubs in vascular processes such as the endothelial regulation of vasomotion, calcium signaling associated with vascular relaxation, smooth muscle cell proliferation, and apoptosis [4–6]. Excess cellular ROS in the vasculature may originate from dysfunctional mitochondria, and this excess ROS precedes vascular inflammation, vascular stiffness, and SMCs apoptosis [7–10]. Excess mitochondrial ROS is also associated with characteristics of type 2 diabetes such as hyperglycemia, decreased antioxidant defense, and insulin resistance [11–15].

In turn, hyperglycemia and insulin resistance also correlate with altered mitochondrial function, dynamics, and morphology [11, 15–20].

Nitric oxide (NO), produced by the enzyme nitric oxide synthase (NOS), regulates not only vascular relaxation [21], but also mitochondrial biogenesis through the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [22–24]. NOS dysfunction is a characteristic of both diabetes and CVD [25–28], and excess ROS contributes to uncoupled NOS (NOS uncoupled from NO production) in diabetes through the inactivation of cofactor tetrahydrobiopterin (BH_4) [25, 29]. Uncoupled NOS is a known additional source of excess cellular superoxide [25]. Our laboratory has shown that disrupted NOS activity is also linked to suboptimal mitochondrial biogenesis signaling downstream [30].

Here, we aimed to characterize the role of NOS in mitochondrial plasticity in aortic primary smooth muscle cells (SMCs) from both control rats (Wistar) and a model of type 2 diabetes (Goto-Kakizaki, GK). We used primary SMCs from the GK model to investigate impaired mitochondrial plasticity based on our report of failed mitochondrial adaptation to exercise in vivo in this model [31]. We hypothesized that impaired NOS activity would result in dampened physiological responses to nutrient stress in GK SMCs as compared to those from Wistar.

2. Material and Methods

2.1. Materials. Dulbecco's Modified Eagles Medium (DMEM, 5 mM glucose, 25 mM glucose) and nonessential amino acids, trypsin, and mammalian protein extraction reagent (M-PER) were obtained from Thermo Scientific Hyclone (MA, USA), and dimethyl sulfoxide (DMSO), sodium chloride, sucrose, and bovine serum albumin were purchased from Fisher Scientific (PA, USA). Penicillin/streptomycin and FBS were procured from Gemini Bioproducts (CA, USA). Hank's Balanced Salt Solution (HBSS) was purchased from Corning Life Sciences (NY, USA). Collagenase, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), sodium pyrophosphate, sodium orthovanadate, sodium fluoride, okadaic acid, 1% protease inhibitor cocktail, dithiothreitol, magnesium chloride, K-lactobionate, taurine, potassium phosphate, HEPES, digitonin, pyruvate, malic acid, glutamic acid, adenosine diphosphate (ADP), succinic acid, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), antibody to β-actin (mouse), phenylephrine and acetylcholine, trypsin inhibitor, and cytochrome c were procured from Sigma-Aldrich (MO, USA). MitoTracker, MitoSOX, DAPI (4′,6-diamidino-2-phenylindole), dihydrochloride, and secondary detection antibodies Alexa Fluor 488 and Alexa Fluor 546 were purchased from Life Technologies (CA, USA). Antibodies to TOM20 (rabbit) and nitrotyrosine (mouse) were procured from Santa Cruz Biotechnology (TX, USA).

Antibodies: antibodies to adenosine monophosphate kinase (AMPK, 1 : 500 rabbit), phosphorylated AMPK (pAMPK, 1 : 1,000, rabbit, Thr172), autophagy-related protein 7 (Atg7, 1 : 500, rabbit), Beclin-1 (1 : 1,000, rabbit), light chain (LC3B-I/II, 1 : 1,000, rabbit), and anti-mouse (1 : 10,000) and anti-rabbit (1 : 10,000) IgG (AP-linked antibodies) were obtained from Cell Signaling (MA, USA). Antibodies to cytochrome c (1 : 1,000, mouse) and eNOS (1 : 200, mouse) and OPA1 (1 : 1,000, mouse) were from BD Biosciences (NJ, USA). Antibody to mitofusin-1 (Mfn1, 1 : 1,000, rabbit), mitofusin-2 (Mfn2, 1 : 500, mouse), uncoupling protein 3 (UCP3, 1 : 500, rabbit), and voltage dependent anion channel 1 (VDAC1, 1 : 1,000, rabbit) were from Abcam (Cambridge, UK). Antibody cocktail to representative subunits of mitochondrial oxidative phosphorylation (OxPhos) complexes I (subunit NDUFA9), II (subunit SDHA), III (subunit UQCRC2), IV (subunit IV), and V (subunit ATP5A) (1 : 1,000, mouse) were obtained from MitoSciences (OR, USA). Antibodies to phosphorylated eNOS (peNOS, 1 : 200, rabbit, S1177), citrate synthase (1 : 1,000, goat), PPARγ coactivator 1 alpha (PGC-1α, 1 : 500, rabbit), and anti-goat IgG (1 : 10,000, AP-linked secondary antibody) were from Santa Cruz Biotechnology (TX, USA). MnSOD antibody (1 : 1,000, rabbit) was procured from EMD Millipore (MA, USA). The antibody to nucleoporin p62 (p62, 1 : 1,000, rabbit) was purchased from Sigma-Aldrich (MO, USA). Fis1 antibody (1 : 500, rabbit) was obtained from Imgenex (CO, USA). Immobilon-P PVDF membrane was from EMD Millipore (MA, USA). CDP-Star Reagent was obtained from New England BioLabs (MA, USA) and Life Technologies (CA, USA). Secondary antibodies (1 : 10,000, mouse, and 1 : 10,000, rabbit) for Western blot detection were purchased from Li-COR (NE, USA). Hoechst dye was obtained from Biotium (Hayward, CA).

2.2. Animals. The use of animals and experimental interventions received prior approval from the Institutional Animal Care and Use Committee at the Denver VA Medical Center. This study followed the principals of animal care as described by the National Institutes of Health. Primary aortic cells were harvested from male 22-week-old Wistar and GK rats (Taconic Biosciences, Inc., NY, USA, and Charles River Laboratories, MA, USA) as previously described [32].

2.3. Measurement of Contractile Function. Aortae were excised, placed in ice-cold physiological saline solution, debrided of loose fat and connective tissue, and prepared for measurement of isometric force as previously described [33–35]. The thoracic segment of aorta was dissected free from surrounding tissues and cut into rings of 2 mm in length. The preparation was then transferred into organ baths containing Krebs solution bubbled with a mixture of 95% O_2 and 5% CO_2. Each aortic ring was mounted between two L-shaped stainless steel hooks, one of which was connected to a force-displacement transducer (Grass Instruments Co., WI, USA). Krebs solution contained (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Basal tension (500 mg) was applied to each ring, and all experiments were performed at 37°C. Tissues were contracted with 80 mmol/L KCl and then contracted with 2 μM phenylephrine. Endothelial dependent relaxation was

stimulated with 20 μM acetylcholine. Data were collected using AcqKnowledge software.

2.4. Cell Isolation, Culture, and Maintenance. Aortae were excised, cleaned, and incubated in euglycemic media (DMEM 5 mM glucose supplemented with 10% FBS, 1% nonessential amino acids, and 1% penicillin/streptomycin) for 1-2 hours at 37°C, followed by an incubation in collagenase buffer (1 mg/mL of collagenase, 1 mg/mL of trypsin inhibitor, and 1 mg/mL of bovine serum albumin in euglycemic media) for 1 hour at 37°C while periodically vortexed. Aortae were finely cut into pieces (approximately 1–3 mm in length) in euglycemic media (20% FBS) and incubated at 37°C overnight. Experiments were conducted between passages 8 and 10, and cells were characterized using α-tubulin. Cells were maintained at 95% air and 5% CO_2.

2.5. Cell Experimentation. SMCs were incubated in 0.1% serum starvation media for 45–48 hours (DMEM 5 mM glucose, 0.1% FBS, 1% nonessential amino acids, and 1% penicillin/streptomycin). SMCs were then preincubated for 30 minutes with normal glucose media NG (DMEM 5 mM glucose, 0.1% FBS, 1% nonessential amino acids, 1% penicillin/streptomycin, and 2% dimethyl sulfoxide (DMSO)) and then incubated for 1 and 4 hours with either NG or high glucose (HG, DMEM 25 mM glucose, recipe as described above). (We refer to normal glucose (NG, 5 mM) for 1 hour after media change as baseline and high glucose (HG, 25 mM) for either 1 or 4 hours as stress response.) Glucose or vehicle (PBS) was directly added into media for 1- and 4-hour incubations for SMCs used for flow cytometry and the ATP assay, and microscopy time course experiments used serum starvation media described above with 25 mM glucose. All experiments were conducted in triplicate.

2.6. Respiration. Mitochondrial respiration was measured using Oroboros Oxygraph-2K (O2k, OROBOROS INSTRUMENTS Corp., Innsbruck, Austria) according to modifications from previously described protocols [31, 36, 37]. Briefly, SMCs were trypsinized using 0.25% trypsin/EDTA and washed and spun (3 minutes at 1,000 g) once each with phosphate buffer saline (PBS) and MiR05 respiration buffer (0.5 mM EGTA, 3 mM magnesium chloride, 60 mM K-lactobionate, 20 mM taurine, 10 mM potassium phosphate, 20 mM HEPES, 110 mM sucrose, and 1 g/L fatty acid free bovine serum albumin). Following the final spin, SMCs were resuspended in MiR06 respiration buffer (MiR05 with 280 IU/mL catalase) and counted using a hemocytometer. Between 0.5×10^6 and 1×10^6 SMCs were placed into a 2 mL chamber of the O2k and permeabilized with 3 μg of digitonin. After respiration rates stabilized, substrates and inhibitors were added to assess respiration rates. Rates were measured following the addition of 5 mM pyruvate, 2 mM malate, and 10 mM glutamate (state 2 PMG), PMG with 2 mM adenosine diphosphate (ADP) (state 3 PMG), PMG, ADP, and 6 mM succinate (state 3 PMGS), and 2 μg/mL oligomycin (state 4 PMGS), and 0.5 μM of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added incrementally until maximal uncoupling (uncoupled state). Cytochrome c (10 μM) was used to determine mitochondrial membrane damage. There were no significant differences in oxygen consumption following cytochrome c addition, indicating intact mitochondrial membranes. Oxygen and all substrates were calibrated to be at saturating concentrations in the chambers with no possibility of rate limitations. The respiration technique described above has been optimized for SMCs. Upon completion of the experiment, SMCs were recounted from the O2k chambers and respiration rates normalized to cell count. An area of consistent respiration rate of 3 minutes or longer was representative of the various states. Respiration control ratios (RCR) were calculated as a ratio of state 3 PMGS/state 4 PMGS.

2.7. ATP Assay. ATP concentrations were measured in cells using the Fluorometric ATP Assay Kit (#ab83355) from Abcam, Cambridge, United Kingdom. Briefly, following the experiment, cells were harvested, counted, and protein-precipitated according to manufacturer instructions. After adjusting the samples' pH to between 6.5 and 8, samples were incubated with the kit reagents according to the fluorometric protocol, and ATP concentrations were normalized to 1×10^6 cells.

2.8. Western Blotting. SMCs were harvested after incubations and protein was measured using Western blotting as previously described [31]. SMCs were harvested after incubations in 4°C mammalian lysis buffer (MPER with 150 mM sodium chloride, 1 mM of EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 500 nM okadaic acid, and 1% protease inhibitor cocktail). After sonication at 4°C, cell lysates were centrifuged at 18,000 ×g at 4°C for 10 min, and the protein concentration of the supernatant was analyzed by Bradford protein assay. Protein samples (15 μg to 40 μg) in Laemmli sample buffer (boiled with 100 mM dithiothreitol) were run on SDS-12% polyacrylamide gels. The resolved proteins were electrophoretically transferred to PVDF membranes, and equivalence of protein loading was initially assessed by staining of membrane-bound proteins by Ponceau S stain. Blots were probed using specific primary antibodies of interest (overnight at 4°C) and followed by either alkaline phosphatase- (AP-) linked or fluorescent secondary (1 : 1000, 1 hr at room temperature). Proteins were detected by chemiluminescence using CDP-Star Reagent on film or Li-COR (Odyssey CLX) Western blot scanner, and densitometric analysis was performed using either Quantity One or Image Studio v3.1. All data is normalized to β-actin protein expression. We detected eNOS monomers and dimers using a 7.5% SDS-page gel transferred to PVDF membrane at 4°C under nonreducing conditions, as described previously with modifications [38]. Samples were prepared with Laemmli buffer without reducing agent and not boiled. Detection and analysis were done as described above.

2.9. Mitochondrial Isolation and Enzyme Activity Assay. Treated SMCs were harvested in 10 mM Tris (pH 7.6).

Isolated mitochondria were obtained by homogenization (10 passes through a syringe with a 25-26 G needle) followed by centrifugation through a 1.5 M sucrose gradient. Final mitochondrial isolates were subjected to 3 freeze/thaw cycles and activity of respiratory chain enzyme complexes I + III and II + III and citrate synthase (CS) was measured spectrophotometrically as previously described [39] on a BioTek Synergy (VT, USA) H1 microplate reader. Enzyme activities were normalized to protein (using the Bradford method).

2.10. Nitric Oxide Synthase Activity Assay. SMCs were seeded at 40,000 to 60,000 cells per well in a 96-well plate. Following a 48-hour serum starvation, SMCs were treated as described and assayed for nitric oxide (NO) evolution according to manufacturer's instructions (Nitric Oxide Synthase Detection System, Sigma-Aldrich, MO, USA, #FCANOS1). Live cells were stained using Hoechst's dye at 0.5 μg/mL for 20 minutes at 37°C prior to reading fluorescence and data expressed as relative fluorescence units (RFU) of NO concentration normalized to live cells.

2.11. Amplex Red Assay. SMCs between passages 3 and 7 were cultured in 5 mM glucose DMEM. Following 48-hour serum deprivation, H_2O_2 accumulation was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (#A22188) Invitrogen, CA, USA. The standard curve was done in live cells to control for quenching. Data are presented in μM equivalents of H_2O_2.

2.12. Flow Cytometry. Treated SMCs were trypsinized, and 150,000 cells were spun for 3 minutes at 1,000 g. Cells were resuspended in MitoTracker (final concentration of 9 nM in HBSS) and incubated at 37°C in darkness for 20 minutes. Cells were spun and resuspended in MitoSOX (final concentration of 5 μM in HBSS) and incubated for 37°C in dark conditions for 15 minutes. DAPI was added for live cell gating, and Beckman Coulter Gallios flow cytometer was used (CA, USA, University of Colorado Cancer Center Flow Core).

2.13. Microscopy and Immunohistochemistry. SMCs were cultured on a glass coverslip, exposed to HG media for 1 or 4 hours, and compared to a NG media baseline. Following glucose exposure, samples were fixed by rinsing once with PBS prewarmed to 37°C and incubated at 37°C for 15 minutes in 37°C 4% paraformaldehyde in PBS. After fixation, samples were then incubated for 15 minutes in 50 mM NH_4Cl in PBS. Finally, samples were stored in PBS at 4°C until staining. Mitochondrial and nitrotyrosine staining were completed by adding TOM20 (1 : 400) and nitrotyrosine (1 : 400) antibodies, respectively, followed by secondary detection antibodies Alexa Fluor 488 (1 : 2,000) and Alexa Fluor 564 (1 : 1,000). DAPI (1 : 1,000) was used to visualize the nuclei. Fixed cells were imaged using an Olympus confocal FV1000 FCS/RICS microscope. Experiments were repeated in triplicate ($n = 3$) on separate days, and three plates were tested at each time point in each experiment, for a total of $n = 9$ plates per time point. In addition, three fields of view were imaged and analyzed on each plate, for a total of $n = 27$ fields of view imaged per experimental time point. Raw images acquired from confocal microscopy were imported into Matlab in 24-bit TIFF format and converted into binary images using a program designed by one of the authors (PMM). To assess differences in mitochondrial morphology, the total mitochondrial network edge length within the field of view was divided by the total number of mitochondria to give an average perimeter per mitochondrion. To assess differences in mitochondrial content, the total area of the mitochondrial network was divided by the total area of cytoplasm to yield the fraction of the cytoplasm filled by mitochondria. Mitochondrial content was found to be correlated with the inverse of average cell size within the field of view in all cell populations at all time points. The fraction of cytoplasm filled by mitochondria was therefore normalized to median cell size using a linear regression in order to prevent aberrant detection of changes in mitochondrial content due to random variation in the size of cells measured between time points. No correlation was observed between average perimeter per mitochondrion and cell size. In addition to average perimeter per mitochondrion and fraction of cytoplasm filled, total network edge length, total network area, total cytoplasm area, total number of nuclei, average network area, and average cytoplasm area per cell were recorded for each field of view. Images which were registered at greater than two standard deviations from the mean value for their experimental group in any of these parameters were discarded as outliers. The mean and standard error of each group were computed from the remaining images.

2.14. Statistics. Either two-tailed Student's t-test or one-way ANOVA was used for data analysis. A p value of less than 0.05 was used as the cutoff for statistical significance in all tests.

3. Results

3.1. Contractility Impairments in GK Rat Aorta. In the representative tracing, phenylephrine (2 μM) evoked a greater contractile response in Wistar aorta than the GK aorta (Figure 1(a)), and addition of acetylcholine (20 mM) resulted in a greater relaxation response in the Wistar aorta than the GK aorta (Figure 1(a)). These differences strongly suggest impaired vascular function in the GK rat.

3.2. NOS Expression. In the representative blot pictured, dimer eNOS is visible at 8.09% higher concentrations in aorta from the 18-week-old GK as compared to the Wistar rats (Figure 1(b)), and 40% higher concentrations of the monomer of eNOS are seen in GK aorta (Figure 1(b)).

3.3. Baseline Mitochondrial Respiration Is Elevated in GK SMCs. The GK SMCs had significantly elevated state 3 respiration rates as well as respiratory control ratio (RCR, state 3 PMGS: state 4 PMGS), an indication of respiration efficiency [40, 41], at baseline as compared to Wistar SMCs ($p < 0.05$, Table 1). Additionally, ATP concentrations were

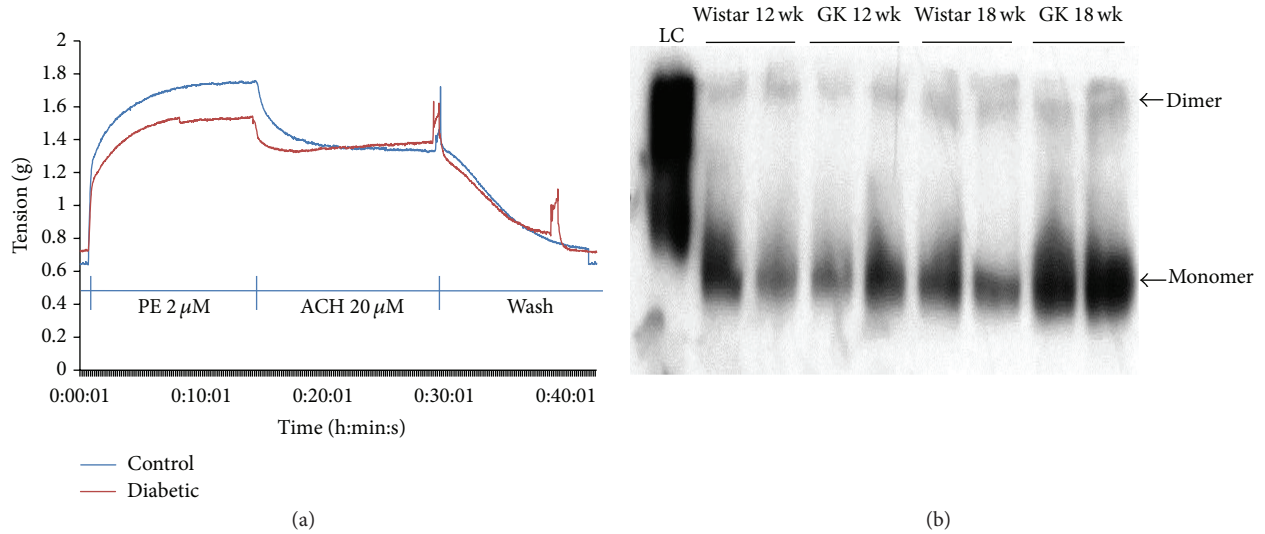


Figure 1: (a) Contractile responses of Wistar (control) and GK (diabetic) aorta smooth muscle. (b) Dimer (coupled) and monomer (uncoupled) eNOS in aorta of 18-week-old Wistar and GK animals. 15–30 μg of protein was loaded onto a 7.5% SDS-page gel and transferred to PVDF membrane at 4°C under nonreducing conditions.

Table 1: Mitochondrial respiration and ATP concentrations as measured with O2k from Oroboros and ATP fluorometric assay. Respiration baseline comparisons are between Wistar ($n = 6$) and GK ($n = 3$) SMCs in NG (5 mM), Wistar, or GK SMCs, passages 8–10. ATP concentrations (μM/1×10^6 cells) are between Wistar ($n = 8$) and GK ($n = 8$). Respiration was measured as oxygen flux (pmol second^{-1} million cells^{-1}) ± standard error of the mean (SEM). $^*p < 0.05$ as measured by Student's t-test.

Respiration states	Wistar	GK
S2 (PMG)	18.35 ± 3.97	29.79 ± 5.38
S3 (PMG)	75.99 ± 19.04	153.88 ± 23.82*
S3 (PMG/S)	187.82 ± 40.96	241.30 ± 36.46
S4 (PMG/S)	54.88 ± 8.33	52.52 ± 4.56
Uncoupled	262.98 ± 46.06	375.15 ± 20.63
RCR	3.35 ± 0.29	4.56 ± 0.45*
ATP	8.64 ± 1.91	7.14 ± 1.08

nonsignificantly decreased in the GK SMCs (Table 1). To further characterize mitochondrial function, the activity of complexes I + III and II + III and citrate synthase was measured. Activity of complexes I + III in GK SMCs at baseline was nonsignificantly less as compared to Wistar SMCs ($p = 0.088$, data not shown).

3.4. Mitochondrial Biogenesis and NOS Signaling in GK SMCs. AMPK expression at baseline was significantly less in GK SMCs as compared to Wistar cells ($p < 0.01$, Figure 2(a)). NO, as a measurement of NOS activity, was significantly elevated in GK SMCs ($p < 0.001$, Figure 2(b)). Phosphorylated eNOS protein expression was significantly elevated in GK SMCs ($p < 0.05$, Figure 2(b)). Unexpectedly, we observed no change in PGC-1α, suggesting a disconnect between eNOS activation and PGC-1α regulation (Figure 2(b)). Mitochondrial complexes II–V (I (subunit NDUFA9), II (subunit SDHA), III (subunit UQCRC2), IV (subunit IV), and V (subunit ATP5A)) were elevated in GK as compared to Wistar SMCs, yet only complex V differed significantly ($p < 0.05$, Figure 2(c)).

3.5. Unstimulated GK SMCs Produced Elevated ROS as Compared with Wistar SMCs. Baseline concentrations of H_2O_2 and superoxide relative to mitochondrial content revealed significantly elevated ROS in the GK cells as compared to the Wistar cells ($p < 0.05$, Figures 3(a) and 3(b)). No differences were seen in MnSOD or UCP3 protein expression (Figure 3(b)), indicators of endogenous antioxidant defenses.

3.6. Mitochondrial Morphology Differed between SMCs at Baseline. Perimeter per mitochondrion was significantly elevated in the GK SMCs at baseline as compared to Wistar SMCs ($p < 0.05$, Figure 4(a)). In accord with this finding, the mean number of mitochondrial bodies per cell was significantly higher in the Wistar SMCs at baseline as compared to the GK SMCs ($p < 0.05$, Figure 4(a)). Fusion markers included OPA1, mitofusin-1 (Mfn1), and mitofusin-2 (Mfn2); fission was examined using Fis1. Expression of fusion molecule Mfn2 was significantly reduced in GK cells as compared to the Wistar SMCs at baseline ($p < 0.05$, Figure 4(b)). In comparison to the Wistar, autophagy targets Atg7, Beclin-1, and LC3-1 were significantly less in GK SMCs ($p < 0.05$, Figure 4(c)).

3.7. Mitochondrial Respiration in the Context of High Glucose Stress. The uncoupled state respiration rate in GK SMCs was significantly decreased following exposure to glucose stress ($p < 0.05$, Table 2), and respiration states 2 and 3 (PMG) showed a nonsignificant decline ($p = 0.05$ for both, Table 2). ATP concentrations were not significantly different following HG exposure (Table 2). No differences were observed in the enzyme activity of mitochondrial complexes I + III and II +

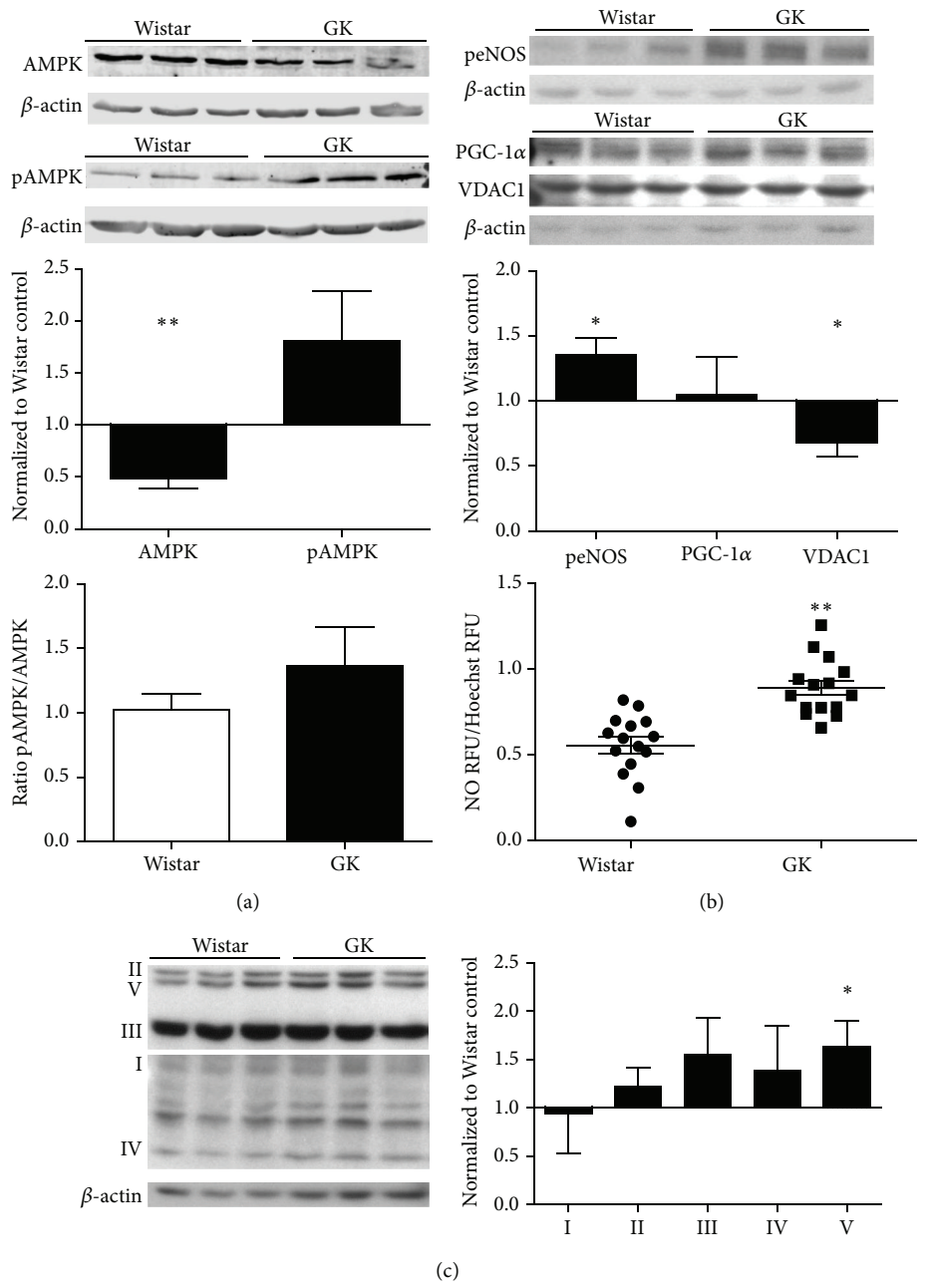


Figure 2: (a) Baseline comparisons of AMPK ($n = 3$), pAMPK ($n = 3$), and AMPK specific activity (pAMPK/AMPK), signaling upstream of mitochondria ($n = 3$) and resultant NO evolution ($n = 4$) (b), and mitochondrial complex differences ($n = 3$) (c) in Wistar and GK SMCs, passages 8–10. Signaling protein expression was measured with Western blot, 15–30 μg protein on an SDS-page gel, and data are normalized to β-actin and expressed as mean fold change from Wistar SMCs + SEM. Significance measured by Student's t-test, $^{*}p < 0.05$. NO ($n = 3$) is expressed as relative fluorescence units (RFU) normalized to RFU of Hoechst live cell staining for cell number. $^{*}p < 0.05$, $^{**}p < 0.01$ as measured by Student's t-test.

III or citrate synthase in Wistar and GK cells in response to high glucose stress (data not shown).

3.8. Signaling Upstream of Mitochondrial Biogenesis and Function Is Disrupted in GK SMCs following Glucose Mediated Stress. There was a significant effect on pAMPK in Wistar and AMPK in GK SMCs with HG incubation ($p < 0.05$, Figure 5(a)). Additionally, phosphorylated eNOS was significantly reduced in GK SMCs after 1 hour and 4 hours of high glucose as compared to normal glucose ($p < 0.05$), with no change in Wistar SMCs (Figure 5(a)). In the GK SMCs, complexes I, II, III, and V were significantly decreased in response to 4 hours of HG treatment ($p < 0.05$ for all, Figure 5(a)).

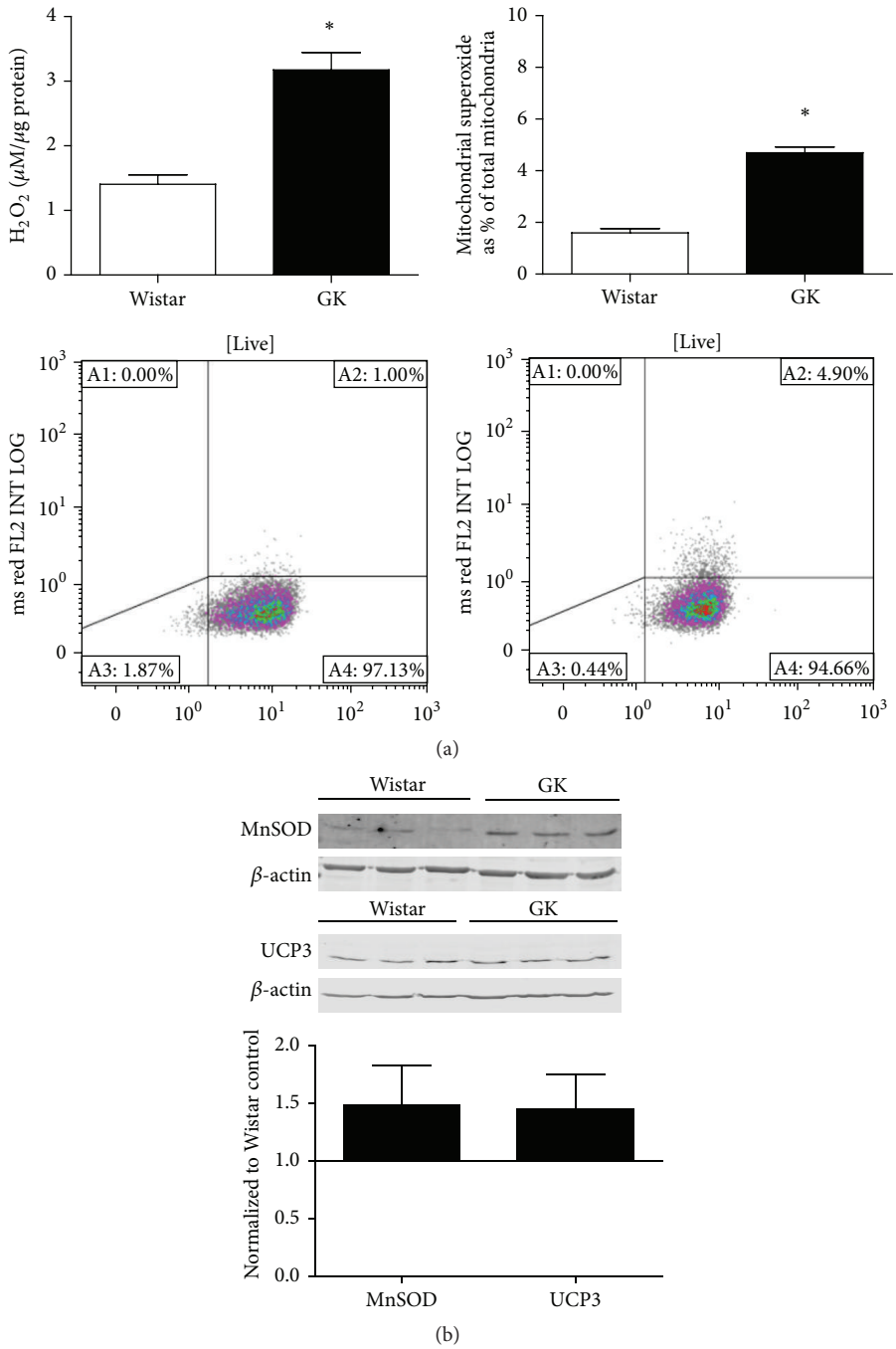


Figure 3: (a) Baseline reactive oxygen species differences between Wistar and GK SMCs in NG (5 mM). Hydrogen peroxide was measured by Amplex Red ($n = 3$), and superoxide was detected as a percentage of total mitochondria using MitoTracker and MitoSOX fluorescence on live cells with flow cytometry ($n = 3$). Representative flow cytometry figures of Wistar SMCs (left) and GK SMCs (right) are presented. (b) Mitochondrial antioxidant status was measured with Western blot, 15–30 μg protein on an SDS-page gel, and data are normalized to β-actin and expressed as mean fold change from Wistar SMCs + SEM. Significance measured by Student's t-test, $^*p < 0.05$.

3.9. ROS Production in GK and Wistar SMCs Was Differently Affected by Glucose Stress. Following 4 hours of HG incubation, mitochondrial superoxide production was significantly increased in the Wistar cells ($p < 0.05$, Figure 5(b)) whereas no changes were observed in GK cells (Figure 5(b)). UCP3 expression in the Wistar SMCs showed a nonsignificant increase following HG treatment ($p = 0.1$, Figure 5(b)), and MnSOD expression in the GK SMCs showed a nonsignificant treatment effect towards decreased expression with 4 hours of HG exposure ($p = 0.07$, Figure 5(b)).

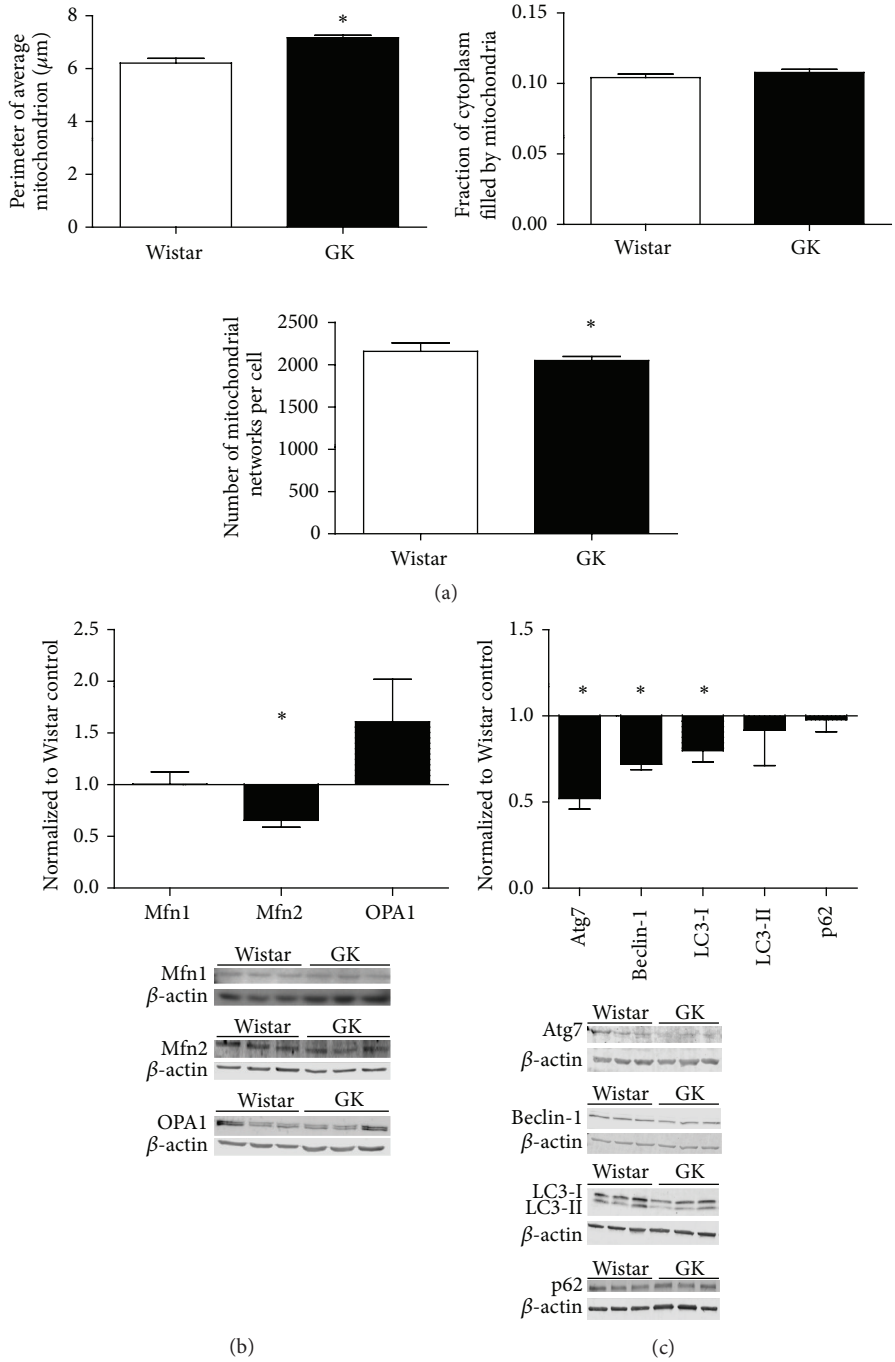


Figure 4: (a) Assessment of baseline mitochondrial morphology. Baseline perimeter per mitochondrion, total mitochondria fill of cytoplasm, and number of mitochondrial networks per cell were measured in fixed Wistar ($n = 3$) and GK ($n = 3$) SMCs using TOM 20 (mitochondria) and nitrotyrosine (cytoplasm). (b) Baseline comparison of mitochondrial dynamics and autophagy regulators (c) in Wistar ($n = 3$) and GK ($n = 3$) SMCs, passages 8–10. Signaling protein expression was measured with Western blot, 15–30 μg protein on an SDS-page gel, and data are normalized to β-actin and expressed as mean fold change from Wistar cells + SEM. Significance measured by Student's t-test, $^*p < 0.05$.

3.10. GK SMCs Display Altered Mitochondrial Dynamics in Response to High Glucose Stress. At all time points measured, the average perimeter per mitochondrion among GK SMCs was significantly greater than in Wistar SMCs ($p < 0.001$, Figure 5(c)). The final average perimeter per mitochondrion in the Wistar SMCs exposed to 4 hours of glucose stress was significantly greater than at baseline ($p < 0.05$, Figure 5(c)). The final average perimeter per mitochondrion in the GK 4-hour group was also significantly greater than baseline ($p < 0.05$, Figure 5(c)). The fraction of cytoplasm filled by mitochondria significantly increased from 1 to 4 hours in GK cells ($p < 0.05$, data not shown). There was a nonsignificant increase of OPA1 protein expression following 1 and 4 hours of HG treatment in the GK SMCs ($p = 0.06$, Figure 5(d)).

Table 2: Mitochondrial respiration and ATP concentrations as measured with O2k from Oroboros and ATP fluorometric assay. Respiration rates and RCR of Wistar and GK SMCs were measured after a 4-hour incubation with normal (5 mM, NG) or high (25 mM, HG) glucose, Wistar ($n = 6$ for NG and $n = 7$ for HG), or GK ($n = 3$ for NG and $n = 5$ for HG) cells, passages 8–10. ATP concentrations (μM/1×10^6 cells) are between Wistar ($n = 9$) and GK ($n = 9$). Respiration was measured as oxygen flux (pmol second^{-1} million cells^{-1}) + standard error of the mean (SEM). $^{*}p < 0.05$, $^{\dagger}p < 0.1$ as measured by Student's t-test.

(a)

Respiration states: Wistar	NG	HG
S2 (PMG)	18.35 ± 3.97	8.61 ± 2.37
S3 (PMG)	75.98 ± 19.04	51.73 ± 12.93
S3 (PMG/S)	187.82 ± 40.96	131.07 ± 9.19
S4 (PMG/S)	54.88 ± 8.33	34.34 ± 1.13
Uncoupled	262.98 ± 46.06	166.38 ± 16.48
RCR	3.35 ± 0.29	3.44 ± 0.21
ATP	8.64 ± 1.91	6.97 ± 1.34

(b)

Respiration states: GK	NG	HG
S2 (PMG)	29.79 ± 5.38	11.96 ± 1.49†
S3 (PMG)	153.88 ± 23.82	90.41 ± 13.78†
S3 (PMG/S)	241.30 ± 36.46	197.96 ± 20.64
S4 (PMG/S)	52.52 ± 4.56	46.17 ± 3.34
Uncoupled	375.15 ± 20.63	246.70 ± 22.60*
RCR	4.56 ± 0.45	4.37 ± 0.11
ATP	7.14 ± 1.08	8.87 ± 1.90

Following a 4-hour exposure to HG, Wistar SMCs showed a significant increase in p62 protein expression as compared to 1 hour of exposure ($p < 0.05$, Figure 5(d)); in response to HG, GK SMCs had a significant decline in protein expression of Atg7 and LC3-II ($p < 0.05$, Figure 5(d)) and nonsignificant decrease in LC3-1 protein expression ($p = 0.08$, Figure 5(d)).

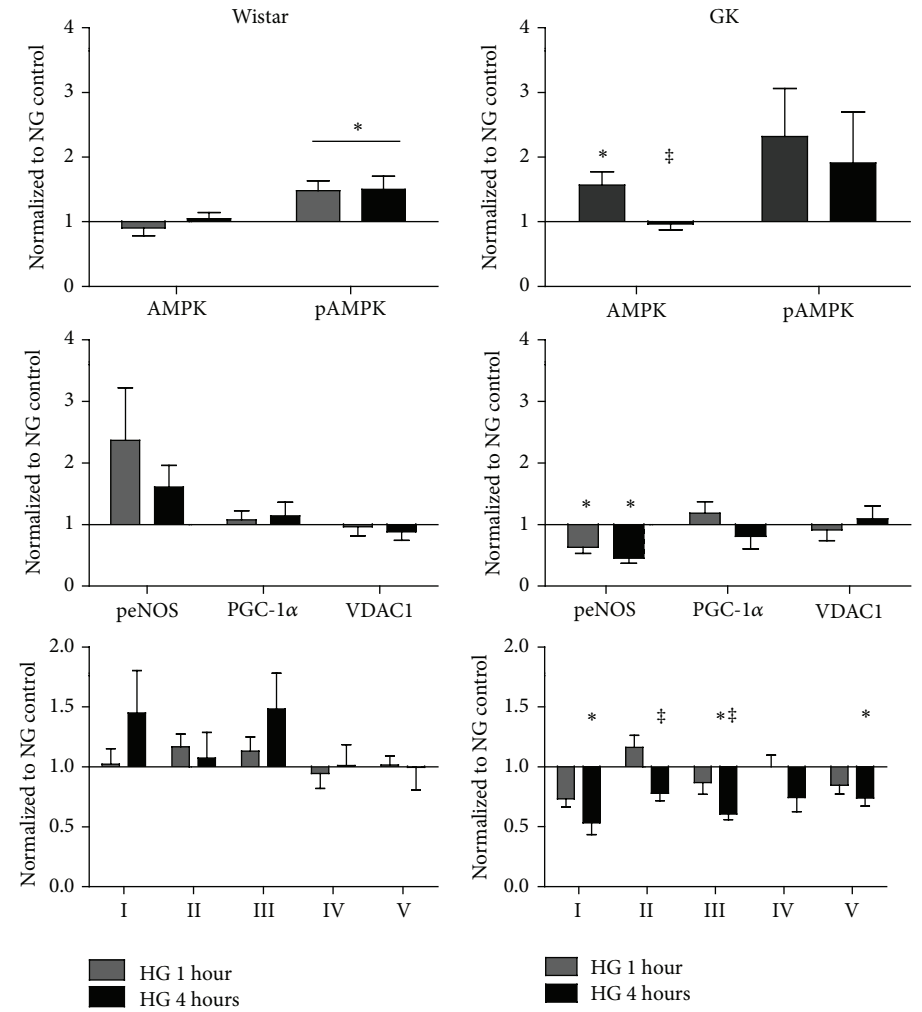
4. Discussion

Two-thirds of individuals with type 2 diabetes die due to cardiovascular disease (CVD), despite control of hyperglycemia and traditional CVD risk factors [1, 42]. In order to address this 3–5-fold excess risk, new therapeutic targets must be identified. The current work further builds upon our previous studies by characterizing alterations in mitochondrial homeostasis in primary SMCs from control (Wistar) and diabetes (GK) rat vasculature in order to more specifically examine the mitochondrial functional implications of our preceding reports. We observe differential mitochondrial and NOS activity, cellular signaling upstream of mitochondrial biogenesis and function, elevated baseline ROS production, and most notably a pattern of different mitochondrial plasticity and ROS in response to glucose induced metabolic stress in GK SMCs ex vivo as compared to Wistar SMCs (Figure 6). Thus, primary GK and Wistar SMCs illustrate persistent cellular mitochondrial differences in culture. One noted observation was the altered expression of mitochondrial complexes in the GK SMCs independent of parallel differences in PGC-1α. This may reflect the activation of alternate pathways leading to mitochondrial biogenesis without the activity of PGC-1α, particularly in states of environmental stress, nutrient excess, or pathology, as described in previous studies utilizing excess pyruvate, a peroxisome-deficient cellular model, and an exercised *ob*/*ob* mouse model of diabetes [43–45].

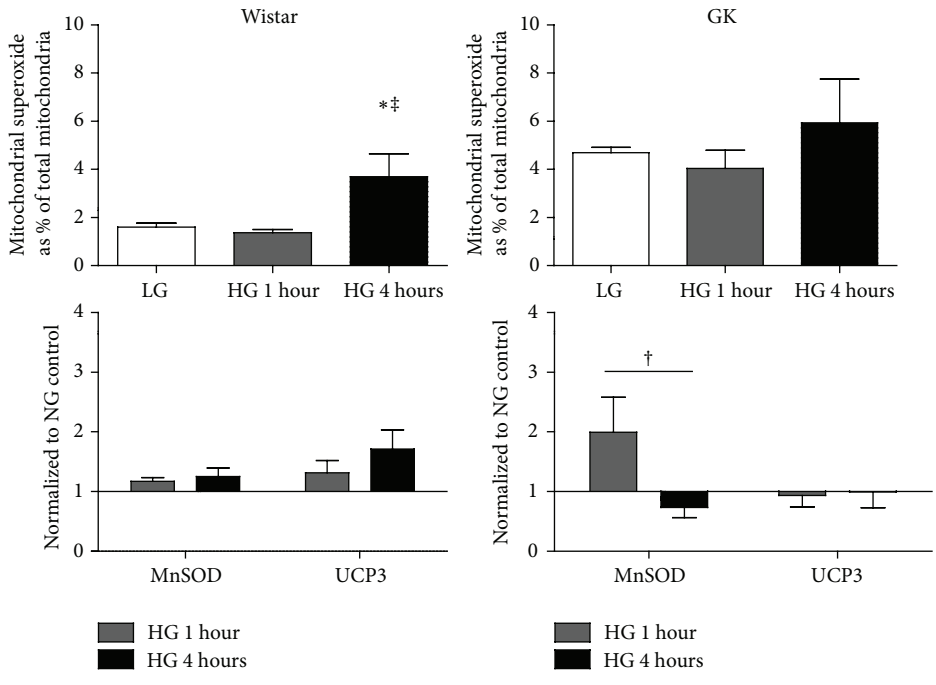
SMCs activation is characterized by increases in oxidative phosphorylation [6]. We assessed mitochondrial respiration in permeabilized cells, which permit evaluation of mitochondrial function separate from intracellular machinery and neutralize the impact of intracellular fuel partitioning [40]. Unexpectedly, GK SMCs cultured in normal glucose displayed greater state 3 and RCR measures of oxidative function compared with Wistar SMCs. Along with elevated baseline ROS in the GK SMCs, these respiratory changes are consistent with the elevated respiration, greater ATP production, and subsequent excess mitochondrial ROS seen in vascular dysfunction [16, 18]. Following exposure to high glucose, we observed decreased states 2 and 3 and uncoupled respiration states in GK SMCs along with a significant increase in perimeter per mitochondria and content. This suggests that baseline mitochondrial functional differences between Wistar and GK are due to altered dynamics, not mass, as mass is increasing in both cell types with nutrient stress. Also, ATP production was not significantly affected by HG exposure, suggesting that the basal levels are stable in these cells in culture.

Interestingly, although NO concentration, a measurement of NOS activity, was elevated in the GK SMCs at baseline, NO concentrations remained unaffected upon treatment exposure in Wistar and GK SMCs despite a significant decline in peNOS. This suggests that although eNOS signaling upstream of mitochondrial function is decreased in the glucose stressed GK SMCs, any resultant effect on NO production may have been overcome in 4 hours. Also, our protein expression measurements represent a snapshot of regulation and may not capture acute signaling or cellular responses. Ultimately, these data support our hypothesis and suggest NOS as a therapeutic target.

There is significant support in the literature for the hypothesis that excess mitochondrial ROS production plays a causal role in diabetic microvascular complications [15]. Nutrient stress derived from both glucose and lipids has been postulated to increase mitochondrial ROS production, presumed secondary to excess nutrient flux through the electron transport chain [11, 15, 46, 47]. We observed significantly elevated baseline ROS, H_2O_2 production, and mitochondrial superoxide in the GK SMCs, indicating innate differences in mitochondrial and total cellular ROS in the GK as compared to the Wistar SMCs, consistent with previous reports in diabetes and endothelial cells [48–53]. In contrast, GK SMCs did not demonstrate the expected increase in ROS generation when exposed to HG. Consistent with this, GK SMCs exposed to HG decreased expression of mitochondrial complexes I, II, III, and V and mitochondrial respiration.



(a)



(b)

Figure 5: Continued.

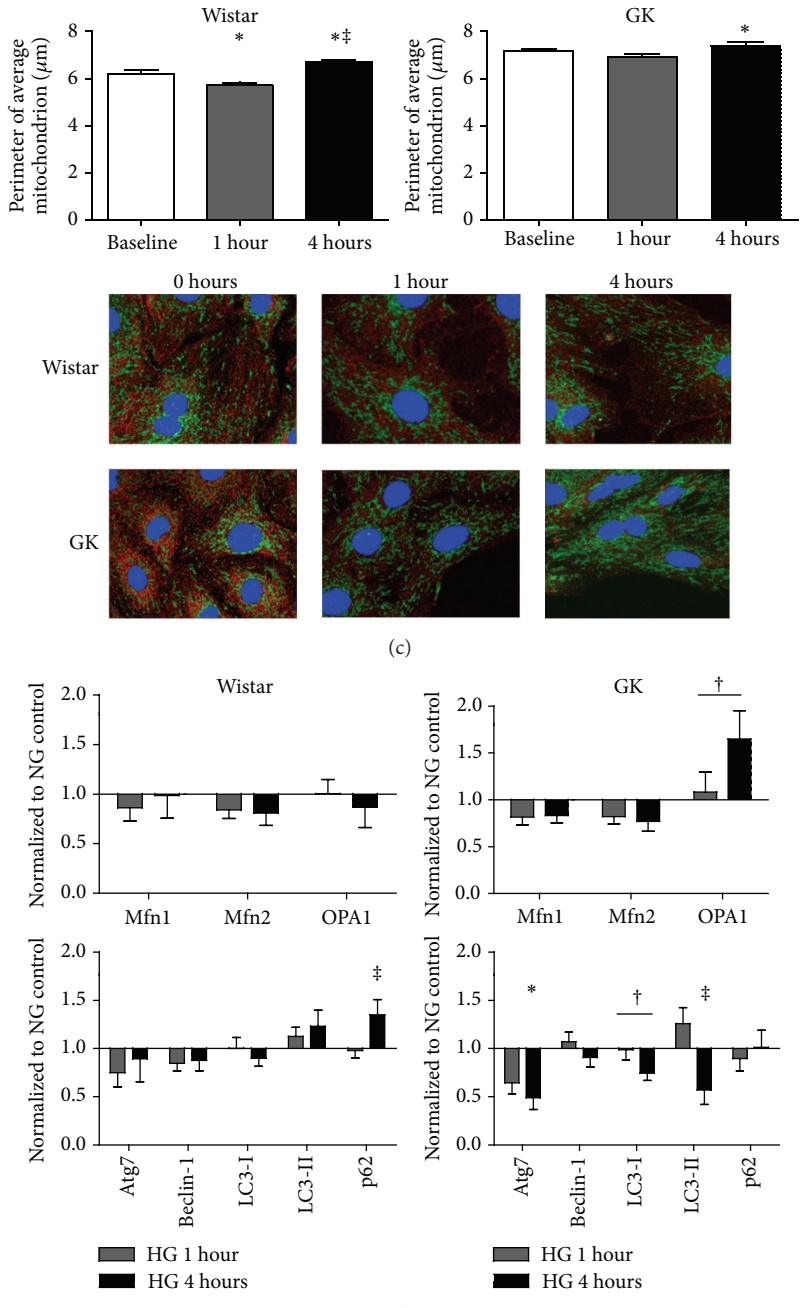


Figure 5: (a) Time course comparisons of AMPK ($n = 3$), pAMPK ($n = 4$), peNOS ($n = 3$), PGC-1α ($n = 3$), and VDAC1 ($n = 3$), and mitochondrial complex expression in Wistar and GK SMCs, passages 8–10. (b) Superoxide (flow cytometry, $n = 3$) and antioxidant status (Western blot, MnSOD $n = 4$, UCP3 $n = 3$) differences between Wistar and GK SMCs in NG (5 mM) or HG (25 mM). Protein expression was measured using Western blot, 15–30 μg protein on an SDS-page gel, and data are normalized to β-actin and expressed as mean fold change from NG + SEM. (c) Assessment of mitochondrial morphology ($n = 3$) during HG (25 mM) time course. Representative photographs of fixed SMCs using TOM 20 (green) and nitrotyrosine (red) are shown. (d) Time course mitochondrial dynamic ($n = 4$) and autophagy ($n = 3$) differences in Wistar and GK SMCs, passages 8–10. Protein expression was measured using Western blot, 15–30 μg protein on an SDS-page gel, and data are normalized to β-actin and expressed as mean fold change from NG + SEM. Significance measured by one-way ANOVA, $p < 0.05$ ($*$ compared to NG, ‡ compared to 1-hour HG), $^{\dagger}p < 0.1$.

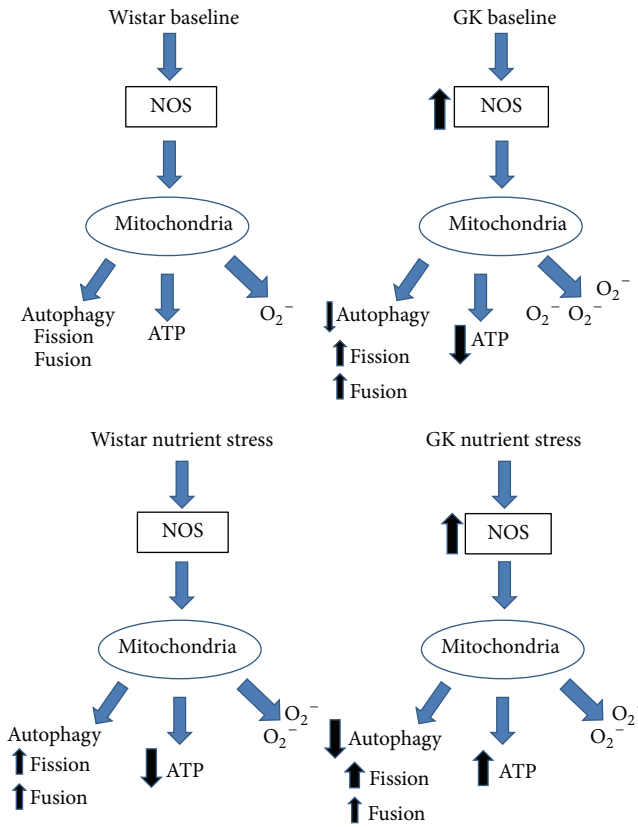


Figure 6: Data summary of cellular metabolic processes. Arrows indicate differences as compared with Wistar SMCs at baseline.

This in vitro finding aligns with our recent report of failed adaptation to an exercise stimulus in the GK aorta [31].

Baseline GK SMCs are metabolically active with activation of mitochondrial biogenesis signaling, mitochondrial ROS production, fusion, and respiration. This increased respiration is different than the general decrease in mitochondrial function observed in skeletal muscle in diabetes [54, 55]. Our finding that oxidative stress and oxidative phosphorylation are elevated in normal glucose media and diminished in high glucose media is consistent with a model where the hyperglycemic state drives lessened mitochondrial function. Importantly, our observation that nutrient stress results in decreased mitochondrial biogenesis signaling suggests that acute metabolic stress, not intrinsic mitochondrial dysfunction, is responsible for the differential adaptation and decreased mitochondrial function. This differential adaptation is reminiscent of our previously reported decrease in mitochondrial respiration and biogenesis signaling observed with exercise mediated stress in diabetes and with L-NAME, an inhibitor of NOS [30, 31, 56].

Alterations in mitochondrial network dynamics have been reported in diabetes [17, 47]. Other studies observe that dysregulated mitochondrial fusion, seen across animal models of cardiovascular disease, is central to this disease progression [6, 17, 20, 57]. Our observation of greater perimeter per mitochondrion is consistent with greater fusion, which aligns with the observed elevation in respiration in the GK SMCs as compared with the Wistar SMCs. We also observed decreased expression of autophagy markers in GK SMCs exposed to nutrient stress, implying that the protective aspects of autophagy may be aberrant in diabetes [58].

The literature supports that diabetes decreases mitochondrial function and increases ROS [17, 31, 47, 54–56]. In contrast, our data indicate that primary SMCs from the diabetes rats have greater mitochondrial content and respiration, NO, eNOS, and cytosolic and mitochondrial ROS and that the decline in mitochondrial function requires exposure to nutrient stress (Figure 6). Altered mitochondrial plasticity in response to excess glucose may be one mechanism underlying vascular smooth muscle cell mitochondrial dysfunction and ROS production in vivo. In conclusion, these data endorse the importance of mitochondrial dysregulation in diabetic vascular disease and suggest a potential role for metabolic stress in the contractile dysfunction (stiffness) observed in diabetes.

Abbreviations

AMPK: Adenosine monophosphate kinase
CVD: Cardiovascular disease
eNOS: Endothelial nitric oxide synthase
GK: Goto-Kakizaki
HG: High glucose
M-PER: Mammalian protein extraction reagent
NG: Normal glucose
NO: Nitric oxide

NOS: Nitric oxide synthase
PGC-1α: Peroxisome proliferator-activated receptor γ coactivator 1α
ROS: Reactive oxygen species
SMCs: Smooth muscle cells.

Conflict of Interests

There is no conflict of interests related to this work for Amy C. Keller, Leslie A. Knaub, P. Mason McClatchey, Chelsea A. Connon, Ron Bouchard, Matthew W. Miller, Kate E. Geary, Lori A. Walker, Dwight J. Klemm, and Jane E. B. Reusch.

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Review Article

Lycopene Deficiency in Ageing and Cardiovascular Disease

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Lycopene is a hydrocarbon phytochemical belonging to the tetraterpene carotenoid family and is found in red fruit and vegetables. Eleven conjugated double bonds predetermine the antioxidant properties of lycopene and its ability to scavenge lipid peroxyl radicals, reactive oxygen species, and nitric oxide. Lycopene has a low bioavailability rate and appears in the blood circulation incorporated into chylomicrons and other apo-B containing lipoproteins. The recent body of evidence suggests that plasma concentration of lycopene is not only a function of intestinal absorption rate but also lycopene breakdown via enzymatic and oxidative pathways in blood and tissues. Oxidative stress and the accumulation of reactive oxygen species and nitric oxide may represent a major cause of lycopene depletion in ageing, cardiovascular disease, and type 2 diabetes mellitus. It has been shown recently that low carotenoid levels, and especially decreased serum lycopene levels, are strongly predictive of all-cause mortality and poor outcomes of cardiovascular disease. However, there is a poor statistical association between dietary and serum lycopene levels which occurs due to limited bioavailability of lycopene from dietary sources. Hence, it is very unlikely that nutritional intervention alone could be instrumental in the correction of lycopene and carotenoid deficiency. Therefore, new nutraceutical formulations of carotenoids with enhanced bioavailability are urgently needed.

1. Introduction

Lycopene is a polyunsaturated hydrocarbon phytochemical present in red fruit and vegetables (papayas, tomatoes, red peppers, watermelons, etc.) and belongs to the tetraterpene carotenoid family [1]. Ingestion of products containing cooked tomato accounts for about 80% of daily dietary intake of lycopene in the developed world [2, 3]. Despite significant individual disparities, the average consumption of lycopene varies between 5 and 7 mg/day in the western world [4]. Due to its distinctive ability to neutralize free radicals, lycopene is believed to confer measurable protection against cancer, atherosclerosis, diabetes, and some inflammatory diseases [5, 6]. Indeed, a growing body of epidemiological evidence suggests that lycopene consumption is associated with decreased risk of various chronic diseases, while lycopene also demonstrates significant antioxidant activity in a number of in vitro and in vivo systems [7]. The multiple biological effects of lycopene are predetermined by the unique chemical structure of the compound and its particular physicochemical properties.

2. Physical and Chemical Properties

Lycopene is a 40-carbon atom acyclic fat-soluble compound containing 13 linearly aligned double bonds, 11 of them being conjugated. It occurs in nature as an all-*trans*-isoform, often referred to as *all-E*-lycopene [8]. Thermal processing as well as intestinal digestion of raw tomato products facilitates *cis*-isomerization of lycopene [9]. In the human body, lycopene is represented predominantly by various *cis*-isomers (referred to as (*Z*)-lycopene) suggesting that *cis*-transformation is essential for efficient intestinal absorption [10]. *cis*-isomerization can also be initiated by exposure of lycopene to heat, light, and oxygen although this can eventually cause irreversible degradation of the lycopene molecule to a number of small end products [8]. C=O bonds are chromogenic and confer a distinctive red color to lycopene crystals. Double bonds are essential to the antioxidant properties of lycopene which are a major functional feature of the compound. Amongst the different carotenoids, lycopene has the highest $^1O_2$ quenching ability, exceeding the antioxidant properties of carotene by at least twofold [11]. (*Z*)-isomers

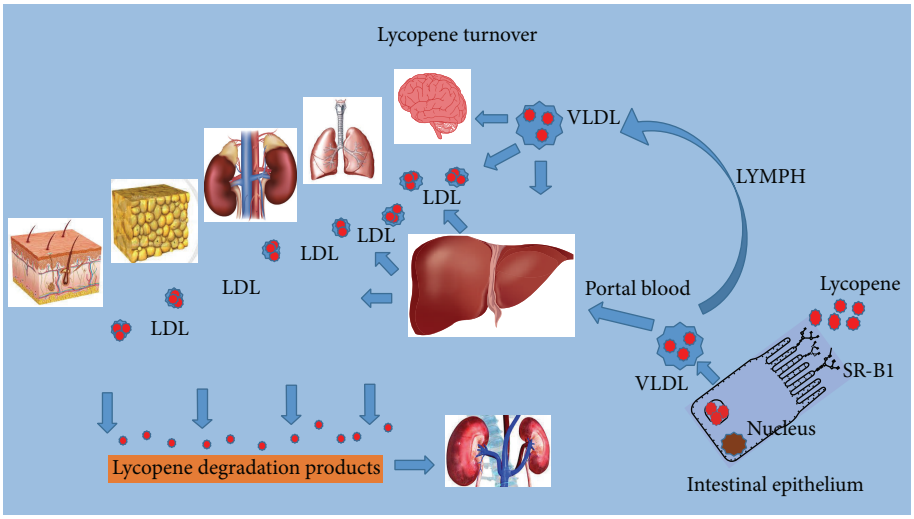

Figure 1

have the greatest antioxidant activity in scavenging lipid peroxyl radicals [8, 11].

3. Bioavailability and Absorption

Bioavailability of lycopene and other carotenoids is poorly understood and studied. It has been shown recently that structural localization of lycopene in the chloroplasts of fruit and vegetables is an important factor limiting bioavailability of lycopene from dietary sources since chloroplasts have high resistance to gastric and intestinal digestion [10]. Thus, food matrix structure predetermines significantly the bioavailability of lycopene. A significant portion of dietary lycopene is excreted from the human body in undigested form and there is no immediate absorption peak in plasma lycopene level after a single tomato meal. However, there is a clear cumulative lycopene absorption spike in the plasma of volunteers after a 5-day ingestion period of lycopene-containing products [12]. As shown in Figure 1, the ingested portion of lycopene released from the food matrix becomes solubilized and emulsified inside the intestinal lumen and is transported with scavenger receptor class B type 1 protein (SR-B1) via the epithelium of small intestine [13]. Lycopene distribution among tissues is very selective. The testes, adrenals, liver, and prostate have the highest lycopene concentration, while other organs are known to have much lower lycopene content [14]. Plasma lipoproteins are major delivery vehicles of carotenoids and lycopene in the human body [15].

Therefore, it is very likely that the differences in lycopene tissue levels are related to variation in tissue expression of lipoprotein receptors and cholesterol transporters.

4. Daily Requirements

There is no consensus on recommended daily dose of lycopene since carotenoids are considered to be nonessential micronutrients. In the industrialized European countries, daily intake of lycopene varies from 0.7 mg (Finland) to 1.3 mg (Germany), while a much higher range (3.7–16.1 mg) is reported for the United States [16]. Higher intake of lycopene at daily dosage up to 100 mg has no side effects in volunteers [17]. No evidence of toxicity of lycopene has been obtained from in vivo studies using laboratory animals [18]. Clinical studies use moderate amounts of lycopene rarely exceeding 10 mg per day. However, in animal experiments daily supplementation up to 200 mg/kg has been reported [19]. Therefore, low toxicity and high tolerance of lycopene open the door to various options in the design of lycopene supplementation protocols.

5. Lycopene in Blood

Factors influencing blood levels of lycopene represented in humans by *cis*-isoforms are not well understood as yet. Among these is geographic location of individuals and health status as well as a number of sociodemographic factors. It has been reported [20] that median lycopene concentration in the plasma of the general population of the USA is 0.59 μmol/L (range 0.07–1.79), whereas average lycopene concentration in the Scandinavian countries, in particular Finland, seems to be lower -0.16 ± 0.11 μmol/L. A very recent study [21] defines world regions with low and high levels of lycopene consumption. Surprisingly, Northern and Western Europe along with Central Africa and the Middle East represent the geographical areas with the lowest lycopene consumption as opposed to the Asian countries which display the highest level of lycopene consumption in the world attributed to high intake of fruit and vegetables [21]. It is also known that age and plasma lipid levels (LDL, total cholesterol, and triglycerides) are inversely correlated to blood lycopene level [20, 22]. Lower lycopene values are reportedly associated with being unmarried, of lower income, and an older nonwhite male [20].

6. Lycopene Depletion

Recent developments in molecular medicine reveal that the plasma level of lycopene and other carotenoids is a function of intestinal absorption rate as well as lycopene utilization in the reactions of biological oxidation in tissues. The antioxidant properties of lycopene and its ability to scavenge lipid peroxyl radicals are attributed to the eleven conjugated double bonds between its carbon atoms. When double bonds are oxidized and broken by reactive oxygen and reactive oxygen species, the lycopene molecule undergoes irreversible nonenzymatic degradation leading to the formation of various oxidative metabolites such as 2-apo-5,8-lycopenal-furanoxide, lycopene-5,6,5′,6′-diepoxide, lycopene-5,8-furanoxide, and lycopene-5,8-epoxide isomers [23, 24]. On the other hand, lycopene level in blood and tissues can be significantly affected by enzymatic degradation leading to the formation of different end products. In particular, enzymatic cleavage of lycopene by lipoxygenase is accompanied by accumulation of 3-keto-apo-13-lycopenone and 15,15′-apo-lycopenal among other minor cleavage products [24]. Depletion of lycopene as well as formation of lycopene oxidative metabolites can be reproduced in cell-free plasma specimens and isolated lipoproteins by introducing into the medium enzymatic systems generating reactive nitrogen species [25].

Taken together, these results suggest that oxidative stress as well as hyperactivity of the endogenous enzymes responsible for generation of reactive oxygen species and nitric oxide may deplete lycopene reserves in human cells and tissues.

7. Lycopene Deficiency in Ageing

Plasma lycopene level can become significantly reduced during the process of ageing. Older individuals show statistically lower lycopene concentration values in blood as compared to younger matching individuals with similar ethnic and dietary background [26]. Intestinal absorption of carotenoids is a complex multistage process which requires a fully intact and functioning gastrointestinal epithelium and subset of various enzymes [27]. Acute and chronic gastritis and abnormal gastric acid secretion as well as deviations in intestinal enzyme spectrum during ageing are considered to be major causes of reduced intestinal carotenoid absorption in older individuals [28]. Moreover, depleted levels of lycopene and other carotenoids in older individuals are believed to reflect age-related changes in the intestinal microbiota, which regulates bioavailability of carotenoids and polyphenols in the large intestine [29]. Although additional research to explain the causes and mechanisms of lycopene deficiency in ageing is required, it is clear now that correction of carotenoid deficiency in older individuals may have an enormous impact on their health status. Recent DNA microarray analysis reveals that lycopene supplementation prevents transcriptional activation of genes implemented in the process of ageing in multiple strains of mice [30]. This antagonizing action was as effective as the effect of well-known SIRT-1 activators such as starvation and resveratrol. Moreover, lycopene has recently been shown to suppress activation of the mTOR/AMPK cascade, a major metabolic pathway linked to ageing [31]. These cutting-edge observations help us to understand the multiple clinical and experimental reports revealing the effects of lycopene on a variety of health conditions associated with ageing. There are numerous pieces of clinical evidence suggesting that lycopene supplementation prevents osteoporosis and incidence of bone fractures [32, 33], improves pulmonary function [34], delays skin ageing [35], and enhances physical performance in elderly patients [36]. The set of clinical consequences of lycopene deficiency is greatly enlightened by the solid body of clinical evidence revealing the crucial role of lycopene in maintaining prostate health and its ability to prevent prostate cancer in elderly males [37, 38].

8. Lycopene and Cardiovascular Disease

Cardiovascular disease (CVD) remains a leading cause of mortality and disability around the world. There is a massive body of epidemiological results suggesting that Mediterranean countries have a lower rate of CVD mortality when compared to other regions of Western Europe and the United States [39]. The reduced rate of CVD mortality can be explained, at least in part, by the dietary culture of the Mediterranean region which includes consumption of large amounts of fruit and vegetables. Tomato-based products represent an essential element of the Mediterranean diet, which motivates many researchers to search for the link between lycopene consumption and occurrence of CVD. Epidemiological studies provide indisputable evidence supporting the direct role of lycopene in prevention of CVD. As has been recently confirmed in the Framingham Heart Offspring Study [40], there is a strong inverse association between lycopene intake and incidence of myocardial infarction, angina pectoris, and coronary insufficiency. Low plasma lycopene levels were reported by many researchers in hypertension, myocardial infarction, stroke, and atherosclerosis [41, 42]. Less convincing results and a more complex landscape emerge when the data from interventional studies on lycopene use in CVD patients are analyzed. There are multiple conflicting reports on how lycopene administration affects the progression of CVD and its outcomes [43, 44]. However, there is a certain degree of reproducibility in scientific reports describing the reduction of cholesterol (LDL and total), upregulation of HDL [45], decrease in carotid artery intima-media thickness [46], and lowering of both plasma markers of oxidative damage [44] and postprandial oxidative stress [47] in patients treated with lycopene. Nevertheless, the effect of lycopene on progression of CVD remains a controversial topic in modern medical science and requires further well-designed clinical studies. The experimental approach brings much more certainty regarding the beneficial role of lycopene in CVD. There are multiple and reproducible reports describing normalization of endothelial nitric oxide synthase activity and nitric oxide level in coronary arteries [48], inhibition of the mevalonate pathway of cholesterol biosynthesis [49], improvement of endothelial function, and attenuation of inflammatory damage [50] as well as improvements in lipoprotein profile and their turnover [48] in different animal models of CVD. These changes may represent a molecular

basis for lycopene action in CVD. Significant discrepancies in the outcomes of clinical and experimental studies verifying the effect of lycopene on CVD can be explained, in our opinion, by significant fluctuations in preexisting levels of lycopene in the blood of subjects enrolled on clinical trials, whereas lycopene concentration in the blood of experimental animals kept on standardized diets tends to stay in a similar range. However, individual variations in plasma lycopene level in general populations may have a significant impact on public health. As recently shown [51], low serum lycopene and total carotenoid levels predict all-cause mortality as well as poor outcomes and rapid progression of CVD in the adult population of the USA. The majority of individuals, even in developed countries, have astonishingly decreased levels of lycopene and total carotenoids in the blood which translates into higher stroke risk [52]. Decreased alpha-carotene and lycopene concentrations in the blood have recently been proposed as possible criteria for prognosis of public health since they are inversely associated with CVD mortality in a highly significant manner [53]. Although carotenoids are considered nonessential micronutrients, there have been some recent attempts to declare desirable plasma concentration levels of carotenoids for public health. As recently proposed, there are 5 cut-off levels for plasma carotenoid levels [54]. According to the results of meta-analysis, a plasma carotenoid level $<1\,\mu$M translates into a very high risk of health consequences. Moderate health risk is proclaimed to be associated with carotenoid concentration in the range 1.5–2.5 μM. Values for carotenoid concentration from 2.5–4 μM suggest a moderate risk, whereas carotenoid concentrations over 4 μM are proposed to have the lowest risk of health consequences. According to the same report, over 95% of the US population falls into the moderate or high risk category of the carotenoid health index.

9. Conclusion

Recent advances in medical and analytical chemistry have allowed pinpointing of multiple nonalimentary diseases and pathological conditions associated with micronutrient deficiencies. It has been increasingly recognized that health conditions associated with oxidative stress (ageing, CVD, and type 2 diabetes mellitus) are accompanied by significant deviations in plasma and tissue levels of many important nutrients, in particular lycopene and other carotenoids [55, 56].

Lycopene is the most powerful antioxidant from the tetraterpene carotenoid family, its anti-radical properties being mediated by eleven conjugated double bonds between carbon atoms. Lycopene is essential in scavenging lipid peroxyl radicals, reactive oxygen species, and nitric oxide. Recent advances in molecular science reveal that the interaction of lycopene with reactive oxygen species leads to irreversible nonenzymatic degradation of lycopene and formation of various oxidative lycopene metabolites. There is also a newly identified enzymatic pathway of lycopene degradation that results in the formation of several end products excreted from the human body. Thus, health conditions accompanied by long-lasting oxidative stress may cause lycopene depletion and require constant and efficient replenishment of carotenoids in the antioxidant "basket" of human cells and tissues. However, lycopene from dietary sources has extremely low absorption and bioavailability rate. It appears in the blood circulation incorporated into chylomicrons and other apo-B containing lipoproteins and requires a sophisticated system of intestinal absorption and distribution among the tissues. Therefore, plasma concentration of lycopene needs to be considered as an integral value reflecting both the intestinal absorption rate of carotenoids and the intensity of lycopene breakdown via enzymatic and oxidative pathways in blood and tissues.

There is alarming prevalence of lycopene and carotenoid deficiency in older individuals and CVD patients as well as widespread carotenoid deficiency in the general population, as revealed by latest research. This illustrates the necessity for a well-designed nutritional strategy and new nutraceutical products capable of normalizing plasma lycopene and carotenoid levels in an efficient manner. However, it is very unlikely that nutritional intervention alone will be sufficient in the correction of carotenoid deficiency. There is a poor statistical association between dietary and serum lycopene levels which can be explained by the limited bioavailability of lycopene from dietary sources. As shown recently, plasma concentration of lycopene, but not dietary intake of lycopene, correlates with predisposition to stroke [53]. It is suggested that even a high intake of lycopene-containing food products may not affect the health outcomes of lycopene and carotenoid deficiency in the general population. Therefore, new nutraceutical formulations of carotenoids and lycopene with enhanced bioavailability are urgently needed. In recent years, new formulations of lycopene and other carotenoids with increased bioavailability have been created using microemulsifying protocols and/or nanodelivery systems [57, 58]. Their implementation could be a milestone development in the prevention and treatment of the health consequences of lycopene and carotenoid deficiency in the general population.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Diabetes-Induced Oxidative Stress in Endothelial Progenitor Cells May Be Sustained by a Positive Feedback Loop Involving High Mobility Group Box-1

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Oxidative stress is considered to be a critical factor in diabetes-induced endothelial progenitor cell (EPC) dysfunction, although the underlying mechanisms are not fully understood. In this study, we investigated the role of high mobility group box-1 (HMGB-1) in diabetes-induced oxidative stress. HMGB-1 was upregulated in both serum and bone marrow-derived monocytes from diabetic mice compared with control mice. In vitro, advanced glycation end productions (AGEs) induced, expression of HMGB-1 in EPCs and in cell culture supernatants in a dose-dependent manner. However, inhibition of oxidative stress with N-acetylcysteine (NAC) partially inhibited the induction of HMGB-1 induced by AGEs. Furthermore, p66shc expression in EPCs induced by AGEs was abrogated by incubation with glycyrrhizin (Gly), while increased superoxide dismutase (SOD) activity in cell culture supernatants was observed in the Gly treated group. Thus, HMGB-1 may play an important role in diabetes-induced oxidative stress in EPCs via a positive feedback loop involving the AGE/reactive oxygen species/HMGB-1 pathway.

1. Introduction

Diabetes mellitus (DM) has been widely recognized as an important modern-day disease, and cardiovascular complications are the leading causes of morbidity and mortality in DM patients. Impaired angiogenesis is thought to be a critical event contributing to the development of cardiovascular complications associated with diabetes [1]. Notably, there is agreement in the literature that endothelial progenitor cells (EPCs), the precursors of endothelial cells, may contribute to angiogenesis and endothelial repair [2, 3]. However, the number of EPCs is reduced and the function is impaired in patients with diabetes, with EPCs found to be defective in vascular repair, thus contributing to the progression of cardiovascular disease in this patients [4–6].

Oxidative stress is a major cause of various pathological processes in DM [7]. Early reports demonstrated that the number of EPCs was negatively correlated with oxidative stress, which resulted in diabetes-related EPC dysfunction [8]. Thus, oxidative stress may represent an important therapeutic target in the prevention of impaired vascular homeostasis in DM. Although discoveries made in the last decade have made it clear that oxidative stress is central to impaired angiogenesis, the endogenous mechanisms remain poorly understood.

Increasing evidence demonstrates that EPCs can be divided into two types: early and late EPCs. Early EPCs, which are generated by the culture of peripheral blood mononuclear cells in medium for approximately 4 days, exhibit higher levels of cytokine release and lower vessel growth compared with late EPCs. Interestingly, late EPCs obtained by long-term culture of early EPCs show greater vasculogenic potential and are regarded as "true EPCs" [9]. Thus, late EPCs were used to evaluate the effects of oxidative stress in this investigation.

It has been established that hyperglycemia promotes reactions between plasma proteins and glucose through a nonenzymatic process, leading to the formation of advanced glycation end productions (AGEs). AGEs are considered to be important mediators of diabetes and diabetic complications. In diabetes, AGEs accumulate in tissues at an accelerated

rate and then contribute, at least in part, to the initiation and development of diabetic cardiovascular complications [10–12]. In the past decade, accumulating evidence has shown that AGEs promote oxidative stress in EPCs and then mediate EPC dysfunction in processes such as migration, tube formation, and apoptosis [13, 14].

High mobility group box-1 (HMGB-1), a nonchromosomal nuclear protein, is ubiquitously expressed in various cells including monocytes, cardiomyocytes, and endothelial cells. Once released into the serum in response to stresses such as high glucose conditions, HMGB-1 functions as a proinflammatory cytokine. Recent studies have indicated that oxidative stress promotes HMGB-1 release in various cells [15], resulting in the induction of reactive oxygen species (ROS) production in cardiomyocytes [16]. However, the role of HMGB-1 in diabetes-induced oxidative stress in late EPCs has received little attention. Thus, we hypothesized that diabetes induces the release of HMGB-1, which subsequently enhances oxidative stress in late EPCs. In the present study, HMGB-1 expression in serum and monocytes of diabetic mice was analyzed, and the role of HMGB-1 in AGE-induced oxidative stress in late EPCs was investigated in vitro.

2. Methods

2.1. Induction and Assessment of Diabetes. The experimental and feeding protocols were approved and conducted in accordance with the laws and regulations controlling experiments on live animals in China and the Asian Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Male C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University (China). Before injection of streptozotocin (STZ), the mice were weighed and the blood glucose was detected in a sample obtained from the tail vein using blood glucose monitoring system. Diabetes was induced in mice by consecutive intraperitoneal injections of STZ (40 mg/kg/day, Sigma-Aldrich, USA) for 5 days. After 3 days of STZ injection, blood glucose was determined as described previously. Mice with blood glucose levels >13.9 mmoL/l in three consecutive measurements were considered to be diabetic. Mice treated with citrate buffer were used as nondiabetic controls. Sixteen mice were divided into the following two groups: normal nondiabetic group (N group, $n = 8$) and diabetic group (DM group, $n = 8$). Nonfasting blood glucose and weight were measured every month until the end of the experiment.

2.2. Measurement of Serum HMGB-1 Levels. At the end of this investigation, the mice were anesthetized and sacrificed by decapitation, and blood was collected for serum separation. Serum HMGB-1 concentrations were determined using a mouse HMGB-1 ELISA kit (USCN Life Science, China) according to the manufacturer's instructions.

2.3. Isolation of Monocytes from Mouse Bone Marrow. After the mice were sacrificed, the femurs and tibias were immediately excised and flushed with cold PBS. After centrifugation at 1,400 rpm for 5 min, the collected cells were filtered and resuspended in red blood cell lysing buffer. After 5 min, the cells were centrifuged at 1,500 rpm for 5 min. After three washing steps, the monocytes were used in Western blot analyses.

2.4. EPC Isolation and Characterization. The protocol was approved by the Ethical Committee of Institutional Ethics Committee of Nanjing University Medical School (China). EPCs were isolated from the peripheral blood of healthy volunteers as previously described [14, 17]. Briefly, peripheral blood mononuclear cells (PBMCs) were collected by Ficoll-Paque PLUS (GE Healthcare Life Sciences, USA) density gradient centrifugation of peripheral blood. PBMCs were resuspended in endothelial cell growth medium-2 (EGM-2) (Lonza, Switzerland) composed of endothelial cell basal medium-2 (EBM-2), 5% fetal bovine serum (FBS), and growth factors containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like factor-1 (IGF-1), hydrocortisone, heparin, and ascorbic acid. After 4 days in culture, medium and nonadherent cells were removed; the medium was replaced every 3 days. After approximately 3 weeks, the "cobblestone" morphology observed by microscope indicated that the cells were late EPCs. These cells (passage < 5) were used in subsequent studies.

To confirm the late EPC phenotype, the cells were incubated with 5 μg/mL 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled low density lipoprotein (Dil-acLDL; Molecular Probes) for 2 h at 37°C and then fixed with 4% paraformaldehyde for 30 min. After being washed three times, the cells were incubated with 10 μg/mL fluorescein-isothiocyanate-conjugated lectin (FITC-lectin; Sigma, USA) for 1 h at room temperature in the dark. Additionally, late EPC surface markers were detected by immunocytochemistry using FITC-CD34 (BD, USA), allophycocyanin-KDR (APC-KDR, R&D, USA), and rabbit anti-CXCR4 (Abcam, UK). Then, goat anti-rabbit Alexa Fluor 488 was used to detect the expression of CXCR4. Nuclei were stained with DAPI. The cells were observed by fluorescence microscopy (Olympus, Japan).

2.5. Western Blot Analysis. After being washed with cold PBS, the cells were resuspended in cell lysis buffer containing a cocktail of protease inhibitors (1 : 100; Sigma, USA). After centrifugation at 15,000 rpm for 10 min, the supernatant was collected and stored at −80°C for later use. The protein concentration of the cell lysate was detected using a BCA protein assay kit (Pierce, USA). Total cell proteins or cell supernatants were mixed with loading buffer and were heated in boiling water for 10 min. The proteins were then separated by SDS-PAGE, electrotransferred, and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 10% dried nonfat milk in 0.1% PBS-Tween-20 (PBST) for 2 h at room temperature and hybridized overnight at 4°C with anti-HMGB-1 (1 : 1,000, Bioworld Technology, USA), anti-p66shc (1 : 1,000, Santa Cruz, USA), and anti-sirt1 (1 : 500, Bioworld Technology, USA). Membranes were then washed with 0.1% PBST and incubated with appropriate secondary

antibodies. The reactions were developed using enhanced chemiluminescence reagents and images were obtained by exposure to film. The bands were analyzed using BioRad Quantity One imaging software.

2.6. Evaluation of Intracellular ROS. Fluorescent probe 2′-7′-dichlorofluorescin diacetate (DCFH-DA; Sigma, USA) was used to measure intracellular ROS generation in late EPCs plated in 6-well plates. After different treatments, the medium was removed and the cells were washed with PBS. The cells were then incubated with 5 μmol/L DCFH-DA probe in serum-free medium at 37°C for 30 min. Intracellular ROS production was detected using a fluorescence microscope (Olympus, Japan).

2.7. Superoxide Dismutase Activity Determination. The superoxide dismutase (SOD) activity in cell supernatants was evaluated using SOD assay kit (Jiancheng Bioengineering Research Institute, China) according to the manufacturer's instructions and was expressed as a percentage compared with the control group.

2.8. Statistical Analysis. Data were expressed as the means ± standard deviation (SD) and were analyzed with one-way ANOVA using SPSS 20.0 software (SPSS lnc., USA). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Diabetes Induced Increased HMGB-1 in Serum and in Bone Marrow-Derived Monocytes from Diabetic Mice. As shown in Figure 1(a), diabetic mice exhibited increased blood glucose compared with normal mice; this high level was stably maintained until the mice were sacrificed. Furthermore, diabetes caused deleterious inhibition of normal weight gain during the present study (Figure 1(b)). Figure 1(c) showed the serum HMGB-1 levels in normal and diabetic mice. STZ-induced diabetic mice showed significantly higher serum HMGB-1 levels than those of normal mice. As monocytes are the main source of serum HMGB-1, HMGB-1 expression in monocytes from normal and diabetic mice was detected by Western blot analysis showing that HMGB-1 protein levels were significantly higher in monocytes from diabetic mice than those from nondiabetic mice (Figure 1(d)).

3.2. AGEs Induced HMGB-1 Upregulation in Late EPCs and in Cell Culture Supernatants. More than 90% of the attached cells that were double positive for Dil-acLDL uptake and lectin staining were characterized as late EPCs. Immunophenotyping revealed that late EPCs expressed CD34 and CXCR4, the surface antigens of progenitor cells, as well as KDR, which is a characteristic surface marker of endothelial cell (Figure 2(a)). AGEs were used to establish an in vitro diabetes model to investigate the role of HMGB-1 in diabetes-induced oxidative stress. HMGB-1 levels were measured in cell culture supernatants and in late EPCs exposed to AGEs. In order to screen the optimal stimulation concentration of AGEs, late EPCs were incubated with 50–400 μg/mL AGEs for 24 h, using 400 μg/mL BSA as a control. As shown in Figure 2(b), AGEs induced the upregulation of HMGB-1 in late EPCs in a dose-dependent manner, and the effect reached the level of statistical significance at 100 μg/mL. In addition, HMGB-1 in cell supernatants was significantly increased by exposure to AGEs at 100 μg/mL or 200 μg/mL (Figure 2(c)). Thus, in subsequent experiments, late EPCs were stimulated with 100 μg/mL AGEs for 24 h.

3.3. Oxidative Stress Was Involved in AGE-Induced Increased HMGB-1 Expression. To investigate the mechanism of AGE-induced oxidative stress in late EPCs, we determined the expression of sirt1 and p66shc in late EPCs. Our results showed that AGE-stimulated EPCs displayed significantly decreased sirt1 expression, and this effect was partly reversed by pretreatment with resveratrol, an activator of sirt1 (Figure 3(a)). p66shc, a critical protein regulating cellular oxidative stress responses, is considered to be the downstream of sirt1. Increased p66shc expression in AGE-stimulated EPCs was abrogated by resveratrol (Figure 3(b)). In addition, treatment with Ex527, a sirt1 antagonist, reversed the effects of resveratrol (Figure 3(b)), suggesting that the sirt1/p66shc pathway modulated oxidative stress in late EPCs in response to AGEs.

We next investigated whether AGEs induced HMGB-1 expression in late EPCs via ROS production by using NAC to inhibit oxidative stress induced in late EPCs by AGEs. The ROS generation in late EPCs demonstrated by DCFH-DA staining was significantly increased by AGE exposure; this effect was abrogated by N-acetylcysteine (NAC) treatment (Figure 3(c)). NAC abolished the stimulatory effect of AGEs on HMGB-1 expression in late EPCs (Figure 3(d)), suggesting that AGEs induce HMGB-1 expression in late EPCs via oxidative stress.

3.4. Inhibition of HMGB-1 Attenuated Oxidative Stress Induced by AGEs in Late EPCs. Glycyrrhizin was used to block the stimulation of HMGB-1 to determine its role in AGE-induced oxidative stress detected by intracellular p66shc expression and superoxide dismutase (SOD) activity in cell culture supernatants. Treatment of late EPCs with glycyrrhizin abrogated AGE-induced upregulation of p66shc expression (Figure 4(a)). Furthermore, the decreased SOD activity induced by AGEs was reversed by the glycyrrhizin administration (Figure 4(b)). Similar to p66shc, AGE-induced dysregulation of sirt1 expression was suppressed by the inhibition of HMGB-1 (Figure 4(c)).

4. Discussion

In the present study, we demonstrate for the first time that AGEs induce HMGB-1 expression in late EPCs via sirt1/p66shc-mediated oxidative stress and that the inhibition of HMGB-1 attenuates sirt1/p66shc pathway activity and oxidative stress in late EPCs exposed to AGEs. Thus, we conclude that HMGB-1 may amplify sirt1/p66shc pathway signaling and oxidative stress reactions in late EPCs induced by AGEs (Figure 5).

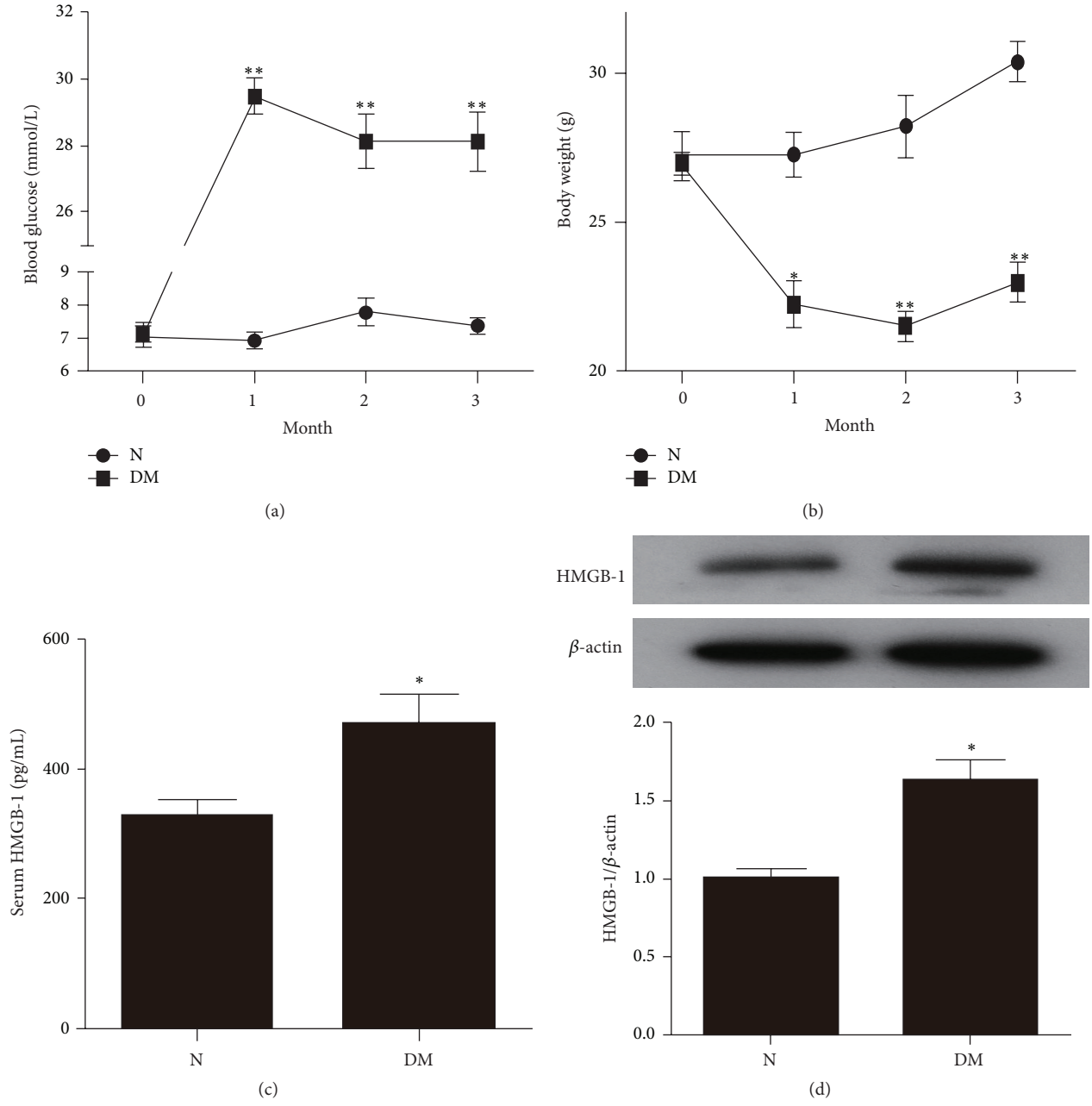

FIGURE 1: Blood glucose, body weight, and HMGB-1 expression in normal and diabetic mice. (a, b) Blood glucose and body weight in normal and diabetic mice. (c) Serum HMGB-1 levels in normal and diabetic mice. (d) Expression of HMGB-1 in bone marrow-derived monocytes in normal and diabetic mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ DM group versus N group ($n = 8$ per group). N: normal mice. DM: diabetic mice.

HMGB-1 is a nuclear DNA-binding protein that regulates gene transcription and maintains the nucleosome structure. Under stress conditions, HMGB-1 is released from the cell and functions as a multifunctional cytokine that contributes to various pathophysiological processes.

Accumulating clinical and experimental evidence demonstrates the presence of elevated serum HMGB-1 in diabetic patients and increased HMGB-1 expression in diabetic animals [18–20]. Furthermore, a recent study showed that plasma HMGB-1 levels were increased in Chinese subjects with pure type 2 DM [21]. In accordance with previous studies, the current investigation demonstrated that serum HMGB-1 levels were significantly higher in STZ-induced diabetic mice compared with their nondiabetic counterparts. It is worth mentioning that increased HMGB-1 expression was also observed in bone marrow-derived monocytes from diabetic mice. It can be speculated that EPCs from diabetic mice also exhibited higher HMGB-1 expression because the EPCs were predominantly mobilized from bone marrow. Although we did not detect expression of HMGB-1 in EPCs from diabetic mice directly, our in vitro studies demonstrated that HMGB-1 expression increased

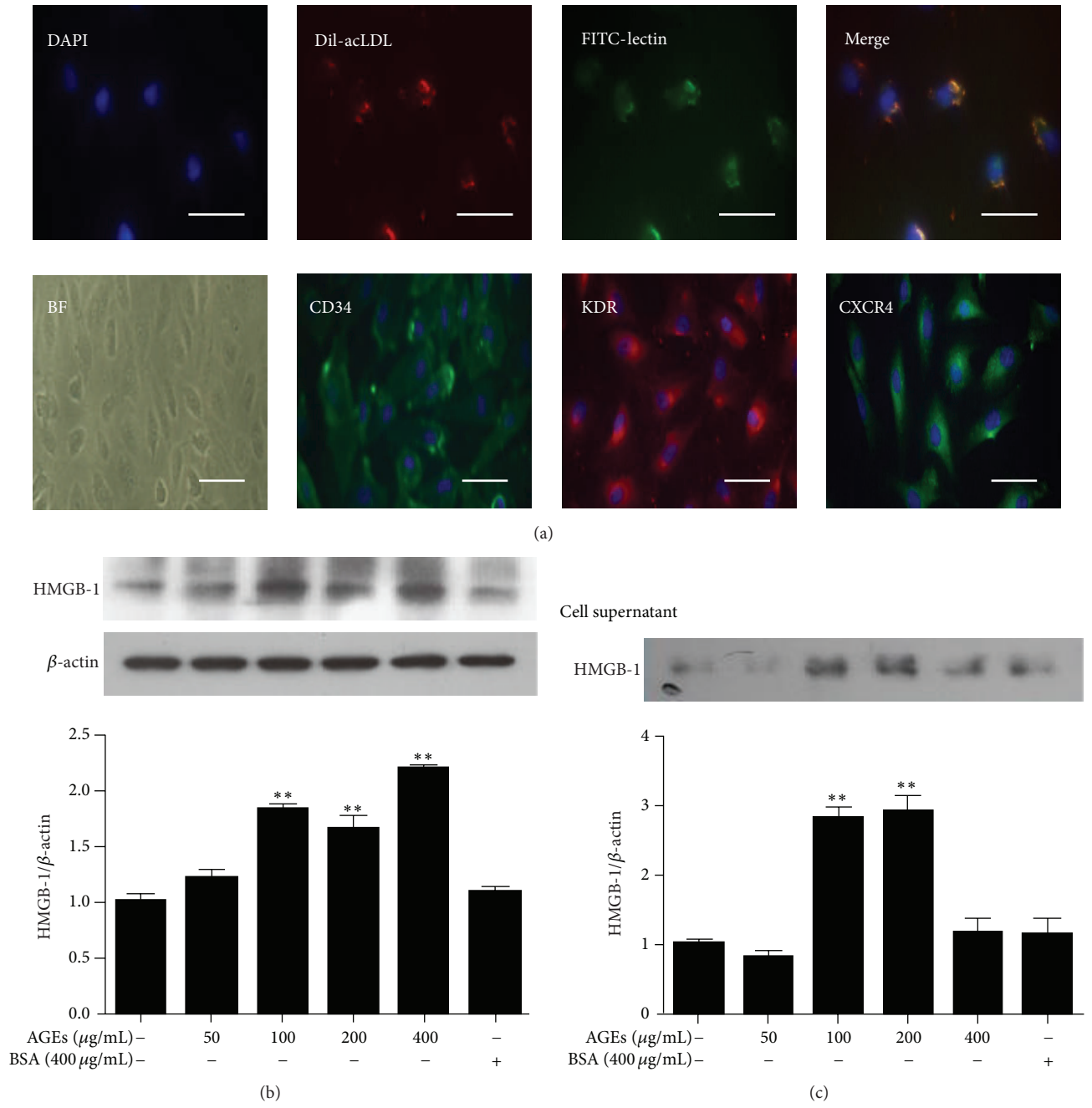

Figure 2: Expression of HMGB-1 in late EPCs and cell supernatants induced by AGEs at different concentrations. (a) Late EPCs were shown to endocytose Dil-acLDL and bind to lectin. Late EPCs showed a typical "cobblestone" morphology in phase-contrast inverted microscopy after approximately 3 weeks of culture and were positive for expression of CD34, KDR, and CXCR4. (b) Effect of AGEs on HMGB-1 expression in late EPCs. (c) HMGB-1 protein expression in cell culture supernatants of late EPCs exposed to AGEs. Scale bar = 20 μm. $^{**}P < 0.01$ versus control group, $n = 3$. BF: bright field; BSA: bovine serum albumin.

significantly and dose dependently in late EPCs following the exposure to AGEs and was accompanied by an increase in the levels detected in cell culture supernatants. This is in accordance with previous reports that hyperglycemia induced HMGB-1 expression in endothelial cells, vascular smooth muscle cells, and cardiomyocytes [22–24]. In addition, the underlying mechanism by which diabetes induces HMGB-1 was investigated in this study.

Oxidative stress has been reported to be involved in active HMGB-1 secretion [15, 25], and HMGB-1 in animal models is inhibited by antioxidants such as resveratrol [19, 26]. As oxidative stress is a central step leading to EPC dysfunction in diabetes and thus contributes to cardiovascular impairment [1, 27], we hypothesize that diabetes induces the upregulation and release of HMGB-1 in late EPCs via oxidative stress. As shown in Figure 3, production of both HMGB-1 and

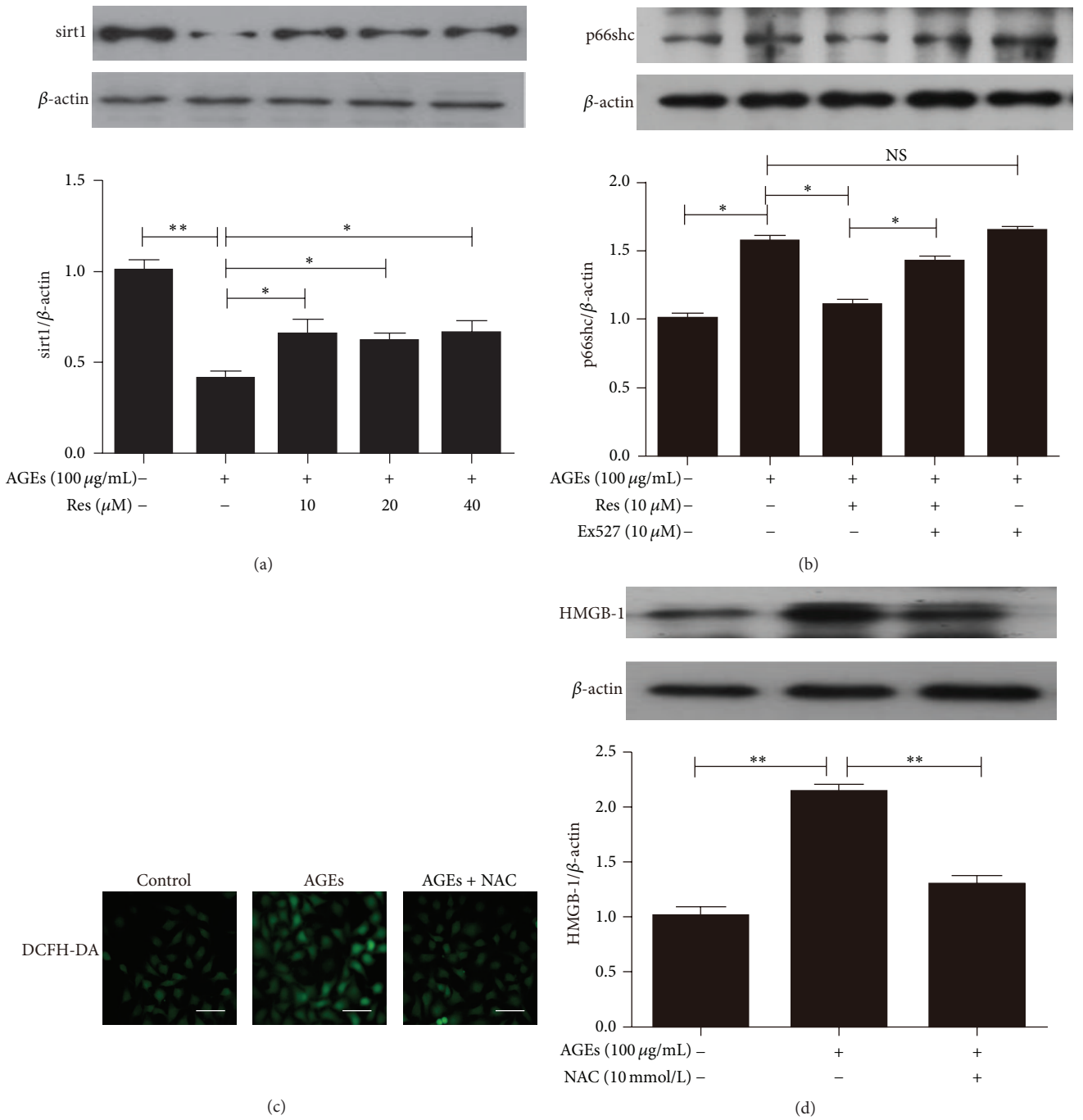

Figure 3: Inhibition of oxidative stress by NAC attenuated HMGB-1 expression in late EPCs. (a) AGE-induced downregulation of sirt1 was partly normalized by resveratrol. (b) Inhibition of sirt1 by Ex527 decreased p66shc expression in AGE-stimulated late EPCs. (c) DCFH-DA staining showing that pretreatment with NAC inhibited ROS production in late EPCs. (d) NAC suppressed HMGB-1 expression in AGE-stimulated late EPCs. $^{*}P < 0.05$ between the two groups and $^{**}P < 0.01$ between the two groups. NS: no difference between the two groups, $n = 3$. Scale bar = 25 μm.

ROS induced by AGEs in late EPCs was partially abolished by treatment with NAC, suggesting that oxidative stress is involved in AGE-induced HMGB-1 expression and that HMGB-1 production occurs downstream of ROS production in late EPCs stimulated by AGEs.

RAGE belongs to an immunoglobulin superfamily of cell surface molecules with the capacity to bind to a number of ligands such as AGEs, HMGB-1, amyloid fibrils, and S-100 proteins and is involved in a variety of cellular functions [28]. It is generally accepted that RAGE activation contributes to

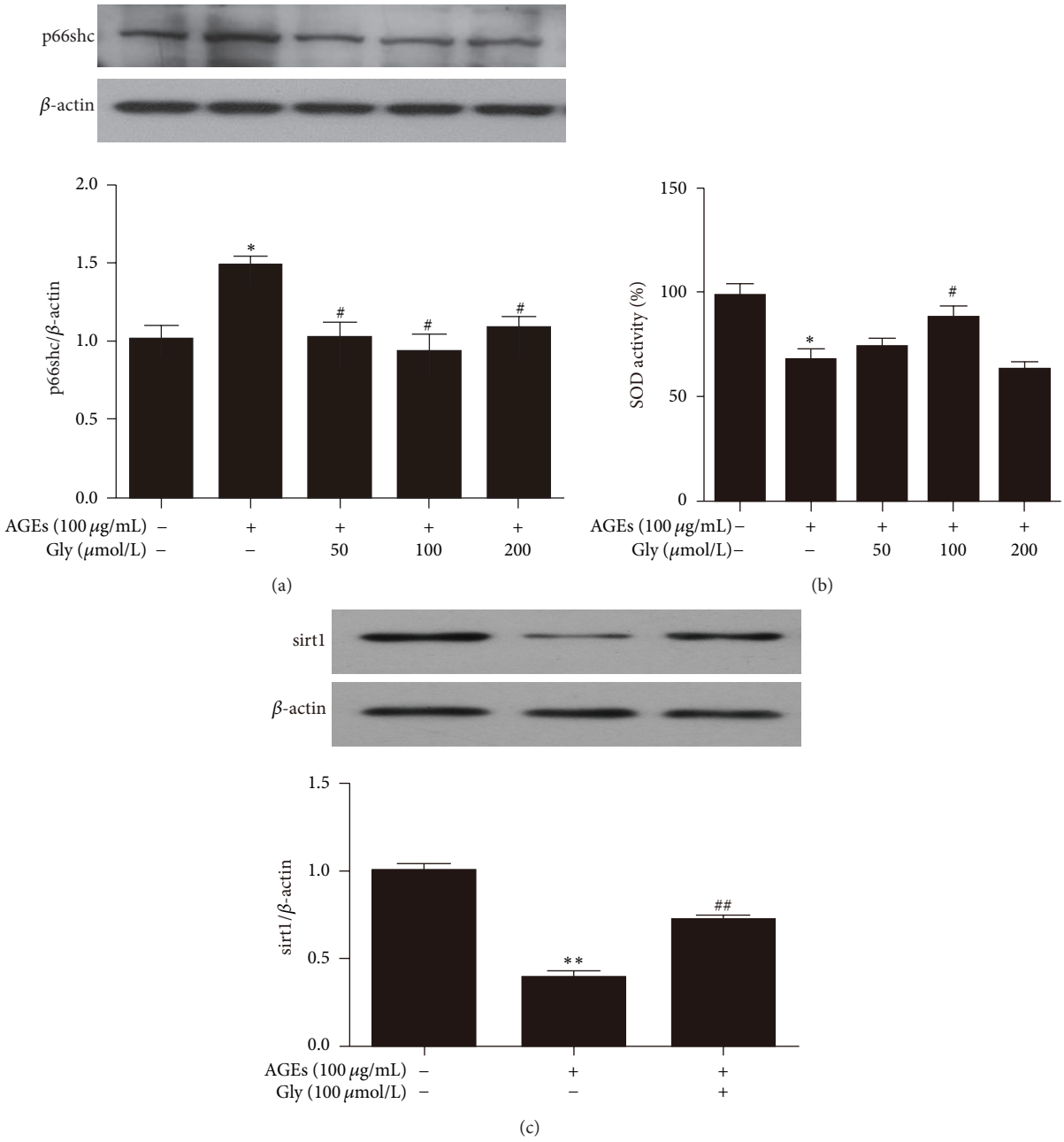

Figure 4: Glycyrrhizin normalized the dysregulated p66shc expression in AGEs treated late EPCs and the dysregulated SOD activity in cell culture supernatants. (a) The increased p66shc expression induced by AGEs was abrogated by glycyrrhizin treatment. (b) Treatment of late EPCs with glycyrrhizin reversed the decreased SOD activity induced by AGEs. (c) Glycyrrhizin attenuated the AGE-induced decrease in sirt1 expression in late EPCs. $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group, $^{\#}P < 0.05$ versus AGEs group, and $^{\#\#}P < 0.01$ versus AGE group, $n = 3$. Gly: glycyrrhizin.

oxidative stress in diabetes [13, 29]. HMGB-1 is a well-known non-AGE ligand for RAGE and can induce endothelial dysfunction [30]; therefore, HMGB-1 may cause increased ROS generation in late EPCs.

Oxidative stress is defined as an imbalance between ROS generation and antioxidant defenses. The adaptor protein, p66shc, promotes ROS production [31, 32], while SOD functions as an intracellular enzymatic antioxidant by scavenging excess oxygen-free radicals. Thus, p66shc expression in late EPCs and SOD activity in cell culture supernatants were determined to evaluate the effects of glycyrrhizin on oxidative stress induced in late EPCs by AGEs. In accordance with the findings of others [33, 34], the present study showed dysregulation of p66shc expression and SOD activity in AGEs-stimulated late EPCs. Furthermore, the changes in p66shc and SOD activity were partly normalized by inhibiting

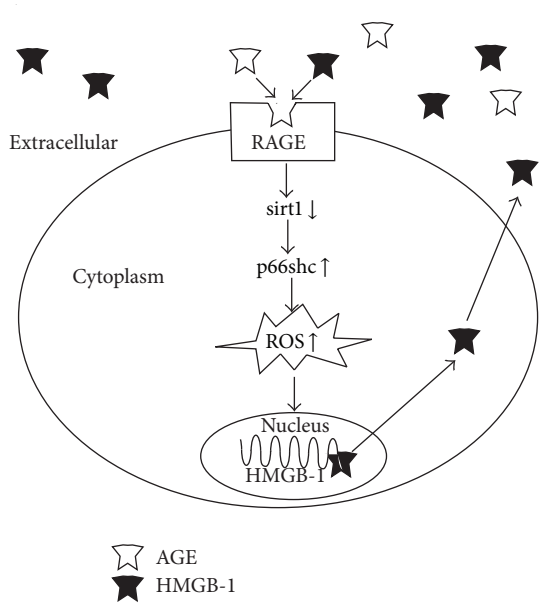

Figure 5: Hypothetical diagram of HMGB-1-mediated enhancement of oxidative stress induced in late EPCs by AGEs. AGEs induced ROS production via the sirt1/p66shc pathway and then promoted HMGB-1 release from the late EPCs. Extracellular HMGB-1 augmented ROS production via binding to RAGE and signaling through the sirt1/p66shc pathway.

HMGB-1 with glycyrrhizin, suggesting that HMGB-1 mediates oxidative stress induced in late EPCs by AGEs. In accordance with this speculation, Feng demonstrated that HMGB-1 mediated endothelial activation by oxidative stress responses via the RAGE pathway [30]. Furthermore, Zhang recently showed that HMGB-1 induced a marked increase in intracellular ROS in cardiomyocytes [16]. All these observations indicate that HMGB-1 stimulates oxidative stress in a variety of cell types.

Sirt1, a mammalian homologue of sir2, was identified as a key mediator of various diseases. Accumulating evidence suggests that sirt1 promotes cellular resistance to oxidative stress associated with diabetes. In the current investigation, we demonstrated that AGE treatment significantly decreased sirt1 expression in late EPCs, while resveratrol, an activator of sirt1, normalized the downregulated sirt1 expression. Furthermore, resveratrol treatment inhibited AGE-induced p66shc expression in late EPCs, although the effect was lost when AGE-stimulated late EPCs were pretreated with the sirt1 antagonist, Ex527. In accordance with previous observations that p66shc modulates oxidative stress in EPCs in response to high glucose [33], our results confirm that AGEs induce increased p66shc expression in late EPCs via downregulation of sirt1.

5. Conclusions

The results of the present study, together with the previous findings, indicate that HMGB-1 is not only an effector of oxidative stress, but also an inducer of oxidative stress in diabetes-induced late EPCs. In addition, targeting HMGB-1 with glycyrrhizin to inhibit oxidative stress is implicated as an attractive therapeutic strategy for impaired function of late EPCs in DM.

Disclosure

Han Wu and Ran Li are co-first author.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Role of Galectin-3 in Obesity and Impaired Glucose Homeostasis

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Galectin-3 is an important modulator of several biological functions. It has been implicated in numerous disease conditions, particularly in the long-term complications of diabetes because of its ability to bind the advanced glycation/lipoxidation end products that accumulate in target organs and exert their toxic effects by triggering proinflammatory and prooxidant pathways. Recent evidence suggests that galectin-3 may also participate in the development of obesity and type 2 diabetes. It has been shown that galectin-3 levels are higher in obese and diabetic individuals and parallel deterioration of glucose homeostasis. Two studies in galectin-3 knockout mice fed a high-fat diet (HFD) have shown increased adiposity and adipose tissue and systemic inflammation associated with altered glucose homeostasis, suggesting that galectin-3 negatively modulates the responsiveness of innate and adaptive immunity to overnutrition. However, these studies have also shown that impaired glucose homeostasis occurs in galectin-3 knockout animals independently of obesity. Moreover, another study reported decreased weight and fat mass in HFD-fed galectin-3 knockout mice. *In vitro*, galectin-3 was found to stimulate differentiation of preadipocytes into mature adipocytes. Altogether, these data indicate that galectin-3 deserves further attention in order to clarify its role as a potential player and therapeutic target in obesity and type 2 diabetes.

1. Introduction

Galectin-3, a chimera-type member of the galectin family, has been recently recognized as an important modulator of biological functions and an emerging player in the pathogenesis of common disease conditions, including cancer [1] and immune/inflammatory [2] and metabolic [3, 4] disorders. Considerable attention has been paid to the role of galectin-3 in the onset and progression of long-term complications of diabetes because of its ability to bind the advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) that accumulate in target organs and exert their toxic effects by triggering proinflammatory and prooxidant pathways [5, 6]. With this respect, galectin-3 was shown to exert a protective effect toward the development of renal disease and atherosclerosis in experimental animal models [3, 4], that is, the opposite of the receptor for AGEs (RAGE), the first identified AGE receptor, which is known to mediate AGE/ALE-induced tissue injury [7] and to be upregulated in the absence of galectin-3 [3, 4]. Recently, galectin-3 has been implicated also in the development of metabolic disorders, such as obesity and type 2 diabetes, though data are still fragmentary [3]. This review article is concerned with current knowledge on the role of galectin-3 in metabolic disorders and the mechanism by which this lectin eventually modulates excess fat mass, adipose tissue and systemic inflammation, and the associated impairment in glucose regulation, with a view to highlighting the unresolved issues that deserve further research.

2. Galectin-3 Structure and Function

Galectin-3 is a 29- to 35-kDa protein consisting of two domains, the C-terminal carbohydrate recognition domain (CRD), with highly conserved residues between galectins,

and the N-terminal domain, with a unique short end continuing into an intervening proline-glycine-alanine-tyrosine-rich repeat motif [8]. Galectin-3 is mainly located in the cytoplasm in quiescent cells, and in the nucleus in replicating cells [9]; it is also secreted into the extracellular space through a nonclassical secretory pathway [10]. Extracellular galectin-3 interacts with the β-galactoside residues of several glycoproteins via the CRD, and increasing concentrations of multivalent glycoprotein ligands drive formation of multimeric structures, which result in higher order lattices. The galectin-glycoprotein lattice has been shown to play a role in the regulation of receptor clustering, endocytosis, and signaling [11]. As a component of the cell surface lattice, galectin-3 also regulates the biogenesis of a subpopulation of clathrin-independent carriers (CLICs) involved in endocytosis of specific cargo proteins [12]. This function most likely represents the mechanism through which galectin-3 regulates tyrosine kinase and TGF-β receptors signaling, thereby controlling important cell functions such as cell migration, transdifferentiation, and fibrogenesis [11, 13]. Moreover, the galectin-3 lattice on the cell surface and its related function in CLIC biogenesis may play a role in AGE/ALE binding, internalization, and degradation [3]. Intracellular galectin-3 interacts with ligands mainly via peptide-peptide associations [8].

These structural features enable galectin-3 to participate in several cell functions via its binding to extracellular and intracellular proteins and make it a broad-spectrum biological response modifier [3, 5]. In addition to serving as an AGE/ALE receptor, galectin-3 is involved in pre-mRNA splicing [14], regulation of cell cycle [15] and Wnt/β-catenin signaling [16], and dual modulation of cell adhesion [17] and immune/inflammatory processes. In particular, galectin-3 has both pro- and anti-inflammatory effects, which depend on factors such as inflammatory setting and target cell/tissue [2, 3]. In acute inflammation, galectin-3 is a key component of the host defenses and promotes onset of the response against microbial infections and may affect immune/inflammatory cells by an autocrine/paracrine mechanism [2]. However, in chronic inflammation, a condition characterizing both diabetes and obesity, galectin-3 exerts some proresolution actions limiting further tissue injury and promoting repair. Relevant anti-inflammatory effects of galectin-3 include stimulation of T-cell apoptosis [18], inhibition of T-cell growth and T helper 1 differentiation [19], downregulation of T-cell receptor-mediated T-cell activation [20], induction of alternative macrophage activation [21], and promotion of efferocytosis of apoptotic neutrophils by macrophages [22]. Moreover, galectin-3 induces epithelial-mesenchymal transition and fibrogenesis, which may favor limitation of inflammation and wound healing [23], unless the fibrotic process proceeds beyond tissue repair [24, 25]. Finally, galectin-3 has been recently shown to restrain the inflammatory response that involves the Toll-like receptor- (TLR-) 2 and 4 and the proinflammatory cytokines interleukin- (IL-) 1-β and tumor necrosis factor-α in macrophages, an effect that contributes to delay the course of Wallerian degeneration in peripheral nerves [26], and to reduce the susceptibility to endotoxin shock [27]. Conversely, galectin-3/TLR-4 interaction has been reported to sustain microglia activation [28].

3. Galectin-3 in Obesity and Impaired Glucose Homeostasis

Obesity and type 2 diabetes are reaching epidemic proportions in the Western world [29] and call for novel pharmacological interventions to complement lifestyle modifications in preventing strategies. The parallel rise in the incidence and prevalence of these two conditions supports the concept that overweight and obesity are powerful risk factors for developing type 2 diabetes [30]. The mechanisms linking excess fat mass, especially at the visceral level, and impaired glucose regulation include adipose tissue macrophage infiltration and deranged lipid metabolism. These changes result in the release of cytokines and free fatty acids which cause insulin resistance and β-cell dysfunction [31]. Several proinflammatory and prooxidant signals have been implicated in the pathogenesis of obesity, including the AGES/ALEs through their receptor RAGE [32].

Recently, a wealth of human studies has provided evidence that galectin-3 levels are increased in subjects with obesity and type 2 diabetes, and animal studies have suggested that galectin-3 may be involved in the onset and progression of these metabolic disorders by acting primarily at the adipose tissue level.

3.1. Human Studies. In the general population, levels of circulating galectin-3 have been shown to correlate positively with age, prevalence of obesity, diabetes, hypercholesterolemia, and hypertension, markers of inflammation, and target organ damage [33, 34], thus indicating a strict relationship of galectin-3 with metabolic disorders and associated risk factors and complications.

A few studies have investigated the relationship between circulating levels of galectin-3, obesity and parameters of glucose metabolism, and insulin sensitivity in patients with diabetes (Figure 1). Weigert et al. [35] reported higher circulating galectin-3 levels in 30 overweight nondiabetic subjects and 30 patients with type 2 diabetes, in comparison with 23 normal-weight controls. All of them showed galectin-3 levels that correlated positively with body mass index (BMI), as well as, in a BMI-dependent manner, with leptin, resistin, IL-6, and age. In the diabetic population, circulating levels of galectin-3 correlated inversely with hemoglobin A_{1c} (HbA_{1c}) and were higher in subjects with C-reactive protein (CRP) values above 5 mg/L. Finally, galectin-3 levels were higher in portal vein blood than in blood from hepatic and systemic veins. This observation suggests that the splanchnic region constitutes a major site for galectin-3 removal, a finding consistent with the scavenging role of this lectin in the hepatic clearance of circulating AGEs [36].

More recently, Yilmaz et al. [37] studied 174 subjects divided into three groups, nondiabetic, prediabetic, and diabetic, based on a 75-g oral glucose tolerance test. Galectin-3 levels were significantly higher in diabetic patients than in prediabetic and nondiabetic groups and higher in the prediabetic than in the nondiabetic group. Moreover, galectin-3 levels correlated with fasting plasma glucose ($r = 0.787$, $P < 0.01$), 2-hour plasma glucose ($r = 0.833$, $P < 0.01$), CRP ($r = 0.501$, $P < 0.01$), and homeostasis model assessment

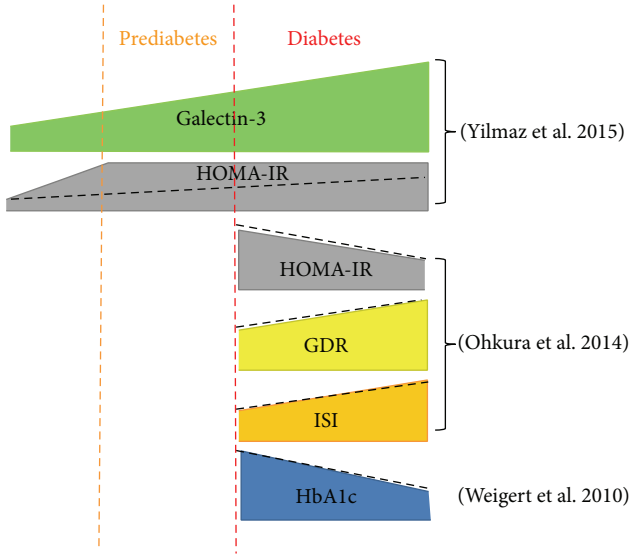


Figure 1: Summary of results of human studies on the role of galectin-3 in impaired glucose regulation and insulin sensitivity. Solid geometric figures indicate levels of each target, whereas dotted black lines indicate positive or negative correlation with galectin-3 levels. In the general population, galectin-3 levels are positively correlated with HOMA-IR, a measure of insulin resistance. Conversely, within the diabetic population galectin-3 levels are negatively correlated to HOMA-IR and HbA_{1c} levels and positively with GDR and ISI. HOMA-IR = homeostasis model assessment of insulin resistance; GDR = glucose disposal rate; ISI = insulin sensitivity index; HbA_{1c} = hemoglobin A_{1c}.

of insulin resistance (HOMA-IR) index ($r = 0.518$, $P < 0.01$). In multivariate logistic regression analysis galectin-3 was an independent predictor of diabetes. Moreover, in receiver operating characteristic analysis, a galectin-3 cut-off value of 803.55 pg/mL was found to diagnose diabetes with a sensitivity of 80.7% and a specificity of 85.5% (area under the curve = 0.912). Yilmaz et al. concluded that galectin-3 is a promising biomarker for early detection of prediabetes and diabetes onset and that it has a role in the progression from prediabetes to diabetes. However, data from this cross-sectional study cannot be construed as evidence of causality between galectin-3 changes and impaired glucose metabolism. In addition, calculation of HOMA-IR index is not the most appropriate method to estimate insulin resistance in this type of studies, where patients with poor glycemic control, low BMI, or severe β-cell dysfunction could be included [38].

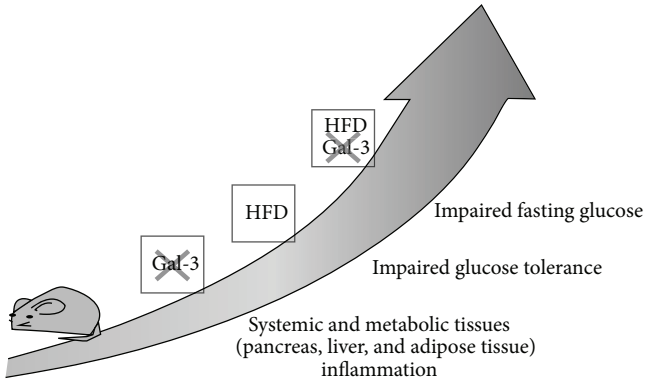
A recent study from Ohkura et al. has investigated the relationship between galectin-3 and insulin sensitivity in 20 patients with type 2 diabetes through the euglycemic-hyperinsulinemic clamp, the gold standard for the assessment of insulin sensitivity, a meal tolerance test (MTT), and HOMA-IR index [39]. Galectin-3 levels correlated positively with the glucose disposal rate ($r = 0.71$, $P < 0.001$), insulin sensitivity index in MTT ($r = 0.62$, $P < 0.005$), and adiponectin concentration ($r = 0.61$, $P < 0.05$) and negatively with the HOMA-IR index ($r = -0.52$, $P < 0.05$) and fasting insulin concentration ($r = -0.56$, $P < 0.01$). In MTT, galectin-3 levels were not significantly associated with the areas under the curve of glucose, insulin, and the insulin/glucose ratio; a negative, but not significant, association was reported with insulin and the insulin/glucose ratio. These data, which should be taken with caution because of the small size of the study, prompted the authors to conclude that galectin-3 affects the concentration of insulin more than that of glucose and that increase of galectin-3 activity in diabetic subjects could improve insulin sensitivity.

These apparently contrasting results might be reconciled by claiming a role for galectin-3 upregulation as an adaptive mechanism to counteract the progression of metabolic derangement by favoring glucose disposal. In this view, galectin-3 levels would increase with development of obesity and diabetes, thus serving as a marker of these disorders, in which this lectin exerts a protective effect toward insulin resistance. This interpretation is consistent with the role of galectin-3 in favoring AGE/ALE disposal [3, 4], as a number of studies in nondiabetic individuals have shown that serum levels of AGEs or of their carbonyl precursors are independent correlates of insulin resistance, as assessed by HOMA-IR index [40].

3.2. Animal Studies. Several experimental studies have investigated the role of galectin-3 in the development of type 2 diabetes (Figure 2) and obesity (Figure 3).

Pejnovic et al. [41] and Pang et al. [42] reported increased fat accumulation and inflammation at the visceral adipose tissue and systemic level in association with altered glucose homeostasis in galectin-3 knockout mice fed a high-fat diet (HFD). Pejnovic et al. also found that galectin-3 knockout mice fed a HFD had increased inflammation in the pancreatic islets associated with accumulation of AGEs [41], which might be responsible for the impaired glucose regulation. This interpretation is consistent with a previous report showing that aminoguanidine prevents decreased glucose-stimulated insulin secretion in islets exposed to high glucose concentrations [43]. These data support the concept that galectin-3 primarily decreases the response of innate and adaptive immunity to overnutrition which leads to adipose tissue inflammation and oxidative stress, thus protecting against the obesity-associated type 2 diabetes [41, 42]. This view is consistent with a major anti-inflammatory effect of galectin-3 in chronic settings [3], the loss of which results in increased macrophage infiltration and release of proinflammatory and prooxidant cytokines at the adipose tissue level that enhances the metabolic derangements associated with fat accumulation. This interpretation is also in keeping with the finding that mice lacking RAGE exhibit an opposite response to a HFD, that is, reduced weight gain and inflammation [44]. However, these data do not explain the mechanism by which galectin-3 limits the expansion of adipose tissue and this interpretation has been challenged by other observations.

Firstly, both Pejnovic et al. [41] and Pang et al. [42] found that blood glucose levels were higher also in galectin-3 knockout fed a standard diet versus the corresponding wild type mice. Moreover, Pang et al. found that, in these animals, increased fat mass and systemic inflammation occurred



(Pang et al. 2013; Pejnovic et al. 2013)

Figure 2: Summary of results of animal studies on the role of galectin-3 in deranged glucose homeostasis and "metabolic" inflammation. Like HFD, galectin-3 ablation induces glucose metabolism dysregulation and metainflammation. Galectin-3 ablation and HFD have cumulative effects in inducing metabolic and inflammatory alterations. Gal-3 = galectin-3; crossed Gal-3 = galectin-3 ablation; HFD = high-fat diet.

after the development of altered glucose homeostasis, which was not accompanied by decreased insulin sensitivity [42], thus suggesting that galectin-3 modulates β-cell function, irrespective of obesity-related inflammation. However, there are conflicting data in the literature on this issue. In fact, previous studies showed that, in rat pancreatic islets exposed to IL-1β, galectin-3 is the most upregulated protein and that its overexpression protects the β-cells from the cytotoxic effect of IL-1β [45]. In contrast, galectin-3 appeared to be crucial for immune mediated β-cell damage, as galectin-3 knockout mice were resistant to multiple low doses of streptozotocin, a classical model of type 1 diabetes [46].

Secondly, at variance with these earlier studies, Baek et al. have recently reported that galectin-3 knockout mice had significantly lower body weight and epididymal white adipose tissue and higher expression of adipose triacylglycerol lipase, the rate-limiting enzyme in fat cell lipolysis, as compared with the corresponding wild type animals after 12-week feeding with a HFD [47]. Conversely, and again at variance with Pejnovic et al. [41] and Pang et al. [42], plasma glucose levels did not differ between the two genotypes, though glucose metabolism was not thoroughly investigated [47]. Interestingly, decreased body size and epididymal white adipose tissue were observed also in galectin-3 knockout mice fed a standard chow for 17 months, with no sign of inflammation [47]. The authors also showed that galectin-3 knockdown significantly reduced the differentiation of 3T3-L1 cells into mature fat cells and the expression of the master regulator of adipocyte differentiation peroxisome-proliferator-activated receptor γ (PPARγ) as well as of CCAAT-enhancer-binding protein (C/EBP) α and C/EBP β [41]. Moreover, galectin-3 was found to interact with PPARγ and to regulate its expression and transcriptional activation, thus suggesting that galectin-3 plays a direct role in adipogenesis through PPARγ regulation [47]. Reduced lipid accumulation was paralleled by decreased fat cell enlargement, indicating that lower adiposity in galectin-3 knockout mice was not due to a decrease in fat cell number [47]. Indeed, Pang et al. found that the mRNA levels of PPARγ were lower in galectin-3 knockout than in wild type mice and that the fat cell size did not differ between the two genotypes, irrespective of diet [42]. The results of the study of Baek et al., though preliminary, point to a stimulatory role of galectin-3 in adipogenesis, with the lack of this lectin resulting in reduced body weight gain in response to HFD and aging. In fact, in human and mouse adipose tissue, galectin-3 is expressed not only by macrophages but also by fat cells, where it is modulated during cell differentiation, with high level in the preadipocyte fraction and almost nil in differentiated fat cells [48]. Moreover, recombinant human galectin-3 was found to stimulate preadipocyte proliferation as well as DNA synthesis [48]. Unfortunately, Baek et al. did not investigate whether feeding a HFD induced adipose tissue and systemic inflammation [47], which appeared to parallel increased fat mass and to drive deranged glucose homeostasis in previous reports from Pejnovic et al. [41] and Pang et al. [42].

Altogether, these contrasting findings point to the need of further investigation on the effect of galectin-3 ablation on adipose tissue morphology and function and glucose homeostasis as well as on the response to a HFD.

4. Adipose Tissue in Type 2 Diabetes: Just a Matter of Quantity? The Role of Galectin-3

The studies in experimental animal models reviewed above have provided new insights in the relationships between adipocyte size and glucose homeostasis. Previous observations point to a critical role of lipid handling capacity of white adipose tissue in the development of type 2 diabetes. This is a key concept of the "adipose tissue expandability" theory, which suggests that increased size of adipocytes, close to a critical volume, favors diversion of lipids to other tissues, cell suffering, and consequent activation of proinflammatory and prooxidant pathways at the tissue and systemic level [49].

This theory has been also used to explain the paradox of the existence of a subset of individuals with normal insulin sensitivity despite being obese, on the one hand, and of lean insulin-resistant/diabetic subjects, on the other hand [50]. In fact, it has been proposed that white adipose tissue inflammation and associated insulin resistance are coupled to adipocyte hypertrophy regardless of fat mass and body weight. However, this traditional view has been recently challenged by the evidence that small, not enlarged, fat cells are associated with the onset of insulin resistance and inflammation in response to overfeeding [51]. Moreover, in obese subjects, small fat cells predominate in individuals with abnormal insulin resistance, whereas larger fat cells are more numerous in insulin-sensitive subjects [52]. Although no significant difference in the mean size of the larger cells was found between the insulin-sensitive and insulin-resistant individuals, insulin resistance was associated with a higher

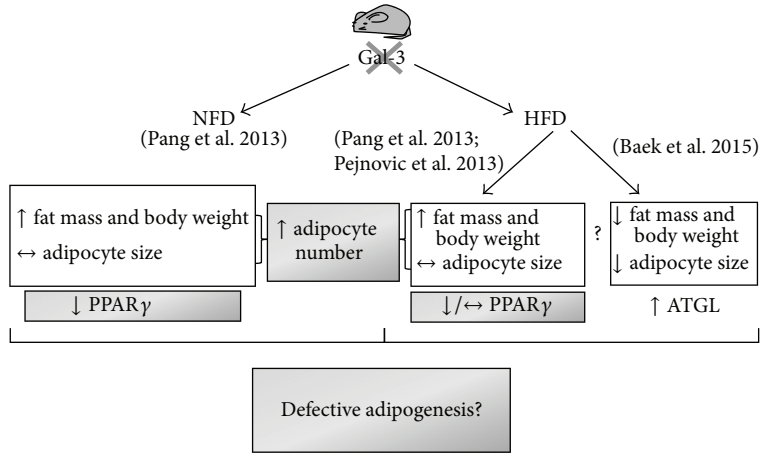


Figure 3: Summary of results of animal studies on the role of galectin-3 in obesity and adipogenesis. Structural and molecular features of adipose tissue of galectin-3 knockout mice, either fed with NFD or HFD. With NFD, galectin-3 ablation is associated with increased adiposity. However, fat cell size is not increased and PPARγ expression is even reduced. With HFD, there are contrasting results on the effect of galectin-3 ablation on fat mass and body weight. However, galectin-3 ablation is always associated with defective size and/or impaired ability of adipocytes to respond with an enlargement to the fat overload. These structural features, together with defects in the expression of adipogenic and lipogenic markers (PPARγ and ATGL), suggest a regulatory role for galectin-3 in adipogenesis. Gal-3 = galectin-3; crossed Gal-3 = galectin-3 ablation; NFD = normal-fat diet; HFD = high-fat diet; PPARγ = peroxisome-proliferator-activated receptor γ; ATGL = adipose triacylglycerol lipase.

ratio of small to large cells [52]. A key point of this study is that the expression of genes related to fat cell differentiation and levels of adiponectin are significantly lower in the insulin-resistant group compared to the insulin-sensitive group, a finding suggesting that the increased number of small fat cells is the consequence of their inability of differentiating into a mature and functional phenotype [52]. Therefore, an alternative hypothesis is that the "metabolic buffer" function of the adipose tissue might be also disrupted by defective adipogenesis which, by reducing the plasticity of adipose tissue in terms of ability to store and metabolize lipids, decreases the threshold of critical volume at which adipocytes cannot properly cope with the metabolic demand.

The studies in galectin-3 knockout mice reviewed above showed that these animals (a) are more susceptible to type 2 diabetes when challenged with a HFD without developing adipocyte hypertrophy and (b) show spontaneous derangement of glucose homeostasis associated with smaller and less mature adipocytes. These data suggest that animals lacking galectin-3 respond to increased fat intake with adipocyte hyperplasia, as the hypertrophic response is impaired [42]. This interpretation is once again consistent with the opposite finding observed in RAGE null mice fed a HFD, which displayed smaller adipocytes than wild type littermates, but reduction in size was much less than reduction in body weight and fat mass, thus suggesting that the hyperplasic response was fundamentally impaired in these mice [44]. These observations make the galectin-3 knockout mice a suitable animal model to test the alternative hypothesis which attributes to small, immature adipocytes a major role in the development of obesity and associated derangement of glucose homeostasis.

5. Conclusions

Recently, galectin-3 has been implicated in the development of type 2 diabetes and obesity. Studies in humans have shown that galectin-3 concentration is higher in obese and diabetic individuals and that it increases in conjunction with unbridled glucose homeostasis. On the other hand, galectin-3 levels correlated positively with insulin sensitivity and negatively with HbA_{1c} levels in patients with type 2 diabetes. Taken together, these data suggest that galectin-3 is a surrogate marker, rather than a mediator of metabolic disorders, in which it might play a protective role, as part of an adaptive response. Moreover, galectin-3 appears to be a modulator of adipogenesis by stimulating differentiation of preadipocytes into mature adipocytes. The absence of fat cell enlargement in galectin-3 knockout mice suggests that this lectin might play a role in the storage capacity of the adipose tissue. Future studies should test the hypothesis that lack of galectin-3 accelerates disruption of glucose metabolism by affecting adipogenesis and lipid storage capacity.

In summary, modulation of galectin-3, an emerging all-out player in metabolic disorders, deserves further scientific attention as a novel marker and therapeutic avenue for the control of metabolic disorders such as diabetes and obesity.

Abbreviations

AGEs: Advanced glycation end products
ALEs: Advanced lipoxidation end products
RAGE: Receptor for AGEs
CRD: Carbohydrate recognition domain
CLIC: Clathrin-independent carriers

IL: Interleukin
TLR: Toll-like receptor
BMI: Body mass index
HbA_{1c}: Hemoglobin A_{1c}
CRP: C-reactive protein
HOMA-IR: Homeostasis model assessment of insulin resistance
MTT: Meal tolerance test
HFD: High-fat diet
PPARγ: Peroxisome-proliferator-activated receptor γ
C/EBP: CCAAT-enhancer-binding protein.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Targeting Pin1 Protects Mouse Cardiomyocytes from High-Dose Alcohol-Induced Apoptosis

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Long-term heavy alcohol consumption is considered to be one of the main causes of left ventricular dysfunction in alcoholic cardiomyopathy (ACM). As previously suggested, high-dose alcohol induces oxidation stress and apoptosis of cardiomyocytes. However, the underlying mechanisms are yet to be elucidated. In this study, we found that high-dose alcohol treatment stimulated expression and activity of Pin1 in mouse primary cardiomyocytes. While siRNA-mediated knockdown of Pin1 suppressed alcohol-induced mouse cardiomyocyte apoptosis, overexpression of Pin1 further upregulated the numbers of apoptotic mouse cardiomyocytes. We further demonstrated that Pin1 promotes mitochondria oxidative stress and loss of mitochondrial membrane potential but suppresses endothelial nitric oxide synthase (eNOS) expression in the presence of alcohol. Taken together, our results revealed a pivotal role of Pin1 in regulation of alcohol-induced mouse cardiomyocytes apoptosis by promoting reactive oxygen species (ROS) accumulation and repressing eNOS expression, which could be potential therapeutic targets for ACM.

1. Introduction

Heart failure continues to be a major public health issue [1]. In the United States, long-term heavy alcohol consumption is the leading cause of nonischemic dilated cardiomyopathy in both genders, known as "alcoholic cardiomyopathy" (ACM) [2, 3]. Generally, patients consuming more than 90 g of alcohol per day for more than 5 years are likely to have asymptomatic ACM, which may develop into symptomatic ACM and signs of heart failure [2, 4].

In the asymptomatic stage, ACM is usually characterized by left ventricular dilation, increased left ventricular mass, and reduced or normal left ventricular wall thickness [3, 5]. Pathologically, previous studies have shown a strong correlation between ACM and cardiomyocyte apoptosis [6]. Apoptotic cardiomyocytes were detected in the heart muscles of individuals with long-term alcoholism, and expression of BAX and BCL-2 was also observed [7, 8]. Studies in animal models also demonstrated that chronic alcohol intake could induce oxidative stress and cellular apoptosis in cardiomyocytes [9, 10]. In a primary cell culture model, alcohol was found to induce reactive oxygen species-mediated apoptosis in a dose-dependent manner in the range of 0–100 mM [8, 11]. However, the molecular mechanism by which alcohol induces apoptosis of cardiomyocytes remains to be investigated.

Peptidyl-prolyl cis-trans isomerase Pin1, a member of the parvulin family of PPIase enzymes, is capable of isomerizing the peptidyl-prolyl bond in specific phosphorylated Ser/Thr-Pro motifs of the substrates, which may lead to profound changes in their activity, stability, phosphorylation status, and protein-protein interactions [12, 13]. Pin1 was originally found to be required for cell division in yeast and human cells. Later studies demonstrated that Pin1 is important for regulation of many other cellular processes, such as gene transcription, cell proliferation, differentiation, and apoptosis

[14]. In addition, since phosphorylation of proteins is an essential signaling mechanism, Pin1 is involved in the Ras signaling pathway and activation of Wnt signaling [15, 16].

With regard to regulation of apoptosis, Pin1 was found to inhibit apoptosis in hepatocellular carcinoma cells and SW620 cells in colorectal carcinoma [17, 18]. In this study, we further investigated the role of Pin1 in regulation of high-dose alcohol-induced cardiomyocyte apoptosis and found that alcohol induced Pin1 expression and activation in a dose-dependent manner in primary mouse cardiomyocytes. We further demonstrated that targeting of Pin1 protects cardiomyocytes from high-dose alcohol-induced apoptosis by regulating mitochondria oxidative stress and endothelial nitric oxide synthase (NOS) expression.

2. Materials and Methods

2.1. Cell Culture, Cell Transfection, and Reagents. Primary cardiomyocytes were isolated from neonatal mouse hearts, as described previously [19]. Briefly, heart tissue was minced and digested, using a collagenase/dispase mixture (Roche, Indianapolis, IN). Tissue fragments were allowed to sediment, and the supernatant-containing suspended cells were preplated for 2 h to remove fibroblasts and endothelial cells. Enriched cardiomyocytes were then cultured in collagen-coated dishes at approximately 1.5×10^5 cells per cm^2. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Harbin Medical University and approved by the Chancellor's Animal Research Committee.

Scrambled and Pin1 siRNAs were purchased from Invitrogen (Carlsbad, CA) and transfected with Lipofectamine RNAiMAX (Invitrogen). Pin1 plasmids were obtained from Addgene (Cambridge, MA). Lipofectamine LTX (Invitrogen) was used for plasmid transfection according to the manufacturer's instructions. Cardiomyocytes (5×10^4 cells/well) were seeded onto 24-well plates and grown overnight to approximately 80% confluence. The cells were transfected with 30 pmol siRNA or 500 ng plasmid and incubated for 48 h, and subsequent experiments were performed after transfection to analyze efficiency, using western blotting.

N-acetylcysteine (NAC) and Mito-TEMPO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from cardiomyocytes, using the TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions, and complementary DNA was then synthesized from 1 μg of total RNA from each sample, using the SuperScript III First-Strand Synthesis System (Life Technologies). mRNA expression levels of Pin1 were measured by qRT-PCR and calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers of oligonucleotides were as follows:

5′-CCGGAATTCATGGCGGACGAGGAGAAG-3′ (forward) and

5′-TGCTCTAGATCATTCTGTGCGCAGGAT-3′ (reverse) for Pin1;

5′-TGGACTCCACGACGTACTCAG-3′ (forward) and 5′-GGGAAGCTTGTCATCAATGGAA-3′ (reverse) for GAPDH.

2.3. Western Blotting. Cardiomyocytes were harvested and lysed on ice for 30 min in RIPA buffer (120 mM NaCl, 40 mM Tris [pH 8.0], and 0.1% NP 40) with proteinase/phosphatase inhibitor (Pierce, Rockford, IL). The lysates were centrifuged at 18,000 g for 15 min at 4°C. The supernatants were collected, and protein concentrations were determined by the BCA method (Pierce). Aliquots of the lysates were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The resolved protein was then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were subsequently incubated with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Pierce), followed by exposure of the membranes to X-ray film. Primary antibodies for Pin1, cytochrome c (Cyt.C), endothelial nitric oxide synthase (eNOS), mHsp70, $p66^{shc}$, and β-actin were purchased from Santa Cruz (Santa Cruz, CA).

2.4. Pin1 Activity Assays. Pin1 activity was analyzed as previously described [20, 21]. Briefly, cardiomyocytes were lysed by sonication in lysis buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 100 mM NaCl, 0.25% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM NaF, 1 mM β-glycerophosphate, and 1 mM ethylene glycol tetraacetic acid) at 4°C. We then prepared a mixture containing 93 μL of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.8), 100 mM NaCl, 2 mM DTT, 0.04 mg/mL of bovine serum albumin), 5 μL of cell lysate (10^5 cells or 0.25 nmol of recombinant Pin1), and 2 μL (20 mg/mL) of trypsin solution. The reaction was started by adding 50 μL (720 μM) of peptide Trp-Phe-Tyr-Ser (PO_3H_2)-ProArg-pNA (NeoMPS), followed by *p*-nitroaniline absorbance at 390 nm for 4 min.

2.5. Cell Viability Assays. Cardiomyocyte viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at 3×10^3 cells per well in 100 μL culture medium in 96-well culture plates. Sterile MTT dye (20 μL) (Sigma) was added to each well. After incubating for 4 h at 37°C, MTT medium mixture was removed, and 200 μL of dimethyl sulfoxide (Sigma) was added to each well. The absorbance representing viable cells was measured by a microplate reader at a wavelength of 490 nm. Cell viability was calculated as a relative ratio of the control.

2.6. Caspase Activity Assays. Cells (1×10^4) were incubated in 96-well plates for 24 h, and caspase-3 activity was measured

using the caspase-3 and caspase-9 assay kits (Ambion, Austin, TX) according to the manufacturer's protocol. Briefly, the cells were incubated with lysis buffer on ice for 10 min and collected via 10,000 g centrifugation for 10 min. Protein (100 μg) from each sample was incubated with specific colorimetric tetrapeptides Asp-Glu-Val-Asp-p-nitroaniline (pNA; specific substrate of caspase-3) or Leu-Glu-His-Asp-pNA (specific substrate of caspase-9) at 37°C for 60 min. The activity of caspase-3 and caspase-9 was quantified using a spectrophotometer at 405 nm, and the data were normalized to the control group.

2.7. TUNEL Staining. TUNEL staining was performed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, cells were grown on chambered culture slides, fixed with 4% PFA for 2 h at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Cells were washed again with PBS and equilibrated with 100 μL equilibration buffer at room temperature for 10 min. Slides were covered with 50 μL of terminal deoxynucleotidyl transferase reaction mixture for 60 min in a humidified chamber. The reaction was stopped with 2x SSC for 15 min. Nuclei were visualized by DAPI staining.

2.8. Mitochondrial Membrane Potential Measurements. Mitochondrial membrane potential ($\Delta\psi$m) was assessed using a TMRE Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, England). Cells were incubated with 100 nM TMRE in the absence or presence of CCCP at 20 μM at 37°C for 15 min and washed with 0.2% BSA in PBS. The cell pellet was collected by centrifugation at 1500 g for 3 min and resuspended in 1 mL of PBS. Fluorescence was measured by a fluorescence plate reader (BioTek, Burlington, VT).

2.9. Measurement of Cyt.C Release from Mitochondria to Cytosol. Release of Cyt.C from mitochondria to cytosol was measured by western blotting, as previously described [22]. Cells (5×10^6) were collected by trypsin-EDTA (0.5%), followed by two washes with cold PBS and lysed in ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris (pH 6.8), 1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 12,000 g at 4°C for 3 min to obtain the supernatants for western blot analysis. Protein concentration in the supernatants was measured using BCA protein assay kit (Thermo Fisher Scientific, Canoga Park, CA).

2.10. Measurement of NO Production and Mitochondrial ROS. Nitric oxide production was assessed by measuring the levels of oxidized forms (nitrites and nitrates) in samples, using a nitric oxide assay kit (Abcam, Cambridge, UK). Mitochondrial ROS levels were analyzed using the Elite Mitochondrial ROS Activity Assay Kit (eEnzyme, Gaithersburg, MD). Cells were harvested and incubated with 100 μL Elite ROS Deep Red stain solution on 96-well plates for 60 min at 37°C. Fluorescence intensity was measured at EX/EM = 650/675 nm.

2.11. Mitochondrial Protein Isolation. Cells were centrifuged at 370 g for 10 min and washed in 10 packed cell volumes of washing buffer (1 mM Tris HCl, pH 7.4, 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$) three times. Cells were then resuspended in 6 packed cell volumes of homogenization buffer (10 mM Tris HCl, pH 7.4, 10 mM KCl, 0.15 mM $MgCl_2$, 1 mM PMSF, and 1 mM DTT) and homogenized for 10 min on ice. Homogenate was transferred into a conical centrifuge tube containing 1 packed cell volume of 2 M sucrose solution. Unbroken cells, nuclei, and large debris were removed by centrifuging at 1,200 g for 5 min twice. The mitochondria were collected by centrifuging at 7,000 g for 10 min. The mitochondrial pellet was resuspended in 3 packed cell volumes of mitochondrial suspension buffer (10 mM Tris HCl, pH 6.7, 0.15 mM $MgCl_2$, 0.25 mM sucrose, 1 mM PMSF, and 1 mM DTT) for further western blot analysis.

2.12. Statistical Analyses. All statistical analyses were performed using SPSS 18.0 software (IBM, Chicago, IL). The significance of differences between groups was estimated by Student's t-test, χ^2 test, or one-way analysis of variance (ANOVA). The data were expressed as the mean ± SEM of three independent experiments. A p value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Alcohol Induced Pin1 Expression and Activation in Cardiomyocytes. We had previously demonstrated that high doses of alcohol induce cardiomyocyte apoptosis [11]. To investigate whether Pin1 plays a role in alcohol-induced apoptosis in cardiomyocytes, we first analyzed Pin1 expression after exposing cells to different concentrations of alcohol (ethanol: 0, 50, 100, or 200 mM). As shown in Figures 1(a) and 1(b), alcohol caused dose-dependent upregulation of Pin1 expression at both the mRNA and protein levels. Moreover, alcohol increased Pin1 activity in a dose-dependent manner (Figure 1(c)).

3.2. Pin1 Regulated Alcohol-Induced Apoptosis in Cardiomyocytes. We investigated whether Pin1 is involved in alcohol-induced cardiomyocyte apoptosis by both loss- and gain-of-function studies. We first transfected mouse cardiomyocytes with control or Pin1 siRNA for 24 h and exposed those cells to alcohol (200 mM) for another 24 h. Knockdown efficiency was confirmed by western blotting (Figure 2(a)). As expected, cell viability decreased by more than 50% in the alcohol-treated group as compared to cells in the control group. However, the viability of Pin1-knockdown cells only decreased by 20% with alcohol treatment (Figure 2(b)). Caspase-9 and caspase-3 activity assays and TUNEL staining consistently showed that alcohol-induced cell apoptosis was inhibited by depletion of Pin1 (Figures 2(c) and 2(d)). Interestingly, ectopic overexpression of Pin1 further enhanced alcohol-induced apoptosis and loss of cell viability (Figures 3(a)–3(d)). Together these results indicate that Pin1 plays an

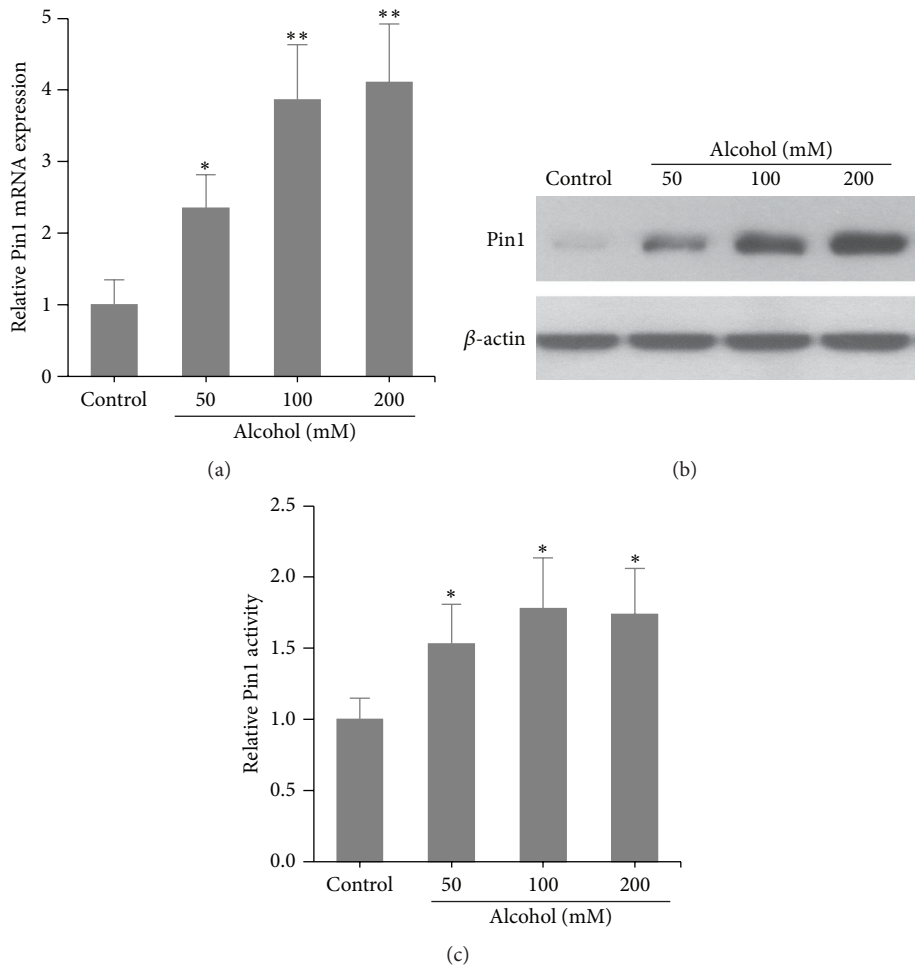

Figure 1: Pin1 expression and activity were upregulated by alcohol treatment of cardiomyocytes. (a) qRT-PCR of Pin1 mRNA expression in cardiomyocytes treated with alcohol (0, 50, 100, or 200 mM) for 24 h. (b) Western blot of Pin1 expression in cardiomyocytes treated with alcohol at indicated concentrations. (c) Pin1 activity assays in cardiomyocytes treated with alcohol at indicated concentrations. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to cells without alcohol treatment.

important role in promoting alcohol-induced apoptosis in cardiomyocytes.

3.3. Knockdown of Pin1 Reduced Alcohol-Mediated Mitochondria Oxidative Stress in Cardiomyocytes. Mitochondrial oxidative signaling contributes to alcohol-induced apoptosis [11], and downregulation of Pin1 can prevent mitochondrial oxidative stress in patients with diabetes [23]. Thus, we investigated whether depletion of Pin1 can prevent alcohol-induced mitochondrial oxidative stress in cardiomyocytes. mCyt.C, mitochondrial membrane potential, and mitochondrial reactive oxygen species (mROS) were examined in control and Pin1-knockdown cardiomyocytes in the presence or absence of 200 mM alcohol. Downregulation of Pin1 significantly reversed mCyt.C release, reduced mitochondrial membrane potential, and stimulated mROS production induced by alcohol treatment (Figures 4(a)–4(c)). We next sought to recapitulate the result by using Pin1 inhibitor Juglone. Unlike Pin1 knockdown cells, cells treated with Pin1 inhibitor Juglone (2.5 μM) demonstrated lower cell viability and higher apoptosis in both condition without or with alcohol treatment (Supplemental Figure 1; see Supplementary Materials available online at http://dx.doi.org/10.1155/2016/4528906). Since Pin1 has been reported to be a strong cytotoxic agent and induce apoptosis in many cell types [24, 25], the apoptosis-inducing activity of Juglone in cardiomyocytes might be through other signaling pathways than inhibiting Pin1. We next overexpressed Pin1 in cardiomyocytes. As expected, alcohol induced mCyt.C release, mitochondrial membrane potential reduction, and ROS production, which were further enhanced by Pin1 overexpression (Figures 4(d)–4(f)). In addition, we tested whether scavenging of ROC could reverse cell viability and apoptosis in alcohol-treated cardiomyocytes. As showed in Figures 4(g) and 4(h), two ROS scavengers (NAC and Mito-TEMPO) all partly rescued alcohol-induced cell death and apoptosis in Pin1-overexpressed cardiomyocytes. Our previous work has found that Pin1 interacts with p-p66Shc which translocates to mitochondria and functions as a redox enzyme to regulate cardiomyocyte apoptosis [11]. To further

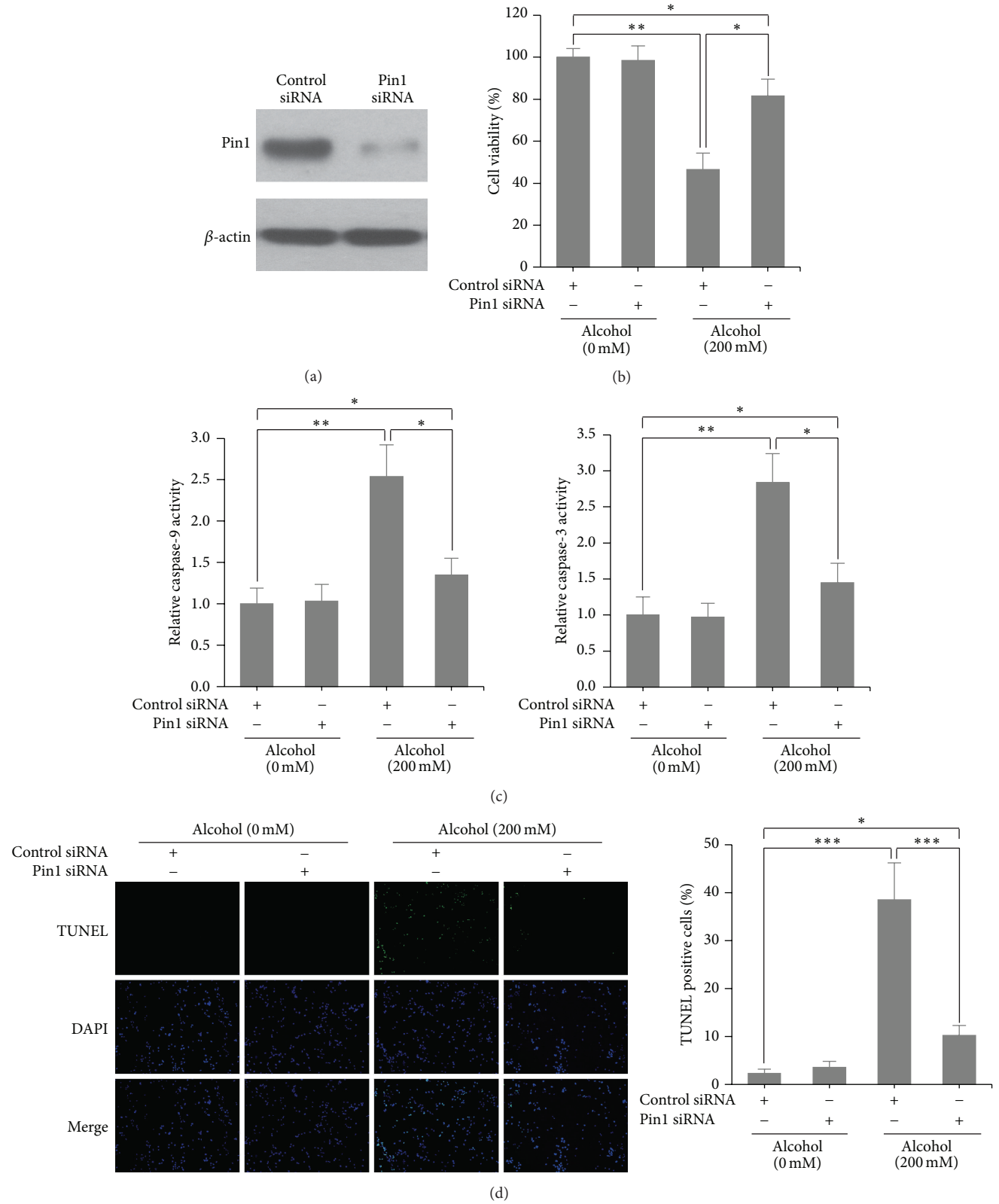

Figure 2: Knockdown of Pin1 inhibited alcohol-induced cardiomyocyte apoptosis. (a) Pin1 protein expression measured by western blotting after Pin1 siRNA transfection. ((b)–(d)) Cell viability assay (b), caspase-9 and caspase-3 activity assays (c), and TUNEL staining (d) in Pin1-knockdown cardiomyocytes treated or untreated with alcohol (200 mM) for 24 h. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

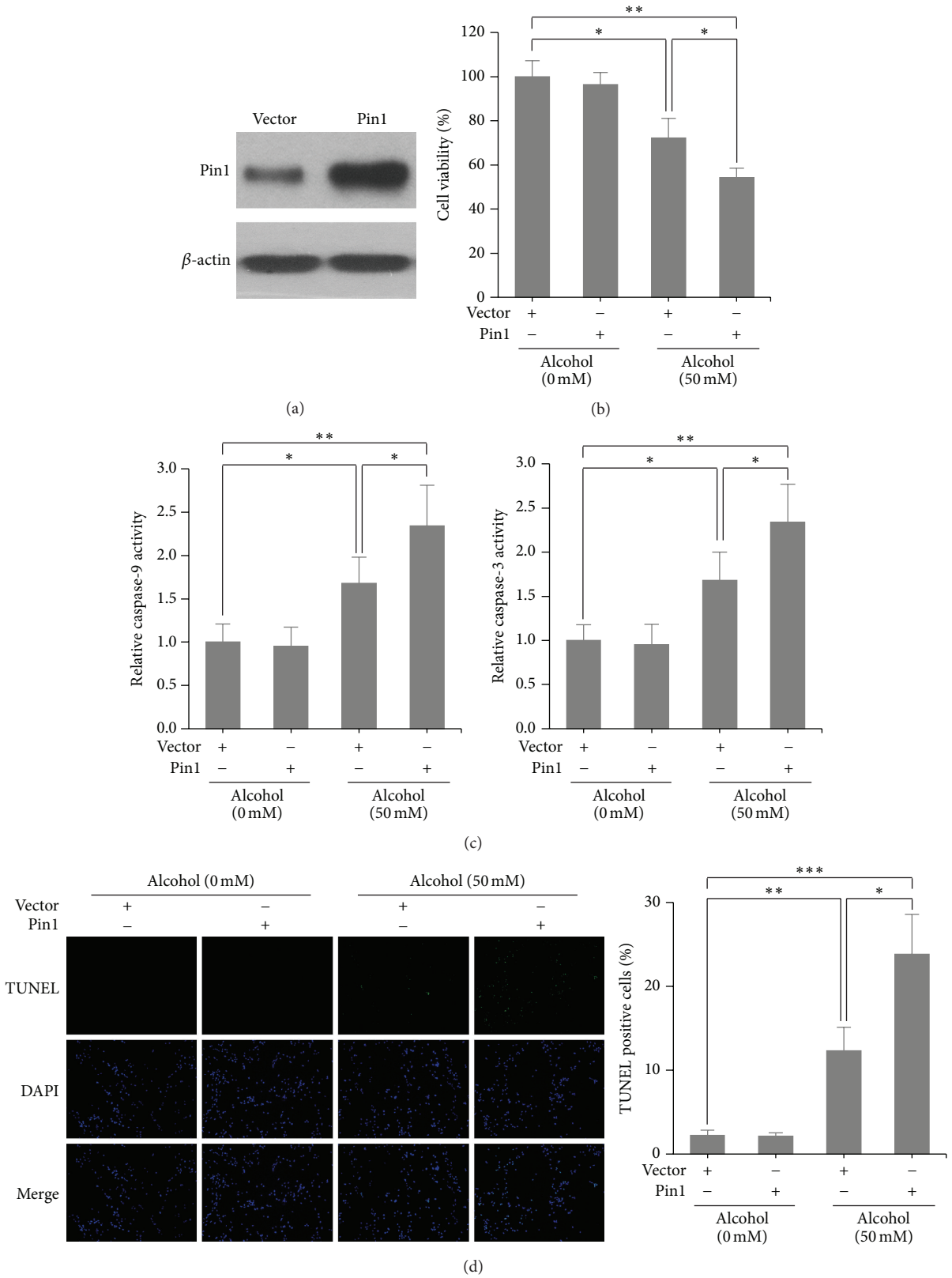

Figure 3: Overexpression of Pin1 enhanced alcohol-induced cardiomyocyte apoptosis. (a) Pin1 protein expression measured by western blotting after Pin1 plasmid transfection. Cell ability (b), caspase-9 and caspase-3 activity assay (c), and TUNEL staining (d) in Pin1-overexpressed cardiomyocytes treated or untreated with alcohol (50 mM) for 24 h. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

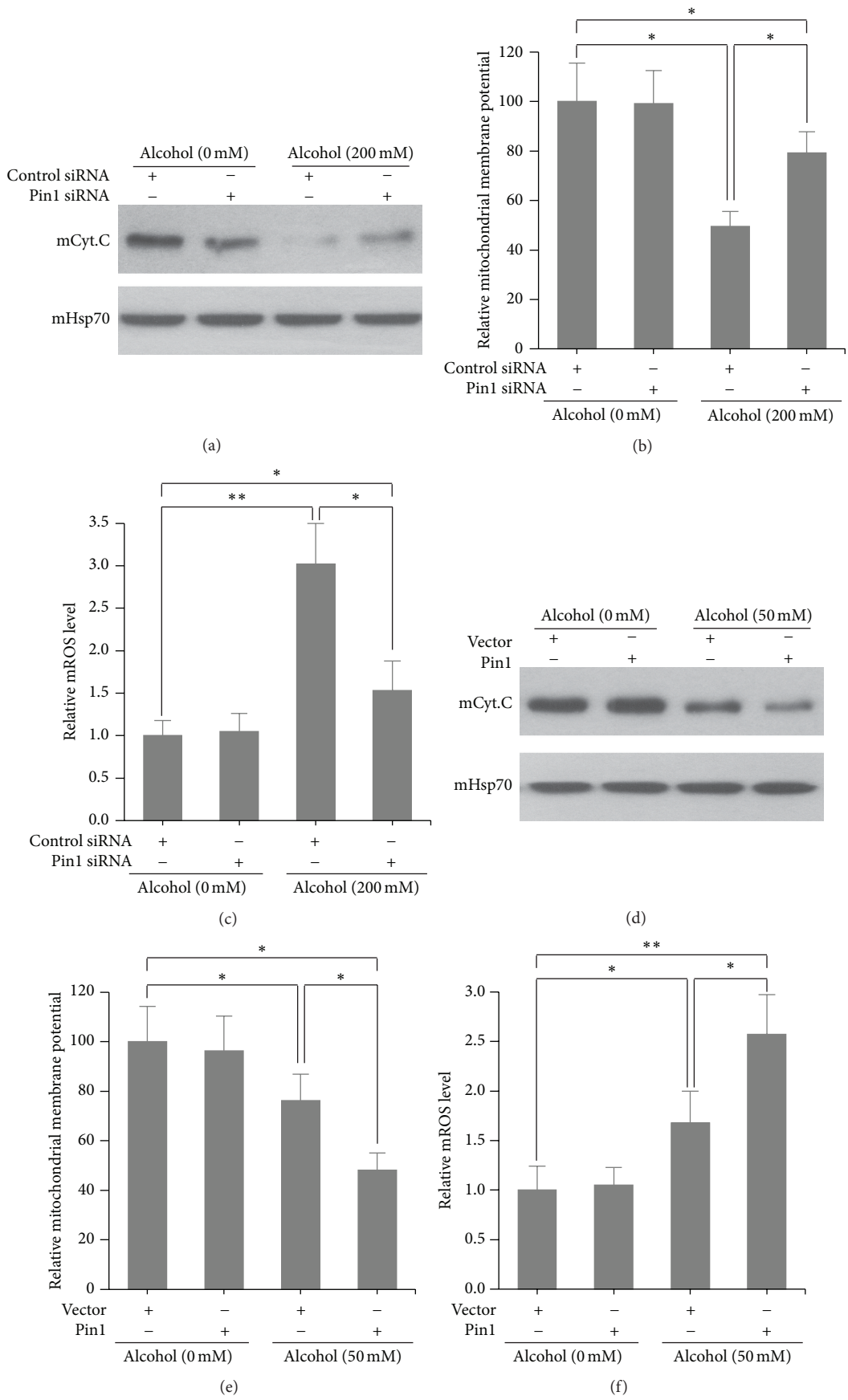

Figure 4: Continued.

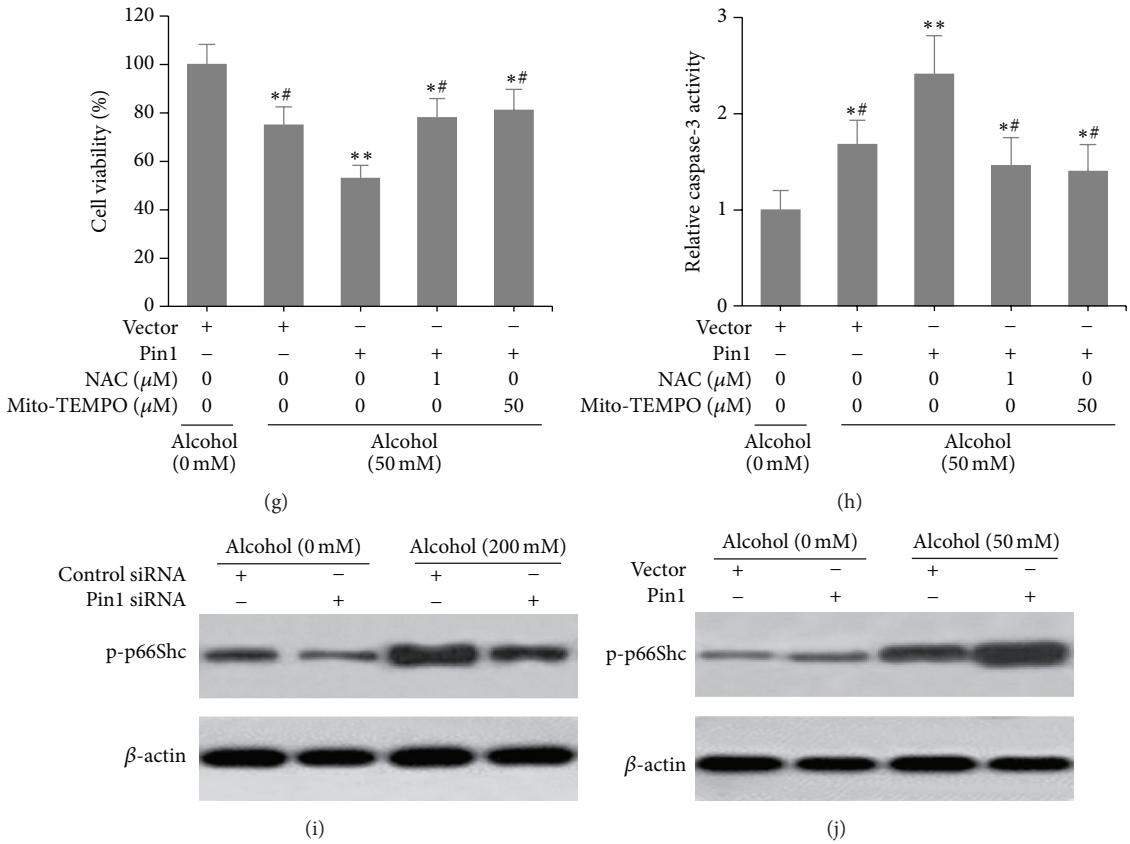

Figure 4: Pin1 enhanced alcohol-mediated mitochondria oxidative stress in cardiomyocytes. (a) Western blot of mCyt.C release in Pin1-knockdown cardiomyocytes treated or untreated with alcohol (200 mM) for 24 h. Relative mitochondrial membrane potential (b) and mROS levels (c) in cells are indicated. (d) mCyt.C levels in Pin1-overexpressed cardiomyocytes treated or untreated with alcohol (50 mM) for 24 h. Relative mitochondrial membrane potential (e) and mROS levels (f) in cells are indicated. $^{*}p < 0.05$ and $^{**}p < 0.01$. Cell viability (g) and caspase-3 activity (h) assays in Pin1-overexpressed cells treated with alcohol (50 mM) and NAC (1 μM) or Mito-TEMPO (50 μM). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with cells untreated with alcohol; $^{\#}p < 0.05$ compared with Pin1 overexpression cells treated with alcohol only. (i) Western blot of mitochondrial p-p66Shc levels in control or Pin1-knockdown cardiomyocytes treated or untreated with alcohol (200 mM) for 24 h. (j) Western blot of mitochondrial p-p66Shc levels in Pin1-overexpressed cardiomyocytes treated or untreated with alcohol (50 mM) for 24 h.

investigate the downstream signaling of Pin1 in alcohol-induced cardiomyocyte apoptosis, we analyzed the mitochondrial p-p66Shc levels in cardiomyocytes treated with or untreated with alcohol in Pin1 knockdown or overexpression cells. As we expected, knockdown of Pin1 reduced and overexpression of Pin increased mitochondrial p-p66Shc level (Figures 4(i) and 4(j)). Taken together, this demonstrates that Pin1 promotes alcohol-mediated mitochondria oxidative stress in cardiomyocytes.

3.4. Pin1 Regulated Alcohol-Mediated NO Production and eNOS Expression in Cardiomyocytes. Since nitric oxide (NO) plays a role in apoptosis of cardiomyocytes [24], we investigated whether alcohol mediates endothelial eNOS expression. As shown in Figures 5(a)–5(c), alcohol inhibited NO production and eNOS levels in a dose-dependent manner. We also tested alcohol-induced NO production and eNOS expression in Pin1-knockdown cells. Compared to control cells, alcohol significantly decreased NO production, while knockdown of Pin1 partly reversed its decrease of production (Figure 5(c)). eNOS expression was consistently rescued by Pin1 depletion in the presence of alcohol (Figure 5(d)). To further confirm Pin1 function in alcohol-mediated NO production and eNOS expression, we overexpressed Pin1 in cardiomyocytes and assessed NO and eNOS levels after alcohol (50 mM) treatment. As shown in Figures 5(e) and 5(f), overexpression of Pin1 further inhibited NO production and eNOS expression, which demonstrates that Pin1 regulates alcohol-mediated NO production by affecting eNOS expression in cardiomyocytes.

4. Discussion

Apoptosis is a mechanism of programmed cell death implicated in the pathogenesis of alcohol-induced left ventricular dysfunction [1]. Several mechanisms have been proposed to explain the role of alcohol during development of ACM, such as upregulated ROS levels and decreased NO production [11, 25, 26]. In this study, we found that alcohol elevated Pin1 expression and activity in mouse primary cardiomyocytes.

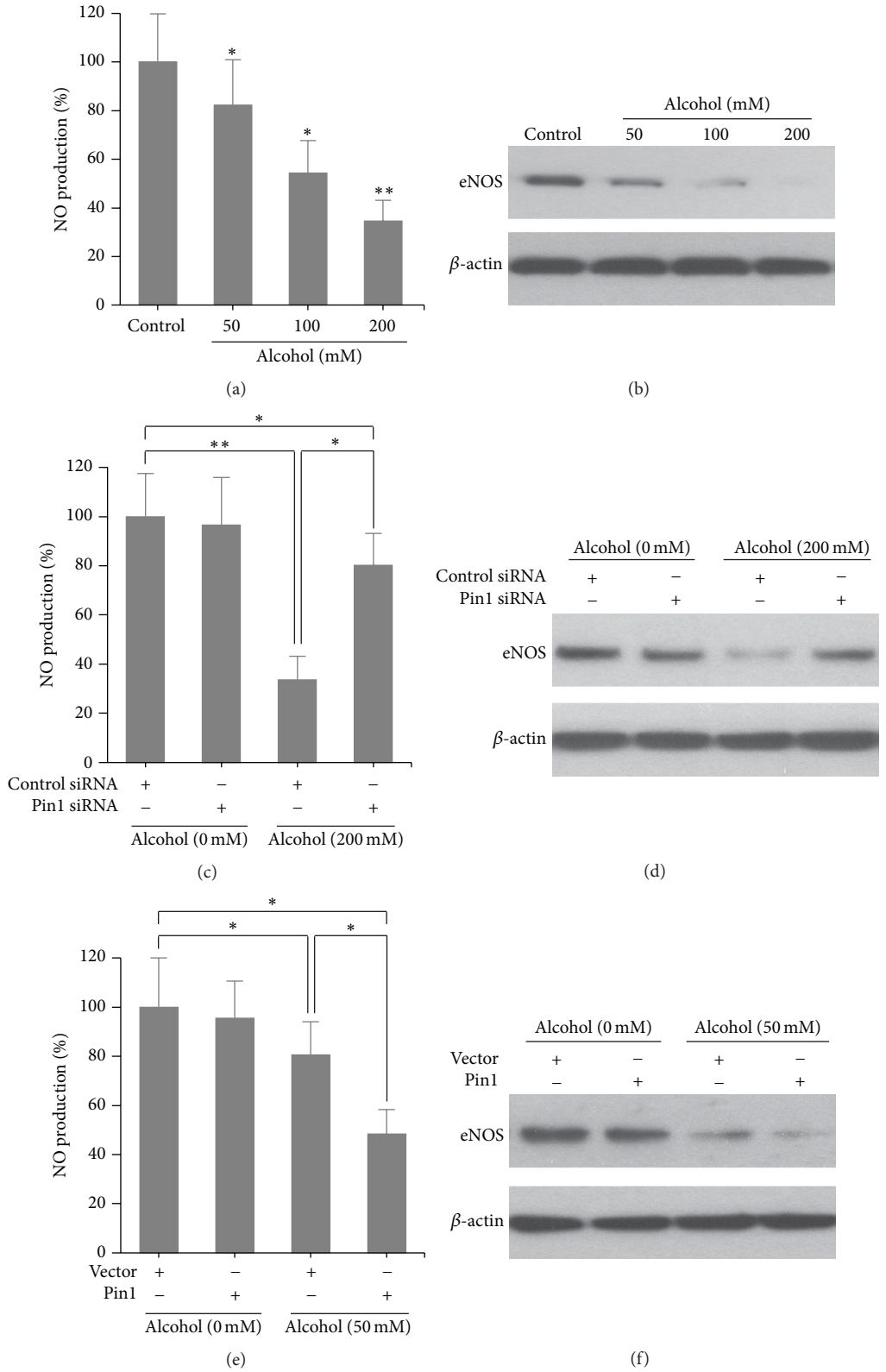

Figure 5: Pin1 reduced the NO production and eNOS expression that were inhibited by alcohol in cardiomyocytes. (a) NO production in cardiomyocytes treated with alcohol (0, 50, 100, or 200 mM) for 24 h. (b) eNOS expression analyzed by western blotting in cells treated with alcohol at indicated concentrations. NO production (c) and eNOS expression (d) in Pin1-knockdown cardiomyocytes treated or untreated with alcohol (200 mM) for 24 h. NO production (c) and eNOS expression (d) in Pin1-overexpressed cardiomyocytes treated or untreated with alcohol (50 mM) for 24 h. $^{*}p < 0.05$ and $^{**}p < 0.01$.

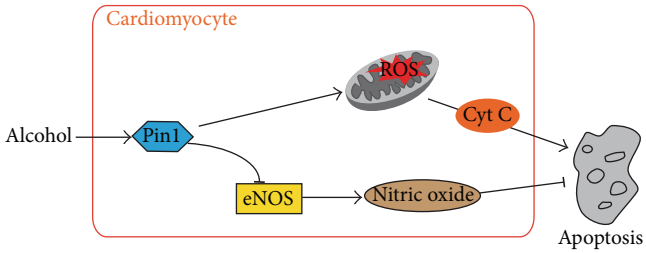

Figure 6: Schematic representation of Pin1 in the process of alcohol-induced cardiomyocyte apoptosis.

Pin1 in turn played a pivotal role in alcohol-induced cardiomyocyte apoptosis by promoting ROS accumulation and repressing eNOS expression (Figure 6).

ROS are free radicals containing oxygen molecules, mostly generated in mitochondria, which contain peroxyl, alkoxyl, superoxide anions, hydroxyl radicals, and oxygen derived nonradical species [27]. Depending on the concentration of ROS, it can be beneficial or harmful to cells and tissues [28]. Normal ROS metabolism plays an essential role in disease resistance and cell-mediated immunity. However, high levels of ROS may cause uncontrolled oxidation of lipids, proteins, and DNA, which finally leads to apoptosis [27]. Previous studies have suggested that high levels of alcohol can induce ROS-mediated apoptosis in cardiomyocytes [8, 11]. Since we found that alcohol treatment stimulated expression and activity of Pin1 in mouse primary cardiomyocytes, we then investigated the role of Pin1 in regulation of alcohol-induced ROS accumulation in mitochondria. Our results showed that depletion of Pin1 significantly reduced the mCyt.C release, loss of mitochondrial membrane potential, and mROS production stimulated by alcohol treatment of cardiomyocytes, which might be mediated by $p66^{shc}$. Conversely, overexpression of Pin1 even enhanced cardiomyocyte apoptosis. Interestingly, the increase of Pin1 activity was not as high as the increase of Pin1 expression level when we treated cardiomyocytes with high-dose alcohol, which indicates that Pin1 might function by interacting with other cofactors.

We also found that Pin1 promoted alcohol-induced cardiomyocyte apoptosis by inhibiting NO production. NO is a free radical, the product of the reaction catalyzed by eNOS [29]. NO levels were originally found to be closely related to inflammatory status, and NO promotes tumor cell proliferation, mobility, and invasiveness [29–31]. With regard to apoptosis, Shen et al. reported that upregulation of eNOS protects cardiomyocytes from apoptosis [25]. By investigating regulation of NO production by Pin1, we demonstrated that knockdown of Pin1 rescued the decrease in eNOS expression and NO production, and ectopic overexpression of Pin1 resulted in less eNOS expression and NO production when the cells were treated with 50 mM of alcohol. However, without alcohol treatment, we did not observe significant changes in NO production by overexpression of Pin1. We suspect that the inhibitory effect of Pin1 on NO production is induced by alcohol. Therefore, the mechanisms by which alcohol regulates Pin1 expression and activity remain to be elucidated. Additionally, since NO and ROS scavenge each other, the effect of Pin1 on NO production may also be affected by alcohol-induced ROS accumulation [32, 33]. Pin1 has been reported to recognize Ser-116 eNOS inhibitory phosphorylation to impair NO release. Therefore Pin1 might regulate eNOS activity and NO production by directly interacting with eNOS [34].

In conclusion, our findings demonstrate that alcohol induces Pin1 expression and activation in a dose-dependent manner in mouse primary cardiomyocytes. We also found that depletion of Pin1 significantly inhibited cardiomyocyte apoptosis by regulating mROS accumulation and NO production. Thus, we found that Pin1 plays a pivotal role in alcohol-induced cardiomyocyte apoptosis, and Pin1 is a potential therapeutic target for left ventricular dysfunction caused by excessive alcohol consumption.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Weimin Li designed the experiments. Yuehong Wang, Zizhuo Li, and Yu Zhang performed the experiments; Yuehong Wang, Wei Yang, Jiantao Sun, and Lina Shan analyzed the data; and Weimin Li and Yuehong Wang prepared the paper.

Acknowledgments

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Research Article

PON-1 Activity and Plasma 8-Isoprostane Concentration in Patients with Angiographically Proven Coronary Artery Disease

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The aim of the study was to estimate association of the extent of angiographically proven coronary artery disease (CAD) with plasma 8-isoprostane F2 (8-iso-PGF2α) levels as a reliable marker of lipid peroxidation and serum activity of paraoxonase-1, which demonstrates the ability to protect against lipid oxidation. The study included 105 patients with angiographically documented CAD (CAD+) and 45 patients with negative results of coronary angiography (CAD−). Compared to the control group CAD+ patients were characterized by increased 8-iso-PGF2α levels ($P = 0.007$) and reduced activity of PON-1 towards paraoxon (PONase, $P = 0.002$) and phenyl acetate (AREase, $P = 0.037$). Univariate correlation analysis indicated that 8-iso-PGF2α concentrations were positively associated with the severity of CAD as evaluated by the Gensini score ($R = 0.41$, $P < 0.001$) while PONase activity ($R = -0.26$, $P < 0.05$) and AREase activity ($R = -0.23$, $P < 0.05$) were inversely correlated with CAD severity. PONase activity and 8-iso-PGF2α concentration remained independent determinant of atherosclerosis severity in multiple linear regression after adjusting for age, gender, smoking habits, hypertension, type 2 diabetes, statin therapy, and HDL-C and TAG concentration (β coefficients −0.267; $P < 0.05$ and 0.368; $P < 0.001$, resp.). The results suggest that PON-1 activity and 8-iso-PGF2α concentration are associated with the presence and extent of coronary stenosis and may be considered additional markers of coronary artery disease.

1. Introduction

The mechanisms of the onset and development of atherosclerosis are still not entirely resolved although oxidation of lipoproteins seems to be essential to this process [1, 2].

Various biomarkers of lipid peroxidation are recently of great interest not only for highlighting pathological mechanisms, but also for clinical applications as biomarkers. Among them isoprostanes, products of nonenzymatic lipid peroxidation, seem to be particularly valuable. Isoprostanes, specifically 8-iso-prostaglandin F2 (8-iso-PGF2α), are recently indicated as the most valid *in vivo* lipids peroxidation biomarkers [3, 4] which themselves exert proatherogenic function by means of their vasoconstrictive, platelet-activating, and mitogenic properties [5, 6].

The second biomarker with sustained interest of researchers is the high-density lipoprotein associated enzyme: paraoxonase-1 (PON-1). This enzyme hydrolyzes aromatic carboxylic acid esters, organophosphates, and oxidized phospholipids, simultaneously destroying biologically active lipids in mildly oxidized lipoproteins, thus protecting them against further oxidation [7, 8]. Several lines of evidence suggest that PON-1 has antioxidant and atheroprotective effects. Genetic deletion of PON-1 in animal models of atherosclerosis is associated with increased oxidation of low-density lipoproteins (LDLs), increased macrophage

oxidative stress, and increased atherosclerotic lesion size [9–11]. Conversely, overexpression of human PON-1 in transgenic mice results in reduction of aortic lesion size and corresponding decreases in oxidized lipid-protein adduct levels [12, 13]. It has been shown that PON-1 activity is associated with accelerated atherosclerosis [14] but is also affected both by genetic polymorphism and by environmental factors including age, lifestyle, and pharmaceutical intervention [15, 16].

Classically, PON-1 activity in serum is named after the substrate used to monitor enzymatic function, namely, paraoxonase activity (using paraoxon as substrate) and arylesterase activity (using phenyl acetate as substrate). A previous report has shown that the phenotype distinguished on the basis of the paraoxonase-to-arylesterase ratio closely corresponds to a common PON-1 polymorphism: Q (Glutamine) or R (Arginine) at codon 192 [15, 17, 18].

The purpose of this study was to test whether PON-1 activity, assessed by its ability to hydrolyse paraoxon or phenyl acetate, as well as plasma 8-iso-PGF2α concentrations can be used as indicators for atherosclerotic processes in coronary arteries. For this purpose we analyzed the association of plasma 8-iso-PGF2α levels and paraoxonase (PONase) and arylesterase (AREase) activity with the extent and severity of angiographically proven coronary artery disease (CAD) assessed by Gensini score.

2. Methods

2.1. Patients. The study group consisted of 150 patients undergoing coronary angiography for suspected CAD at the Medical University of Gdańsk (Poland). All subjects were in stable condition. None of the subjects had sustained a myocardial infarction within 6 months prior to taking part in the study. Patients with acute coronary syndrome or hepatic or renal disorders were excluded. The study was approved by the Independent Ethics Committee of the Medical University of Gdańsk and all patients gave their informed consent.

2.2. Coronary Angiography. Coronary angiography was performed using the transradial or femoral approaches in all recruits. The severity and extent of coronary atherosclerosis were quantified for each patient using the Gensini score, an assessment with prognostic significance for predicting the incidence of death or other cardiovascular events [19]. The Gensini score was assigned according to a previously described protocol [20]. Patients were divided into two groups: those with CAD (Gensini score ≥1; CAD+) and those without (Gensini score = 0; CAD−) according to angiographic results.

2.3. Laboratory Measurements. Blood samples were obtained between 7 and 8 a.m. on the day of and prior to coronary angiography following an overnight fast. Samples (serum and plasma) were separated after collection by centrifugation at 1000 ×g for 15 min and stored at −80°C pending analysis.

Total cholesterol (TC) and triacylglycerols (TAG) were measured in serum using standard enzymatic colorimetric tests. High-density lipoprotein cholesterol (HDL-C) was determined following precipitation of apolipoprotein B containing lipoproteins; LDL cholesterol level (LDL-C) was calculated using the Friedewald formula.

8-Iso-PGF2α was analyzed in plasma using an enzyme immunoassay kit (Cayman Chemical Company, USA). Data are expressed in pg/mL. The intra- and interassay coefficients of variation were 7.6 and 8.8%, respectively.

Paraoxonase (PONase) and arylesterase (AREase) activity were measured in serum based on paraoxon and phenyl acetate hydrolysis, respectively, according to procedure described earlier [17, 21]. The intra- and interassay coefficients of variation were 4.5 and 6.7%, for the PONase activity assay, and 2.8 and 5.5% for the AREase activity assay, respectively.

2.4. Statistics. All statistical analyses were performed using STATISTICA software, version 10. The Shapiro-Wilk test was used to test the distribution of variables that followed a Gaussian pattern. Continuous variables were expressed as mean ± SD (standard deviation) or medians with 25th and 75th percentiles. Student's unpaired t-test or the Mann-Whitney U test was used to assess the differences between two groups. Pearson's chi-squared test was used to compare categorical variables. Univariate correlations were assessed using standardized Spearman coefficients. Skewed variables, like PONase and 8-iso-PGF2α, were log-transformed to normal distribution for multiple linear regression analysis. Multilinear regression was assessed using standardized β coefficients. P values below 0.05 were considered statistically significant.

3. Results

The result of coronary angiography confirmed atherosclerosis in 105 patients; 45 patients received a negative result.

Clinical characteristics of patients with angiographically proven coronary artery disease (CAD+) and patients with negative results from coronary angiography (CAD−) are shown in Table 1. The groups were matched for sex, age, BMI, smoking habit, preexisting hypertension, diabetes, and metabolic syndrome. Statins were being taken by 90% of patients with confirmed atherosclerosis and by 55% in the group with negative results of angiography. Compared to the CAD− group, CAD+ patients had significantly lower mean concentrations of total cholesterol by 14%, LDL cholesterol (LDL-C) by 19%, HDL cholesterol (HDL-C) by 15%, and Apo AI by 12%. Concentrations of triacylglycerols and Apo B were similar in both groups (Table 1).

Patients with angiographically proven coronary artery disease, compared to patients with negative results, were characterized by significantly lower PONase activity (median with 25th and 75th percentiles: 123 (93–193) versus 201 (11–272)) and AREase activity (median with 25th and 75th percentiles: 103 (80–123) versus 109 (96–136)) (Figure 1).

Figure 2 shows the distribution of paraoxonase (PONase) versus arylesterase (AREase) activity in all study populations. This relationship enabled the extraction of 3 groups (PON-1 phenotypes) of patients with different relative enzyme activity

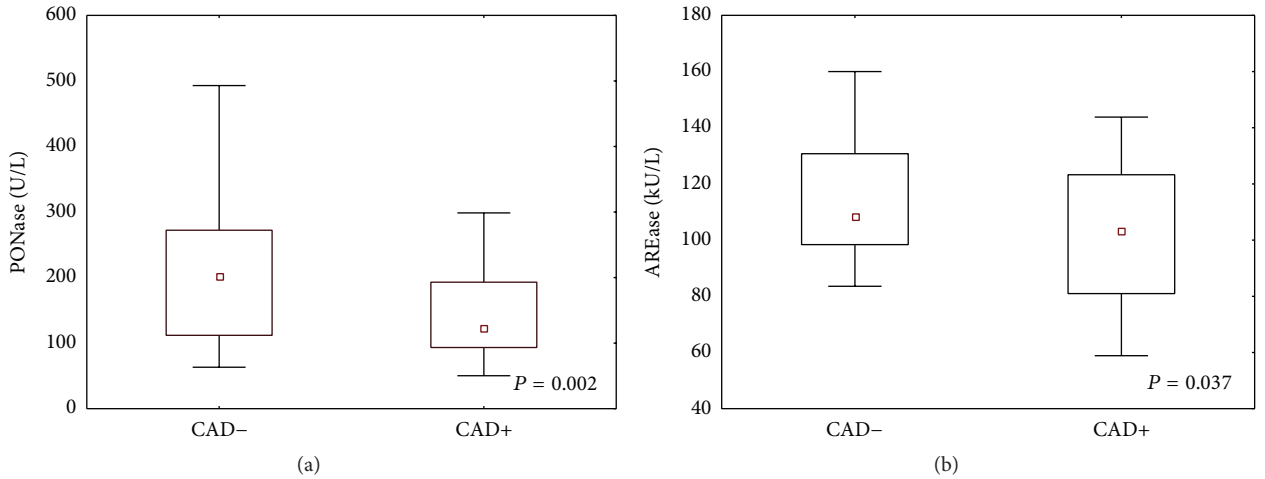

Figure 1: PONase activity (a) and AREase activity (b) in patients with coronary artery disease (CAD+) and patients with negative result of coronary angiography (CAD−). Values are presented as medians (25–75th percentiles, 5–95th percentiles) and assessed using the Mann-Whitney U test.

Table 1: Characteristics of patients with coronary artery disease (CAD+) and patients with negative result of coronary angiography (CAD−).

	CAD− $N = 45$	CAD+ $N = 105$	P value
Gender, M/F	20/25	41/64	0.537***
Age (years)	63 ± 10	65 ± 10	0.252*
BMI (kg/m^2)	27 ± 4	28 ± 5	0.664*
TAG (mg/dL)	102 (76–141)	107 (80–136)	0.882**
TC (mg/dL)	196 ± 40	168 ± 41	<0.001*
HDL-C (mg/dL)	52 ± 13	44 ± 11	<0.001*
LDL-C (mg/dL)	125 ± 33	101 ± 37	<0.001*
Apo AI (g/L)	1.7 ± 0.3	1.5 ± 0.3	<0.001*
Apo B (g/L)	0.84 ± 0.17	0.79 ± 0.24	0.283*
Apo B/Apo AI	0.52 ± 0.13	0.54 ± 0.18	0.512*
Current smokers (%)	48%	66%	0.053***
Diabetes (%)	22%	26%	0.566***
Hypertension (%)	68%	79%	0.181
Metabolic syndrome (%)	42%	64%	0.143***
Statin therapy (%)	55%	90%	<0.001***

Values are presented as mean ± SD or as median (25th and 75th percentiles).
*Student's t-test; **Mann-Whitney U test; ***Pearson's chi-squared test.

Table 2: PON-1 phenotypes frequencies.

PONase/AREase	<1.5	1.5–4	>4
N	79	63	8
PONase/AREase	0.8 ± 0.2	2.5 ± 0.5	5.1 ± 0.7
Paraoxonase (PONase) [U/L]	98 ± 33	222 ± 62	465 ± 51
Arylesterase (AREase) [kU/L]	117 ± 22	92 ± 22	92 ± 12
CAD− (%)	42	47	11
CAD+ (%)	57	40	3

CAD+ patients with coronary artery disease and CAD− patients with negative result of coronary angiography; PONase and AREase activity are presented as mean ± SD.

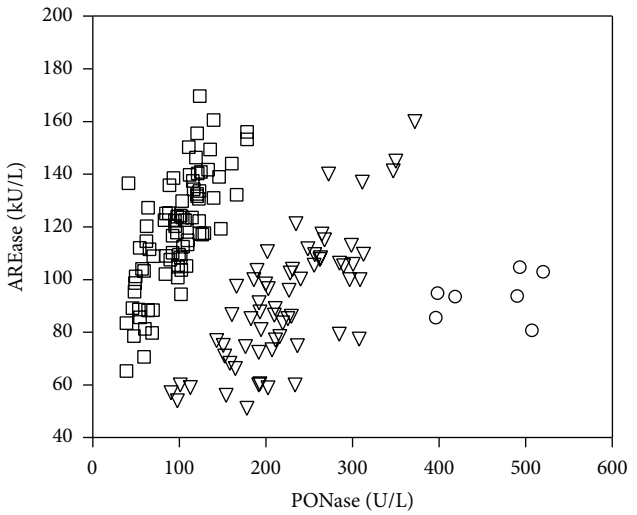

Figure 2: PONase activity towards AREase activity: squares: PONase/AREase < 1.5; triangles: PONase/AREase = 1.5–4.0; and dots: PONase/AREase > 4.0.

in the two different substrates (PONase versus AREase ratio less than 1.5, between 1.5 and 4.0, and greater than 4.0) as shown in Table 2. The percentages of phenotypes in CAD+ and CAD− participants were similar. In both groups, the PONase versus AREase ratio of most patients was less than 1.5; the smallest number of patients had a PONase/AREase value above 4 (Table 2).

Patients with angiographically proven coronary artery disease had significantly higher concentrations of 8-iso-PGF2α compared to patients with negative results (median with 25th and 75th percentiles: 125 (89–173) versus 99 (71–133)) (Figure 3). There was no significant correlation between the activity of PON-1 and plasma 8-iso-PGF2α concentration

Table 3: 8-Iso-PGF2α concentration according to serum arylesterase and paraoxonase activity quartiles.

	Paraoxonase (PONase) [U/L]				Arylesterase (AREase) [kU/L]			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Quartile 1	Quartile 2	Quartile 3	Quartile 4
All subjects								
Range	<98	98–135	135–224	≥225	<87	87–107	107–122	≥123
8-Iso-PGF2α [pg/mL]	134 (130–196)	114 (89–157)	103 (74–146)	104* (69–154)	140 (100–200)	103 (70–162)	117 (74–148)	118 (83–168)
CAD+								
Range	<98	98–123	123–192	≥193	<83	83–103	103–123	≥123
8-Iso-PGF2α [pg/mL]	145 (88–158)	113 (78–161)	112 (88–158)	123 (84–169)	142 (103–205)	122 (89–184)	121 (75–157)	114 (76–168)
CAD−								
Range	<112	112–201	201–271	≥272	<98	98–108	108–130	≥130
8-Iso-PGF2α [pg/mL]	124 (70–170)	92 (49–125)	92 (74–130)	110 (71–137)	133 (80–153)	95 (69–133)	103 (74–119)	92 (72–149)

8-Iso-PGF2α concentrations are presented as median (25th and 75th percentiles); *$P < 0.05$ compared with Quartile 1; CAD+ patients with coronary artery disease and CAD− patients with negative result of coronary angiography.

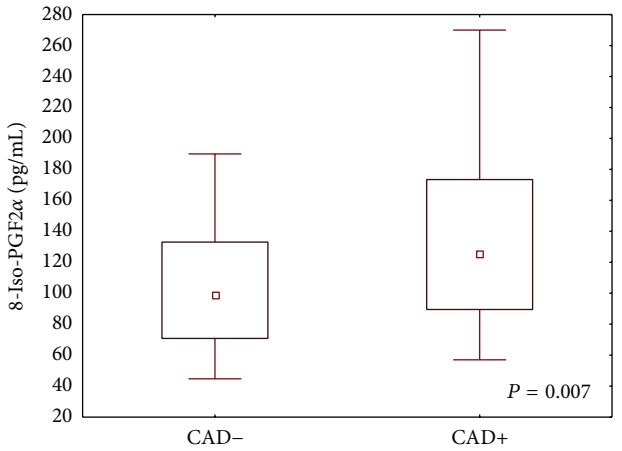

Figure 3: Plasma 8-iso-PGF2α concentrations in patients with coronary artery disease (CAD+) and patients with negative result of coronary angiography (CAD−). Values are presented as medians (25–75th percentiles, 5–95th percentiles) and assessed using the Mann-Whitney U test.

in both the CAD+ patients and CAD− patients as also in the whole group of studied population. However evaluation of 8-iso-PGF2α concentration according to serum AREase and PONase activity quartiles had shown that the patients with lowest PONase activity have higher 8-iso-PGF2α level compared to the patients with the highest PONase activity (Table 3).

Analyzing the impact of traditional risk factor on the PON-1 activity and isoprostane levels we noticed the significant higher 8-iso-PGF2α level in CAD+ patients with hypertension compared to these without hypertension; similarly 8-iso-PGF2α levels were higher in smoking patients relative to nonsmoking patients. We did not observe the impact of diabetes on isoprostane concentrations. However diabetic patients had clearly reduced PONase activity compared to nondiabetic subjects (Table 4).

To evaluate coronary artery stenosis and determine the severity of CAD, we used angiography results drawn up on the basis of the Gensini score.

Univariate correlation analysis indicated that 8-iso-PGF2α concentrations were positively associated with Gensini scores (Figure 4(a)), whereas PONase and AREase activity were inversely correlated with the severity of CAD (Figures 4(b) and 4(c), resp.). 8-Iso-PGF2α concentrations and PONase activity remained an independent determinant of atherosclerosis severity in multiple linear regression after adjusting for age, gender, smoking habits, preexisting hypertension, diabetes, statin therapy, and HDL-C and TAG concentration (Table 5).

4. Discussion

The major finding of our study is the demonstration of decreased activity of PON-1 and increased concentration of 8-iso-prostaglandin F2 in patients with coronary artery disease (CAD) proved by angiography and establishment of the correlation between these factors and the severity of CAD.

Studies on PON-1 activity are based on quantifying its wide range of enzymatic activities in breaking down *in vitro* substrates such as paraoxon-paraoxonase (PONase) and phenyl acetate-arylesterase (AREase) activity. We have shown a significant reduction of PONase and AREase activity in a group of patients with angiographically proven coronary artery disease (CAD+) compared to patients with negative results of coronary angiography (CAD−) (Figure 1). The reduced activity of PON-1 is consistent with previous reports and confirms suggestions concerning the antiatherosclerotic properties of the enzyme [22, 23]. Moreover, our results

Table 4: Concentration of 8-iso-PGF2α and PONase and AREase activity in patients with and without traditional risk factors.

	8-Iso-PGF2α [pg/mL]	P value**	Paraoxonase (PONase) [U/L]	P value**	Arylesterase (AREase) [kU/L]	P value*
Hypertension/nonhypertension						
All subjects	122 (80–170)/101 (79–137)	0.124	125 (93–202)/186 (104–234)	0.065	105 ± 26/112 ± 24	0.159
CAD+	134 (94–195)/101 (78–141)	0.045	121 (89–177)/152 (100–233)	0.501	102 ± 20/110 ± 26	0.218
CAD−	95 (69–138)/112 (80–133)	0.295	183 (99–272)/210 (201–311)	0.484	113 ± 23/114 ± 23	0.841
Diabetes/nondiabetes						
All subjects	120 (74–162)/116 (80–163)	0.894	104 (65–163)/153 (102–240)	<0.001	105 ± 25/108 ± 26	0.827
CAD+	126 (100–164)/120 (89–178)	0.694	104 (69–176)/125 (99–202)	0.049	102 ± 26/104 ± 23	0.761
CAD−	110 (71–119)/95 (72–135)	0.293	116 (64–140)/227 (127–306)	0.004	115 ± 25/115 ± 23	0.704
Smoking/nonsmoking						
All subjects	126 (89–178)/113 (70–137)	0.006	123 (93–201)/182 (109–264)	0.012	102 ± 26/112 ± 24	0.044
CAD+	140 (89–198)/116 (73–141)	0.042	131 (90–225)/119 (88–191)	0.074	99 ± 26/108 ± 24	0.153
CAD−	117 (90–138)/89 (66–133)	0.223	163 (96–238)/228 (140–300)	0.073	114 ± 20/117 ± 24	0.680

Values are presented as mean ± SD or as median (25th and 75th percentiles). *Student's t-test; **Mann-Whitney U test.

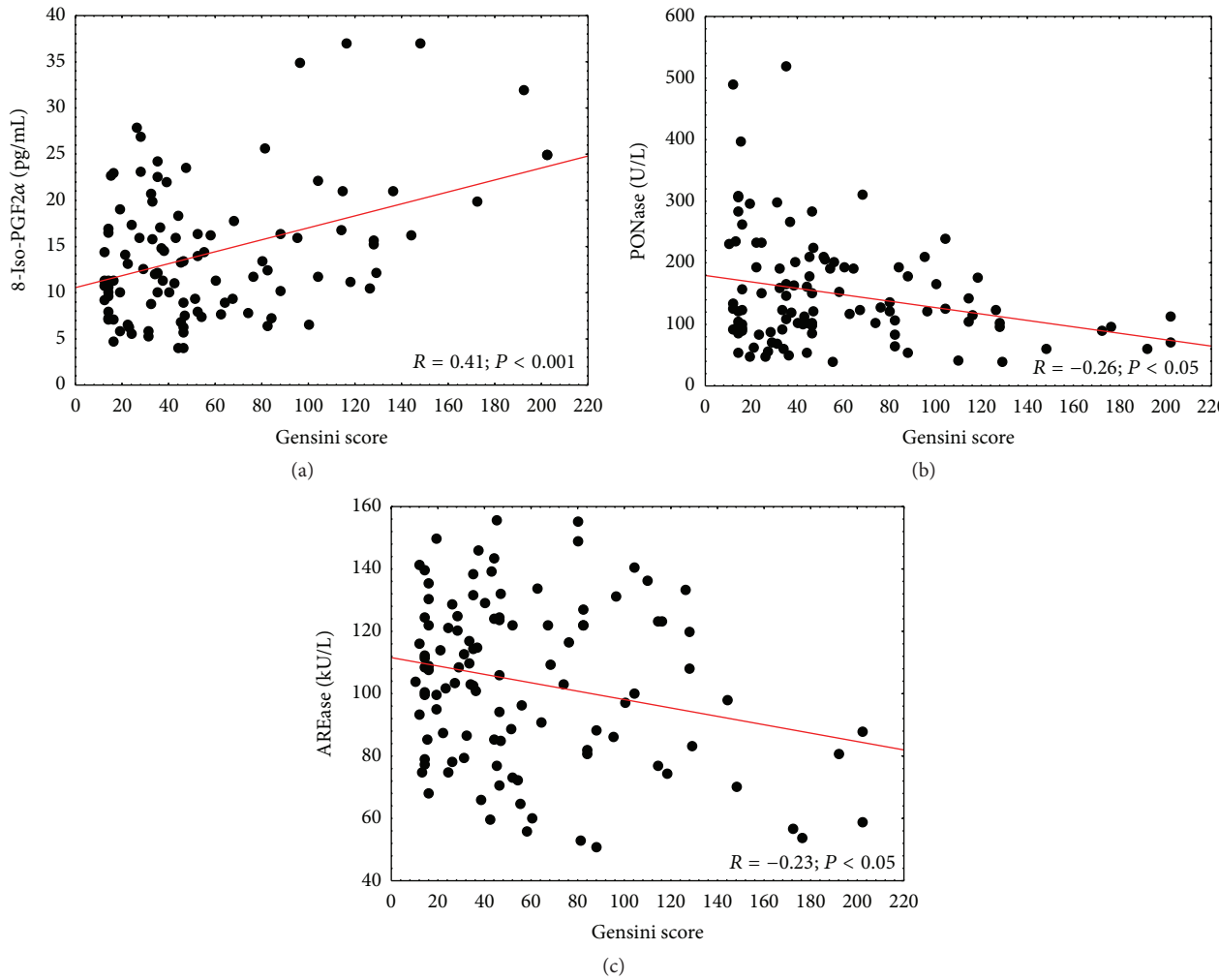

Figure 4: Correlations between Gensini score and (a) 8-iso-PGF2α concentrations, (b) PONase activity, and (c) AREase activity.

Table 5: Multiple linear regression analysis for Gensini score[a].

	β	SE	P value
8-Iso-PGF2α*	0.368	0.09	<0.001
PONase*	−0.267	0.10	0.01
AREase	−0.227	0.79	0.097

β: standardized beta coefficients; SE: standard error; [a]adjusted for age, gender, smoking habits, hypertension, type 2 diabetes, statin therapy, and HDL-C and TAG concentration; *log-transformed values.

show that the correlation between these two types of PON-1 activities is not simple [17]. The presentation of PON-1 activity results as a ratio of paraoxonase to arylesterase activities enables the extraction of three PON-1 phenotypes (Figure 2). The unique relationship between the two PON-1 activity measurements is probably the result of the stronger impact of genetic polymorphism on paraoxonase activity. A previous report has shown that the phenotype distinguished on the basis of the paraoxonase-to-arylesterase ratio closely corresponds to a common PON-1 polymorphism: Q (Glutamine) or R (Arginine) at codon 192 [15, 17, 18]. Several studies have examined the relationship between the PON 192 genotype and cardiovascular disease. Results are not consistent: whereas some have reported a correlation between the PON 192 allele and disease [24, 25], other studies have failed to find a connection, showing at the same time the decreased paraoxonase activity regardless of the PON-1 genotype [20, 26]. In our study, the percentage of phenotypes in the CAD+ and CAD− groups was similar (Table 2), which may support the hypothesis that the polymorphisms influence paraoxonase activity although this is clearly not crucial to the antiatherosclerotic properties of PON-1.

Apart from reduced PON-1 activity, CAD patients also were characterized by increased isoprostane levels (Figure 3). Isoprostanes are a class of biologically active products of arachidonic acid peroxidation that provides a reliable index of lipid peroxidation. Theoretically, a large number of isoprostanes types can be generated, but most interest has focused on F2 isoprostanes and in particular on 8-iso-prostaglandin F2 (8-iso-PGF2α). A previous study suggested

increased concentrations of circulating 8-iso-PGF2α in coronary atherosclerosis development [27–30]. Clejan et al. suggested that accelerated isoprostane formation is intensified in CAD and predisposes patients to acute coronary syndrome [31]. Vassalle et al. showed that 8-iso-PGF2α concentrations are higher in CAD patients with multivessel disease compared to patients with single-vessel disease [32, 33]. Gross et al. showed an association between increased concentrations of circulating 8-iso-PGF2α and coronary calcification [30].

Our findings of elevated 8-iso-PGF2α levels in CAD+ patients are in agreement with earlier studies and support the hypothesis that an important role is played by the oxidative process in atherosclerosis.

Angiography, as the gold diagnosis standard for the coronary artery, is used not only to confirm the disease but also to assess the severity of atherosclerotic lesions using, among others, the Gensini score. One of the major discoveries of our study, after assessment of the severity of atherosclerosis using the Gensini score, was that 8-iso-PGF2α levels were positively, and PON-1 activity was negatively, correlated with the severity of coronary artery disease (Figure 4). Although, according to earlier reports [29, 34, 35], we have shown the impact of traditional risk factors for 8-iso-PGF2α concentration and activity of PON-1 (Table 4), PONase activity and 8-iso-PGF2α concentration remained independent determinants of atherosclerosis severity in multiple linear regression after adjusting age, gender, smoking habits, hypertension diabetes, statin therapy, and HDL-C and TAG concentration (Table 3). This confirms the hypothesis that the oxidative processes not only initiate the process of atherosclerosis but are also involved in the progression of the disease [23]. We failed to find simple correlation between paraoxonase activity and 8-iso-PGF2α concentration; however the patients with lowest paraoxonase activity have higher 8-iso-PGF2α level compared to the patients with the highest paraoxonase activity (Table 4), which may confirm, as previously suggested [36], possibility that these two opposing processes responsible for maintaining oxidant-antioxidant balance exert a reciprocal influence on one another.

Elevated levels of triglycerides and decreased levels of HDL are well-documented epidemiological risk factors for cardiovascular disease [37], which confirms the results of our work. However, many studies show that evaluation of HDL quality may have an impact on the prediction of CAD progression similar to the evaluation of numbers of HDL particles by measuring HDL cholesterol [38, 39]. In the development of atherosclerosis, HDL particles can lose their protective properties through changes in the oxidative processes or the loss of parts of molecules. The level of 8-iso-PGF2α, which assesses the severity of oxidant processes and the activity of PON-1, as an enzyme closely linked with HDL particles, can be valuable parameters for estimating the antiatherogenic properties of HDL.

Approximately 90% of the CAD+ study population had undergone statin therapy, which may explain the lower levels of LDL cholesterol and total cholesterol in patients with atherosclerosis compared to the CAD− group, in which 55% of patients had received statin drug therapy.

We did not observe a correlation between Gensini scores on one hand and LDL and total cholesterol level on the other hand. The impact of lipid-lowering therapy on the activity of PON-1 and 8-iso-PGF2α levels remains in question. Although some clinical studies showed that PON-1 activity increased and lipid peroxidation decreased in patients undergoing lipid-lowering therapy, no consensus has been reached [35, 40]. In our study, the small number of patients without statin therapy did not enable us to draw conclusions; however, patients with angiographically proven coronary artery disease, compared to patients with negative results of coronary angiography, were characterized by lower PON-1 activity and higher 8-iso-PGF2α concentration despite a higher percentage of statin users.

In summary, we have shown that oxidative stress plays an important role in the pathogenesis and development of CAD. The present finding suggests that impaired PONase activity and elevated 8-iso-PGF2α can be additional important biomarkers of coronary atherosclerosis development and in the future may be considered a potential pharmacological strategy for reducing CAD and preventing the progression of cardiovascular atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Markers of Antioxidant Defense in Patients with Type 2 Diabetes

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Aims. Diabetes is considered a state of increased oxidative stress. This study evaluates blood concentrations of selected markers of antioxidant defense in patients with type 2 diabetes. *Methods*. The study included 80 type 2 diabetes patients and 79 apparently healthy controls. Measured markers included ferric reducing ability of plasma (FRAP), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), γ-glutamyltransferase (GGT) and uric acid serum, and plasma and/or hemolysate levels. *Results*. FRAP, uric acid, CRP, and GGT levels were significantly higher in patients with diabetes. Plasma and hemolysate GR was significantly higher whereas GPx activity was significantly lower in patients with diabetes. There were no significant differences in antioxidant defense markers between patients with and without chronic diabetes complications. Fasting serum glucose correlated with plasma GPx, plasma and hemolysate GR, FRAP, and serum GGT, and HbA1c correlated with serum GGT. Only FRAP and serum uric acid were significantly higher in obese (BMI > 30 kg/m^2) patients with diabetes than in nonobese patients. *Conclusions*. Some components of antioxidant defense such as GR, uric acid, and GGT are increased in patients with type 2 diabetes. However, the whole system cannot compensate for an enhanced production of ROS as reflected by the trend toward decreased erythrocytes GSH.

1. Introduction

Persistent hyperglycemia secondary to insulin resistance and diminished insulin secretion in type 2 diabetes leads to progressing organ injuries known as late or chronic diabetes complications. It is believed that one of the underlying causes of microvascular and macrovascular diabetes complications is oxidative stress [1].

There are several suggested mechanisms linking hyperglycemia with increased production of reactive oxygen species (ROS). These mechanisms include increased mitochondrial synthesis of superoxide anion radical (O_2^-) [2], activation of the NF-κB signaling pathway leading to inflammatory reaction and increased ROS production in phagocytes [3], increased glucose flux through the polyol pathway, and formation of the advanced glycation end products (AGE) enhancing oxidative stress [4].

In general, oxidative stress is caused by an imbalance between ROS generation and antioxidant defense mechanisms eliminating the superoxide anion radical and similar compounds. Antioxidant defense system consists of a series of specific enzymes, metal binding proteins, and a number of low molecular weight antioxidants such as ascorbate, cysteine, glutathione, and urate. Some elements of this system are measured in blood as markers of antioxidant defense. Diabetes is considered a state of increased oxidative stress but the published data referring to impairment of antioxidant defense in diabetic patients are contradictory [5, 6].

The aim of this study was to evaluate blood concentrations of selected markers of antioxidant defense in patients with type 2 diabetes and to assess the relationship between antioxidant defense and glycemic control and the presence of diabetes complications.

Table 1: Demographic and biochemical characteristics of studied patients with type 2 diabetes and controls.

Parameter	Type 2 diabetes $n = 80$	Control group $n = 79$
Age [years]	59.5 ± 11.6	55.2 ± 11
Gender		
Women	40 [50]	40 [51]
Men	40 [50]	39 [49]
Diabetes duration [years]	8.5 (4.0–12)	—
BMI	31.54 ± 5.73	29.26 ± 8.43
Hypertension	77 [88.7]	—
Obesity (BMI > 30 kg/m^2)	44 [55]	32 [41]
No complications	38 [47.5%]	—
Complications	42 [52.5%]	—
Glucose [mmol/L]	6.75 (5.25–9.35)	4.4 (4.0–4.9)
HbA1c [%] [mmol/mol]	8.5 (7.25–10.0) 69 (56–86)	—
Creatinine [μmol/L]	66.8 (58.2–82.0)	—
e-GFR (MDRD) [mL/min/1.73 m^2]	94.8 (75–116.4)	—
TCH [mmol/L]	4.38 (3.82–5.27)	5.06 (4.09–5.9)
LDL [mmol/L]	2.21 (1.81–3.15)	2.94 (2.22–3.75)
HDL [mmol/L]	0.82 (0.71–1.00)	1.29 (1.06–1.24)
TG [mmol/L]	2.55 (1.74–3.75)	1.47 (1.08–1.87)
Albuminuria [mg/L]	2.51 (0.0–8.03)	—

Data are expressed as mean ± SD, median and interquartile range, or percentage of frequency [%], as appropriate.

2. Materials and Methods

The study included 80 patients with type 2 diabetes—40 females and 40 males, aged from 30 to 80 years (mean 59.5 ± 11.5), hospitalized in the Department of Metabolic Diseases, University Hospital, Krakow, Poland. Mean disease duration from diagnosis was 8.5 years (4–12). In 77 (88.7%) patients arterial hypertension was diagnosed. Among studied patients 41 had chronic complications of diabetes including nephropathy in 15 patients, retinopathy in 21, neuropathy in 29, and diabetic foot in 6 patients. Exclusion criteria included the fifth stage of chronic kidney disease (GFR < 15 mL/min/1.73 m^2), inflammatory diseases, cancer, and systemic diseases.

The control group was composed of 79 healthy subjects matched according to age (mean 55.2 ± 11; range 30–85 years) and sex with the studied patients (Table 1).

Healthy subjects were recruited from the individuals who went through regular medical check-up in the Department of Diagnostics, University Hospital, Krakow, Poland. They were selected among individuals with no history of cancer and systemic diseases, healthy at the time of study, and whose laboratory findings did not show inflammatory disease, prediabetes, or diabetes.

Participation required informed consent signed by all subjects enrolled both into the studied and into the control group. The study had the approval of Jagiellonian University Bioethics Commission number KBET/139/09/.

Blood samples for the study purposes were collected using S-Monovette-serum and S-Monovette-EDTA tubes (SARSTEDT AG&Co, Nümbrecht, Germany). Routine laboratory tests including fasting glucose, lipid profile, gamma-glutamyltransferase activity, and creatinine and uric acid concentrations were performed in serum. HbA1c levels were measured in whole blood.

The K_2-EDTA blood samples were used for measurements of selected antioxidant defense markers in plasma (the ferric reducing ability of plasma (FRAP), glutathione peroxidase (GPx), and glutathione reductase (GR) activity) and in hemolysate (glutathione peroxidase (GPx) and glutathione reductase (GR) activity and reduced glutathione concentration (GSH)). These blood samples were centrifuged for 10 minutes at 3000 ×g and the obtained plasma was separated, aliquoted to microtubes, and stored frozen at −80°C from 1 to 12 months until testing. Remaining erythrocytes were separated from plasma, washed three times with isotonic saline, and hemolyzed by a fourfold dilution with deionized water. The obtained erythrocytes suspension was frozen and stored overnight at −80°C. Then hemolysate samples were thawed and centrifuged at 3000 ×g for 10 minutes to remove cell debris. The hemolysate samples were aliquoted to microtubes and stored frozen at −80°C for 1–12 months before use.

Hemoglobin concentration in hemolysate was measured using the cyanmethemoglobin method [7]. After mixing of hemolysate samples with diluted potassium ferricyanide and potassium cyanide solution at a slightly alkaline pH to form the stable cyanmethemoglobin, the absorbance was measured at 540 nm using microplate EL-800 reader (BioTek Instruments, Inc., USA). Hemolysate reduced glutathione concentration was determined using the method described by Beutler et al. employing 5,5′-dithiobis(nitrobezoic) acid forming with glutathione thiol groups colored adduct, with spectrophotometric measurement at 412 nm [8]. Glutathione peroxidase activity was measured in plasma and hemolysate based on the decrease of NADPH absorbance at 340 nm according to the method of Paglia and Valentine [9]. Similarly, glutathione reductase activity was measured in plasma and hemolysate using the method based on the reduction of glutathione (GSSG) in the presence of NADPH, which is oxidized to $NADP^+$ [10]. Total antioxidant capacity was measured as the ferric reducing ability of plasma (FRAP). In this method ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form at low pH by the plasma antioxidants. Reduced Fe^{2+}-TPTZ takes on an intense blue color, with the peak absorbance at 593 nm [11]. All spectrophotometric measurements for the determination of glutathione peroxidase and glutathione reductase activity and the concentration of reduced glutathione were performed on the MaxMat PL analyzer (Maxmat SA, Montpellier, France).

Statistics. Normality of distribution of obtained variables was checked using the Shapiro Wilk test. Data are expressed as mean ± SD, median and interquartile range, or percentage of frequency, as appropriate. Relationships between paired

Table 2: Comparison of antioxidant defense markers in patients with type 2 diabetes and controls.

Parameter	Type 2 diabetes	Control group	p
FRAP [mmol/L]	0.97 ± 0.21	0.84 ± 0.16	**<0.001**
GPx_{plasma} [U/L]	235.7 (93.2–359.9)	644.5 (466.0–798.8)	**<0.001**
$GPx_{hemolysate}$ [U/gHb]	43.8 ± 21.5	55.4 ± 25.9	**0.002**
GR_{plasma} [U/L]	74.5 ± 21.3	43.7 ± 13.5	**<0.001**
$GR_{hemolysate}$ [U/gHb]	33.0 (26.9–38.5)	22.62 (16.2–29.1)	**<0.001**
GSH [μmol/L]	0.87 (0.46–2.23)	0.92 (0.56–1.20)	0.117
Uric acid [μmol/L]	364.3 ± 117.9	293.5 ± 85.7	**<0.001**
GGT [U/L]	23.5 (11.5–37.5)	10.5 (7.0–17.0)	**<0.001**
CRP [mg/dL]	1.95 (0.85–5.72)	0.87 (0.46–2.23)	**<0.001**

Data are expressed as mean ± SD and median and interquartile range.

parameters (continuous variables) were analyzed by Pearson product moment correlation coefficient. Variables with positively skewed distribution were log transformed before the correlation analysis. Differences between groups were assessed by t-test for variables with positively skewed distribution and Mann-Whitney U test for nonparametric variables. p values less than 0.05 were taken as statistically significant. All analyses were performed using a standard statistical package Statistica 9.0 (StatSoft Inc.) and Microsoft Office Excel 2007 spreadsheet (Microsoft Corporation).

3. Results

The characteristics of the studied groups are shown in Table 1. The studied group was characterized by hyperglycemia and elevated glycated hemoglobin. Mean values of lipid profile parameters did not fit treatment goals recommended by diabetes associations including the Polish Diabetes Society [12]. Mean BMI value was elevated and 55% of patients were obese (BMI > 30 kg/m^2).

The results of antioxidant defense markers measurements in patients with diabetes and controls are shown in Table 2.

In subjects with type 2 diabetes significantly lower levels of glutathione peroxidase and higher levels of glutathione reductase both in plasma and hemolysate were found. The trend towards decreased hemolysate reduced glutathione concentrations in the group with diabetes compared with controls (0.87 (0.46–2.23) μmol/L versus 0.92 (0.56–1.20) μmol/L) was observed.

Total antioxidant capacity of plasma assessed by the FRAP, serum uric acid, and GGT, also involved in antioxidant defense, were significantly higher in patients with diabetes (Table 2). Plasma uric acid concentration was significantly correlated with FRAP both in patients and in the control group (Figures 1 and 2).

Fasting serum glucose correlated with glutathione peroxidase in plasma ($r = -0.2816$, $p < 0.001$), glutathione reductase in plasma and hemolysate ($r = -0.2816$, $p < 0.001$; $r = 0.6010$, $p < 0.001$), FRAP ($r = 0.3816$, $p < 0.001$), and GGT ($r = 0.4565$, $p < 0.001$). Moreover correlation between GGT and HbA1c ($r = 0.4179$, $p < 0.001$) was found in diabetic patients.

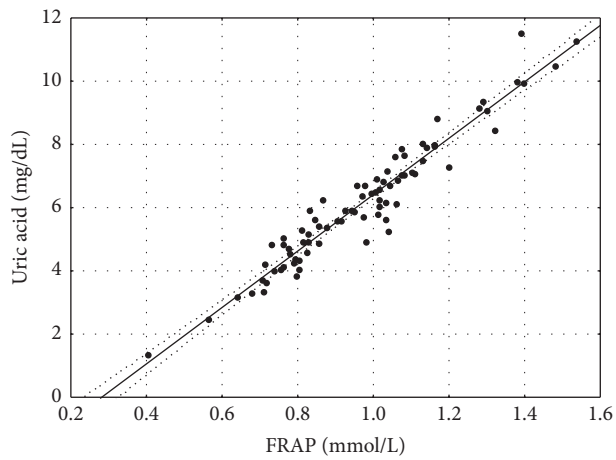

Figure 1: Correlation between FRAP and uric acid in people with type 2 diabetes ($r = 0.963$; $p \leq 0.001$).

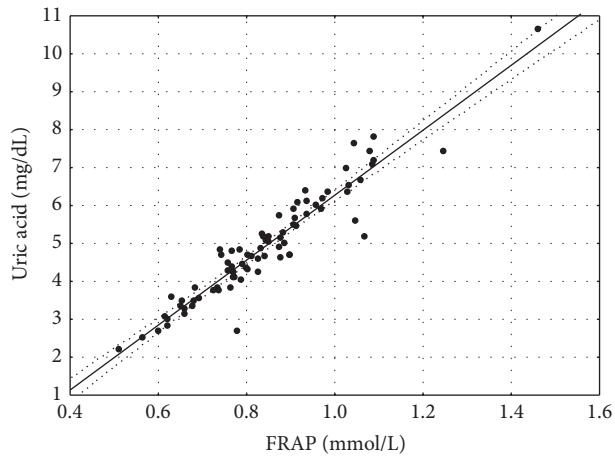

Figure 2: Correlation between FRAP and uric acid levels in the control group ($r = 0.948$; $p \leq 0.001$).

In patients with chronic diabetes complications (at least one) no significant differences in antioxidant defense markers as compared to those without complications were found

Table 3: Comparison of selected clinical and biochemical parameters in patients with and without chronic diabetes complications.

Parameter	Complications ($N = 42$)	No complications ($N = 38$)	p
Age [years]	60.9 ± 9.0	57.9 ± 13.9	0.009
BMI	30.92 ± 5.24	32.22 ± 6.22	0.315
Diabetes duration [years]	10 (7–16)	5 (1–9)	**<0.001**
Mean daily glucose [mmol/L]	6.3 ± 1.0	6.3 ± 1.1	0.651
HbA1c [mmol/mol]	69.4 (58.5–74.9)	77.1 (51.9–92.4)	0.372
[%]	8.5 (7.5–9.0)	9.2 (6.9–10.6)	
Creatinine [μmol/L]	66.4 (59.4–76.0)	67.8 (56.1–83.0)	0.891
FRAP [mmol/L]	1.00 ± 0.22	0.94 ± 0.20	0.512
GPx_{plasma} [U/L]	234.5 (89.2–60.5)	236.9 (98.4–59.3)	0.950
$GPx_{hemolysate}$ [U/gHg]	43.1 ± 22.0	44.6 ± 21.1	0.819
GR_{plasma} [U/L]	73.1 ± 18.2	75.5 ± 24.5	0.063
$GR_{hemolysate}$ [U/gHg]	33.1 (27.1–38.6)	32.8 (26.7–37.9)	0.969
Glutatione [μmol/L]	0.88 (0.53–1.18)	0.94 (0.71–1.21)	0.402
Uric acid [μmol/L]	381.0 ± 131.0	345.2 ± 71.4	0.170
GGT [U/L]	22.5 (12–38)	25.5 (10.0–34.0)	0.711
CRP [mg/L]	1.7 (0.5–4.4)	2.7 (1.1–10.0)	0.117

Data are expressed as mean ± SD, median and interquartile range, or percentage of frequency [%], as appropriate.

(Table 3). However, for uric acid correlation with albuminuria was found ($r = 0.46$, $p = 0.02$).

The relationship between metabolic syndrome features present in studied patients and antioxidant defense markers was also evaluated. No significant differences in these markers levels between obese (BMI > 30 kg/m^2) and nonobese patients with diabetes were found except for the FRAP and uric acid concentrations significantly higher in obese (FRAP: 1.03 ± 0.2 mmol/L versus 0.89 ± 0.18 mmol/L; $p = 0.002$; UA: 6.7 ± 2.0 μmol/L versus 5.4 ± 1.7 μmol/L, $p = 0.003$) and significantly correlated with BMI (FRAP: $r = 0.593$, $p < 0.001$; UA: $r = 0.415$, $p < 0.001$). Hypertensive patients with diabetes had significantly higher glutathione reductase activity in hemolysate as compared to those without hypertension (38.6 U/L versus 32.7 U/L, $p = 0.03$).

4. Discussion

Diabetes is a state of increased oxidative stress, which is one of the mechanisms leading to development of chronic diabetes complications [1]. While it is believed that diabetes is associated with an increased production of ROS, the reports of the antioxidant defense in diabetes are contradictory [5, 6]. In this study markers of antioxidant defense in patients with type 2 diabetes were evaluated. The study included a heterogeneous group of patients with type 2 diabetes—with and without chronic complications, and a part of patients had obesity, hypertension, and other features of the metabolic syndrome. Results obtained in this study showed that patients with diabetes had significantly higher antioxidant defense activity reflected by FRAP, glutathione reductase and gamma-glutamyltransferase activity, and uric acid levels. Glutathione peroxidase activity measurements in plasma and hemolysate yielded significantly lower results in the patients group (Table 2). Similarly, an inverse relationship between glutathione peroxidase and glutathione reductase in patients with type 2 diabetes and the control group was observed by Kumawat et al. [13]. In another study decreased activity of both glutathione peroxidase and glutathione reductase in type 2 diabetes was found [14].

The low activity of GPx could be directly explained by the low content of GSH found in patients with type 2 diabetes, since GSH is a substrate and cofactor of GPx. Enzyme inactivation could also contribute to low GPx activity. GPx is a relatively stable enzyme, but it may be inactivated under conditions of severe oxidative stress. Inactivation of this enzyme may occur through glycation governed by prevailing glucose concentration [15]. Increased activity of GR may be a compensatory response to oxidative stress. Changes in glutathione peroxidase and glutathione reductase activity found in this study can be considered an adaptation of antioxidant defense against increased production of ROS. However, this adaptation seems to be ineffective. Reduced GSH level in red blood cells reflects generalized decrease in intracellular content of this compound. The obtained results showed that patients with type 2 diabetes had lower GSH content in erythrocytes than observed in the control group. However, this difference was not statistically significant. The trend found in our results is consistent with the report of Aaseth and colleagues, who found reduced levels of glutathione in erythrocytes of subjects with obesity and poorly controlled type 2 diabetes [16]. Similarly, decrease in intracellular glutathione levels in patients with type 2 diabetes was reported by Livingstone in his recent review [17]. These results indicate that patients with type 2 diabetes have lower concentration of intracellular GSH, which increases the susceptibility of cells to the damaging effects of ROS.

There were no significant differences in oxidative defense markers between patients with and without chronic diabetes complications.

Plasma glutathione reductase, plasma glutathione peroxidase, FRAP, and GGT were associated with fasting glucose concentration, which indicated the association of higher level of antioxidant defense with hyperglycemia and subsequent ROS production. This activation of antioxidant defense seems to be associated with short-term glycemia fluctuations, because no relationship between antioxidant defense markers and HbA1c was found.

Metabolic syndrome includes risk factors for development of type 2 diabetes—hypertension and atherogenic dyslipidemia (elevated triglycerides and decreased HDL cholesterol concentrations). In obese patients serum uric acid levels were significantly higher as compared with those without obesity. We also observed significant positive correlation between BMI and serum uric acid concentrations and negative with plasma glutathione peroxidase activity. Decreased glutathione peroxidase activity associated with increased BMI is reported as a feature of oxidative stress in obese individuals [18].

In the whole studied group significant correlation between uric acid concentration and plasma total antioxidant capacity both in patients ($r = 0.963$) and in the control group ($r = 0.948$) was found. This observation confirms the well-known relationship between the level of antioxidant capacity measured as FRAP and plasma uric acid [11]. Moreover, serum uric acid concentrations correlated significantly with HDL cholesterol (negatively) and triglycerides (positively) levels. Similarly, increased levels of uric acid were reported by Chen et al. to be associated with abnormal levels of HDL [19]. Increase in uric acid concentrations is a well-known abnormality observed in metabolic syndrome. The underlying mechanisms are not fully explained. Recently it was demonstrated that leptin may influence hyperuricemia associated with obesity. Uric acid is considered a risk factor for diabetes complications. The study by Hovind et al. showed that uric acid is an independent risk factor for the development of diabetic nephropathy in type 1 diabetes. Moreover, in this study correlation between serum uric acid and albuminuria was found [20]. Similar results were reported for patients with type 2 diabetes [21]. Significant correlation between serum uric acid and albuminuria was demonstrated in our study but the limitation is the small number of albuminuric patients studied. Regardless of these relationships uric acid remains an important component of antioxidant defense.

Another enzyme involved in glutathione metabolism and the overall antioxidant defense system is GGT responsible for glutathione uptake from the extracellular fluid into the cells to maintain its constant level. Recently the relationship between plasma GGT activity and obesity with the risk for type 2 diabetes was reported [22]. In our study the mean GGT activity was significantly higher in diabetes individuals but without significant correlation with BMI.

Monitoring antioxidant defenses may also be important in clinical practice. Since the increased antioxidant defense may alleviate oxidative stress, supplementation with antioxidants has been considered an attractive potential therapy. This approach has been evaluated in numerous clinical trials assessing the effect of antioxidants supplementation on the risk for diabetes, glycemic control, and the development of chronic complications. However, the results of these studies are contradictory and do not allow us to draw consistent conclusion [23–29]. Nevertheless, our results indicating insufficient activation of antioxidant defense in type 2 diabetes may argue for the usefulness of such supplementation.

In summary, results obtained in this study show that some components of antioxidant defense such as glutathione reductase, uric acid, and GGT are increased in type 2 diabetes. However, this system seems to be ineffective as reflected by the trend toward decreased reduced glutathione content in erythrocytes. Although increased, the whole system of antioxidant defense cannot compensate for an enhanced production of ROS in diabetes, which results in oxidative stress. These data may indicate the usefulness of therapies enhancing antioxidant defense.

Abbreviations

FRAP:	Ferric reducing ability of plasma
GP:	Glutathione peroxidase
GR:	Glutathione reductase
GGT:	γ-Glutamyltransferase
CRP:	C-reactive protein
HBA1c:	Glycated hemoglobin
ROS:	Reactive oxygen species
O_2^-:	Superoxide anion radical
AGE:	Advanced glycation end products
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$:	Oxidized nicotinamide adenine dinucleotide phosphate
GSH:	Reduced glutathione
GSSG:	Glutathione disulfide (oxidized glutathione)
BMI:	Body mass index
UA:	Uric acid
HDL:	High-density lipoprotein
LDL:	Low-density lipoprotein
TG:	Triglyceride
TCH:	Total cholesterol
NF-κB:	Nuclear factor kappa B
GAPDH:	Glyceraldehydes -3-phosphate dehydrogenase
G-6PDH:	Glucose-6-phosphate dehydrogenase
IGT:	Impaired glucose tolerance.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

EGCG Attenuates Uric Acid-Induced Inflammatory and Oxidative Stress Responses by Medicating the NOTCH Pathway

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Background. The aim of this study is to investigate whether (-)-epigallocatechin-3-gallate (EGCG) can prevent the UA-induced inflammatory effect of human umbilical vein endothelial cells (HUVEC) and the involved mechanisms in vitro. *Methods*. HUVEC were subjected to uric acid (UA) with or without EGCG treatment. RT-PCR and western blots were performed to determine the level of inflammation marker. The antioxidant activity was evaluated by measuring scavenged reactive oxygen species (ROS). Functional studies of the role of Notch-1 in HUVEC lines were performed using RNA interference analyses. *Results*. UA significantly increased the expressions of IL-6, ICAM-1, TNF-α, and MCP-1 and the production of ROS in HUVEC. Meanwhile, the expression of Notch-1 and its downstream effects significantly increased. Using siRNA, inhibition of Notch-1 signaling significantly impeded the expressions of inflammatory cytokines under UA treatment. Interestingly, EGCG suppressed the expressions of inflammatory cytokines and the generation of ROS. Western blot analysis of Notch-1 showed that EGCG significantly decreased the expressions of inflammatory cytokines through Notch-1 signaling pathways. *Conclusions*. In summary, our findings indicated that Notch-1 plays an important role in the UA-induced inflammatory response, and the downregulation of Notch-1 by EGCG could be an effective approach to decrease inflammation and oxidative stress induced by UA.

1. Introduction

Hyperuricemia has been reported to increase the risk of gout, renal diseases, and cardiovascular diseases, such as hypertension and atherosclerosis [1]. Though its etiology is unclear, hyperuricemia can be secondary either to an exaggerated production of UA that follows high cellular metabolic conditions or to a low renal excretion in patients with renal function impairment [2, 3]. The tissue accumulation of UA leads to the formation of crystalline deposits, which triggers endothelial dysfunction and the outset of an inflammatory response [2, 4]. Inflammation is known to play a role in the progression of various disorders such as hypertension, atherosclerosis, and diabetes [5–11]. Useful topical treatment for hyperuricemia is limited.

Emerging evidence confirms that Notch signaling play a key role in inflammatory response involved in the pathogenesis of cardiovascular diseases [12–14]. Notch ligands and receptors have been reported to increase in damaged myocardial and vessels [15–17]. However, little is known about the association between UA levels and the expression of Notch-1.

EGCG is a flavanol derived from green tea extracts (GTE) and recently has been explored in disease therapy because of its antioxidant, anti-inflammatory, and anticancer effects [18–21]. Previous studies have shown that EGCG-mediated cardioprotection against the H_2O_2-induced oxidative stress by Akt/GSK-3β/caveolin signaling in vitro and in vivo [22]. In addition, EGCG inhibits cytochrome c release and activation of pro-caspase-3 to improve cigarette smoke-induced myocardial dysfunction [23]. EGCG increases production of NO via a phosphatidylinositol (PI) 3-kinase/Akt-medicating endothelial nitric oxide synthase (eNOS) in vascular endothelial cells and enhance insulin sensitivity to improve endothelial function [24, 25]. Recent study has shown that EGCG regulates Notch signaling to inhibit the proliferation of colorectal cancer cells [26]. However, the role of EGCG on the effect of hyperuricemia has been unresolved.

In this study, we evaluated that treatment of HUVEC with UA activated inflammatory response and oxidative stress.

Next, we examined the potential mechanism which revealed that Notch-1 was essential for UA-induced inflammatory response and oxidative stress. Further experiments indicated that EGCG mediated vascular endothelial cells protection in an UA-induced inflammatory response and oxidative stress through Notch pathway, at least partly.

2. Materials and Methods

2.1. Materials. UA and EGCG were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG stock solution was prepared in sterile double distilled water at 20 mM. UA was dissolved in deionized water and filtered and was free of crystals (by polarizing microscopy).

2.2. Cell Culture and Cell Transfection. The human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (Rockville, MD, USA). HUVEC were grown in EGM-2 Bullet kit (Lonza, Basel, Switzerland) containing penicillin G (100 Units/mL; Sigma-Aldrich) and streptomycin sulfate (100 μg/mL; Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2. HUVEC were cultured with UA (8 mg/dL) for another 24 hours after pretreating with or without EGCG (20μM).

Small interference RNA (siRNA) targeting Notch-1 (numbered 1–3) and a scrambled siRNA were designed and purchased from GenePharma Co., Ltd. (Shanghai, China). The effective complementary (upper) sequences in the two RNA duplexes were 5′-GCACGCGGAUUAAUUUGCAdT-dT-3′ and 5′-UGCAAAUUAAUCCGCGUGCdTdT-3′. The full cDNA of Notch-1 was cloned in the pcDNA3.1 vector (Invitrogen) and was confirmed by DNA sequencing. For transient silencing, 5×10^5/mL cells were seeded onto 60 mm dish and transfected with Notch-1 siRNA (50 nmol/L) using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to manufacturer's protocol. The medium was replaced with complete medium and incubated at 37°C for 48 hours before further analysis. The transfection efficacy was evaluated by western blot.

2.3. Measurement of Intracellular ROS Production by Fluorescence Spectrophotometry. To determine whether the ROS levels of HUVEC are affected by the presence of UA or EGCG, a chemiluminescence assay was assessed using CellROX Green Reagent (Life technologies). The examined HUVEC were seeded onto 24-well plates and treated with various processing. CellROX Green Reagent was incubated to each well at a concentration of 10 μmol/L and mixed vigorously for 1 hour at 37°C. Fluorescence of CellROX was measured with a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany) at 488 nm of excitation wavelength and 510 nm of emission filter. The semiquantified ROS was expressed as relative fluorescent units (RFU) and all experiments were performed in duplicate and repeated three times.

2.4. RNA Extraction and Quantitative Real Time PCR (qRT-PCR). Total RNA from cells was extracted using Trizol (Invitrogen Corp) following the manufacturer's protocol. cDNA was synthesized using 500 ng of total RNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The resulting first-strand cDNA was amplified in a final volume of 20 μL containing 10 pmol of each primer by One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa, Shuzo, Japan). The oligonucleotide primers that were used for the PCR amplifications were synthesized by Shanghai Sangon Technologies, Inc. (Sangon, China) and are listed as follows: forward primer: Hes1 forward primer: 5′-CCTGTCATCCCCGTCTACAC-3′, reverse primer: 5′-CACATGGAGTCCGCCGTAA-3′; MCP-1, forward primer: 5′-CAGCCAGATGCAATCAATGCC-3′, reverse primer: 5′-TGGAATCCTGAACCCACTTCT-3′; NF-κB, forward primer: 5′-GAAGCACGAATGACAGAGGC-3′, reverse primer: 5′-GCTTGGCGGATTAGCTCTTTT-3′; ICAM-1, forward primer: 5′-TTGGGCATAGAGACC-CCGTT-3′, reverse primer: 5′-TGGAATCCTGAACCC-ACTTCT-3′; TNF-α, forward primer: 5′-CCTCTCTCT-AATCAGCCCTCTG-3′, reverse primer: 5′-GAGGACCTG-GGAGTAGATGAG-3′; GAPDH, forward primer: 5′-ACA-ACTTTGGTATCGTGGAAGG-3′, reverse primer: 5′-GCCATCACGCCACAGTTTC-3′. A melting curve analysis was performed for each of the primers used, and each showed a single peak indicating the specificity of each of the primers tested. All values were calculated using the delta Ct method and expressed as the change relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The amplification conditions were 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s.

2.5. Western Blot Analysis. Cell lysates were rinsed with ice-cold PBS and prepared using lysis buffer with 1% Triton, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 1% deoxycholate and protease inhibitors cocktail (Roche). Protein concentration was determined by Bradford protein assay kit (Bio-Rad) and equal amount of protein was separated by 12% SDS-PAGE using Bio-Rad apparatus. The membranes were blocked for 2 hours in 5% skim milk at room temperate after protein was transferred to PVDF membrane (Millipore, Billerica, MA, USA). The proteins were incubated with the following primary antibodies: IL-6 and TNF-α (Merck Millipore, Bedford, MA, USA), ICAM-1, Hes5 and MCP-1 (Abcam, Cambridge, Mass, USA), Hes1 (OriGene Technologies, Rockville, MD, USA), Hey1 and Hey2 (Proteintech Group, Chicago, IL, USA), and phospho-p65 (CST, Chicago, IL, USA). Anti-GAPDH antibody (Bioworld, Nanjing, China) was used as the loading control in all western blots. The secondary antibodies, anti-mouse and anti-rabbit IgGs conjugated to horseradish peroxidase, were obtained from Kangchen (Shanghai, China). Finally, protein bands were visualized using Supersignal West Femto Substrate (Pierce, Rockford, IL, USA).

2.6. Statistical Analysis. The results were presented as means ± SD. The statistical significance of differential findings was statistically evaluated using GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). Statistical significance for comparisons between groups was determined

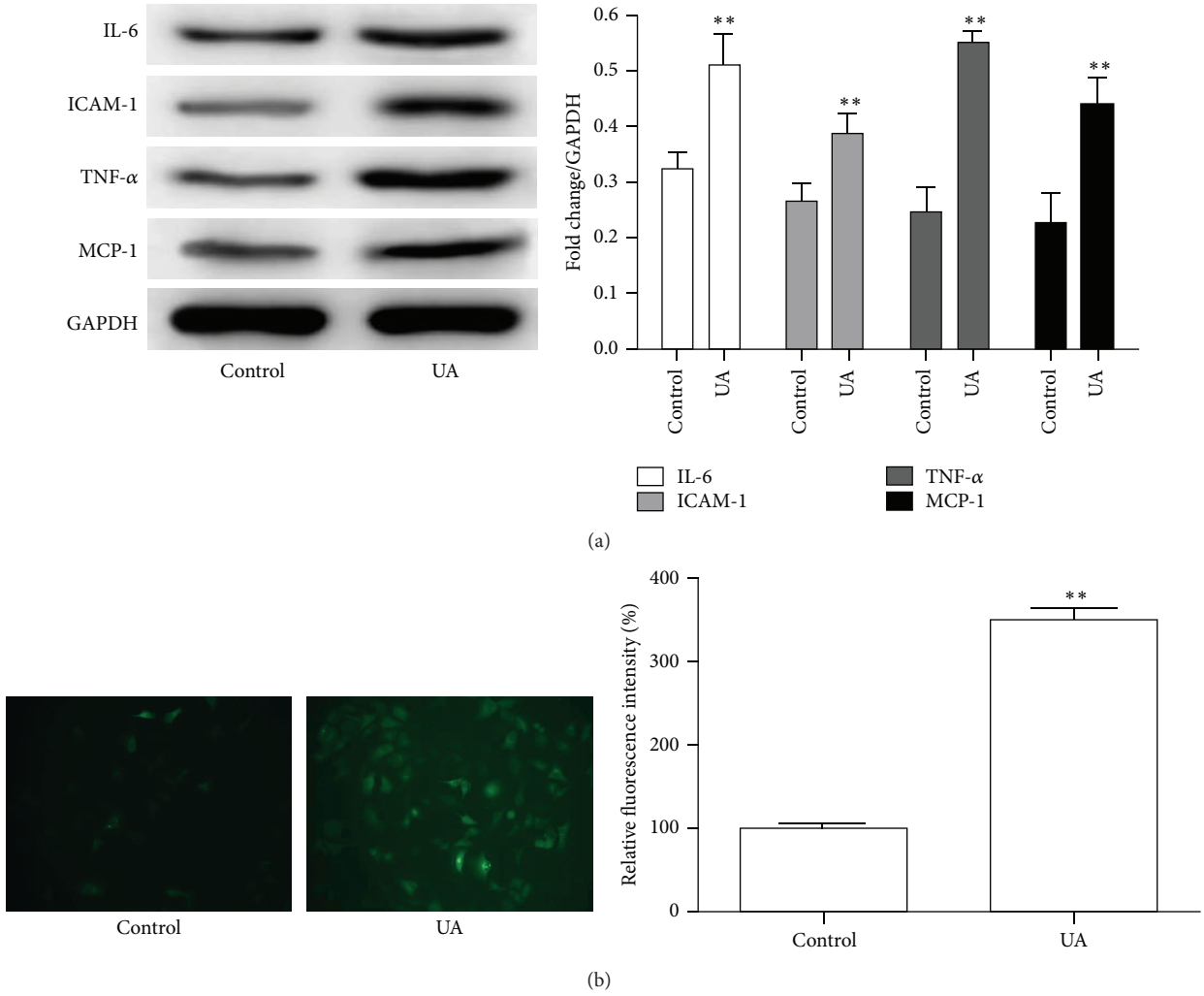

Figure 1: Effect of UA on the expression of IL-6, ICAM-1, MCP-1, and TNF-α and the ROS production in HUVEC. (a) Western blot analysis for IL-6, ICAM-1, MCP-1, and TNF-α in HUVEC after incubation with UA (8 mg/dL). (b) Representative images showing that intracellular ROS production was detected using CellROX Green Reagent. Histogram illustrating ROS production showed a different response compared with UA. $^{**}P < 0.01$.

using Student's paired two-tailed t-test or analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Results

3.1.1. High Uric Acid Level Induces Inflammatory Responses and Oxidative Stress in HUVEC. Previous studies indicated that hyperuricemia was associated with hypertension, systemic inflammation, and cardiovascular disease mediated by endothelial dysfunction and pathologic vascular remodeling [27]. To investigate the effects of UA on HUVEC, we examined the expression of inflammatory chemokines by western blot. UA remarkably increased the expression of IL-6, ICAM-1, MCP-1, and TNF-α at a concentration of 8 mg/dL (Figure 1(a)) in HUVEC. Recent studies have shown that inflammatory response induced the generation of reactive oxygen species (ROS) in an NADPH oxidase-dependent manner in endothelial cells [28]. Then we further investigated the effects of UA on the ROS production. As shown in Figure 1(b), UA significantly increased the ROS production in HUVEC (3.28 ± 0.34-fold, $P = 0.010$).

3.1.2. High Uric Acid Level Upregulates Notch-1 Expression and Activates NOTCH Signaling. Upregulation of Notch-1 played important roles in inflammatory reaction [13, 29]. To investigate whether Notch-1 is regulated by UA, we examined Notch-1 expression by introducing UA. As presented in Figure 2(a), UA induced intracellular Notch-1 levels in a dose-dependent manner, and maximal stimulation was achieved at 8 mg/dL ($P < 0.05$). The expression of Notch-1 induced by UA was also time-dependent, being significantly higher than that of control by 8 hours, peaking after 24 hours of stimulation ($P < 0.05$; Figure 2(b)). To further examine

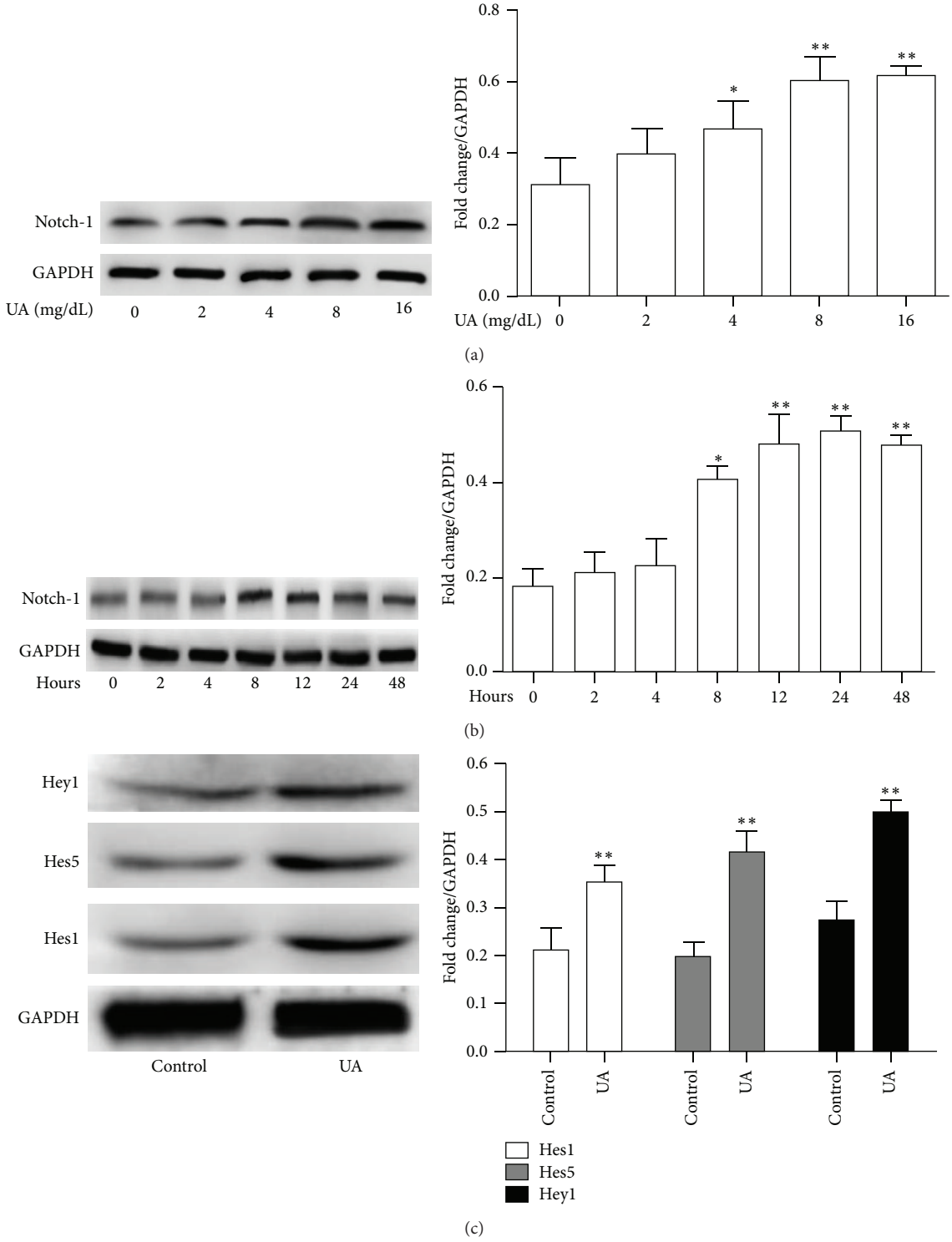

Figure 2: Dose- (a) and time-dependent (b) effect of UA on Notch1 expression in HUVEC. (a) UA enhanced the expression of Notch1 at concentrations of 8 mg/dL or higher compared with control. (b) UA-induced expression of Notch1 peaked at 8 hours and remained elevated at 48 hours. (c) Western blot analysis of Hes1, Hes5, and Hey1 protein expressions in HUVEC treated with UA (8 mg/dL) for 8 hours.

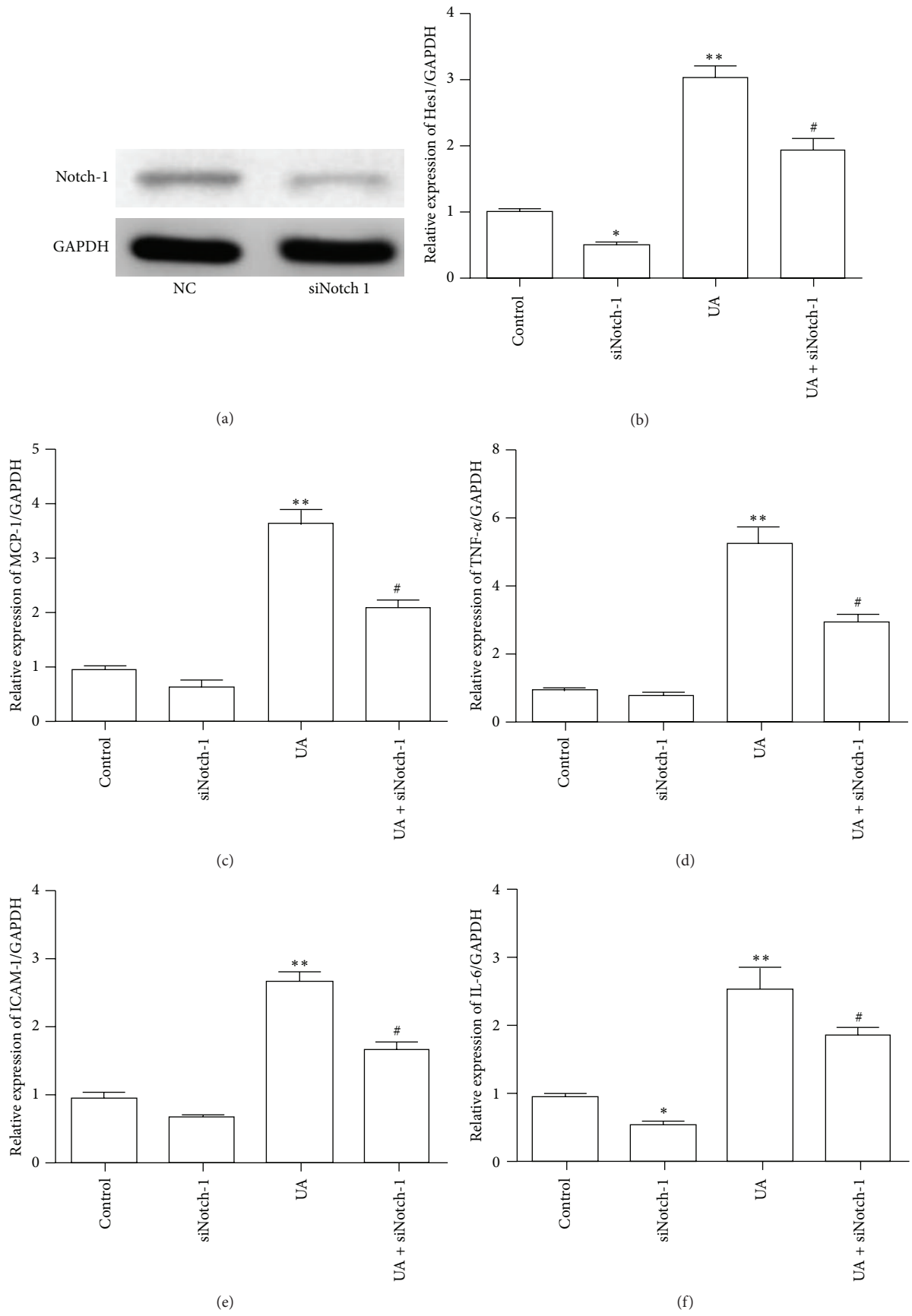

Figure 3: Continued.

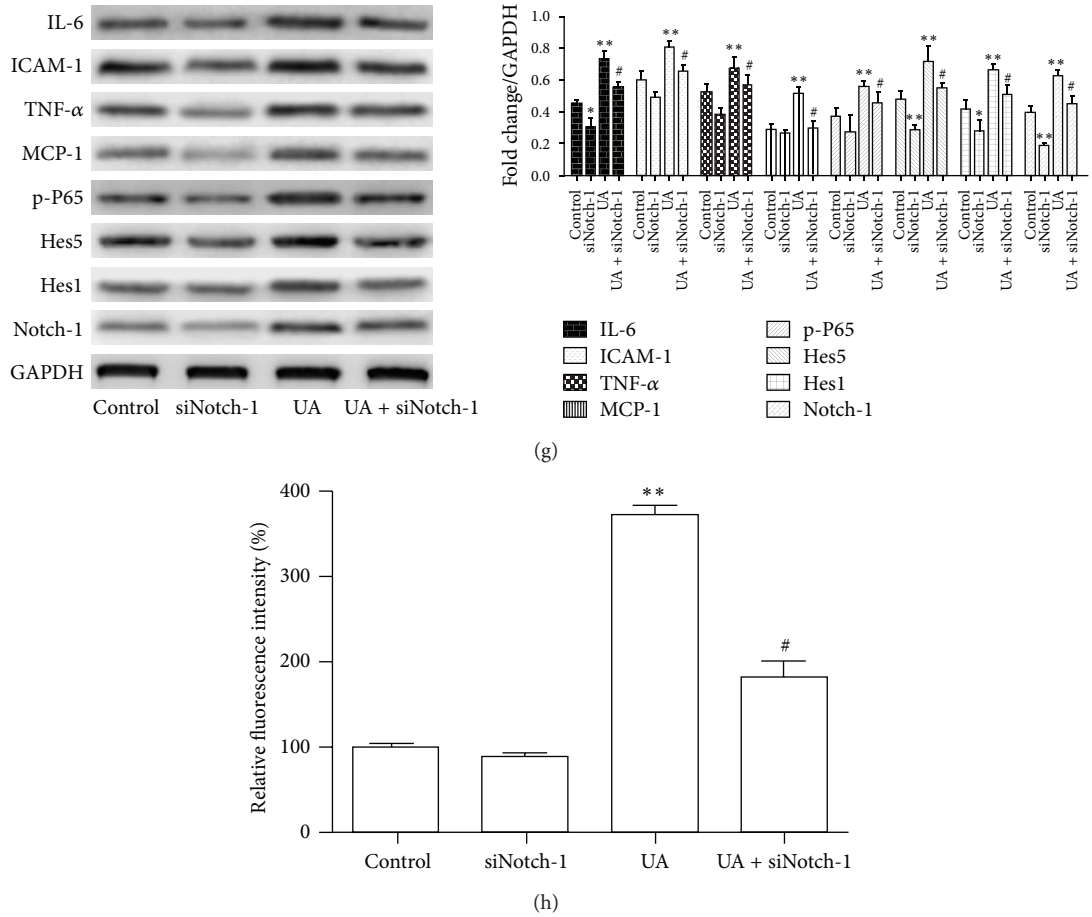

Figure 3: Notch signaling pathway was involved in UA-induced inflammatory reaction and oxidative stress. (a) The expression of Notch1 was confirmed by western blotting analysis in HUVEC transfected with Notch1 siRNA. (b–f) The Hes1, MCP-1, TNF-α, ICAM-1, and NF-κB mRNA were detected qRT-PCR in HUVEC with treatment as indicated. (g) Immunoblot analysis showing effects of UA with or without Notch1 inhibition by siRNA on the expression of Notch-1, IL-6, MCP-1, ICAM-1, TNF-α, Hes1, Hes5, and p-P65 in HUVEC. (h) Quantitative analysis of intracellular ROS production for the HUVEC with the indicated treatment. $^{*}P < 0.05$, $^{**}P < 0.01$.

the activation of NOTCH signaling after UA treatment, Hes1, Hes5, Hey1, and Hey2 protein expression were evaluated by western blot analysis. As shown in Figure 2(c), UA increases the protein levels of Hes1, Hes5, and Hey1, but not of Hey2 in HUVEC (data not shown). These data suggested that NOTCH signaling pathway was involved in damage induced by UA.

3.1.3. NOTCH Silencing Restricts UA-Induced Inflammatory Responses and Oxidative Stress. We had previously reported that UA led to increased mRNA expression of inflammatory chemokines, such as MCP-1, ICAM-1, P65, and TNF-α. Next, to further investigate whether activation of the NOTCH signaling pathway was involved in UA-induced inflammatory reaction, we transfected cells with Notch-1-specific siRNA and western blot analysis confirmed effective knockdown of Notch-1 (Figure 3(a)). As expected, downregulation by siRNA decreased the mRNA levels of IL-6, MCP-1, ICAM-1, P65, and TNF-α (Figures 3(b)–3(f)). To confirm these results, we also analyzed the protein levels and found that downregulation of Notch-1 expression resulted in decreased expression of p-P65, Hes1, IL-6, MCP-1, ICAM-1, and TNF-α, which was induced by UA (Figure 3(g)). Furthermore, we examined the ROS production when Notch-1 knockdown and our results showed that Notch-1 silencing significantly decreased the ROS production compared to control in HUVEC (Figure 3(h)). These results clearly indicated that Notch-1 plays a crucial role in UA-induced inflammatory reaction and oxidative stress.

3.1.4. EGCG Attenuate the Effect of UA. Studies have shown that the EGCG plays an important role in antioxidant and anti-inflammatory effects in multiple physiological processes [19, 20, 25]. To assess the biological activities of EGCG on HUVEC damaged by UA, we detected the protein levels of IL-6, MCP-1, ICAM-1, TNF-α, Notch-1, Hes1, Hes5, and Hey1 and the results showed that EGCG could significantly inhibit the expression of inflammatory chemokines (Figure 4(a)). Additionally, consistent with previous results, we found that preincubation with EGCG significantly suppresses ROS production induced by UA. Thus, these data indicated that EGCG effectively reduced inflammatory responses and

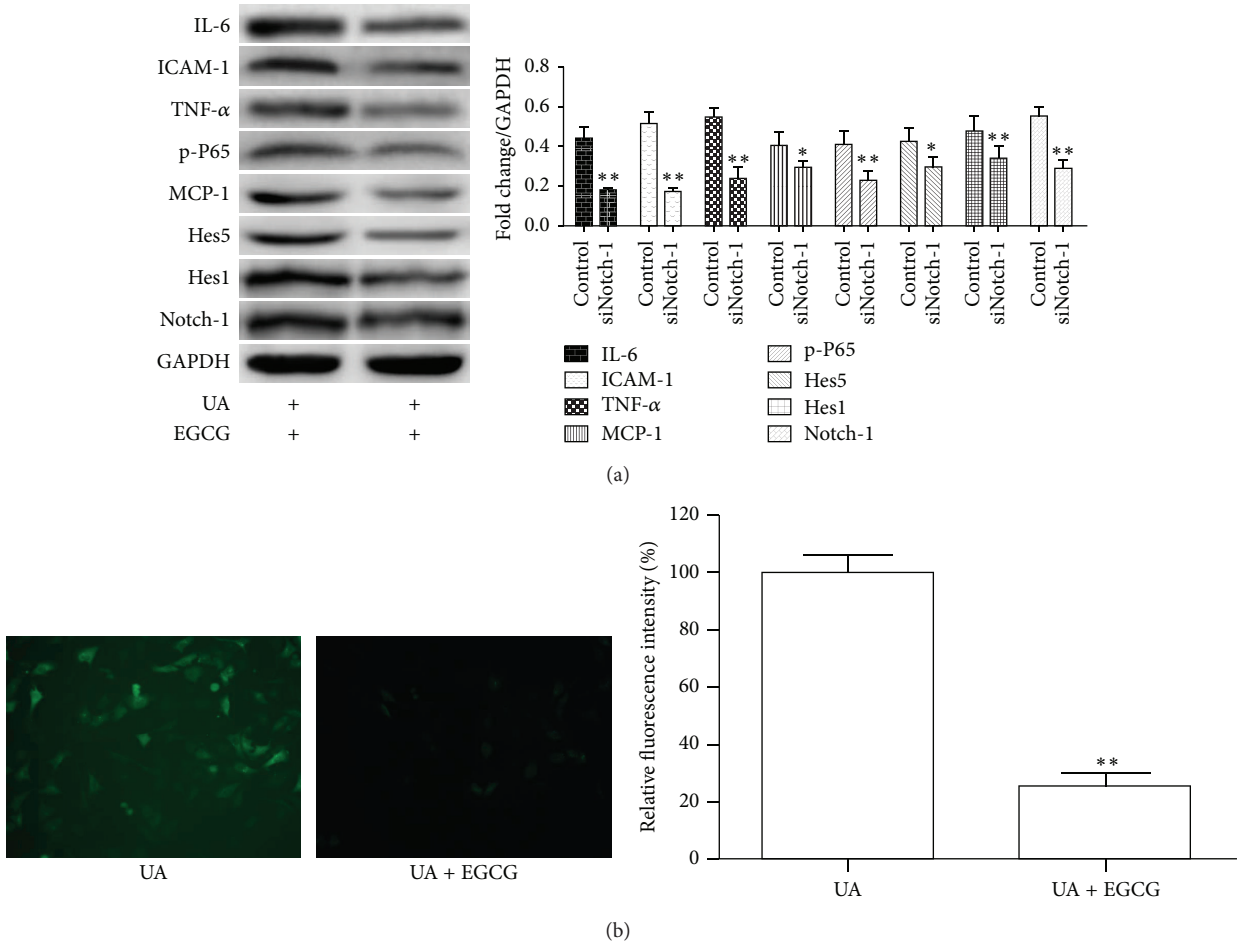

Figure 4: A cell model illustrating cardioprotection of EGCG on UA-induced inflammatory reaction and oxidative stress in HUVEC. (a) Immunoblot analysis reporting the protein levels of Notch1, IL-6, MCP-1, ICAM-1, TNF-α, Hes1, Hes5, and p-P65 in whole cell lysates of HUVEC with treatment as indicated. (b) Representative fields of intracellular ROS production in HUVEC treated with UA with or without EGCG. Corresponding densitometric analysis showed the relative ROS production. $^{**}P < 0.01$.

oxidative stress and might thus help in patients with hyperuricemia.

3.1.5. Overexpression of Notch-1 Reduced Protection of EGCG in HUVEC. To further investigate whether EGCG regulates Notch-1-mediated inflammatory responses and oxidative stress, we transfected cells with pcDNA3.1-Notch-1 plasmid. Western blot analysis showed that the protein level of Notch-1 expression was significantly increased in HUVEC compared to control vector transfected cells (Figure 5(a)). Moreover, to prove that the overexpression of Notch-1 could inhibit the EGCG decreased downstream protein, we performed western blotting analysis. As we expected, the protein level of IL-6, MCP-1, ICAM-1, TNF-α, Notch-1, Hes1, and Hes5 was reversed after Notch-1 overexpression (Figure 5(b)). Consistent with above results, there was no significant difference in HUVEC transfected with Notch-1 with or without EGCG (Figure 5(c)). In summary, our results indicated that EGCG regulated UA-induced cell damage partly through mediating NOTCH signaling pathway.

3.2. Discussion. Previously, we have shown the cardioprotection of EGCG against inflammatory lesions induced by UA via the NF-κB signaling pathway. In the current study, we further investigated the effects of UA and EGCG on inflammatory responses and oxidative stress in HUVEC. We found that Notch-1 was involved in UA-induced inflammatory responses and oxidative stress. Moreover, we found that pretreatment with EGCG significantly decreased the expression of Notch-1 and its downstream genes. When we were writing this paper, Jatuworapruk et al. reported that EGCG could modestly lower serum uric acid (SUA) level and significantly elevated serum antioxidant capacity in healthy individuals [30]. These results, for the first time, provided a mechanistic link between the high levels of UA and the antioxidant and anti-inflammatory effects of EGCG.

Recent studies have shown that hyperuricemia characterized by high serum uric acid level was associated with hypertension, gout, systemic inflammation, and cardiovascular disease mediated because of endothelial dysfunction and pathologic vascular remodeling [1, 2, 13, 27]. In the present study, treating the HUVEC with UA, we found that UA

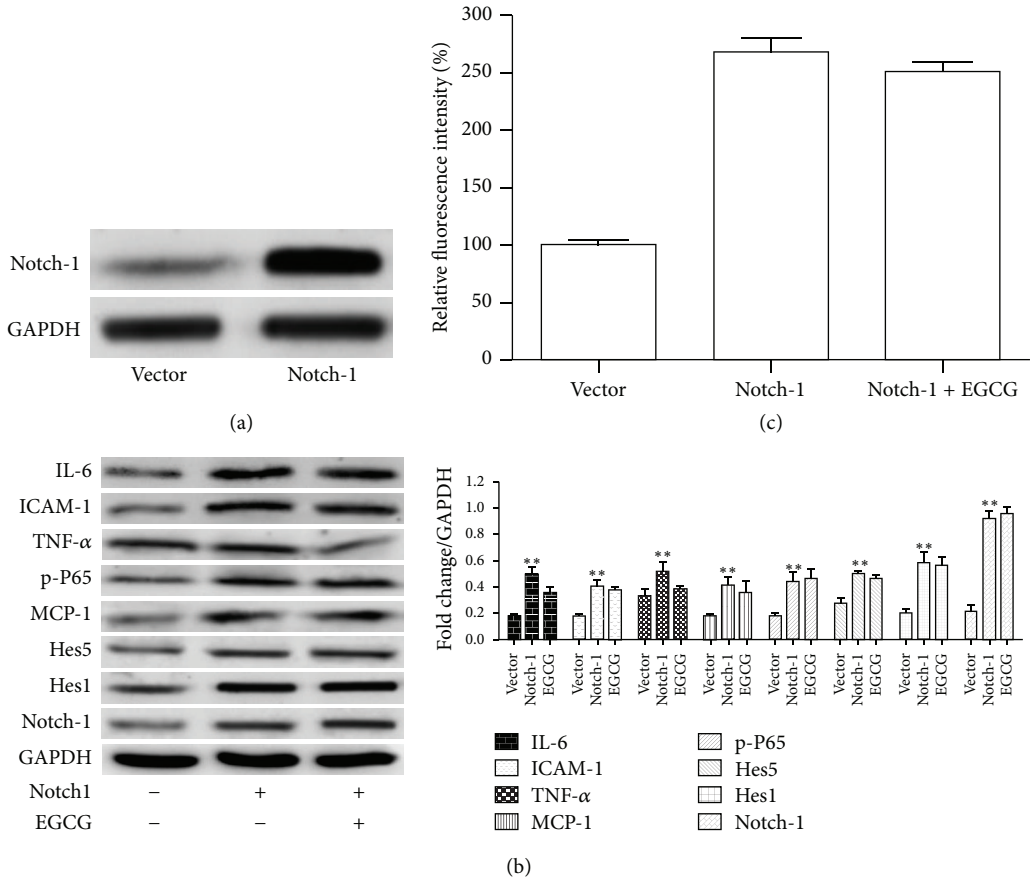

Figure 5: Effects of EGCG on UA-medicated Notch inflammatory signaling pathway. (a) Western blotting analysis of Notch1 was performed to assess the Notch1 transfection in HUVEC. (b) Representative western blot images of proteins of the Notch pathway and inflammatory chemokines detected in whole-cell lysates with antibodies indicated. HUVEC were transfected with Notch1 cDNA with or without EGCG in medium containing UA (mg/dL). (c) Quantitative analysis of intracellular ROS production for the HUVEC with the indicated treatment.

significantly increased the inflammatory cytokines and ROS production. Recent evidence has demonstrated that NOTCH signaling played an important role during inflammation in cardiovascular disorders [31]. However, the roles of NOTCH signaling in HUVEC were not well understood. Then we focused on the effect of UA on the Notch-1 expression. Our findings indicated that UA activated Notch-1 in a dose- and time-dependent manner. NOTCH target genes Hes1, Hes5, Hey1, and Hey2 were further determined at protein level and the expression of Hes1, Hes5, and Hey1, but not Hey2, was significantly increased. To further assess UA-induced inflammatory responses and oxidative stress involved in NOTCH signaling, we knockdown Notch-1 expression by siRNA. Inhibition of Notch-1 expression remarkably decreased the downstream genes of Notch-1 and inflammatory chemokines both at mRNA and protein levels. Our results suggested that the effects of UA in HUVEC resulted in inflammatory responses and oxidative stress involved in altering the expression of Notch-1 and its downstream genes.

Previous study showed that EGCG has potent properties of antioxidant and radical-scavenger [19, 24, 25]. Chen et al. have reported that EGCG could protect H9c2 rat cardiomyoblasts against H_2O_2-induced oxidative stress via the Akt/GSK-3β/β-catenin and caveolae signaling [32]. It reminds us whether EGCG could reduce the expression of inflammatory chemokines and generation of ROS. As we expected, EGCG pretreatment reduced the expression of inflammatory chemokines and prevented the release of ROS. Recent study has shown that EGCG inhibited the Notch signaling to suppress the proliferation of colorectal cancer cells [26], in order to determine whether the molecular mechanism by which EGCG inhibited inflammatory responses and oxidative stress of HUVEC cell involved in NOTCH signaling proteins. Supporting this, overexpression of Notch-1 attenuated the effects of EGCG and enhanced the expression of inflammatory chemokines and the release of ROS. The results of this study could be useful for patient with hyperuricemia in order to improve the life quality.

4. Conclusions

In summary, our current findings suggested that EGCG inhibited the UA-induced inflammatory responses and oxidative stress through Notch-1 medicating inflammatory chemokines and ROS production. However, further investigations are required to determine the real effect in vivo.

Disclosure

The funding sources had no role in study design, data collection, analysis and interpretation, the writing of the report, or the decision to submit the paper for publication.

Conflict of Interests

The authors declare that there is no conflict of interests.

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