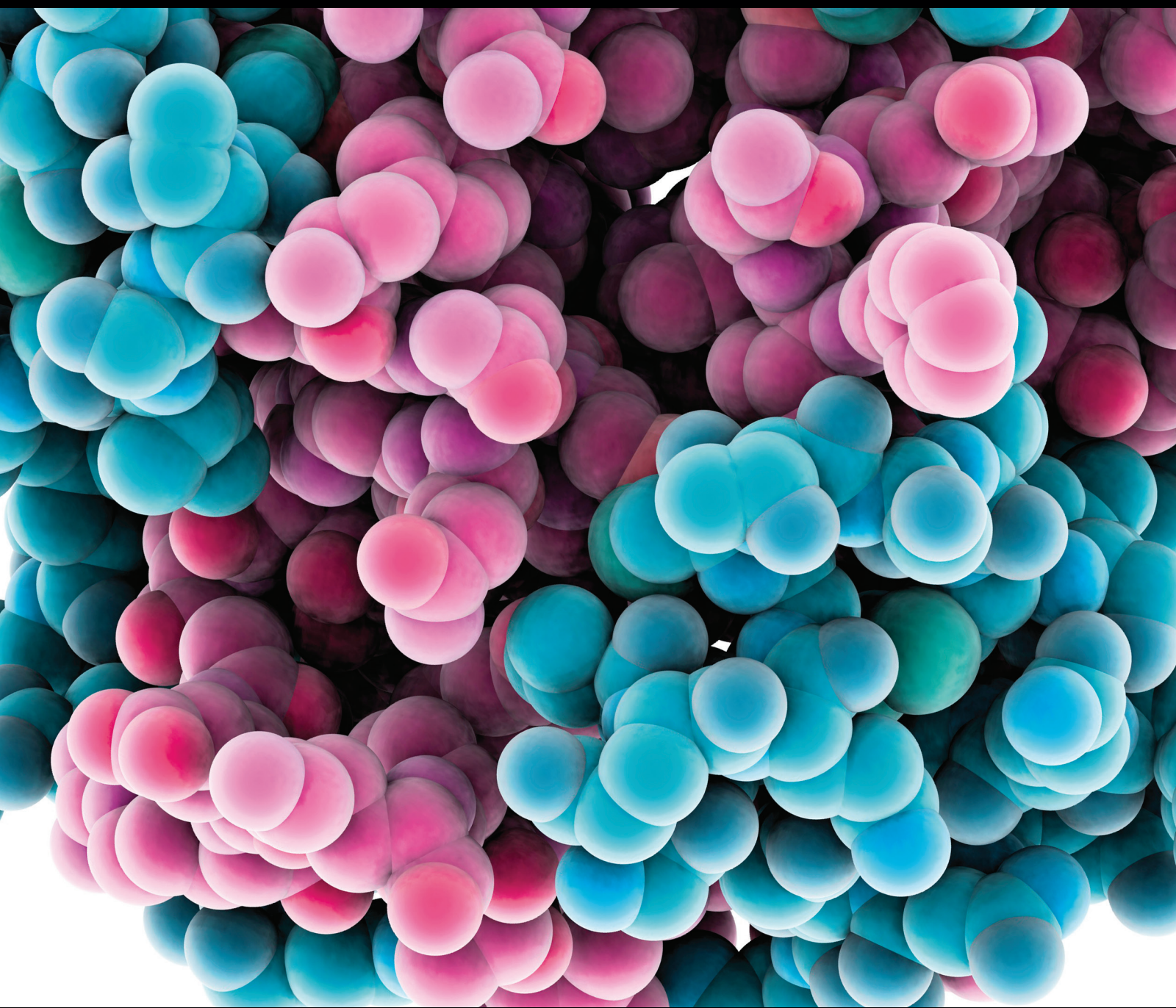
Journal of Diabetes Research

Targeting Oxidative Stress in Diabetic Complications: New Insights

Lead Guest Editor: Hao Wu
Guest Editors: Lu Cai, Judy de Haan, and Robertina Giacconi



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Editorial Board

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Editorial

Targeting Oxidative Stress in Diabetic Complications: New Insights

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Diabetes is a serious disease, and the number of adults affected by diabetes is predicted to grow worldwide from 422 million (estimated number in 2014) to 642 million by 2040. Diabetes causes high morbidity and mortality through its complications, with oxidative stress known to be a major contributor. The present special issue, which includes 3 original research articles and 4 review papers, is devoted to novel insights into targeting oxidative stress in diabetic complications.

New strategies designed to attenuate diabetes-induced oxidative stress, through the blockage of reactive oxygen species (ROS) generation and the enhancement of antioxidant-scavenging activity, are revealed in the original research articles published in this special edition. NADPH oxidase 4 (NOX4) is a key factor that generates ROS and contributes to the pathogenesis of diabetic kidney disease (DKD). Inhibition of NOX4 improves DKD. In the report by F. Hu et al., early growth response protein 1 (EGR1) was found to be a transcription factor involved in the regulation of the *Nox4* gene and responsible for the diabetes-induced renal oxidative stress. This study highlights that EGR1 is a potential novel target in future management of DKD. Diabetes-induced advanced glycation end products (AGE) lead to aberrant angiogenesis in the brain. The study by A. Alhusban et al. showed that silymarin, a milk thistle seed extract, attenuated AGE-induced aberrant angiogenesis by glycogen synthase kinase-3β-mediated inhibition of VEGF release. In addition to blocking ROS generation, activation of the nuclear factor-erythroid-2-related factor 2 (NRF2) antioxidant pathway is another effective approach. K. Shukla et al. reported that the aldose reductase inhibitor fidarestat alleviated oxidative stress during hyperglycemic stress via activation of NRF2 signaling, the effect of which enhanced the antioxidant-scavenging capacity.

The review articles in the present special issue have updated the findings of several complications of diabetes, such as cardiomyopathy, nephropathy, polyneuropathy, and cognitive dysfunction. B. Yan et al. have provided an overview of antioxidant natural products in the prevention of diabetic cardiomyopathy, with an emphasis being on key targets of natural products. Y. Yang et al. reviewed the effect of serotonin and its receptor on the induction of oxidative stress in DKD, suggesting the targeting of this axis as a therapeutic strategy for DKD and macrovascular complications. The review by S. Sifuentes-Franco et al. summarized the role of oxidative stress in diabetic polyneuropathy. Finally, Y. Cao et al. reviewed the multiple functions of sirtuin 1 in diabetic cognitive dysfunction, including its antioxidant activity.

Collectively, the present special issue reviews previous work and provides new findings in diabetic complications,

with an emphasis on targeting oxidative stress. We hope that this special issue brings new insight for future management of diabetic complications.

Acknowledgments

We would like to express our gratitude to all the authors and the reviewers for their contribution to this special issue.

Hao Wu
Lu Cai
Judy B. de Haan
Robertina Giacconi

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Research Article

Early Growth Response 1 (Egr1) Is a Transcriptional Activator of NOX4 in Oxidative Stress of Diabetic Kidney Disease

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Background. NADPH oxidase 4 (NOX4) plays a major role in renal oxidative stress of diabetic kidney disease (DKD). NOX4 was significantly increased in Egr1-expressing fibroblasts, but the relationship between Egr1 and NOX4 in DKD is unclear. *Methods*. For the evaluation of the potential relationship between Egr1 and NOX4, both were detected in HFD/STZ-induced mice and HK-2 cells treated with TGF-β1. Then, changes in NOX4 expression were detected in HK-2 cells and mice with overexpression and knockdown of Egr1. The direct relationship between Egr1 and NOX4 was explored via chromatin immunoprecipitation (ChIP). *Results*. We found increased levels of Egr1, NOX4, and α-SMA in the kidney cortices of diabetic mice and in TGF-β1-treated HK-2 cells. Overexpression or silencing of Egr1 in HK-2 cells could upregulate or downregulate NOX4 and α-SMA. ChIP assays revealed that TGF-β1 induced Egr1 to bind to the NOX4 promoter. Finally, Egr1 overexpression or knockdown in diabetic mice could upregulate or downregulate the expression of NOX4 and ROS, and α-SMA was also changed. *Conclusion*. Our study provides strong evidence that Egr1 is a transcriptional activator of NOX4 in oxidative stress of DKD. Egr1 contributes to DKD by enhancing EMT, in part by targeting NOX4.

1. Introduction

Because of its high incidence and mortality rates, diabetic kidney disease (DKD) has become the most severe microvascular complication of diabetes mellitus [1]. In recent years, renal tubule fibrosis has been shown to crucially involved in the development of DKD [2]. Epithelial-mesenchymal transition (EMT), a characteristic of renal tubule fibrosis, eventually leads to renal fibrosis. During EMT, the epithelial cells lose their characteristic properties and gain mesenchymal features, resulting in upregulation of the mesenchymal protein alpha-smooth muscle actin (α-SMA) [3].

Genetic factors, abnormal glucose metabolism, and glomerular hemodynamic changes are also involved in DKD development. Among them, oxidative stress is a common mechanism [4]. NADPH oxidase 4 (NOX4), a member of the NADPH oxidases, has been confirmed to be the most important factor in DKD by generating superoxide and other reactive oxygen species (ROS) [5]. TGF-β1, a crucial pathogenic factor in DKD, can strongly induce NOX4 expression and is involved in the myofibroblast transformation [6]. Local activation of the renin-angiotensin system by angiotensin is regarded as the strongest factor contributing to NOX4 activation [7]. NOX4 is primarily expressed in renal tubule cells, and its upregulation indicates the degree of renal tubular injury in DKD [7]. Apocynin (Apo), a NOX inhibitor, has been shown to effectively alleviate kidney injury [8, 9].

Early growth response factor 1 (Egr1), a member of the immediate early response family, is highly expressed in a variety of kidney cells, including kidney proximal tubular

epithelial cells [10]. Recent studies have indicated that Egr1 plays a crucial role in the pathogenesis of renal fibrosis by activating the promoters of collagen 1α1 (COL1A1), cartilage oligomeric matrix protein (COMP), periostin, matrix metalloproteinase 2 (MMP2) [11], tissue metallopeptidase inhibitor 1 (TIMP1), and osteopontin (OPN). Previous research in our department revealed that Egr1 has a crucial effect on the development of DKD by binding to the TGF-β promoter [12].

Using genome-wide expression profiling, Bhattacharyya [13] identified 647 genes whose expression was substantially changed by Egr1 in Egr1-expressing fibroblasts. One of these genes was NOX4, which showed an > eightfold increase. The increased expression of NOX4 caused by chronic hypoxia in pulmonary artery smooth muscle cells was mediated by Egr1 [14, 15]. In systemic sclerosis patients, increased expression of NOX4 enhances oxidative stress and further promotes fibrosis, during which elevation of Egr1 may play an important role [16, 17]. However, no studies have explored the direct relationship between Egr1 and NOX4. Through bioinformatics analyses, we discovered that the NOX4 gene contains putative Egr1-binding sites in its promoter region. To test this hypothesis, we investigated the regulation of NOX4 by Egr1 both in vitro and in vivo. The direct relationship between Egr1 and NOX4 was explored via chromatin immunoprecipitation (ChIP).

2. Materials and Methods

2.1. Animals. Male C57BL/6J mice (Animal Center of Guangdong province, 3-4 w) weighing 15-16 g received a high-fat diet (protein 26.2%, fat 34.9%, and carbohydrate 26.3%) for four weeks and then received a single injection of streptozotocin (STZ) (120 mg/kg, i.p., in citrate buffer, pH = 4.5, MP Biomedicals). Blood glucose was measured once a week, and a sustained blood glucose level of >16.7 mM for 16 weeks was considered an indicator of hyperglycemia. Control mice were injected with an equal volume of sodium citrate. The renal weight index was evaluated. At the end of 2 weeks of STZ injection, 30 mice were randomly assigned to one of five groups: HFD/STZ-induced diabetic mice (DM; $n = 6$), pcDNA3-Egr1-treated diabetic mice (EDM; $n = 6$), pcDNA3-vector-treated diabetic mice (PDM; $n = 6$), pGPU6-vector-treated diabetic mice (GDM; $n = 6$), and pGPU6-shEgr1-treated diabetic mice (shEDM; $n = 6$). EDM, PDM, GDM, and shEDM mice were administered the corresponding plasmid once a week for four weeks (12–16 w) via rapid injection of a large volume of DNA solution through the tail vein [18]. Animals were fed an HFD in a specific pathogen-free facility. Control and DM mice were sacrificed 12 weeks after modeling. EDM, PDM, GDM, and shEDM mice were sacrificed 16 weeks after modeling. Animal studies were conducted in accordance with the established institutional and state guidelines for the care and use of laboratory animals.

2.2. Metabolic Profile Analysis. At the end of the study, urine samples for 24 h were collected in metabolism cages. Blood samples were collected from the orbital sinus after inhalation of CO_2 and fasting for 8 h. Triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, glucose, creatinine, and glycated hemoglobin (HbA1c) were detected using ELISAs (Shanghai Fanke Biotechnology Co., Ltd.) according to the manufacturer's instructions. Urinary albumin was determined by ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA). Renal cortices were collected, quickly frozen in liquid nitrogen, and then stored at −80°C for later analysis.

2.3. Cell Culture and Transfection. Human proximal tubular epithelial (HK-2) cells were cultured as described previously [19]. Depending on the experiments, HK-2 cells were treated with recombinant human TGF-β1 (10 ng/mL; Gibco, New York, NY, USA), pENTER-Egr1 plasmid (2 μg; Vigene Biosciences, Shandong, China), and small interfering RNA targeting Egr1 (siEgr1, 50 nM, RiboBio, Guangzhou, China). Transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen, USA) following the manufacturer's instructions when cells were cultured to approximately 50–60% confluence in 12-well plates. Cells were harvested 48 h after transfection. TGF-β1 (10 ng/ml) was added to the culture medium 24 h before cell collection.

2.4. RNA Isolation and Real-Time Quantitative PCR (RT-qPCR). Total RNA was extracted from renal cortices and HK2 cells with TRIzol (Dingguo, Beijing, China) according to the manufacturer's instructions. After reverse-transcription using *M-MLV* reverse transcriptase (Invitrogen, Carlsbad, CA, USA), the gene expression levels were determined by a Roche 480 thermal cycler using 40 ng of cDNA, SYBR Select Master Mix (Invitrogen), and the respective primers (Invitrogen, Table 1). The cycling conditions were described previously [12]. The relative mRNA expression level of each gene was calculated by the comparative $2^{-\Delta\Delta Ct}$ method, with β-actin as the reference.

2.5. Western Blotting Assays. Total protein was extracted from renal cortices and HK-2 cells using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of protein (20–50 μg) were electrophoresed in 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA, USA) and then were transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, MA, USA). PVDF membranes were blocked with 5% skim milk in 0.1% Tris-buffered saline with Tween-20 for 1 h and then incubated overnight with a primary antibody against Egr1 (1 : 400 dilution; Santa Cruz Biotechnology), NOX4 (1 : 200 dilution; Santa Cruz Biotechnology), or α-SMA (1 : 800 dilution; Santa Cruz Biotechnology). Finally, PVDF membranes were incubated with peroxidase-conjugated secondary antibody (1 : 15,000 dilution; LI-COR Biosciences, NE, USA) for 1 h at room temperature. Fluorescence was obtained using an Odyssey infrared imaging system (LI-COR) and quantified by ImageJ software.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). Total protein was extracted from renal tissues using normal saline and a grinding machine. The samples were centrifuged for 10 min at 3000 ×g at 2–8°C within 30 min of collection and

Table 1: Sequences of primers for quantitative RT-PCR used in this study.

Gene	Primers
hEgr1	(f) CTGACCGCAGAGTCTTTTCCTG (r) TGGGTGCCGCTGAGTAAATG
mEgr1	(f) CCTTTTCTGACATCGCTCTGAA (r) CGAGTCGTTTGGCTGGGATA
hNOX4	(f)CTTTTGGAAGTCCATTTGAG (r) CGGGAGGGTGGGTATCTAA
mNOX4	(f) ACAATCTTCTTGTTCTCCTGCT (r) CATCCTTTTACCTATGTGCCG
hβ-actin	(f) CCCTGGACTTCGAGCAAGAGAT (r) GTTTTCTGCGCAAGTTAGG
mβ-actin	(f) CGAGCGTGGCTACAGCTTCA (r) AGGAAGAGGATGCGGCAGTG
hα-SMA	(f) ATCCTCCCTTGAGAAGAGTT (r) ATGCTGTTGTAGGTGGTTTC
mα-SMA	(f) TGGATCAGCGCCTTCAGTTC (r) GGCCAGGGCTAGAAGGGTA

Table 2: Primer sequences for PCR of ChIP-enriched DNA used in this study.

Gene	Primers
hNOX4	(f) ATCTGGAGGCTCTGCTGGTA (r) GGCATGCTGTGAGAAGTTCA

stored at −20°C or −80°C. The expression levels of NOX4, α-SMA, and ROS were detected using an ELISA (Shanghai Fanke Biotechnology Co., Ltd.) according to the manufacturer's instructions.

2.7. Kidney Histology and Immunohistochemistry. Kidney tissue was first embedded in paraffin and then cut into 4 μm thick sections. Sections were stained with Masson's trichrome by standard protocol. To observe location and expression of target protein in kidney tissue, the sections were dealt with a series of steps below: dewaxing, closing endogenous peroxidase by hydrogen peroxide, antigen repairing, normal serum closing, dropping the first antibody(Egr1-, NOX4-, and α-SMA-specific antibodies), dropping biotinylated secondary antibody, dropping triple antibody (SAB complex), dropping tris Anti (SAB complex), and staining by hematoxylin. The pathological sections were observed under light microscope. Five high magnification (400x), perspectives were randomly selected from each samples. Semiquantitative analysis was performed adopting Image Pro-plus 6.0 software.

2.8. Chromatin Immunoprecipitation (ChIP). ChIP was carried out using a ChIP-IT® Express kit (Active Motif, Bedford, MA, USA) according to the protocols. Anti-Egr1 antibody (Santa Cruz Biotechnology) or control IgG was used to cross-link protein DNA as shown in Table 2 (Sangon Biotech, Shanghai, China). RT-qPCR was used to detect the enrichment effect. We calculated the fold enrichment using the slope of the standard curve.

Table 3: Metabolic Profile Analysis of mouse parameters.

	Ctrl	DM
Glucose (mM)	5.65 ± 0.34	22.38 ± 1.13[b]
HbA1c (%)	5.31 ± 0.40	9.83 ± 0.56[b]
Urine microalbumin (μg/24 h)	6.23 ± 0.55	78.75 ± 4.06[b]
Renal weight index (mg/kg)	6.42 ± 0.45	15.57 ± 1.12[b]
Body weight (g)	27.31 ± 1.97	23.30 ± 1.46
Creatinine (mM)	177.67 ± 12.54	174.15 ± 2.31
CH (mM)	3.77 ± 0.24	6.12 ± 0.58[b]
TG (mM)	5.06 ± 0.40	7.54 ± 0.51[b]
LDL-C (mM)	2.12 ± 0.12	4.06 ± 0.23[b]

Mean ± SD; $n = 6$; [a]$P < 0.05$ and [b]$P < 0.01$ versus control

2.9. Dual-Luciferase Reporter Gene Assay. NOX4 promoter (-2000-0) luciferase reporter plasmid was constructed adopting the GV238-REPORT vector (GENE, Shanghai, China). 293 T cells were seeded in 24-well plates and transfected with 0.5 μg of GV238 plasmid, 0.2 μg of β-gal plasmid, pENTER-Egr1 plasmid (0, 0.2, 0.4, 0.6, 0.8, and 1.0μ g), and pENTER-vector plasmid (1.0, 0.8, 0.6, 0.4, 0.2, and 0 μg). Lipofectamine® 3000 (Invitrogen, CA, USA), OptiMEM (Gibco, CA, USA), and β-gal were transfected as a transfection control. Cells were harvested 48 h after transfection and analyzed adopting luciferase assay kits (Beyotime, China). All experiments were performed in triplicate.

2.10. Statistical Analysis. Values are expressed as the mean ± SD. Two-tailed Student's t-test was used for two independent sample comparisons. Statistical analysis was performed using SPSS 13.0 software (IBM, IL, USA). The statistically significant level was set at $P < 0.05$.

3. Results

3.1. In Vivo Correlation between EMT in DM and Elevated Expression of Egr1, NOX4, and ROS. Based on the data shown in Table 3 and Masson's trichrome staining (Figure 1(g)), our animal model was successfully constructed. We examined renal cortices in mice at 12 weeks because a previous study showed that Egr1 was statistically higher in DM mice than in controls at this age [20].

Egr1 mRNA levels were fivefold higher in kidneys of DM mice than in kidneys of the control mice as shown by quantitative RT-PCR assays (Figure 1(a)). Egr1 protein levels were substantially higher in DM mice than in control mice as indicated by Western blotting assays (Figures 1(b) and 1(c)) and immunohistochemistry (Figure 1(g)). Furthermore, quantitative RT-PCR assays revealed that NOX4 and α-SMA expression was upregulated in the kidneys of DM mice compared to control mice (Figure 1(a)), which was further confirmed by Western blotting assays (Figures 1(b) and 1(c)), ELISAs (Figures 1(d) and 1(f)), and immunohistochemistry (Figure 1(g)). ROS, which are produced by

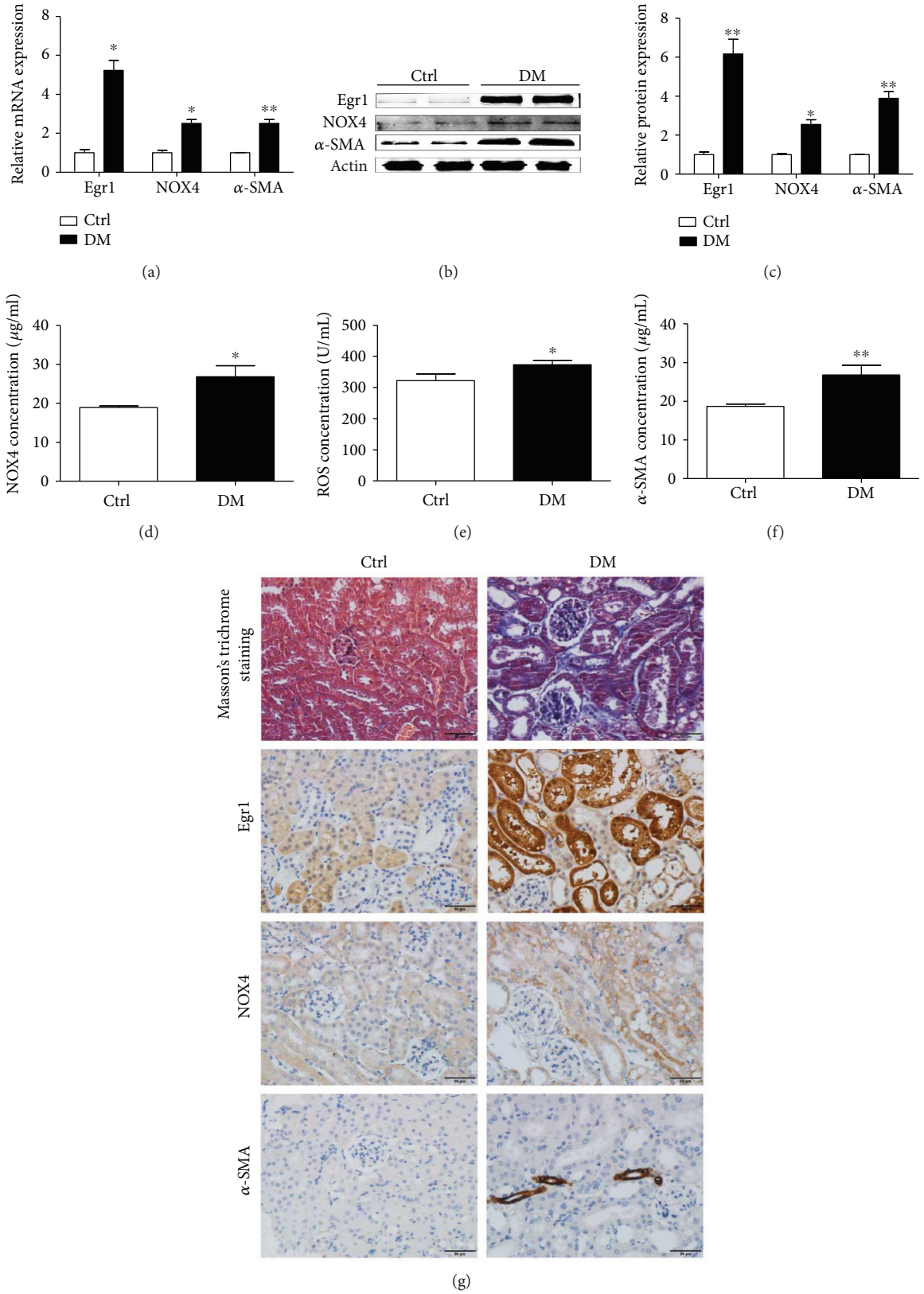

Figure 1: Egr1, NOX4, ROS, and α-SMA expression in control and HFD/STZ-induced diabetic mice at 12 weeks. (a) Levels of Egr1, NOX4, and α-SMA mRNA were measured using RT-qPCR. (b) Levels of Egr1, NOX4, and α-SMA protein were measured using Western blotting assays. (c) Semiquantitative levels of Egr1, NOX4, and α-SMA protein. (d) NOX4 concentration, (e) ROS concentration, and (f) α-SMA concentration were measured using ELISAs. The results are expressed as foldchange over baseline (control group). Values are the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. (g) Masson staining and Immunohistochemical staining of Egr1, NOX4, and α-SMA.

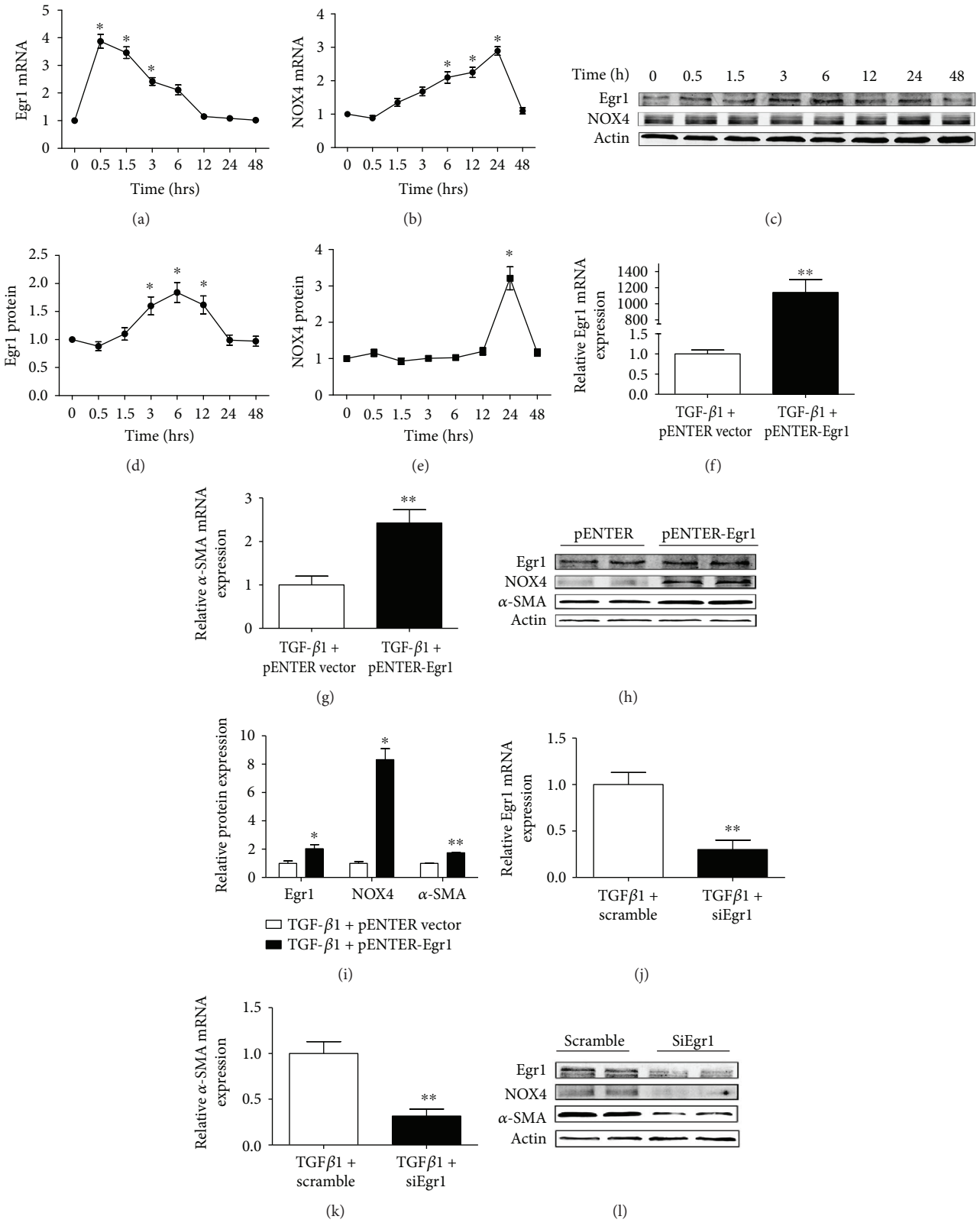

Figure 2: Continued.

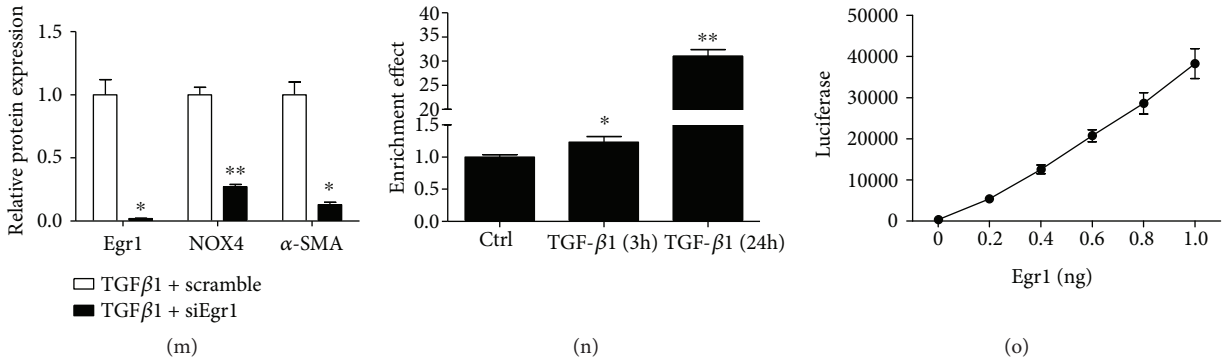

Figure 2: Changes in NOX4 expression with overexpression and knockdown of Egr1 in TGF-β1-treated HK-2 cells. (a, b, and c) Egr1 and NOX4 protein were measured using Western blotting assays in HK-2 cells treated with TGF-β1 (10 ng/mL) at 0 h, 0.5 h, 1.5 h, 3 h, 6 h, 12 h, 24 h, and 48 h. (d and e) Semiquantitative levels of Egr1 and NOX4 protein. (f) Cells were treated with either pENTER-Egr1 overexpression plasmid or with a pENTER vector for 48 h and then exposed for 3 h and 24 h to TGF-β1 (10 ng/mL). Levels of Egr1 were detected at 3 h, and levels of NOX4 and α-SMA were detected at 24 h after TGF-β1 (10 ng/mL). Levels of Egr1 mRNA were measured by RT-qPCR. (g) Levels of α-SMA mRNA were measured by RT-qPCR. (h and i) Levels of Egr1, NOX4, and α-SMA protein were measured using Western blotting assays. (j) Cells were either silenced with siEgr1 or treated with a scrambled control RNA for 48 h and then exposed for 24 h to TGF-β1 (10 ng/mL). Levels of Egr1 mRNA were measured using RT-qPCR. (k) Levels of α-SMA mRNA were measured using RT-qPCR. (l and m) Levels of Egr1, NOX4, and α-SMA protein were measured using Western blotting assays. The above results are expressed as foldchange over baseline. Values are the mean ± SD. $^*P < 0.05$ and $^{**}P < 0.01$ versus control group. (n) ChIP test to explore Egr1 binding to the NOX4 promoter. ChIP was performed using an anti-Egr1 antibody or a negative control IgG antibody in HK-2 cells treated for 3 h and 24 h with TGF-β1 (10 ng/mL). Immunoprecipitated DNA was subjected to RT-qPCR using specific NOX4 primers that included the Egr1 binding sites. (o) Dual-luciferase reporter gene assay to explore Egr1 binding to the NOX4 promoter.

NOX4, were shown to be increased in DM mice by ELISA (Figure 1(e)). In addition, NOX4 and α-SMA were mostly located in the renal tubule, while Egr1 was located in the glomerulus and renal tubule by immunohistochemistry (Figure 1(g)).

3.2. Changes in NOX4 Expression with Overexpression and Knockdown of Egr1 in TGF-β1-Treated HK-2 Cells. To explore whether Egr1 and NOX4 further contribute to the DKD process, we tested Egr1 and NOX4 expression levels in TGF-β1-treated HK-2 cells. We chose HK-2 cells as the model because both Egr1 and NOX4 are present in these cells. TGF-β1-treated HK-2 cells are a classic cell model of DKD. We found increased Egr1 and NOX4 mRNA and protein levels under stimulation with recombinant TGF-β1 (10 ng/mL) (Figures 2(a)–2(e)).

Then, we explored the changes in NOX4 expression with overexpression and knockdown of Egr1 in TGF-β1-treated HK-2 cells. Transfection of cultured HK-2 cells with a plasmid encoding Egr1 (pcDNA3-Egr1) increased the expression of Egr1 (Figure 2(f)). The NOX4 protein expression was increased by eightfold (Figure 2(h) and 2(i)), and α-SMA was subsequently increased (Figures 2(g)–2(i)). In addition, transfection of cultured HK-2 cells with siEgr1 decreased the expression of Egr1 (Figure 2(j)). Subsequently, the NOX4 and α-SMA protein levels decreased (Figure 2 L-M). Next, HK-2 cells were stimulated with TGF-β1 (10 ng/mL) for 3 h and 24 h, and a ChIP assay was performed. TGF-β1 strongly induced Egr1 to bind to the NOX4 promoter over time. No enrichment was observed in the control IgG group (Figure 2(n)). The same results could be obtained by dual-luciferase reporter gene assay (Figure 2(o)).

3.3. Changes in NOX4 Expression with Overexpression and Knockdown of Egr1 in DM Mice. Hydromechanics, which has been effectively used for transfecting mice with plasmids for thirty years [21], was used to induce Egr1 overexpression or knockdown in DM mice in this study. Egr1 mRNA and protein expression increased 5.2-fold and 3.3-fold in the EDM group compared with the vector group, respectively (Figures 3(a)–3(c) and 3(g)). Subsequently, the NOX4 and α-SMA mRNA and protein levels increased (Figures 3(a)–3(c) and 3(g)). The NOX4, ROS, and α-SMA protein levels measured by ELISAs also showed the same results (Figures 3(d)–3(f)). In addition, Egr1 levels were decreased in the SEDM group (Figure 4(a)). NOX4 was subsequently significantly reduced (Figure 4(a)). α-SMA, the molecular signature of renal myofibroblasts, was also decreased (Figure 4(a)). Similar results were obtained using ELISAs, Western blotting, and immunohistochemistry (Figures 4(b)–4(g)).

4. Discussion

DKD is a serious complication of diabetes mellitus. Currently, there is no effective treatment for DKD [22, 23]. Thus, there is an urgent need to identify novel targets. Oxidative stress is considered the most common pathway promoting kidney injury in DKD [24, 25]. The glucose-TGF-β1-NOX4-ROS pathway has been accepted worldwide. In the present study, we further extended the previous conclusion and first demonstrated that Egr1 can bind to the NOX4 promoter and is involved in the development of DKD. Importantly, we demonstrated that Egr1 knockdown mice showed alleviation of EMT due to downregulation of NOX4.

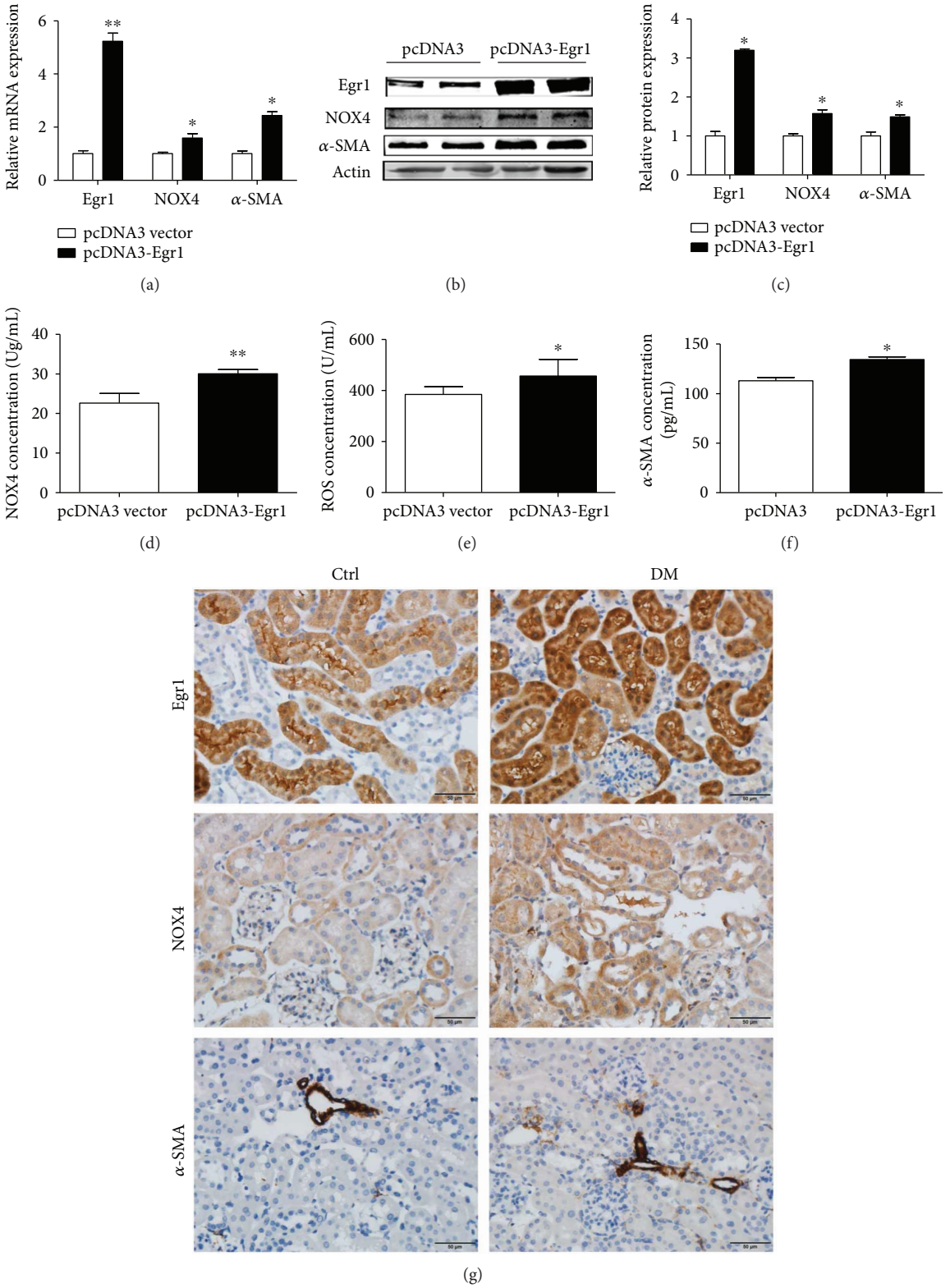

Figure 3: Egr1, NOX4, and α-SMA expression in HFD/STZ-induced diabetic mice transfected with pcDNA3-Egr1 plasmid. Diabetic mice were treated with either pcDNA3-Egr1 overexpression plasmid or with a pcDNA3 vector once a week for four weeks (12–16 w) via rapid injection of a large volume of DNA solution through the tail vein. (a) Levels of Egr1, NOX4, and α-SMA mRNA were measured using RT-qPCR. (b and c) Levels of Egr1, NOX4, and α-SMA protein were measured using Western blotting assays. (d) NOX4 concentration, (e) ROS concentration, and (f) α-SMA concentration were measured using ELISAs. The results are expressed as foldchange over baseline (pcDNA3 vector group). Values are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the pcDNA3 vector group. (g) Immunohistochemical staining of Egr1, NOX4, and α-SMA.

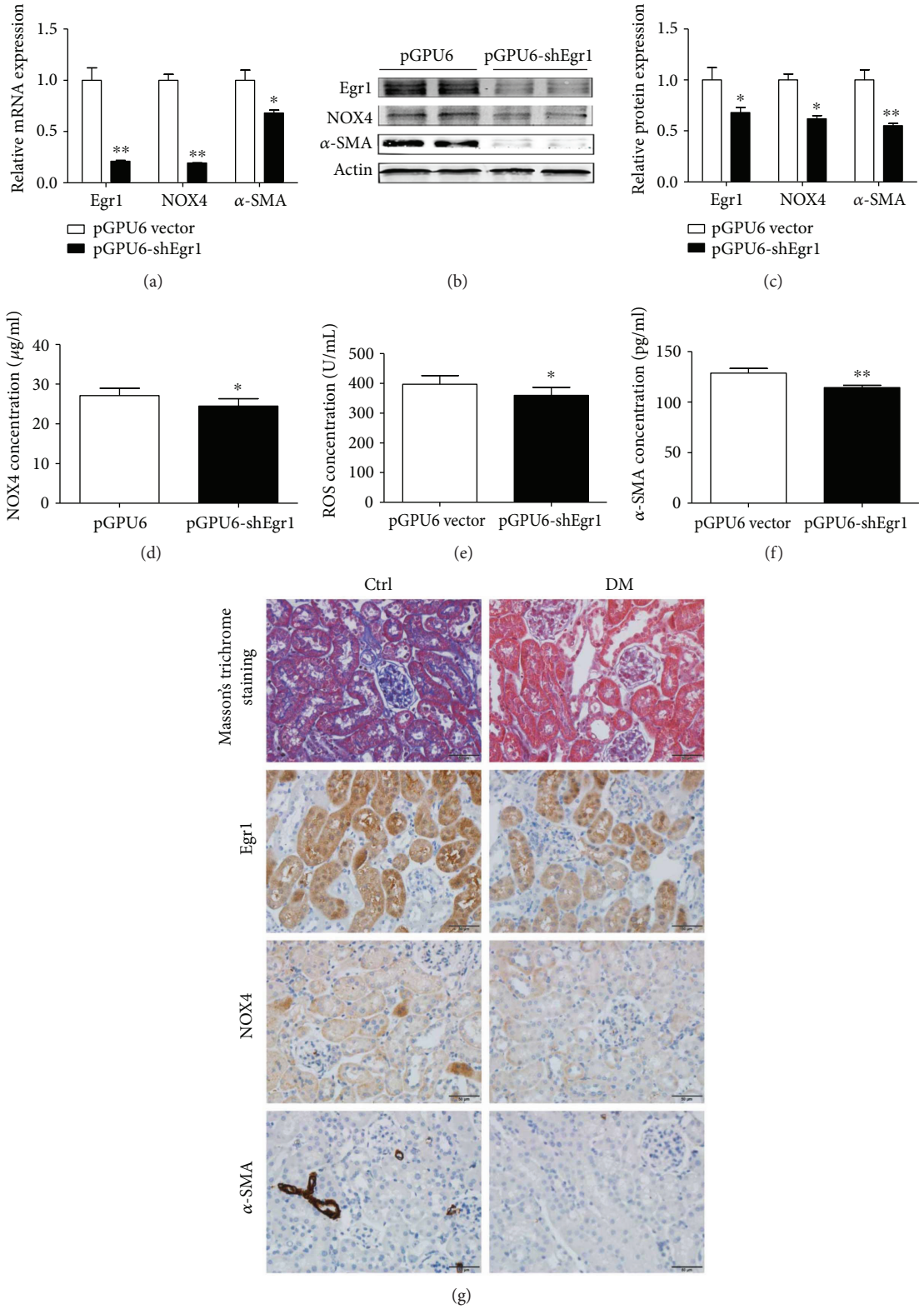

Figure 4: Egr1, NOX4, and α-SMA expression in HFD/STZ-induced diabetic mice transfected with pGPU6-shEgr1 plasmid. Diabetic mice were treated with either pGPU6-shEgr1 silencing plasmid or with a pGPU6 vector once a week for four weeks via rapid injection of a large volume of DNA solution through the tail vein. (a) Levels of Egr1, NOX4, and α-SMA mRNA were measured using RT-qPCR. (b and c) Levels of Egr1, NOX4, and α-SMA protein were measured using Western blotting assays. (d) NOX4 concentration, (e) ROS concentration, and (f) α-SMA concentration were measured using ELISAs. The results are expressed as foldchange over baseline (pGPU6 vector group). Values are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the pGPU6 vector group. (g) Masson staining and immunohistochemical staining of Egr1, NOX4, and α-SMA.

Egr1 expression is low or undetectable in resting cells. However, its expression can be elevated rapidly and transiently following exposure to various extracellular stimuli. Previous work from our department has revealed a change in Egr1 following high glucose and TGF-β1 stimulation in NRK-52E, HMC, and HK-2 cells [12, 19, 26]. This paper reports similar results. Egr1 is induced in a rapid and transient manner via the negative feedback of NGFI-A binding protein 2 (Nab 2). Egr1/Nab 1 and Nab 2 can be recognized by the inhibitory domain of Egr1 protein [27]. Then, cofactors recruit the inhibitory nucleosomal remodeling and deacetylation (NuRD) complex to promoter-bound Egr1 and ultimately repress Egr1-dependent transcriptional activity [28]. Egr1 is regarded as a fibrosis factor in kidney fibrosis. Ho et al. [29] found that Egr1 knockout mice exhibited alleviated fibrosis and inflammatory changes; in addition, primary HK-2 cells with knockout of Egr1 exhibited an attenuated reaction to TNF-α and TGF-β. However, there is little research regarding the contribution of Egr1 to fibrosis in DKD. Recent studies have shown increased Egr1 gene and protein expression in DM and rats [19, 20] but did not determine whether Egr1 promoted kidney fibrosis in DKD. Using Egr1 and shEgr1 plasmid-treated DM mice, we showed here that Egr1 can promote the development of DKD.

There have been many studies investigating the association between NOX4 and kidney injury. However, the conclusions in different models vary. Renal NOX4 has been suggested to be either protective or not involved in UUO and 5/6Nx models [19, 20], while it has been definitively shown that NOX4 can promote DKD development [30]. NOX4 exhibits very low constitutive activity, which can be highly upregulated in response to various stimuli, such as high glucose, TGF-β, and AngII [31, 32]. In the present study, both NOX4 mRNA and protein were overexpressed in the diabetic renal cortex and TGF-β1-treated HK-2 cells, indicating that NOX4 is fully activated in the diabetic kidney. These results suggest that NOX4 is involved in the development of DKD. Our findings are similar to those reported in a previous study.

Then, we explored the relationship between Egr1 and NOX4 in vivo and in vitro. NOX4 expression increased following treatment of HK-2 cells and diabetic kidney mice with the Egr1 plasmid. Conversely, NOX4 decreased following Egr1 knockdown in HK-2 cells and diabetic kidney mice. A direct relationship between Egr1 and NOX4 has not been previously reported. We are the first to show that Egr1 can directly bind to the NOX4 promoter to improve DKD. These results offer further evidence that Egr1 can promote kidney fibrosis in addition to binding to the promoters of COL1A1, COMP, periostin, MMP2, TIMP1, and OPN. Surprisingly, we found that knockdown of Egr1 in mice led to decreased α-SMA expression, a characteristic of EMT. Our research extends previous work by providing strong evidence that Egr1 contributes to DKD by promoting NOX4 expression. Therefore, we concluded that Egr1 knockdown can ameliorate DKD in part by decreasing NOX4. Thus, Egr1 may be a possible target for DKD treatment.

However, our study has some limits. Microalbuminuria, the inflammation index, and other factors in addition to α-SMA should be examined to explore and confirm the nephroprotective role of Egr1 in DKD. Additionally, an Egr1 inhibitor should be used to treat DM mice to obtain more convincing results. These experiments will be part of subsequent work performed in this department.

5. Conclusion

Our research demonstrates that Egr1 is a transcriptional activator of NOX4 in DKD. Egr1 contributes to DKD by enhancing EMT, in part by targeting NOX4.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Silymarin Ameliorates Diabetes-Induced Proangiogenic Response in Brain Endothelial Cells through a GSK-3β Inhibition-Induced Reduction of VEGF Release

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Diabetes mellitus (DM) is a major risk factor for cardiovascular disease. Additionally, it was found to induce a dysfunctional angiogenic response in the brain that was attributed to oxidative stress. Milk thistle seed extract (silymarin) has potent antioxidant properties, though its potential use in ameliorating diabetes-induced aberrant brain angiogenesis is unknown. Glycogen synthase kinase-3β is a regulator of angiogenesis that is upregulated by diabetes. Its involvement in diabetes-induced angiogenesis is unknown. To evaluate the potential of silymarin to ameliorate diabetes-induced aberrant angiogenesis, human brain endothelial cells (HBEC-5i) were treated with 50 μg/mL advanced glycation end (AGE) products in the presence or absence of silymarin (50, 100 μM). The angiogenic potential of HBEC-5i was evaluated in terms of migration and *in vitro* tube formation capacities. The involvement of GSK-3β was also evaluated. AGE significantly increased the migration and tube formation rates of HBEC-5i by about onefold ($p = 0.0001$). Silymarin reduced AGE-induced migration in a dose-dependent manner where 50 μM reduced migration by about 50%, whereas the 100 μM completely inhibited AGE-induced migration. Similarly, silymarin 50 μg/mL blunted AGE-induced tube formation ($p = 0.001$). This effect was mediated through a GSK-3β-dependent inhibition of VEGF release. In conclusion, silymarin inhibits AGE-induced aberrant angiogenesis in a GSK-3β-mediated inhibition of VEGF release.

1. Introduction

Diabetes mellitus (DM) is a wide-spread chronic metabolic disease characterized by elevated blood glucose either due to insulin resistance (type II) or insulin deficiency (type I) [1–3]. Around 8.5% of adults aged 18 and older had elevated blood glucose in 2014 [4]. The prevalence of diabetes is increasing over the world and the expected number of patients to be diagnosed with DM is estimated to reach 366 million by the year 2030 [5].

Diabetes mellitus is a well-known major risk factor for the development of cardiovascular disease, including coronary artery disease (CAD), stroke, and peripheral artery disease [1]. About 80% of deaths among diabetics are due to atherosclerosis-related diseases [6, 7]. The majority of diabetes-associated complications are caused by its effect on both small and large blood vessels. Additionally, diabetes was found to alter angiogenesis in a tissue-dependent manner [8].

Angiogenesis is a multistep process in which new blood vessels are formed from preexisting ones [9]. This process is highly regulated by a balance between pro- and antiangiogenic factors to meet the metabolic demand of the body [10]. Angiogenesis has been shown to play an essential role in many physiological conditions, such as wound healing and growth [11]. Brain angiogenesis is involved in a multitude

of brain functions, including learning [12] and recovery after ischemic insults [13–16]. Additionally, the endothelial cells of the brain have been found to release a number of growth factors, including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and stromal-derived factor (SDF) that has been shown to be involved in recovery after CNS ischemic insults [17].

Diabetes has been found to impair angiogenesis in the peripheral vascular beds [6, 7, 18]. In contrast, it was found to have a proangiogenic response in the brain and retina [6, 8, 19–21]. In the brain, diabetes-induced proangiogenic response is characterized by generation of immature and fragile blood vessels [8]. This dysfunctional angiogenic response has been linked to a higher incidence of hemorrhagic transformation [8]. Prakash et al., 2012, demonstrated that diabetes-induced altered angiogenic response is mediated through oxidative stress-induced VEGF expression and release [8]. This finding suggests a potential role of antioxidants in ameliorating diabetes-induced dysfunctional angiogenesis.

Silymarin, extracted from the dry seeds of milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)], is a mixture of at least seven flavonolignans, silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin, and the flavonoid taxifolin [22]. It has been used for hundreds of years for liver diseases such as liver cirrhosis and chronic hepatitis [23]; clinical studies confirmed its hepatoprotective properties [24].

Silymarin has been widely studied for its profound biological activities particularly in cancer chemoprevention and hepatoprotection areas [25–27]. The biological effects of silymarin were also assessed in areas such as Alzheimer's disease [28], Parkinson's disease [29], and diabetes [30].

Silymarin with its potent antioxidant activity, low toxicity, and cellular protective effects compared with placebo encourages the exploration of its therapeutic potential uses [24].

Gadad et al., 2013, demonstrated the ability of silymarin to overcome diabetes-induced reduction in the migration of human dermal microvascular cells [31]. It is still unknown whether silymarin would have the ability to counteract diabetes-induced proangiogenic response in the brain given the tissue-dependent effect of diabetes on angiogenesis.

Recently, diabetes has been found to increase the expression and activity of glycogen synthase kinase-3β (GSK-3β). GSK-3β in turn has been found to play a significant role in regulating the expression of angiogenic factors such as VEGF and the angiogenic process in endothelial cells [32]. It is still unknown whether antioxidants like silymarin would alter the activity of GSK in endothelial cells or not. Accordingly, this study aims at evaluating the inhibitory potential of silymarin on diabetes-induced aberrant angiogenesis.

2. Materials and Methods

2.1. Cell Culture. Human brain microvascular endothelial cell line HBEC-5i (ATCC® CRL-3245™) was purchased from ATCC (ATCC; Manassas, VA) and was cultured in DMEM:F12 purchased from Euroclone (Euroclone S.p.A; Pero, Italy) supplemented with microvascular endothelial cell growth kit-BBE (ATCC; Manassas, VA). The cells used in the study were p1-5.

2.2. Treatments. Silybum marianum seed extract (silymarin) was obtained from Indena S.p.A. (Milan, Italy) (lot number 27691). The extract was analyzed for its content of eight individual bioactive components using a validated UHPLC-tandem mass spectrometry method [33]. The extract content of taxifolin, isosilychristin, silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B was found to be 4.13, 1.22, 11.60, 7.02, 10.29, 15.68, 4.92, 2.52%, respectively, which sum to a total analyte content of 57% [33].

To prepare the stock solution, silymarin was dissolved in 1 mL of DMSO that was further diluted with serum-free media to a final concentration of 12.5 μM. Silymarin was used in two concentrations (50 and 100 μM). Advanced glycation end (AGE) products were purchased from Tocris (Tocris; Minneapolis, MN) and were reconstituted with serum-free DMEM:F12 to a final concentration of 250 μg/mL. The concentration used in the experiments was 50 μg/mL according to published literature [34, 35]. GSK-3β inhibition was achieved using 10 nM of SB-216763 (Tocris; Minneapolis, MN) that was dissolved in 0.0001% DMSO. DMSO was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology; Dallas, TX). The cells were treated with silymarin 30 min before AGE application, whereas SB-216763 was applied 30 min before silymarin application.

2.3. VEGF ELISA. The concentration of VEGF released into the media was measured using a commercially available ELISA kit purchased from Abcam (Abcam; Cambridge, MA) according to the manufacturer recommendations. Briefly, conditioned media was collected 16 h after the application of different treatments and centrifuged at 10,000 rpm for 10 min. The supernatant was isolated and stored in −60°C until the time of analysis.

2.4. Angiogenesis Assays

2.4.1. Cell Migration. The migratory capacity of HBEC-5i was measured using the wound healing assay. Cells were cultured in a 12-well plate to about 80% confluence before being serum starved for 24 h. A scratch was introduced in the cell monolayer using a 1 mL pipette tip, and the media was replaced by fresh serum-free DMEM:F12 media. The different treatments were sequentially added according to the abovementioned order. Images of the scratch edges were captured using a digital camera mounted on an inverted microscope at baseline and 16 h after treatment. The migration rate was determined by measuring the distance between the scratch edges using National Institutes of Health ImageJ software at both time points. The migration rate was calculated by subtracting the scratch width at 16 h from the width measured at baseline and dividing it the width at baseline. The wound recovery rate was presented as a percentage of the recovery rate of the control.

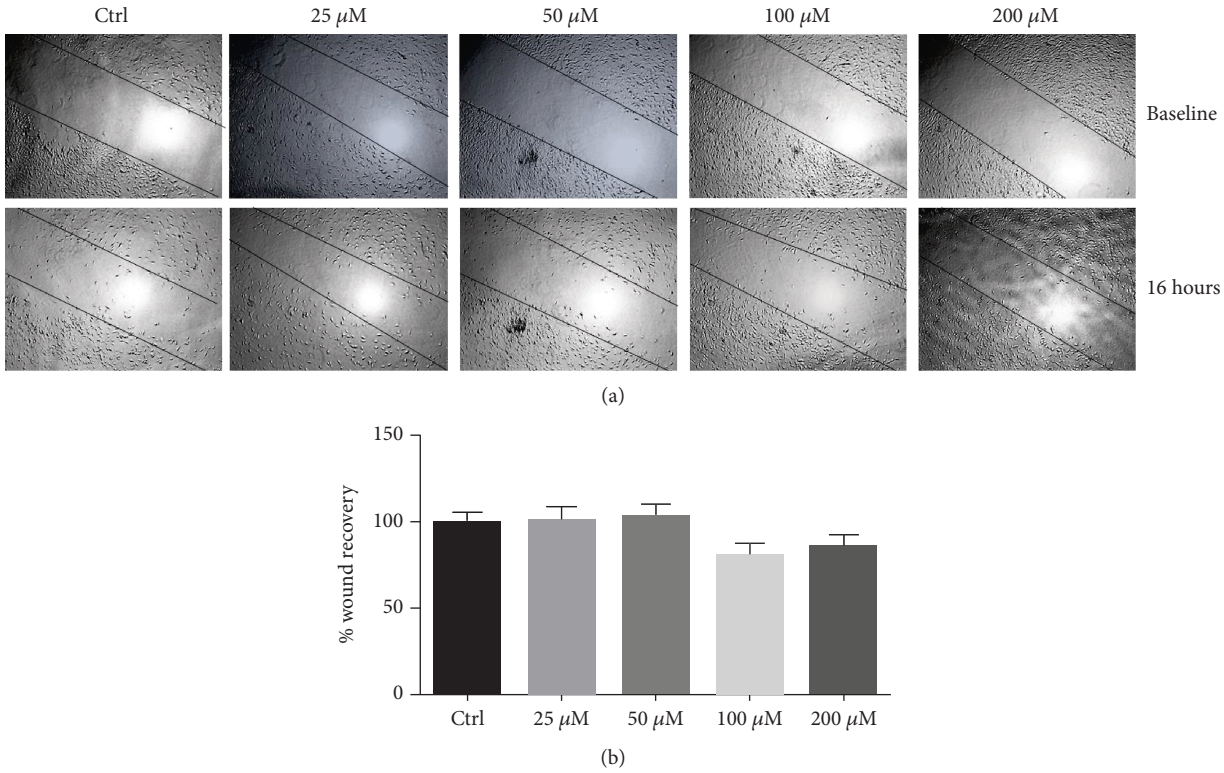

Figure 1: The effect of silymarin on the migratory potential of untreated HBEC-5i. Human brain endothelial cells were treated with a range of silymarin concentrations, and the migration rate was measured. Representative images of the migrated cells (a) and a quantification of the migratory potential (b). Silymarin did not alter the migration rate of endothelial cells. Data are presented as mean ± SEM; ∗ indicates a significant difference from control. $n = 3$.

2.4.2. Tube Formation Assay. The tube formation potential of HBEC-5i was determined using the *in vitro* tube formation assay according to published literature [32]. Briefly, 20,000 cells/well were added to a Cultrex® basement membrane extract-coated 96-well plate. The plate was coated with Cultrex basement membrane extract purchased from Trevigen (Trevigen; Gaithersburg, MD). To coat the plates, basement membrane extract was allowed to thaw at 4°C for 24 h before the experiment and 50 μL were added per well of a prechilled 96-well plate. The treatments were applied as mentioned above. The tube formation rate was measured by counting the number of tube-like structure at 6 and 8 h after treatment application in three nonoverlapping images of each well.

2.5. Statistical Analysis. All experiments were repeated at least three times in duplicates. Statistical significance was determined using one-way ANOVA followed by the post hoc Tukey test. All statistical analyses were carried out using GraphPad Prism version 6; GraphPad Software, La Jolla, California. All values were reported as mean ± standard deviation error of mean (SEM). Statistical significance was considered as $p < 0.05$.

3. Results

3.1. Silymarin Does Not Affect Endothelial Cell Migration Rate. To investigate the ability of silymarin to directly alter the angiogenic potential of untreated HBEC-5i, a range of silymarin concentrations (0–200 μg/mL) was applied to HBEC-5i (Figures 1(a) and 1(b)). Silymarin in doses up to 200 μg/mL did not have an appreciable effect on the migration of HBEC-5i when assessed 16 hours posttreatment.

3.2. Advanced Glycation End Products Increased the Migration and Tube Formation Rates in HBEC-5i in a Time-Dependent Manner. Diabetes has been found to increase the migration and tube formation capacity of brain endothelial cells [8]. Furthermore, the incidence of diabetes-induced complications has been found to increase with time [6, 18]. Accordingly, we assessed whether the effects of AGE would mimic the effects of diabetes on the behavior of HBEC-5i. AGE induced a onefold increase in the migratory rate of HBEC-5i ($p < 0.0001$) when assessed 16 hours posttreatment. (Figures 2(a) and 2(b)). Additionally, AGE induced a modest increase in the tube formation rate of HBEC-5i when measured at 6 h. Two hours later, AGE induced a 25% increase in the tube formation rate of endothelial cells ($p < 0.05$) (Figures 2(c) and 2(d)).

3.3. Silymarin Inhibited AGE-Induced Migration in a Dose-Dependent Manner. To assess the ability of silymarin to counteract AGE-induced effects in HBEC-5i, cells were treated with silymarin in two different doses (50 and 100 μM). Silymarin at the 50 μM concentration reduced AGE-induced HBEC-5i migration by about 50% when assessed 16 hours posttreatment (Figures 3(a) and 3(b)).

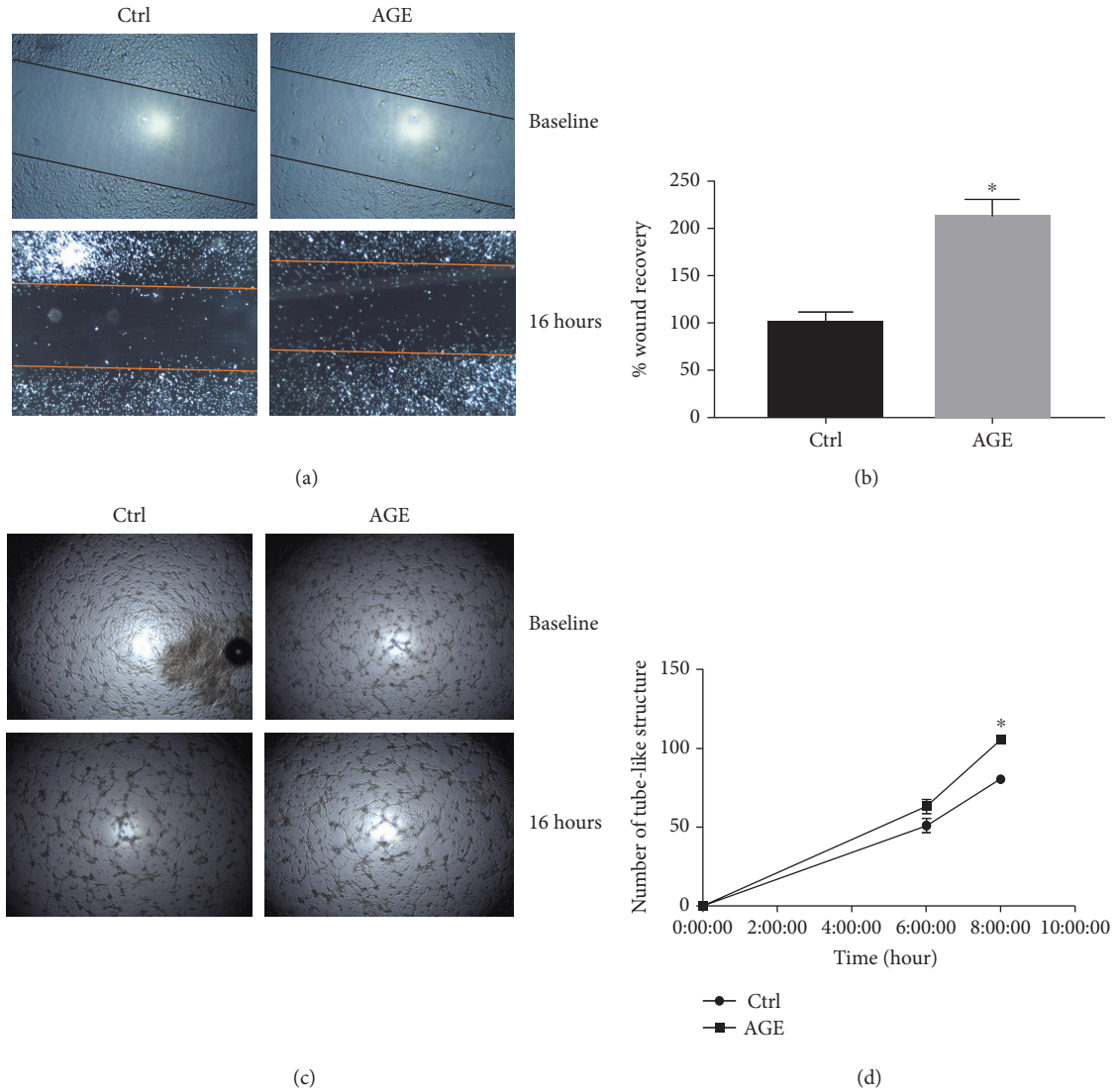

Figure 2: Advanced glycation end products induce a proangiogenic response in endothelial cells. Endothelial cells were treated with 50 μg/mL of AGE, and the migration and tube formation rates of the cells were assessed. AGE increased the migration rate of endothelial cells as well as the rate of tube formation in a time-dependent manner. Representative images of the migrated cells (a) and a quantification of the migratory potential (b). Representative images of the tube formation potential of endothelial cells (c) and its quantification (d). $*$ indicates a significant difference from control. Data are presented as mean ± SEM. $n = 3$.

Doubling the concentration of silymarin completely blunted AGE-induced migration when assessed 16 hours posttreatment (Figures 3(a) and 3(b)).

3.4. Silymarin Inhibited AGE-Induced Tube Formation in HBEC-5i in a Concentration-Dependent Manner. Silymarin at the 50 μM concentration blunted the angiogenic potential of AGE-treated HBEC-5i ($p < 0.001$) (Figures 3(c) and 3(d)). Similarly, treatment with the higher concentration level of silymarin (100 μg/mL) resulted in a more pronounced reduction of the tube formation rate in AGE-treated HBEC-5i ($p < 0.001$). The tube formation rate in the cells treated with 100 μM was significantly lower than that in the AGE-treated group and the low-dose silymarin-treated group (Figures 3(c) and 3(d)). Interestingly, both concentrations of silymarin reduced the tube formation rate of AGE-treated HBEC-5i as compared to the control.

3.5. The Role of GSK-3β-Mediated Signaling in AGE-Induced Migration of HBEC-5i and Silymarin-Induced Amelioration of its Effects. To investigate the involvement of GSK-3β-mediated signaling in AGE-induced effects in HBEC-5i and its amelioration by silymarin, the activity of GSK-3β was inhibited using the compound SB-216763 (10 nM) in AGE-treated cells. GSK-3β inhibitor blunted the AGE-induced migratory response in HBEC-5i when assessed 16 hours posttreatment ($p < 0.05$) (Figure 4(a)). The migratory response of untreated HBEC-5i was not affected by GSK-3β inhibition. Interestingly, GSK-3β inhibition in silymarin-treated HBEC-5i did not alter silymarin-induced effects (Figure 4(a)).

3.6. Silymarin Ameliorates AGE-Induced Angiogenic Response by Reducing VEGF Release. Treatment with AGE induced a onefold increase in VEGF release from endothelial

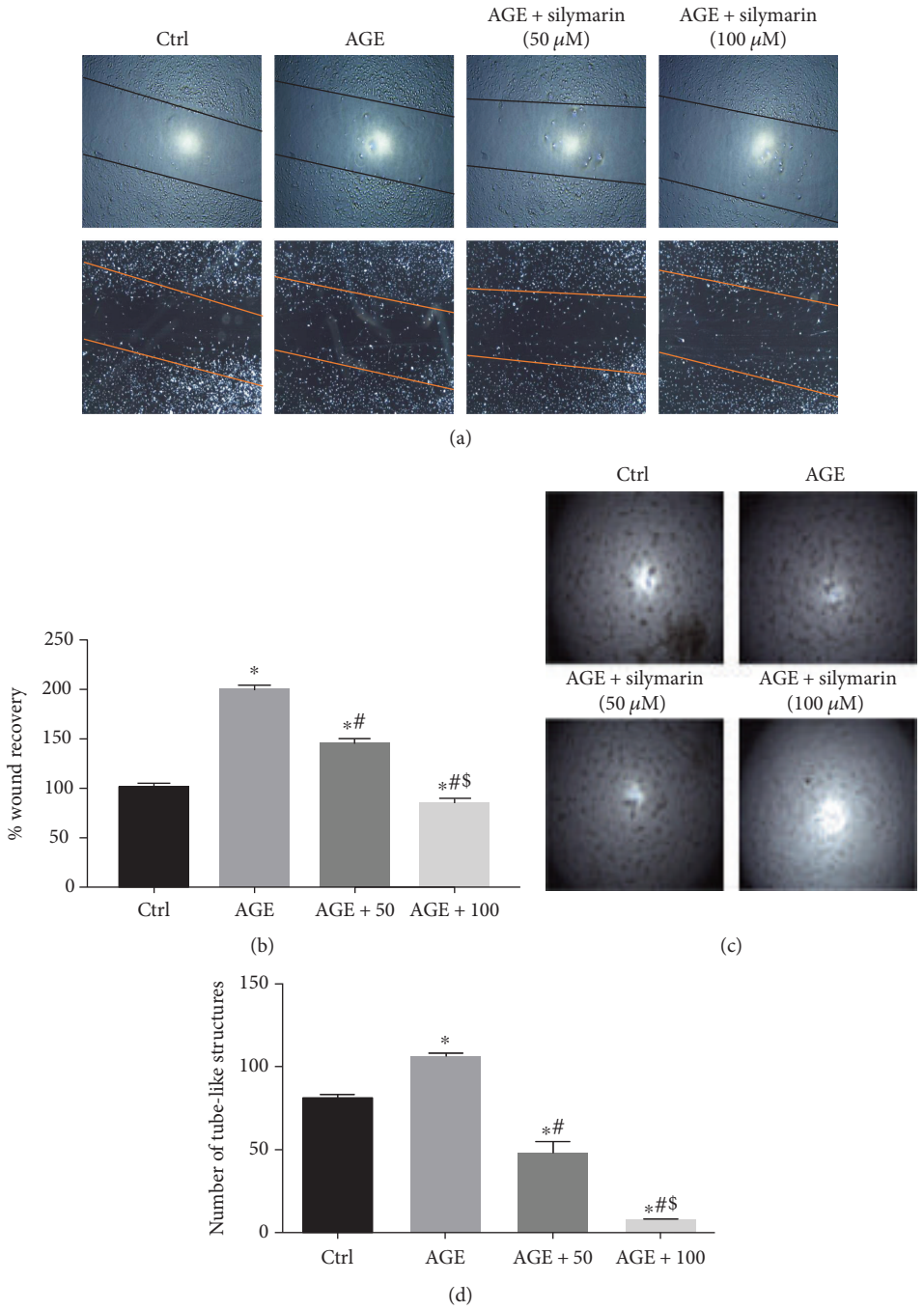

Figure 3: Silymarin inhibited diabetes-induced proangiogenic response in brain endothelial cells. Advanced glycation end products (50 μg/mL) increased the migration rate of hBMECs. Treatment with silymarin inhibited AGE-induced brain endothelial cell migration in a dose-dependent manner. Representative images of the migrated cells (a) and a quantification of the migratory potential (b). Similarly, silymarin inhibited AGE-induced angiogenic response in human brain endothelial cells in a dose-dependent manner. Representative images of the tube formation potential of endothelial cells (c) and its quantification (d). Data are presented as mean ± SEM, $n = 3$–6. **Significantly different* as compared to control; $^{\$}$*significantly different* as compared to AGE; $^{\#}$*significantly different* as compared to AGE + 50 μg/mL-treated cells.

cells ($p < 0.05$) (Figure 4(b)). Treatment with silymarin blunted the AGE-induced VEGF release ($p < 0.05$).

3.7. Silymarin-Induced Inhibitory Effect on VEGF Release May Be Associated with GSK-3β Inhibition. Glycogen synthase kinase-3β inhibition blunted AGE-induced VEGF release from HBEC-5i ($p < 0.05$) (Figure 4(b)). Similarly, silymarin inhibited AGE-induced VEGF release. Inhibiting GSK-3β in HBEC-5i treated with both silymarin and AGE did not alter VEGF release compared with AGE and silymarin cotreatment. This finding suggests the involvement of GSK-3β in silymarin-induced effects.

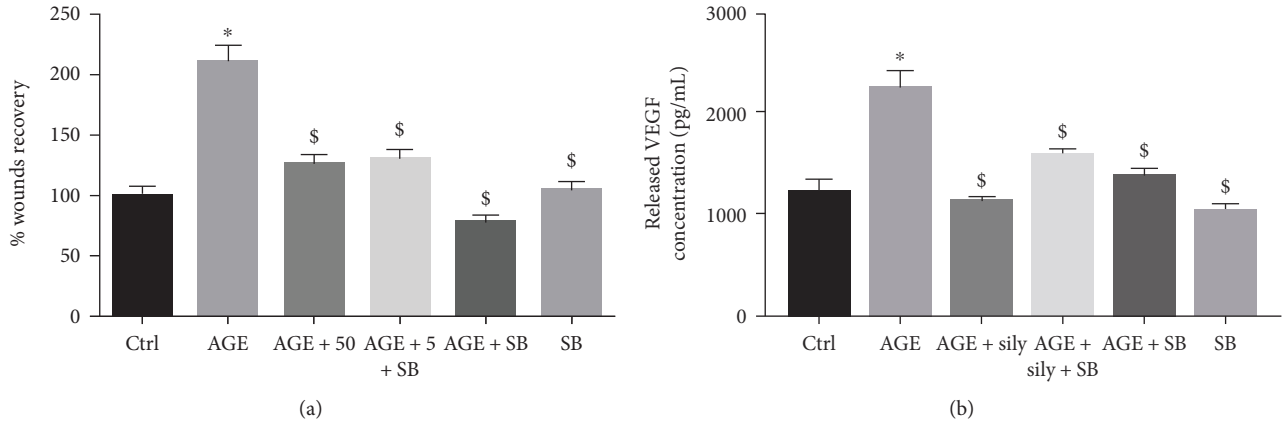

Figure 4: Silymarin inhibits diabetes-induced proangiogenic response in a GSK-3β inhibition-dependent reduction of VEGF release. Treatment with silymarin or GSK-3β inhibition using 10 nM of SB-216763 reduced AGE-induced migration in HBEC-5i. Cotreatment with both GSK inhibitor and silymarin resulted in a comparable inhibition to what is achieved in either of them (a). Treatment with AGE induced a onefold increase in VEGF release. This increase was blunted with silymarin cotreatment. GSK inhibition blunted AGE-induced VEGF release and did not alter silymarin-induced VEGF release inhibition (b). $n = 3$. **Significantly different* as compared to control; $^\$$*significantly different* as compared to AGE.

4. Discussion

The results obtained in this study demonstrated, for the first time, that silymarin ameliorates diabetes-induced angiogenesis in brain endothelial cells through a GSK-3β-mediated inhibition of VEGF release. Silymarin reduced both the migration and tube formation rate in HBEC-5i in AGE-treated cells. In contrast, silymarin did not have any appreciable effect in untreated cells except at very high doses. Furthermore, we demonstrated the essential role of GSK-3β in diabetes-induced dysfunctional angiogenesis. GSK-3β inhibition inhibited diabetes-induced migration and VEGF release.

In this investigation, we used advanced glycation end (AGE) products to model diabetes *in vitro*. There are plenty of methods to model diabetes *in vitro* [8, 34, 35]. Additionally, AGE and its receptor (RAGE) are implicated in a variety of pathological conditions that may not involve hyperglycemia [6, 9, 18, 34–42]. In contrast, AGE has been widely accepted as a substance that can induce the detrimental effects of diabetes and model diabetes *in vitro* [34, 35, 40]. Furthermore, the observed effects of AGE in endothelial cells are consistent with the reported effects of diabetes in the same type of cells [8]. Accordingly, we are confident that our model is valid and truly reflects the effects of diabetes in endothelial cells.

Data from experimental studies demonstrated the ability of diabetes to induce a proangiogenic state in the brain [8, 14]. This angiogenic response is characterized by the formation of nonperfused fragile blood vessels [8, 14–16]. Additionally, diabetes was found to impair the neuroprotective effects of brain endothelial cells [34, 35]. Collectively, these data demonstrate the detrimental effect of diabetes on brain endothelial cells. Additionally, they explain the larger infract, poor outcome, and impaired recovery after ischemic insults among diabetic individuals [14, 15, 43, 44]. Accordingly, ameliorating diabetes-induced angiogenesis would offer an intriguing target to prevent diabetes-induced aggravation of CNS ischemic insults.

Diabetes is associated with a high level of oxidative stress [19, 20, 45, 46]. Prakash et al., 2012, demonstrated the ability of antioxidants to inhibit diabetes-induced angiogenesis using FeTPPS [8]. This compound accelerates the degradation of peroxynitrite and thus reduces diabetes-induced oxidative stress [8, 47]. Unfortunately, this compound is highly toxic and [8, 47]. Additionally, it has been found that epicatechin can reduce diabetes-induced pathologic angiogenesis in the retina [19]. These data in addition to other highlight the potential of antioxidants to ameliorate diabetes-induced abnormal angiogenesis [8, 14–16, 19, 21, 43, 45, 46, 48, 49]. An essential prerequisite for the success and applicability of this approach is the use of safe antioxidants.

Silymarin is a natural antioxidant with a long history of safety and efficacy [23, 24, 26, 30, 50]. Gadad et al., 2013, tested the ability of silymarin wafers to inhibit diabetes-induced impairment of human dermal microvascular cell migration [31]. Although interesting, their work focused on the migratory capacity of human dermal microvascular endothelial cells. Additionally, they did not characterize the molecular pathway of silymarin-induced effect. Furthermore, the type of the cells they used is essential in wound recovery after peripheral injuries but cannot extrapolate to endothelial cells from other vascular beds. Accordingly, assessing the ability of silymarin to ameliorate diabetes-induced angiogenesis and the involved molecular pathway is of utmost importance to establish the potential use of silymarin in preventing diabetes-induced dysfunctional angiogenesis in the brain.

In this investigation, we tested the hypothesis that silymarin ameliorates diabetes-induced abnormal angiogenesis in the brain in a GSK-3β-mediated inhibition of VEGF release. Similar to what has been reported by Gadad et al., 2013, silymarin inhibited diabetes-induced alteration in endothelial cell migration [31]. In contrast, to what has been reported in human dermal microvascular cells, silymarin

reduced diabetes-induced migration in brain endothelial cells in a dose-dependent manner. This discrepancy is mainly related to the differential effect of diabetes on microvascular endothelial cells [8]. Prakash et al., 2012, reported a proangiogenic effect of diabetes on endothelial cells of the brain and retina [8]. In contrast, endothelial cells from peripheral vascular beds exhibit an impaired migratory potential in response to diabetes [8]. This impaired migratory potential is the main cause of impaired wound healing in diabetic patients [8]. Accordingly, silymarin normalizes the endothelial cell response to diabetes.

In this investigation, we expanded the work reported by Gadad et al., 2013 [31], by assessing the ability of silymarin to ameliorate diabetes-induced alteration in the ability of endothelial cells to form tube-like structures. Our data demonstrated, for the first time, that silymarin reduces diabetes-induced tube formation potential of endothelial cells. Interestingly, both concentrations of silymarin reduced the capacity of endothelial cells to less than that observed in untreated endothelial cells. In contrast, the low dose of silymarin (50 μg/mL) reduced the migration of AGE-treated endothelial cells by about 75% as compared to untreated endothelial cells. The high concentration of silymarin completely blunted AGE-induced migration and reduced it to the level detected in the untreated cells. A similar differential effect on the migration and tube formation in brain endothelial cells has been reported previously in response to angiotensin II [32]. Alhusban et al., 2013, reported that 1 μM of angiotensin II reduced the angiogenic potential of brain endothelial cells while the migratory capacity was not affected by the same concentration [32].

Diabetes was found to increase VEGF expression and release in primary brain microvascular endothelial cells [8]. Furthermore, treatment with an anti-VEGF prevented diabetes-induced angiogenesis [8]. Similarly, diabetes increased VEGF release in the human brain microvascular endothelial cell line used in this study. Silymarin reduced diabetes-induced VEGF release. Accordingly, silymarin-induced normalization of the angiogenic response in HBEC-5i is mediated through silymarin-induced inhibition of VEGF release. This finding highlights the potential utility of silymarin as an intervention to reduce VEGF release. It is still unknown whether this effect is mediated through a reduction of VEGF expression or an inhibitory effect on VEGF secretion from HBEC-5i.

Glycogen synthase kinase-3β (GSK-3β) has been shown to function as a signaling node in the brain [51]. It integrates signals from outside and inside the cell and modulates the expression of growth factors such as VEGF [51]. Furthermore, it modulates the activity of intracellular adhesion molecules [51]. Accordingly, GSK-3β has an essential role in angiogenesis, neurogenesis, and recovery after CNS ischemic insults [13, 51]. Diabetes was found to increase GSK-3β activity [52, 53]. It is still unknown whether GSK-3β is involved in diabetes-induced dysfunctional angiogenesis. Our results showed that GSK-3β inhibition antagonizes diabetes-induced migration of HBEC-5i. This finding highlights a potential role of GSK-3β in diabetes-induced dysfunctional angiogenesis. Additionally, inhibiting GSK-3β in silymarin-treated cells did not alter silymarin-induced inhibition of diabetes-induced migration. This finding suggests that silymarin-induced effects may be induced through GSK-3β inhibition. Confirming this finding requires assessing the phosphorylation level of GSK-3β in silymarin-treated cells and comparing it to that in silymarin-untreated cells using western blotting. Currently, we are in the process of conducting this investigation.

5. Conclusion

Silymarin inhibits AGE-induced brain angiogenesis in a dose-dependent manner. This inhibitory effect is induced through a GSK-3β-mediated inhibition of VEGF release. Additionally, we demonstrated the potential role of GSK-3β in diabetes-induced abnormal angiogenesis.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

The Role of Oxidative Stress, Mitochondrial Function, and Autophagy in Diabetic Polyneuropathy

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Diabetic polyneuropathy (DPN) is the most frequent and prevalent chronic complication of diabetes mellitus (DM). The state of persistent hyperglycemia leads to an increase in the production of cytosolic and mitochondrial reactive oxygen species (ROS) and favors deregulation of the antioxidant defenses that are capable of activating diverse metabolic pathways which trigger the presence of nitro-oxidative stress (NOS) and endoplasmic reticulum stress. Hyperglycemia provokes the appearance of micro- and macrovascular complications and favors oxidative damage to the macromolecules (lipids, carbohydrates, and proteins) with an increase in products that damage the DNA. Hyperglycemia produces mitochondrial dysfunction with deregulation between mitochondrial fission/fusion and regulatory factors. Mitochondrial fission appears early in diabetic neuropathy with the ability to facilitate mitochondrial fragmentation. Autophagy is a catabolic process induced by oxidative stress that involves the formation of vesicles by the lysosomes. Autophagy protects cells from diverse stress factors and routine deterioration. Clarification of the mechanisms involved in the appearance of complications in DM will facilitate the selection of specific therapeutic options based on the mechanisms involved in the metabolic pathways affected. Nowadays, the antioxidant agents consumed exogenously form an adjuvant therapeutic alternative in chronic degenerative metabolic diseases, such as DM.

1. Introduction

Distal sensorimotor polyneuropathy is considered the most frequent diabetic polyneuropathy (DPN) and is the most prevalent chronic complication of diabetes mellitus (DM) [1]. It is possible that DPN is present in 10% of patients with an initial diagnosis of type 2 DM. In fact, emerging data suggest that DPN can occur before the development of hyperglycemia in the diabetic range in people with metabolic syndrome or altered tolerance to glucose [2]. The DPN can affect ~50% of patients with long-term DM [3]. The prevalence of DPN increases with age and history of the disease and is typically characterized by deficient control of glycemia [4]. The objective of the present review was to describe the mechanisms of functional and structural damage in DPN, the role and participation of oxidative stress, the oxidative stress of the endoplasmic reticulum, the behavior of the antioxidants, the effect on mitochondrial function, and autophagy in DPN.

2. Functional and Structural Damage to the Nervous Tissue in DPN

The potential mechanisms that lead to the damage of the nervous tissue in DPN include the activation of the different pathways: (a) the polyol pathway (glucose metabolism), (b) the deposit of end-products of advanced glycosylation, (c) the poly(ADP-ribose) polymerase, (d) the hexosamine pathway, and (e) the protein kinase C pathway. All these pathways are activated in the state of hyperglycemia (Figure 1).

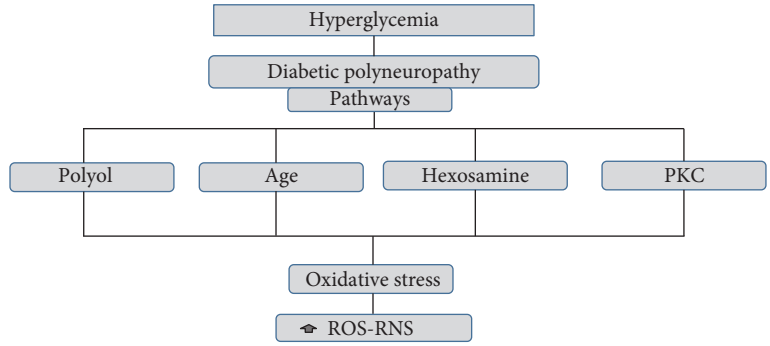

Figure 1: Interaction of hyperglycemia pathways with oxidative stress in DPN.

Each one of the pathways has the capacity to produce vascular insufficiency and oxidative stress [5]. The hyperglycemic state triggers the increase in the production of mitochondrial and cytoplasmic ROS, which, in conjunction with the deregulation of the antioxidant defenses, activates new pathways capable of producing oxidative damage in DPN [6, 7].

3. Oxidative Stress

Free radicals such as hydroxyl radical (HO•), nitric oxide (•NO), peroxynitrite (ONOO−), superoxide anion (O_2•−), nitrogen dioxide (•NO_2), peroxyl radicals (ROO•), and lipid peroxyl (LOO•) are highly reactive, unstable molecules that have an unpaired electron in their outer shell. ROS comprehends free radical and nonradical molecules. Nonradicals include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and lipid peroxide (LOOH), among others. H_2O_2 is a major ROS in cells and can diffuse long distances crossing membranes and causing cell damage at high concentrations by reacting with transition metals (copper, iron (Fe), and cobalt) yielding HO• via the Fenton reaction [8]:

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + HO^- + HO\bullet \quad (1)$$

ROS and reactive nitrogen species (RNS) are formed during normal metabolic activity in a variety of biochemical reactions and cellular function. Their beneficial effects occur at low concentrations and involve physiological roles in cellular signaling systems. For example, H_2O_2 is produced in response to cytokines and growth factors and is involved in regulating immune cell activation and vascular remodeling in mammals [9]. NO• is generated *in vivo* by specific NO synthases (NOS) and the nitrate-nitrite-NO pathway and is a critical regulator of vascular homeostasis, neurotransmission, and host defense [10]. Excessive NO• production, under pathological conditions, leads to detrimental effects of this molecule on tissues, which can be attributed to its reaction with superoxide anion (O_2•−) to form ONOO−. ONOO− is 1000 times more potent as an oxidizing compound than H_2O_2 [11].

The main sources of ROS are the mitochondrial electron transport chain and enzymatic reactions catalyzed by NOS, NADPH oxidases, xanthine oxidase, and hemeperoxidase enzymes, such as myeloperoxidase. The nonenzymatic production of O_2•− occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (Flavin's or iron sulfur clusters) or by xenobiotics previously reduced. Ubisemiquinone autoxidation (ubisemiquinone donates one electron to molecular oxygen yielding O_2•− and ubiquinone) is the major source of O_2•− in mitochondria, and because the ubiquinone or coenzyme Q pool faces both the intermembrane space and the mitochondrial matrix, O_2•− is vectorially released into both compartments. O_2•− released in the intermembrane space can cross the outer mitochondrial membrane into the cytosol through the porin protein. Furthermore, the mitochondrial electron transport chain contains several redox centers that may leak electrons to oxygen [12].

Under physiological conditions, the steady-state formation of ROS and RNS is normally balanced by a similar rate of consumption by antioxidants. Thus, oxidative stress results from the overproduction of ROS in the organism that exceeds the endogenous antioxidant capacity for them to be eliminated. The oxidative and nitrosative stress induced by hyperglycemia is considered one of the primary links between DM and diabetic complications [13]. The mechanism by which hyperglycemia leads to the generation of ROS is primarily due to autooxidation of the glucose and the glycosylation of proteins. The persistent increase in ROS and RNS favors the presence of oxidative and nitrosative stress, with the capacity to produce endothelial dysfunction, insulin resistance, and alterations in the number and functions of the pancreatic β-cells, favoring the appearance of micro- and macrovascular complications of DM [14]. The ROS and the RNS cause structural deterioration of the macromolecules (carbohydrates, proteins, lipids, and nucleic acids) causing loss of function [15]. Also, the ROS and the RNS are capable of activating cellular signaling cascades that lead to the transcription of genes that facilitate the development of diabetic complications. The nuclear factor-κB (NF-κB) is a nuclear transcription factor that can be activated by the increase in ROS, resulting in the transcription of proinflammatory proteins that aggravate the conditions of the illness. The chemokines and proinflammatory cytokines like the monocyte chemoattractant protein-1 (MCP-1) of the macrophages, the tumor necrosis factor-α (TNF-α), and the interleukins (interleukin-1β and interleukin-6) are implicated in the progression and complications of DM [16].

The increase in ROS and RNS, together with the significant reduction of the antioxidant defense mechanisms in the neurons, contributes to the clinical manifestations of DPN, which include the deterioration of nervous blood flow, endoneurial hypoxia, deterioration of the motor conduction and nerve sensation, degeneration of the peripheral nerves, sensorial loss, axonal atrophy of the large myelinated fibers, and characteristic neuropathic pain [17].

3.1. Oxidative Stress of the Endoplasmic Reticulum. The functions of the endoplasmic reticulum (ER) include the protein synthesis and transport, protein folding, lipid biogenesis, maintenance of calcium homeostasis, and the participation of other crucial cellular functions [18]. The ER can control and maintain cellular homeostasis acting as a sensor for stressors in the intra- and extracellular medium, on providing a platform for the interaction between environmental signals and the basic biological cellular functions, and acting as an intersection to integrate multiple responses to stress [19]. The interruption of cellular homeostasis can lead to the gradual reduction in the function of a determinant organ on decreasing the capacity to respond to physiological stress. In fact, it has been suggested that the interruption in ER homeostasis is involved in the pathogenesis of DM and its complications [20]. Study of the behavior of the ER in DPN emerges as an interesting opportunity to investigate the functions of the ER and its signaling network in relation to DM, to develop possible therapeutic strategies [21]. The state of hyperglycemia has the ability to induce oxidative stress of the ER through the accumulation of unfolded or poorly folded proteins into the lumen. When oxidative stress is extreme, or of lengthy duration, the unfolded protein response can be overwhelming, unchaining diverse apoptotic processes including the factor 2 associated with the receptor of the tumor necrosis factor (TNF) and the kinase 1 regulator of the apoptosis signal through activation of the c-Jun N-terminal kinase [22, 23], liberation of calcium from the cytosol, depolarization of the mitochondrial membrane, and the liberation of cytochrome c [24], with excision of the procaspase 12 [25]. Other mechanisms that are altered include the perfusion of nerves [26], the C-peptide release [27], the appearance of dyslipidemia with an increase in the levels of circulating unsaturated fatty acids [28], a decrease in the levels of the glycolysis and intermediaries of the tricarboxylic acid cycle [29], and alterations of the redox state and calcium homeostasis [30]. Furthermore, alterations in the mitochondrial energy metabolism in the neurons of the dorsal root ganglia modulated by the heat shock protein 70 and the ciliary neurotrophic factor are produced [31, 32].

3.2. Oxidative Damage to Peripheral Nerves in DPN. The increase in ROS and RNS is capable of causing damage to the lipids present in the myelinated structures of the nerves, resulting in the loss of axons and interruption of the microvasculature in the peripheral nervous system [33]. The oxidative damage to the peripheral nerves causes hyperexcitability in the afferent nociceptors and the central neurons, causing the generation of spontaneous impulses in the axons and the dorsal root ganglia of the nerves, causing neuropathic pain [34].

3.3. Oxidative Damage to DNA in DPN. The oxidative stress that can be produced by the persistent hyperglycemic state in type 2 DM leads to modifications in the mitochondrial genetic material (mtDNA) and the nuclear DNA (nDNA) [35]. The persistence of oxidative damage to the mtDNA is capable of causing mutations in the mitochondrial genome, causing mitochondrial dysfunction that unchains an increase in ROS, forming a vicious cycle within the mitochondria, producing intense oxidative damage that can lead to cell death [36–38]. The mitochondria are the primary source of the production of ROS and RNS and are the first organelles to suffer oxidative damage, putting the cells that are highly susceptible, like neurons, at risk while favoring the progression of DPN. The axons are highly susceptible to the metabolic and endothelial imbalances that lead to the progression of DPN because the axons normally contain a large number of mitochondria. Thus, oxidative damage favors mitochondrial damage of the DNA, mitochondrial dysfunction, and axonal degeneration [39]. On the other hand, H_2O_2 has the capacity to move into the cellular nucleus and subsequently the HO• is generated through the Fenton reaction. Then, the reaction of the HO• with the bases of the DNA strand, such as guanine, leads to the generation of radical adducts, then by one electron abstraction, the 8-hydroxy-2′-deoxyguanosine (8-OH-dG) is formed. Other bases of DNA react with HO• in a similar manner; however, the 8-oxodG product is the most abundant and it is relatively easily formed and is promutagenic. In normal conditions, the 8-OHdG can be repaired by the endonuclease 8-oxoguanine DNA glycosylase (hOGG1) enzyme through mechanisms of base excision [40]. However, experimental studies in animals and clinical studies have demonstrated that there are elevated levels of the 8-OHdG marker and deficiency of the DNA repair enzyme in patients with type 2 DM with DPN [41, 42]. The ROS induce activation of the poly(ADP-ribose) polymerase 1 (PARP-1) that undertakes an important role in the repair of damaged DNA through a costly process of energy consumption that causes rapid depletion of the nicotinamide adenine dinucleotide (NAD^+) with a concomitant decrease in ATP production [43]. Therefore, the control of oxidative damage to the DNA seems to be an important therapeutic target in patients with type 2 DM with DPN (Figure 2).

In neurons and other cell types, steady-state ATP production is necessary for ion homeostasis, particularly for impulse conduction, as maintenance and post-impulse restoration of the membrane potential are dependent on the activity of Na^+/K^+ ATPase. Therefore, ATP depletion elicits sodium to accumulate intracellularly and potassium to diffuse out of the cell causing cell swelling and dilation of the endoplasmic reticulum [44].

4. The DAMPs in Diabetes Mellitus

The damage-associated molecular patterns (DAMPs) are intracellularly sequestered molecules involved in the

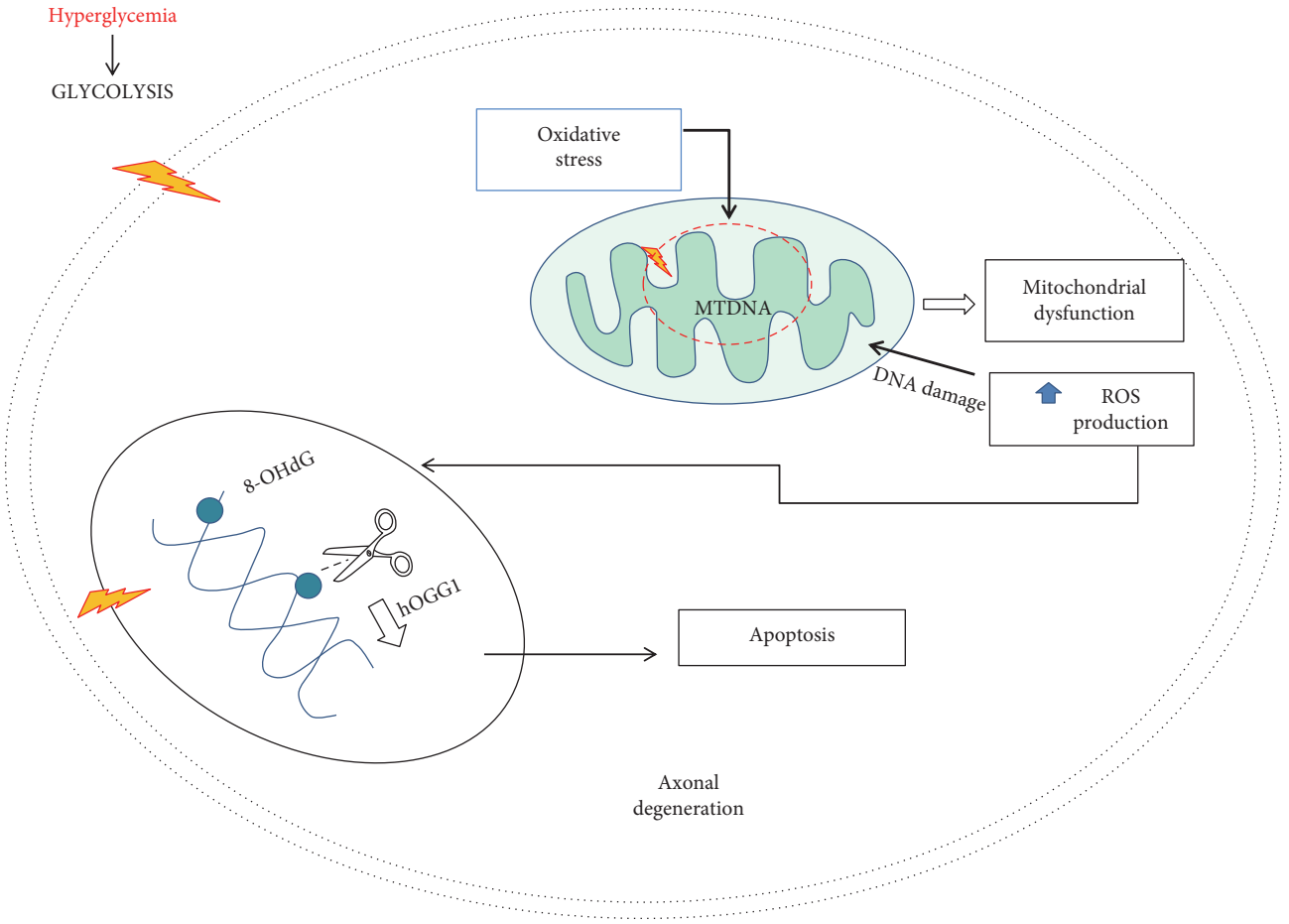

Figure 2: Hypothetical drawing of the effect of hyperglycemia on the increase of oxidative metabolism that induces damage to the mtDNA, which leads to mitochondrial dysfunction. The increase in the production of ROS augments damage to the nDNA with the generation of the product 8-OHdG and the decrease in repair of the DNA in DPN, which can ultimately cause axonal degeneration and cell death.

pathogenesis of many human diseases. DAMPs are characterized by being hidden from recognition by the immune system under normal physiological conditions. However, under conditions of stress or cell injury, they may be actively secreted by stressed immune cells or by stress cells in which the neoantigens bind to natural immunoglobulin M (IgM) antibodies. DAMPs can be passively released into the extracellular environment of dying cells or when the extracellular matrix is damaged [45]. DAMPs are recognized by cells of the receptor recognition pattern (PRR) of the innate immune system, including macrophages, leukocytes, dendritic cells, vascular cells, fibroblasts, and epithelial cells for the purpose of promoting proinflammatory and profibrotic pathways [46]. PRRs include RIG-I-like receptors, NOD-like receptors, and Toll-like receptors (TLR) to activate intracellular signaling cascades resulting in the production of cytokines and immunomodulators released from immune cells [47]. In metabolic diseases such as DM, class V DAMPs play a crucial role. This class of DAMPs can be generated by intracellular stress even in nondying cells. This can occur due to minimal metabolic disturbances of homeostasis within the intra/extracellular microenvironment observed in type 2 DM, metabolic syndrome, and obesity. Primary ER disturbances elicit different classes of DAMPs which, through recognition by PRR cells, promote innate inflammation of immune tissue resulting in cell dysfunction. It is essential to consider that metabolism and innate immunity are linked since both systems involve the recognition of exogenous stressors. However, proper management leads to the maintenance of the individual homeostasis of each individual. Recent studies reveal molecular associations between immunity and metabolism because they could be substantial therapeutic targets for sterile inflammatory diseases such as type 2 DM [48]. Therefore, type 2 DM represents the prototype of an innate immune disease where sterile autoinflammatory processes induced by PRR cells trigger dysfunction of β-cells and favor cell death (pyroptosis) [49]. It is currently argued that metabolic insults such as insulin resistance, prolonged hyperglycemia, and increased free fatty acids (depletion of calcium levels in the ER) lead to excessive stimulation of insulin production by associated β-cells with the accumulation of proinsulin in the ER [50]. Proinsulin overload leads to alterations in ER homeostasis, resulting in accumulation of newly synthesized unfolded or misfolded proteins in the ER lumen that may be considered class V DAMPs [51]. Metabolic disturbances favor ER depletion associated with oxidative

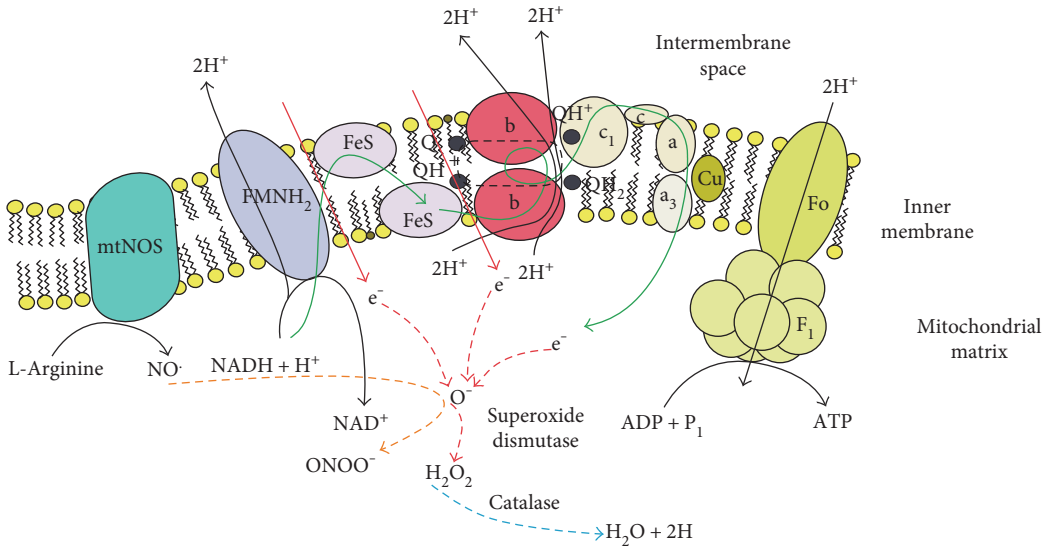

Figure 3: Formation of reactive oxygen and nitrogen species in mitochondria. The process is mediated by oxidative phosphorylation and the activity of the mitochondrial NO synthase: in physiological conditions the production of ROS and RNS are reduced by multiple steps that involved SOD, GPx and catalase. When the mitochondria suffers an insult the increase of the leakage of electrons to the matrix leads to an overload to the capacity of the enzymatic systems and leads to toxicity of the cell. Black arrows: vectors of reactions and products. Green arrows: the physiological pathway for formation of oxidative stress. Red arrows: leakage of electron to matrix. Dotted red and orange arrows: pathophysiological pathway for formation of ROS and RNS.

stress [52]. The intersection and crosstalk between the innate immune system, stress of ER, and the machinery of the inflammamosome seem to regulate the quality, intensity, and duration of innate proinflammatory and proapoptotic immune responses [53]. It is clear that further studies are required to determine whether the DAMP axis reflects an innate immune pathway that contributes to the pathogenesis of metabolic inflammatory diseases such as DM and its involvement in PND [54].

Mitochondrial DNA (mtDNA) contains a higher frequency of hypomethylated cytosine-phosphate-guanine motifs which are natural ligands for PRR and, therefore, can be recognized by the innate immune system [55]. The mtDNA is highly sensitive to ROS-induced damage, and oxidative stress promoted the fragmentation of mtDNA. It has been shown that after induction of mitochondrial damage by oxidative stress, mtDNA fragments of low molecular weight were released to cytosol via the permeability transition pore [56]. Then, mtDNA fragments can serve as DAMPs when liberated into the extracellular space [57]. Interestingly, mtDNA that escapes from autophagy cell autonomously leads to TLR 9-mediated inflammatory responses. This mechanism might work in inflammation-related diseases such as diabetes mellitus. [58]. In fact, high levels of mtDNA have been reported in peripheral blood mononuclear cells in patients with type 2 diabetes [59] and diabetic retinopathy [60].

5. The Role of the Mitochondria in DPN

Mitochondria are the primary source of cellular oxidants, taking into account that about 2% of molecular oxygen is not completely reduced to water at the electron transport chain and, therefore, is the primary site for the potential overproduction of ROS and a prime target of cumulative oxidative damage. The mitochondria play a critical role in the regulation of the metabolic imbalance observed in DM, since both H_2O_2 and ONOO− can cross the mitochondrial membranes and damage macromolecules in other cellular regions [61]. An increase in the levels of O_2•− in the mitochondrial electron transport chain as a result of the hyperglycemic state that favors the increase of oxidative stress has been reported [62]. Other metabolic pathways involved in ROS production, which augments the oxidative stress in DM, are the synthesis of metabolites through the xanthine oxidase pathway, the production of neurotransmitters, and the detoxification of the xenobiotics through the cytochrome P450 system and the NADPH oxidase [63].

Because diabetic cells exhibit high glucose content, excess of glucose-derived pyruvate is oxidized through the tricarboxylic acid cycle which causes higher levels of electron donors (NADH and $FADH_2$) to the electron transport chain. This exceeds the capacity of the electron transport chain and blocks the electron transfer in the ubiquinol-cytochrome c reductase complex, causing the electrons to return to coenzyme Q. Thus, an increasing level of O_2•− is observed. O_2•− is a relatively small anion; in fact, the hydration shell of the superoxide anion is relatively small, with only four protons being strongly coupled to the unpaired electrons. The superoxide dismutase (SOD) enzyme degrades this oxygen-free radical to H_2O_2, which is then converted to H_2O and O_2 by other enzymes such as catalase and glutathione peroxidase [64] (Figure 3). H_2O_2 affects lipids and intramembranous proteins. It is a ROS whose biological actions are governed by its chemical reactivity towards biological targets, among which are metalloenzymes such

as hemoperoxidases and amino acid residues sensitive to oxidants such as cysteine [65].

HO• is actively involved in lipid peroxidation and is associated with the genesis of harmful factors involved in many chronic degenerative diseases [66]. HO• can attack macromolecules (lipids, nucleic acids, and amino acids). Phenylalanine can be converted enzymatically into a physiological para-tyrosine. The attack of HO• on phenylalanine can produce para-tyrosine, meta-tyrosine, and ortho-tyrosine. The target and ortho-tyrosine are considered markers of HO•-induced damage. The use of resveratrol to treat patients with type 2 DM leads to decreased urinary excretion of ortho-tyrosine and concomitantly improves insulin signaling and sensitivity to this hormone [67]. Thus, the administration of resveratrol may be an attractive therapeutic tool along with strict metabolic control in patients with DM and chronic complications of DM.

6. Nitric Oxide

The production of •NO occurs from the L-arginine by the nitric oxide synthase (NOS) (Figure 3). The NOS has four isoforms: neuronal (nNOS), inducible (iNOS), endothelial (eNOS), and mitochondrial (mtNOS) [68]. The •NO is implicated in physiological processes like vasodilation, the modulation of nociception, the immune function, neurotransmission, and the excitation-contraction coupling [69]. The •NO is considered an atypical neurotransmitter and a second messenger in the nervous system [70] or as a hormone [71]. The majority of the effects of •NO are mediated through activation of the guanylate-cyclase enzyme that produces cyclic guanosine-3,5-monophosphate (cGMP) [29]. The •NO has pronociceptor properties in the neural crest and in the dorsal root ganglia that positively regulate as a result of cutaneous or visceral inflammation and by the peripheral lesions of the fibers. This effect could be potentiated or inhibited by the •NO donors [72].

O_2•− interacts with •NO, forming the potent ONOO− that attacks several biomolecules, conditioning the production of a modified amino acid: nitrotyrosine [73]. Nitrotyrosine was initially considered a specific marker of ONOO− generation, but other pathways such as mieloperoxidase may also induce nitrosation of tyrosine. Nitrotyrosine is often described as a stable marker of oxidative/nitrosative stress [74]. Nitrosative stress-induced damage plays a crucial role in multiple interrelated aspects of the pathogenesis of DM and its complications. In the state of hyperglycemia, it stimulates the production of ONOO− capable of damaging the vascular endothelium and the peri-neuro in DPN [75]. Angiotensin II is also capable of inducing intraendothelial ONOO− production and activation of poly(ADP-ribose) polymerase (PARP) [76]. Angiotensin II is capable of inducing direct prooxidant effects on the vascular endothelium. The effects of angiotensin II are mediated in part by the formation of intraendothelial ROS through the family of nonphagocytic NAD(P)H oxidase subunits. ROS produced after angiotensin II-mediated stimulation have the ability to exert direct oxidative effects through pathways such as mitogen-activated protein kinases, tyrosine kinases, and transcription factors that promote inflammation, hypertrophy, remodeling, and angiogenesis [77]. Inhibition of angiotensin II by the angiotensin-converting enzyme (ACE) *in vivo* seems to reduce the formation of ONOO− [78]. Neutralization of RNS or inhibition of PARP activation pathways may emerge as a new approach, first as experimental therapy of DM, even for the prevention or reversal of complications caused by DM.

7. Mitochondrial Dysfunction in DPN

Mitochondria are the major sites of adenosine triphosphate (ATP) synthesis by the processes of oxidative phosphorylation. Mitochondria also mediate amino acid biosynthesis, fatty acid oxidation, steroid metabolism, calcium homeostasis, and ROS production and detoxification. Often, the mitochondria accumulate in the synapses and play a predominant role in synapse maintenance through attenuation of the Ca^{2+}. A lot of neurons depend on the mitochondria, and so there is a strong link between neuronal dysfunction and mitochondrial dysfunction [79]. The indicators of mitochondrial dysfunction present in neurodegenerative illnesses include ultrastructural changes, inhibition of the respiratory chain, decrease in ATP production, an increase in the production of FR, deletions of the mtDNA, loss of calcium buffer effect, and loss of the mitochondrial membrane potential [80]. Mitochondria are dynamic bodies that constantly divide and fuse within the cell as the environment demands [81]. These processes can facilitate formation of new mitochondria, repair of defective mitochondrial DNA through mixing, and redistribution of mitochondria to sites requiring high-energy production [82]. Both processes effectively lower the percentage of defective mitochondria in the cell and ensure stability in cellular proliferation; indeed, metabolism, energy production, calcium signaling, reactive oxidative species production, apoptosis, and senescence all depend on the balance of fission and fusion. Conversely, dynamic distortion (i.e., excessive fragmentation/elongation) results in inefficiencies in cell functioning, if not cell death [83, 84]. Mitochondrial dynamics is a tightly regulated cellular process, with sophisticated molecular machinery involving GTPases. Fission is regulated by at least two proteins: a large GTPase, dynamin-like protein 1 (Drp1), and a small molecule, Fis1, and fusion involves three large mitochondrial transmembrane proteins localized to the outer membrane: mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy protein 1 [82, 85]. One model of mitochondrial fission suggests that the Drp1 is formed into rings or spirals that surround the external mitochondrial membrane with the help of hFis1 and other cofactors and regulators yet to be discovered. It is thought that GTP hydrolysis causes a conformational change in Drp1 that drives the fission event of the external mitochondrial membrane [86]. The excess of mitochondrial fission is an early and important event in neurodegenerative illnesses. The oxidative and nitrosative stress appear to play a predominant role as inductors of mitochondrial fission [87]. Several studies suggest that the damage to DNA and hyperglycemia can stimulate mitochondrial fission and indicate that the aberrant activation of components of the cellular

cycle in postmitotic neurons plays an important role in the regulation of the mechanics of mitochondrial fission [88, 89]. Damage to the DNA is an event that can unchain mitochondrial fission, which can contribute to neuronal loss [90]. Mitochondrial fusion requires components of the external and internal membrane. Mfn1 and Mfn2 facilitate fusion of the external membrane in mammals, probably through transinteractions that promote the curvature and fusion of the membrane [91]. Some studies suggest that the GTPase is the principle mediator of fusion of the internal membrane and of the maintenance of the mtDNA in mammals. The mutations in the proteins of mitochondrial fusion give way to greater mitochondrial fragmentation, which could favor the appearance of neurodegenerative illnesses such as Parkinson's, Alzheimer's, and Huntington's diseases, among others [92, 93]. The dorsal root ganglion neurons (DRGs) exposed to hyperglycemia present with mitochondrial dysfunction, fragmented mitochondria, and an increase in the expression of Drp1 and oxidative stress [94]. Hyperglycemia stimulates an increase of the Drp1/Bax complexes, which mediate apoptotic mitochondrial fragmentation [95].

8. Autophagy

Autophagy is a catabolic process induced by oxidative stress that involves the delivery of cytoplasmic materials to the lysosome for degradation and component recycling. It is considered a protector of the cells against diverse factors of stress and routine wear and tear and is characterized by the sequestration of organelles/senescent or damaged proteins, forming autophagosomes to recycle those products [96]. Autophagy is involved in the elimination of cells that have suffered programmed cell death type 1 (classic) and in one form of nonapoptotic cell death or cell death type 2. Therefore, autophagy protects the cells on promoting cell death, depending on the state and cellular environment in which they are found [97]. The increase in ROS is essential for autophagy to prosper because in the presence of ROS it is possible to control the Atg4 activity, a family of cysteine proteases that are necessary for the formation of autophagosomes [98]. Autophagy can be inhibited by the regulator protein the mammalian target of rapamycin (mTOR) [99]. Recently, it was reported that the activation of PARP-1 induced by the ROS promotes autophagy through the activation of AMP-activated protein kinase (AMPK), likely by suppression of the mTOR [43]. The deregulation of autophagy is related to pathologies like cancer, myopathies, neurodegenerative illnesses, heart diseases, liver diseases, gastrointestinal disturbances, and the complications of DM [100]. Autophagy can be categorized in three classes: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy [101]. The primary focus of the macroautophagy involves the formation of autophagosomes (double-membrane vesicles) in a multistep process. The autophagosomes combine with the liposomes and degrade the content through diverse acid hydrolases. This process is mediated by more than 30 autophagy-related proteins (Atg). Macroautophagy consists of two subsets: autophagy of specific organelles and selective macroautophagy. Although substantial progress has been made in the understanding of the complex mechanisms that regulate autophagy, many interactions involved in the control of the process have not yet been adequately described [102]. The ROS inhibit the activity of the mTOR signaling protein on invoking the dephosphorylation of the Atg13, the activation of the serine/threonine protein kinase ULK, and the recruitment of the focal adhesion kinase family-interacting protein of 200 kD (FIP200). The ULK-Atg13-FIP200 complex plays a critical role in the formation of autophagic double-membrane vacuoles in forming autophagosomes capable of disposing cellular waste. Microautophagy has been discussed little in chronic degenerative diseases [43].

8.1. Autophagy in DPN. Numerous metabolic and cellular alterations in neural tissue because of DM have been described, including the state of dyslipidemia, the excessive generation of ROS and RNS, and, obviously, the state of hyperglycemia [103]. These alterations cause mitochondrial and cytosolic oxidative stress with the generation of abnormal glycated proteins and dysfunctional mitochondrial proteins [104]. These alterations are a growing field of research which suggests that autophagy occurs as a cytoprotective response [105]. Autophagy in neural tissue has been described as a mechanism of cleansing that eliminates the damage caused by cellular stressors [106]. Mounting evidence shows that autophagy plays a potentially significant role in the pathophysiology of DPN, which requires additional research to completely understand the mechanisms that unchain the induction of autophagy in the nerves of diabetics and the relationship with neuronal injury during the natural history of DPN. Still pending to answer are numerous questions with regard to the relative contribution of the different stress factors in the process of autophagy and the cascade of interactions of autophagy with other cellular signals [66].

Rapamycin, an immunosuppressive drug that induces autophagy, has the ability to affect other aspects of cellular function, and it could be a focus of therapeutic interest in DM and its complications [107]. It has been reported that rapamycin improves tolerance to glucose in experimental animals fed with a diet rich in fats supplemented with branched chain amino acids, but not with high fat diets [108], which suggests the possible role of rapamycin or rapamycin-related compounds in type 2 DM [109]. Thus, the development of treatments that favor the cytoprotective effect of autophagy in the complications of DM is a potentially promising research path.

9. Management Alternatives in DPN

Currently, an absolute cure has not been defined for DPN or any other complications of DM, although some medications are conventionally useful. However, it is interesting to consider the pathophysiological links between hyperglycemia and oxidative stress. As well, the superior adjuvant effect of the antioxidants and FR scavengers continues to be essential in the prevention of DPN in diabetic patients.

9.1. Glycemia Control. Achieving control of stable glycemia is the only, most effective, and most difficult goal to achieve therapeutic target for the management of the complications of DM. According to reports from the follow-up study, "Diabetes Control and Complications and Epidemiology of Diabetes Interventions and Complications" (EDIC), the intensive glycemic control designed to achieve nearly normal blood glucose levels, implemented early in the course of the diabetes, delays the development of DPN in patients with type 1 DM, without obtaining the same results in type 2 DM [110]. To date, an absolute cure for DN has not been defined. Although some drugs are conventionally used, some may be found in which some aspects of the pathophysiological links with oxidative stress are known.

9.2. Antioxidants. Antioxidants diminish or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions, thus reducing its capacity to damage. Antioxidants may act as radical scavengers, peroxide decomposers, hydrogen donors, electron donors, singlet oxygen quenchers, enzyme inhibitors, or metal-chelating agents [111]. Their effect depends on concentration [112], polarity, and the medium [113], and also the presence of other antioxidants [114]. In fact, antioxidants may act from directly scavenging free radicals to increasing antioxidative defenses. There are several types of antioxidants in cells: dietary antioxidants (vitamins A, C, and E), endogenous antioxidant enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase (GPx), glutathione S-transferase (GST), and peroxiredoxins), and antioxidant molecules (glutathione (GSH), coenzyme Q, ferritin, bilirubin, uric acid, lipoic acid, melatonin, carotenoids, and flavonoids). Under physiological conditions, these molecules and enzymes work synergistically and together with each other to protect the cells [115]. The SOD dismutates the $O_2\bullet-$ to form H_2O_2 upon which acts as the catalase or the GPx to produce water. The GST converts the reactive electrophilic species to form easily excretable hydrophilic products as a result of the conjugation with GSH. Vitamins C and E and the alpha-lipoic acid are involved in the elimination of the products of lipoperoxidation (LPO) [116]. As well, the flavonoids are capable of eliminating FR [117]. Some specialized proteins have regulator functions of the redox signaling with an antioxidant effect, like the peroxiredoxins (Pxr), thioredoxins (Trx), and glutaredoxins (Grx), with intracellular effects on the ROS and RNS [118]. The members of these families of proteins are ubiquitously expressed in all organisms, tissues, types of cells, and organelles. Some of these proteins can also move between cellular compartments and the extracellular space [119].

The stoichiometric number of antioxidants that capture FR by an antioxidant molecule and the effectiveness of FR scavenging can be evaluated by performing *in vitro* tests. The biological functions of antioxidants have been widely evaluated for their effects on the expression of antioxidant enzymes. For example, γ-tocopherol is a relatively mild ROS scavenger when compared to α-tocopherol. However, the oxidized product, γ-tocopheryl-quinone, reacts readily with the thiols to release the nuclear factor (Nrf-2) resulting in the expression of antioxidant enzymes such as hemooxygenase-1 [120].

There are several existing strategies with the use of different antioxidants to manage DPN. The choice of antioxidant depends on its chemical structure and concentration, the type of DPN, and stage of the illness, its severity, and the prevalence and primary causes from which it originated [121]. The antioxidants have different mechanisms and action sites through which they exert their biochemical effects and improve nerve dysfunction produced by oxidative stress in DPN.

9.3. Metformin. Metformin is a widely prescribed oral antidiabetic agent that reduces the production of hepatic glucose and improves peripheral sensitivity to insulin. The antihyperglycemic mechanisms of action of metformin include decrease of the absorption of glucose by the small intestine, increase in glucose uptake by the cells, decrease in concentrations of fatty acids free in plasma, and inhibition of gluconeogenesis through the activation of protein kinase activated by the AMP (AMPK). Other mechanisms of action of metformin are related to its antiatherosclerotic action, hypotensive and anticarcinogenic action, and its impact on the endothelial function in the veins. The pleiotropic actions of metformin include the impact on plasma lipid profiles, the decrease of oxidative stress, and the increase in the fibrinolytic activity in the plasma. Metformin is actively transported to the hepatocytes and the renal tubular epithelium by organic cation transporters 1 and 2 coded by the corresponding SLC22A1 and SLC22A2 genes, respectively. The transporter of the multi-antimicrobial extrusion protein 1 (MATE1) coded by the gene SLC47A1 facilitates the excretion of metformin through bile and urine [122]. It seems that metformin reduces the accumulation of autophagic vesicles and death of the pancreatic β-cells in patients with type 2 DM. These effects can be associated with the restored expression of the lysosome membrane-associated protein-2 [123]. Metformin is capable of inhibiting the mTOR pathway independently of the AMPK, and it promotes the generation of and elimination of autophagic vesicles [124].

9.4. Vitamins. The antioxidant vitamins A, C, and E are ingested with food and are capable of directly neutralizing and detoxifying FR and interacting with the recycling processes to create reduced forms of the vitamins [125]. The antioxidant vitamins have diverse biological activities in stimulating the immune system and preventing genetic changes through inhibiting the oxidative damage to DNA [126]. There is little information on the role of vitamin C in DPN, though there is evidence that it normalizes the concentration of sorbitol in blood and diminishes LPO and regenerates GSH in DM [127].

Vitamin E or tocopherols react with the OH• to form a stable phenolic radical that is reduced to a phenol by the ascorbate and the NADPH enzyme dependent on the reductase enzymes [70]. Vitamin E has a preventative effect in diabetic complications through the decrease in LPO, although without demonstrating significant improvement in symptomatology of the

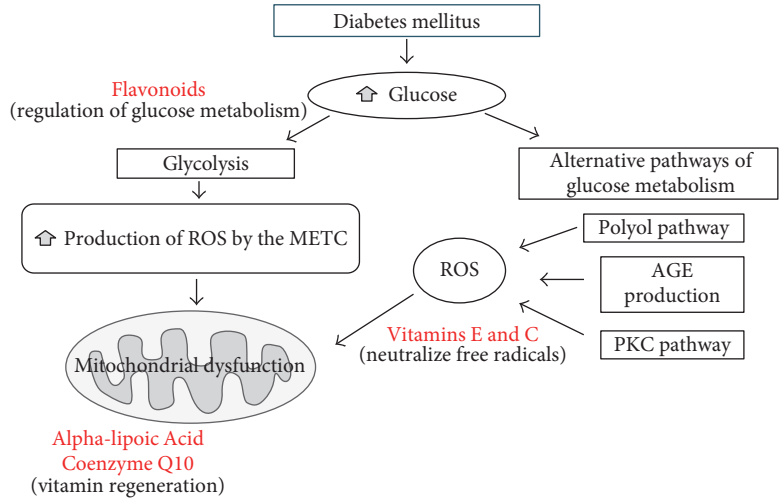

Figure 4: We show the theoretical mechanism of how hyperglycemia favors the activation of several metabolic pathways that favor the production of ROS causing mitochondrial dysfunction. The beneficial action of antioxidants in the regeneration of antioxidant vitamins and the effect of flavonoids in the regulation of hyperglycemia.

micro- and macrovascular complications despite reducing the markers of oxidative stress [128]. Vitamin E output is directed toward DPN because it has the ability to reduce neuropathic pain through modulating oxidative stress in the dorsal root ganglia [129]. Supplementation with vitamin E has been reported to significantly reduce blood glucose levels and glycated hemoglobin and has a neuroprotector effect in the myenteric nerves without affecting the intestinal area, the thickness of the intestinal wall, or muscular tone [130, 131].

L-Methylfolate, the active form of folic acid, is 7 times more bioavailable than folate and 3 times more effective in reducing homocysteine levels than folic acid [132]. Although the role of folic acid in vascular disease is not well established, active folate (5-methyltetrahydrofolate) can regenerate tetrahydrobiopterin (BH4). L-Methylfolate plays a role as an enzymatic cofactor for the conversion of the guanidinium nitrogen of L-arginine (L-Arg) to NO [133].

Benfotiamine is a synthetic lipid form of thiamine (B1) developed in Japan in the late 1950s to treat alcoholic neuropathy, sciatica, and other painful nerve conditions. Benfotiamine increases intracellular levels of thiamine di-phosphate (transketolase cofactor). This enzyme reduces AGE and LPO by directing its substrates to the pentose phosphate pathway. Reduction of AGE has been shown to contribute to the prevention of macro- and microvascular endothelial dysfunction in individuals with type 2 DM. Prospective cohort studies involving folic acid, benfotiamine, and its metabolites in PND patients will yield interesting results [134].

9.5. Flavonoids. The flavonoids are the largest and most important group of polyphenolic compounds. The flavonoids are widely distributed in plants, fruits, vegetables, grains, roots, stems, flowers, tea, and wine [135]. The antidiabetic properties of the flavonoids are primarily based on their effect on diverse molecular objectives and in the regulation of various pathways, like the reduction of apoptosis, improvement of proliferation of the pancreatic β-cells, promoting the secretion of insulin through regulation of glucose metabolism in hepatocytes on improving hyperglycemia, and decreasing insulin resistance, inflammation, and oxidative stress in adipocytes and skeletal myofibrils. They also favor the uptake of glucose by the skeletal muscle and adipose tissue [136]. Some subclasses of flavonoids can eliminate FR and chelate metals [137]. Taurine, acetyl-L-carnitine, and acetylcysteine have also reportedly demonstrated reducing the progression of DPN [15]. Polyphenols are potent antioxidants capable of contributing to the prevention of type 2 DM through its anti-inflammatory, antimicrobial, and immunomodulating properties. Citrus fruits contain polyphenols that have antioxidant and antidiabetic activity. Citrus polyphenols are mainly contained in the shell and have the ability to capture free radicals, in addition to antioxidant activity [138] (Figure 4).

9.6. Aldose Reductase Inhibitors. Inhibitors of aldose reductase in humans belong to the superfamily of aldo-keto-reductase proteins, characterized by catalyzing and limiting the polyol pathway of glucose metabolism by reducing glucose to sorbitol. Inhibitors of aldose reductase also reduce a wide range of aldehydes by detoxifying toxic lipids generated by oxidative stress by combining them with glutathione [139]. Accelerated flow of sorbitol through the polyol pathway has been implicated in the pathogenesis of secondary diabetic complications such as PND [140]. Previously, it was reported that the administration of albinase reductase inhibitors sorbinil or fidarestat in diabetic rats was able to correct depletion of glutathione and ascorbate induced by DM. At the same time, they are capable of correcting the negative regulation of SOD enzyme activity and the accumulation of LPO products in the peripheral nerves of the formation of $O_2\bullet-$ *vasa nervorum* of the retina associated with oxidative and nitrosative stress with the ability to inhibit the accumulation of poly(ADP-ribose), a marker of PARP activation in the diabetic nerve and retina [141]. Although in experimental animals, aldose reductase inhibitors have demonstrated the potential inhibition of secondary diabetic

complications, none of the aldose reductase inhibitors have been subjected to phase III clinical trials for the prevention of PND [142]. Recent studies suggest that increasing the polyol pathway could alter the NADPH/NADP ratio and attenuate GPx the GR by decreasing the reduced glutathione/oxidized glutathione which would cause oxidative stress [143]; it is interesting to explore the role of inhibition of aldose reductase in PND patients with a minimum follow-up of 5 years.

9.7. Free Radical Scavengers

9.7.1. Alpha-Lipoic Acid. Alpha-lipoic acid is a hydrophilic and lipophilic acid that can be synthesized by plants and animals where it is metabolized to dihydrolipoic acid when captured by the cells [144]. The alpha-lipoic acid and the dihydrolipoic acid are potent eliminators of FR and are involved in the regeneration of vitamins C and E and the GSH in the cell. Alpha-lipoic acid is also a cofactor for the production of diverse mitochondrial enzymes [145]. The ingestion of alpha-lipoic acid at a dose of 200–600 mg can provide up to 1000 times the quantity of available alpha-lipoic acid present in a regular diet. Preclinical and clinical data indicate that alpha-lipoic acid is safe and can be bioavailable in moderate doses. Gastrointestinal absorption of alpha-lipoic acid is variable and requires the consumption of food: its intake is recommended 30–60 min before food or 120 min after a meal [76]. It is rapidly absorbed and reaches maximum levels in the blood in 30–60 min, with a parenteral half-life of 30 min [146], and its consumption is considered safe in liver and kidney diseases [147]. One study done with alpha-lipoic acid over a four-year period reported that it is well tolerated in mild-moderate DPN and demonstrated significant clinical improvement and prevented the progression of the neuropathic disturbances without, at the end, impacting improvements in the neurophysiologic tests [148].

9.7.2. Resveratrol. Resveratrol is a stilbene compound and a phytoalexin. It is abundantly present in red wine, berries, red grapes, blueberries, peanuts, teasadori, hops, pistachios and grape juice, and cranberry. The antihyperglycemic effects of resveratrol appear to be the result of increased action of the glucose transporter on the cytoplasmic membrane. Neurons are extremely susceptible to damage induced by oxidative stress due to their high rate of oxygen consumption and low levels of antioxidant defense enzymes. The protective actions of resveratrol in DPN are attributed to its intrinsic FR scavenger properties. However, many other associated or separate mechanisms have recently been proposed such as the upregulation of Nrf2, SIRT1, and inhibition of the NF-κB transcriptional factor with a beneficial effect against nerve dysfunction [149]. Resveratrol emerges as an interesting management alternative to glycemic control in patients suffering from DPN.

10. In conclusion

The fundamental characteristic of patients with DPN is hyperglycemia with the capacity to unchain multiple and diverse processes among which are oxidative stress, RE oxidative stress, oxidative damage to DNA, mitochondrial dysfunction, alterations in the physiology of autophagy, the deregulation of endogenous antioxidants, and the variable effect of exogenous antioxidants in relation to metabolic control. Oxidative stress induced by hyperglycemia is mediated by several widely identified traditional signaling pathways, which at the same time are interesting therapeutic targets: (a) polyol, (b) hexosamine, (c) protein kinase C, (d) advanced glycosylation end-products, and (e) glycolysis. Management alternatives of these alterations emerge as interesting therapeutic targets in the study of the mechanisms of action at the molecular level as the FR scavengers and some nutrients with an antioxidant effect, always trying to correct the state of hyperglycemia.

Conflicts of Interest

There are no conflicts of interest to report.

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Review Article

Antioxidative Effects of Natural Products on Diabetic Cardiomyopathy

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Diabetic cardiomyopathy (DCM) is a common and severe complication of diabetes and results in high mortality. It is therefore imperative to develop novel therapeutics for the prevention or inhibition of the progression of DCM. Oxidative stress is a key mechanism by which diabetes induces DCM. Hence, targeting of oxidative stress-related processes in DCM could be a promising therapeutic strategy. To date, a number of studies have shown beneficial effects of several natural products on the attenuation of DCM via an antioxidative mechanism of action. The aim of the present review is to provide a comprehensive and concise overview of the previously reported antioxidant natural products in the inhibition of DCM progression. Clinical trials of the antioxidative natural products in the management of DCM are included. In addition, discussion and perspectives are further provided in the present review.

1. Introduction

Diabetes mellitus (DM) is one of the most common metabolic disorders, encountered in human populations worldwide. The number of adult diabetic patients was 285 million in 2010, and it is estimated to increase to 439 million by 2030 [1]. Persistent hyperglycemia can cause damage to various organs, including the heart, via different modes of action [2]. Amongst the numerous complications of DM, cardiovascular complications namely, hypertension, coronary heart disease, and diabetic cardiomyopathy (DCM) are the main causes of morbidity and mortality. DCM accounts for nearly 80% of the mortality noted in diabetic patients [3]. DCM is initially defined as the presence of abnormal myocardial structure and function in the absence of coronary artery disease, hypertension, and valvular disease [4]. Recent studies have proposed that DCM would be a result of a long-lasting process in which the myocardium is affected at a very early stage by metabolic changes prior to the diagnosis of DM [5]. This process progresses rapidly by the incidence of myocardial ischemia [5].

The clinical features of DCM include diastolic dysfunction at an early stage and systolic dysfunction at a late stage which result in reduced left ventricular function, early heart failure, myocardial fibrosis, and death [6]. This procedure is not accompanied by hypertension or coronary heart disease. Some patients may have no symptoms and/or mild diastolic dysfunction at the early stage, while with the progression of DCM, the patients may develop the following symptoms: shortness of breath, fatigue, weakness, and ankle edema [7].

The main cause of the pathological change of DCM is microangiopathy, which results in cardiac structural and functional alterations, such as apoptosis of the myocardium, myocardial interstitial fibrosis, and perfusion abnormality of the heart muscles. It was reported that capillary basement membrane thickening and microaneurysms were observed in patients with DCM [8, 9]. Once the myocardial interstitial fibrosis has developed, it cannot be reversed and a poor

prognosis of the diseases is frequently expected. Consequently, it is imperative to identify appropriate therapeutic targets notably at the early stage of DCM.

The pathogenesis of DCM has not been fully elucidated. Various biological processes have been shown to account for the pathogenesis and progression of DCM, including, but not are limited to, oxidative stress, cardiomyocyte apoptosis, disordered calcium handling, endoplasmic reticulum stress, myocardial insulin resistance, endothelial dysfunction, mitochondrial dysfunction, and autophagy [10, 11]; amongst which, oxidative stress is believed to be a key mechanism through which DM induces DCM. Despite a few reports regarding the antioxidative effects of natural products on DCM, a systematic review, to date, has not been provided. Here, we summarize the previous findings and provide perspectives and indications for future studies.

2. Role of Oxidative Stress in DCM

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen, including peroxides, superoxide, hydroxyl radical, and singlet oxygen [12]. Mitochondrion is the main "factory" in which DM produces excessive mitochondrial superoxide [13]. The DM-induced overproduction of mitochondrial superoxide leads to increased formation of advanced glycosylation end products (AGEs), expression of the receptor for AGEs (RAGE), and activation of protein kinase C (PKC), the polyol pathway, and the hexosamine pathway [14]. In case of the excess ROS not being balanced and/or removed via the action of endogenous antioxidative enzymes and/or exogenous antioxidant molecules, an increased oxidative stress occurs, which can result in damage to proteins, lipids, and DNAs in cardiomyocytes [15]. These detrimental effects eventually lead to the remodeling of the diabetic heart, followed by its dysfunction (Figure 1).

3. Signaling Pathways in the Regulation of Oxidative Stress in DCM

The excess production and inefficient removal of ROS causes the induction of oxidative stress. The improvement of the antioxidative mechanisms and the suppression of the oxidative stress are considered as key targets in the treatment of DCM. Key factors, such as nuclear factor erythroid 2-related factor 2 (Nrf2), RAGE, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), and peroxisome proliferator-activated receptor (PPAR), have notably been investigated with regard to the inhibition of oxidative stress (Figure 1).

3.1. Nrf2 Signaling. Nrf2 is a member of the cap 'n' collar family of proteins. The gene encoding Nrf2 belongs to a subset of basic leucine-zipper (bZip) genes that was reported to act as an essential regulator of antioxidative activity and electrophilic signaling [16]. Nrf2 can promote the expression and production of detoxification enzymes and antioxidant proteins, which contribute to the clearance of ROS and the restoration of the prooxidant/antioxidant balance [17–19]. Nrf2 combines with Kelch-like ECH-associated protein 1 (Keap1), which can rapidly degrade Nrf2 through ubiquitination by proteasome [20]. Certain chemical inducers, such as heavy metals, oxidizable diphenols, and Michael acceptors, can modify the cysteine residues in Keap1 that act as nucleophiles and activate Nrf2 by suppressing the degradation of the protein [20]. Under physiological conditions, Nrf2 combines with Keap1 in the cytoplasm, whereas under oxidative stress conditions, Nrf2 dissociates from Keap1 and translocates to the nucleus. The activated Nrf2 protein then binds antioxidant-responsive elements within the promoter regions of the antioxidant genes and induces transcription of a series of antioxidant enzymes, including NADPH quinone oxidoreductase (NQO1), glutathione-S-transferase (GSH), heme oxygenase-1 (HO-1), and γ-glutamylcysteine synthetase [18, 21–23] (Figure 1). Nrf2 is a key protective factor in a multitude of diseases, such as cancer [24], chronic degenerative pathology [25], metal-induced toxicities [26], and angiotensin II-induced apoptosis of testicular cells [27]. Recent studies demonstrated that Nrf2 was essential in the prevention of high glucose-induced oxidative damage in cardiomyocytes, endothelial cells and vascular smooth muscle cells [28–30], and in animal models of DCM [31]. It was reported that *Nrf2* knockout mice were prone to develop severe cardiomyopathy in a streptozotocin-induced diabetic model compared with wild-type mice [31]. These findings confirmed the protective function of Nrf2 in DCM. Therefore, the activation of Nrf2 is considered as a promising therapeutic target for the treatment of DCM.

3.2. RAGE Signaling. RAGE is a multiligand cell surface receptor, which can be activated by a wide range of ligands, such as AGEs [32] and amphoterin [33]. RAGE is expressed in numerous normal cell types, including cardiomyocytes [34], endothelial cells [35], mononuclear phagocytic cells [36], and vascular smooth muscle cells [37]. Under diabetic condition, the DM-induced formation of AGEs binds RAGE that is expressed on the cell membrane of cardiomyocytes and endothelial cells, leading to the production of ROS, proinflammatory cytokines, and the activation of nuclear factor kappa B (NF-κB). NF-κB, in turn, activates the expression of RAGE [38], resulting in more severe oxidative damage. AGEs/RAGE is positively involved in the activation of NOX [39]. It has been shown that the AGE/RAGE-induced ROS interaction with NOX to generate more ROS in human endothelial cells isolated from patients with type 1 diabetes (T1DM) [39]. The data demonstrated a cross-talk between NOX and AGE/RAGE signaling, as a positive feedback loop (Figure 1). The studies proposed that the inhibition of the AGE/RAGE signaling pathway can effectively reduce DM-induced oxidative stress, thereby ameliorating DCM.

3.3. NOX Signaling. NOXs contribute to the production of superoxide and hydrogen peroxide (H_2O_2) under pathological conditions [40]. There are 7 vascular NOX isoforms in total; amongst which, NOX1, NOX2, and NOX4 are highly expressed in the diabetic heart [41]. NOX4 is the major

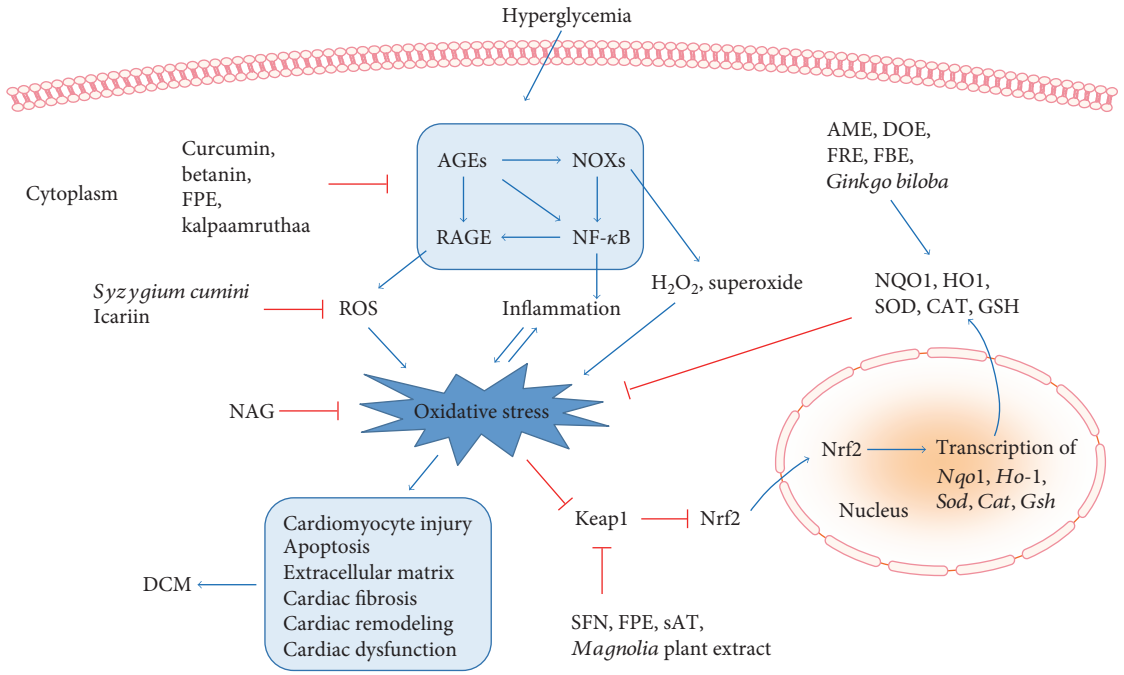

Figure 1: Role of antioxidative natural products in diabetic cardiomyopathy. Diabetes causes the formation of AGEs, leading to the activation of NOXs and RAGE, the effects of which induce overproduction of ROS, H2O2, and superoxide, followed by enhanced oxidative stress. AGEs can activate NF-κB both directly and indirectly through NOXs, resulting in inflammation, a status that positively amplifies oxidative stress and vice versa. Consequently, the diabetes-elevated oxidative stress can cause cardiomyocyte injury, apoptosis, accumulation of extracellular matrix, cardiac fibrosis, remodeling, and dysfunction, all of which are hallmarks of DCM. These effects can be blocked or blunted by several natural products, functioning through different targets. Curcumin, betanin, FPE, and kalpaamruthaa were reported to inhibit the AGE/RAGE/NOX/NF-κB pathway. *Syzygium cumini* and icariin decreased the formation of ROS. NAG had the ability to diminish diabetes-induced oxidative stress. In addition, several natural products were shown to elevate antioxidant capacity, via activating Nrf2 antioxidant system. SFN, FPE, sAT, and Magnolia plant extract inactivated Keap1, the key negative regulator of Nrf2, leading to the release of Nrf2. This effect facilitated nuclear translocation of Nrf2, resulting in the transcription of various antioxidant genes, such as *Nqo1*, *Ho-1*, *Sod*, *Cat*, and *Gsh*. As a result, these antioxidants were increased in the cytoplasm, acting as scavengers for the diabetes-induced excessive free radicals. AME, DOE, FRE, FBE, and *Ginkgo biloba* were reported to elevate the activity of these antioxidants. Collectively, the natural products, functioning either through blocking the formation of oxidative stress or through enhancing the scavenging activity, ameliorated DCM in experimental models. AGEs: advanced glycosylation end products; AME: *Aegle marmelos* leaf extract; CAT: catalase; DCM: diabetic cardiomyopathy; DOE: *Dendrobium officinale* extract; FBE: *Ficus racemosa* stem bark extract; FPE: Flos Puerariae; GSH: glutathione; HO-1: heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; NAG: North American ginseng; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NOX: NADPH oxidase; NQO1: NADPH quinone oxidoreductase; Nrf2: nuclear factor erythroid 2-related factor 2; RAGE: receptor for AGEs; ROS: reactive oxygen species; sAT: *Aralia taibaiensis*; SFN: sulforaphane; SOD: superoxide dismutase; $\uparrow$: activation or improvement; $\perp$: inhibition or downregulation.

NOX isoform that is expressed in cardiomyocytes [42] and has been demonstrated to be an important source of ROS (Figure 1). NOX4 is localized in the endoplasmic reticulum [43] and nucleus [44], interacting with NADPH as an electron donor, producing H_2O_2 or superoxide [45].

Increased NOX4 expression was found in the left ventricles of streptozotocin- (STZ-) induced diabetic rats [41]. This result was further confirmed in high glucose-cultured cardiomyocytes [41]. Moreover, treatment of high glucose-cultured cardiomyocytes with antisense NOX4 abrogated the high glucose-induced ROS production. In addition to the protective effect of NOX4 inhibition on high glucose- (HG-) induced cardiomyocyte injury, the beneficial effect of this approach was found in HG-treated neonatal cardiac fibroblasts as well [46], the result of which is in line with the finding that NOX4 plays an essential role in the differentiation of myofibroblasts [47, 48]. Hence, these studies suggest that the NOX4-inhibiting approach could be a promising strategy in the prevention of DCM.

4. Antioxidative Role of Natural Products in DCM

The antioxidative effect of natural products on the attenuation of DCM has been extensively investigated in recent years [49], showing promising outcomes. These natural products and their functions and mechanisms are listed below and in Table 1 and summarized in Figure 1.

4.1. Sulforaphane (SFN). SFN, initially isolated from broccoli sprouts, is a well-known activator of Nrf2 [50] and was intensively studied for its effects in diabetic complications in recent years [18, 31, 51–54]. SFN activates Nrf2

Table 1: Effects of natural products on diabetic cardiomyopathy.

Name	Model	Dose	Target	Effect	Ref.
Sulforaphane	STZ-induced diabetic C57BL/6J mice	0.5 mg/kg/d, for 3 months	Nrf2	Cardiac oxidative damage ↓, inflammation ↓, hypertrophy ↓, fibrosis ↓, and dysfunction ↓	[56]
	HFD/STZ-induced diabetic C57BL/6J mice	0.5 mg/kg/d, for 4 months	Nrf2	Cardiac LKB1/AMPK pathway ↑, lipotoxicity ↓, fibrosis ↓, inflammation ↓, and dysfunction ↓	[57]
	HFD/STZ-induced diabetic C57BL/6J WT and *Nrf2* KO mice and 129 s WT and *Mt* KO mice	0.5 mg/kg/d, for 4 months	Nrf2	Cardiac MT ↑, HO-1 ↑, NQO1 ↑, oxidative damage ↓, inflammation ↓, fibrosis ↓, hypertrophy ↓, and dysfunction ↓	[31]
Curcumin	STZ-induced diabetic Wistar rats	200 mg/kg/d, for 6 weeks	Free radicals	Myocardial capillary sclerosis ↓	[61]
	STZ-induced diabetic Wistar rats	100 or 200 mg/kg/d, for 16 weeks	AGEs/RAGE, NOX subunits, and SOD	Myocardial dysfunction ↓, cardiac fibrosis ↓, AGE accumulation ↓, oxidative stress ↓, inflammation ↓, apoptosis ↓, phosphorylation of Akt and GSK-3β ↑	[62]
	High glucose-treated neonatal rat cardiomyocytes	10 μmol/L, for 30 min	NOX subunits	HG-induced oxidative stress and apoptosis ↓	[65]
	STZ-induced diabetic Sprague-Dawley rats	100 mg/kg/d, for 8 weeks	PKC, NOX subunits, and TGF-β	Blood glucose ↓, cardiac oxidative stress ↓, lipid peroxidation ↓, antioxidant activity ↑, cardiomyocyte hypertrophy ↓, myocardial fibrosis ↓, left ventricular dysfunction ↓	[63]
	STZ-induced diabetic rats	20 mg/kg/d, for 45 days	HO-1 ↑	Expression of ANP, MEF2A, MEF2C, and P300 ↓, left ventricular function ↑	[64]
Icariin	STZ-induced diabetic Sprague-Dawley rats	30 or 120 ml/kg/d, for 8 weeks	Mitochondrial ROS	Myocardial collagen deposition ↓, ventricular hypertrophy ↓, body weight loss ↓, cardiac function ↑	[68]
Flos Puerariae	STZ-induced diabetic C57BL/6J mice	100 or 200 mg/kg/d, for 10 weeks	Expression of NOX and the antioxidants SOD and GSH	Cardiac remodeling↓, apoptotic cardiac cell death ↓	[69]
Betanin	High fructose feed-induced diabetic Sprague-Dawley rats	25 or 100 mg/kg/d, for 60 days	AGEs/RAGE, oxidative stress, and NF-κB	Cardiac fibrosis ↓, TGF-β1 and CTGF ↓	[70]
Chrysin	STZ-induced diabetic Wistar rats	60 mg/kg, for 28 days	PPAR-γ	Cardiac CAT ↑, MnSOD ↑, GSH ↑, AGEs/RAGE ↓, oxidative stress ↓, apoptosis ↓, cardiac dysfunction ↓	[71]
Aralia taibaiensis	High glucose-treated H9c2 cells	25, 50, or 75 μg/ml	Nrf2	Apoptosis ↓, ROS ↓, and oxidative damage ↓	[75]
Magnolia plant extract	High-fat diet-induced obese C57BL/6 mice	BL153 at 5 or 10 mg/kg/d, for 24 weeks	Not indicated	Cardiac lipid accumulation ↓, inflammation ↓, oxidative stress ↓, and apoptosis ↓.	[76]
	High-fat diet-induced obese C57BL/6 mice	4-O-methylhonokiol at 0.5 or 1.0 mg/kg/d, for 24 weeks	Nrf2/HO-1, Akt2	Cardiac oxidative stress ↓, lipid accumulation ↓, hypertrophy ↓, and dysfunction ↓	[78]
Abroma augusta L. leaf	STZ/nicotinamide-induced type 2 diabetic rats	100 or 200 mg/kg/d, for 4 weeks	Not indicated	Hyperglycemia ↓, hyperlipidemia ↓, membrane disintegration ↓, cardiac oxidative stress and oxidative stress-induced cell death ↓	[80]

Table 1: Continued.

Name	Model	Dose	Target	Effect	Ref.
Aegle marmelos leaf extract	Alloxan-induced diabetic rats	200 mg/kg/d, for 14 days	GSH, CAT, and SOD	Cardiac necrosis ↓ and inflammation ↓	[81]
Dendrobium officinale extract	STZ-induced Kunming diabetic mice	300 mg/kg/d, for 8 weeks	SOD	Cardiac MDA ↓, lipid accumulation ↓, and the expression of inflammatory and fibrotic factors ↓	[82]
Fermented rooibos extract	H_2O_2-treated cardiomyocytes isolated from the hearts of STZ-induced rats	1 or 10 μg/ml, for 6 hours	GSH	ROS generation ↓, apoptosis ↓	[84]
Ficus racemosa stem bark extract	STZ-induced diabetic Wistar rats	200 or 400 mg/kg/d, for 8 weeks	SOD	Cardiac MDA ↓	[85]
Ginkgo biloba	STZ-induced diabetic rats	100 mg/kg/d, for 3 months	SOD	Creatine kinase activity ↑, myofibril loss ↓, reduction of myocyte diameter ↓	[88]
Kalpaamruthaa	HFD/STZ-induced diabetic Sprague-Dawley rats	200 mg/kg/d, for 28 days	NOX, eNOS	Cardiac lipid peroxides ↓, proinflammatory cytokines ↓, matrix metalloproteinase-2 and matrix metalloproteinase-9 ↓, cardiac remodeling ↓	[89]
	HFD/STZ-induced diabetic Sprague-Dawley rats	200 mg/kg/d, for 28 days	PKC-β/Akt	Cardiac lipid accumulation ↓, chromatin condensation and marginalization ↓, hepatic antioxidants ↑, insulin resistance ↓, blood glucose ↓	[90]
	HFD/STZ-induced diabetic Sprague-Dawley rats	200 mg/kg/d, for 28 days	Cardiac expression of protease-activated receptor-1	Pancreatic antioxidants ↑, pancreatic lipid peroxides and carbonyl content ↓, markers of injury in the plasma, heart, and liver↓	[91]
North American ginseng	STZ-induced diabetic C57BL/6J type 1 diabetic mice or db/db type 2 diabetic mice	200 mg/kg/d, for 2 or 4 months	Oxidative stress	Cardiac extracellular matrix proteins and vasoactive factors ↓, hypertrophy ↓, dysfunction ↓	[92]
Pongamia pinnata	STZ/nicotinamide-induced type 2 diabetic rats	100 mg/kg/d, for 4 months	Not indicated	Cardiac SOD ↑, GSH ↑, MDA ↓, remodeling, dysfunction ↓, biomarkers for cardiac injury ↓, blood glucose ↓	[93]
Syzygium cumini	High glucose-treated H9c2 cells	9 μg/ml	ROS	Hypertrophy ↓, accumulation of extracellular matrix ↓	[94]

AGEs: advanced glycosylation end products; AMPK: 5′AMP-activated protein kinase; ANP: atrial natriuretic peptide; CAT: catalase; CTGF: connective tissue growth factor; eNOS: endothelial nitric oxide synthase; GSH: glutathione; GSK-3β: glycogen synthase kinase 3 beta; HFD: high-fat diet; HG: high glucose; HO-1: heme oxygenase-1; KO: knockout; LKB1: liver kinase B1; MDA: malondialdehyde; MEF2A: myocyte-specific enhancer factor 2A; MEF2C: myocyte-specific enhancer factor 2C; MT: metallothionein; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NOX: NADPH oxidase; NQO1: NADPH quinone oxidoreductase; Nrf2: nuclear factor erythroid 2-related factor 2; PKC-β: protein kinase C-beta; PPAR-γ: peroxisome proliferator-activated receptor-gamma; RAGE: receptor for AGEs; ROS: reactive oxygen species; SOD: superoxide dismutase; STZ: streptozotocin; WT: wild type; ↑: activation or improvement; ↓: inhibition or downregulation.

through inactivation of Keap1, via modifying specific residues within Keap1 protein [55].

In a STZ-induced mouse model of DM, treatment with SFN for either 3 months or 6 months significantly activated Nrf2 signaling and prevented DM-induced cardiac oxidative damage, inflammation, hypertrophy, fibrosis, and dysfunction [56]. Nrf2 played a crucial role in the protective effect of SFN, at least on HG-induced fibrotic response in cultured cardiomyocytes, since SFN lost this effect in the presence of *Nrf2* siRNA [56]. Similarly, in a mouse model of type 2 diabetes (T2DM), SFN was able to activate Nrf2 antioxidant signaling, the effect of which restored the oxidative stress-induced inhibition of liver kinase B1/5′ AMP-activated protein kinase (LKB1/AMPK) signaling pathway and prevented T2DM-induced lipotoxicity and cardiomyopathy [57]. The crucial role of Nrf2 in mediating the protection by SFN against DCM was further demonstrated by using Nrf2 knockout mice [31]. Moreover, metallothionein, a potent antioxidant [58], was identified to be a downstream target of Nrf2 and predominantly mediated SFN's protective effects on diabetic nephropathy [18] and DCM [31]. The effect of SFN has been tested in a double-blind clinical trial of T2DM, showing a significant improvement of insulin resistance [59]. In addition to the improvement of insulin resistance [59], SFN has the advantage of ameliorating DCM and therefore has a good potential for the use in future clinical trials of DCM.

4.2. Curcumin. Curcumin is a natural compound isolated from turmeric and has been widely used in indigenous medicine. Attention has been paid to the antioxidative effect of curcumin on DCM [60]. Curcumin was found to reduce myocardial capillary sclerosis [61]; attenuate cardiac tissue damage, myocardial cell hypertrophy, and apoptosis; reduce extracellular protein accumulation; and preserve left ventricular function [62–64] in the hearts of STZ-induced diabetic rats. Mechanistically, curcumin was found to increase HO-1 [64], catalase (CAT), superoxide dismutase (SOD), and GSH [61]. In addition, the ability to reduce the expression of the NOX subunits p22 phox, p47 phox, p67 phox, and gp91 phox could also account for curcumin's protective effects on DCM [62, 63]. In HG-cultured neonatal rat cardiomyocytes, curcumin suppressed the expression of the NOX subunit Rac1 and reduced HG-induced oxidative stress, the effect of which inhibited the HG-induced apoptotic cell death [65].

Although curcumin was found to have antioxidative effects on DCM, the exact target through which curcumin exerted the functions remained unclear. Gene knockout and silencing approaches could aid the investigation of the exact mechanism of antioxidative function induced by curcumin. Furthermore, curcumin exhibits a poor bioavailability in plasma and in target tissues which may hinder its therapeutic efficacy [22]. Therefore, the improvement of the pharmacokinetics and the increase in the plasma concentration of curcumin are significant therapeutic targets in the application of curcumin for the treatment of DCM. Recently, C66, a curcumin derivative with much a higher bioavailability [22, 66], was found to activate Nrf2 and its downstream antioxidants in the kidneys and aortas of the STZ-induced diabetic mice [22, 67]. Nrf2 played a prominent role in the C66 protection against diabetic nephropathy, since C66 lost partial, but significant, protection against the DM-induced renal damage [22] in the *Nrf2* knockout mice. Future studies need to focus on the bioavailability and off-target effects of curcumin.

4.3. Icariin. Icariin was reported to inhibit mitochondrial oxidative stress and increase SOD activity in the hearts of the STZ-induced diabetic rats. Icariin was shown, in this study, to reduce myocardial collagen deposition, inhibit ventricular hypertrophy, reverse the DM-induced body weight loss, and improve cardiac function [68]. These results may indicate the efficiency of inhibiting mitochondrial ROS generation and increasing antioxidant capacity in ameliorating DCM. However, blood glucose levels were not indicated by this report, the result of which might be important to know whether the amelioration of DCM was caused by the icariin reduction of oxidative stress or by the amelioration of DM.

4.4. Flos Puerariae (FPE). FPE was shown to inhibit gp91 phox and p47 phox, the two subunits of NOX in the hearts of STZ-induced C57BL/6J diabetic mice [69]. Additionally, FPE inhibited the DM-induced ROS generation and enhanced the activity of SOD and glutathione peroxidase (GSH-Px), maintained myocardial structure, and attenuated DM-induced apoptotic cardiac cell death [69]. Thus, FPE had the capability to both inhibit NOX expression and upregulate the expression of antioxidants in the hearts of the diabetic mice. Despite the speculation of c-Jun N-terminal kinase and P38 mitogen-activated protein kinase to be the target of FPE, further investigations are needed to elucidate the mechanism of this natural product in the amelioration of DCM.

4.5. Betanin. Betanin, extracted from natural pigments, was shown by Han et al. to have a protective effect against high fructose feed-induced diabetic cardiac fibrosis in Sprague-Dawley rats [70]. The DM-induced expression of the cardiac profibrotic factors transforming growth factor β (TGF-β)1 and connective tissue growth factor were significantly inhibited by betanin. The further mechanistic study revealed the efficacy of betanin in inhibiting the AGE/RAGE signaling, oxidative stress, and NF-κB under the diabetic condition. Given that cardiac fibrosis is a hallmark of DCM and contributes to cardiac dysfunction, the remarkable effect of betanin on the inhibition of fibrotic signaling should attract attention for the future studies on DCM.

4.6. Chrysin. Chrysin is a natural flavonoid that has antioxidative activity [71]. In a rat model of myocardial injury induced by STZ followed by isoproterenol injection, chrysin was found to activate PPAR-γ; upregulate CAT, MnSOD, and GSH; inhibit AGE/RAGE signaling and oxidative stress; and attenuate apoptosis [71]. These results provided evidence for PPAR-γ activation in the potential management of DCM. However, more studies are needed to clarify the exact role of chrysin and other PPAR-γ activators in ameliorating DCM.

4.7. Aralia taibaiensis (sAT). sAT, with antioxidative property [72, 73], is a traditional Chinese medicine that is frequently used in patients with DM [74]. Recently, sAT was reported to activate Nrf2 signaling and reduce HG and glucose oxidase-induced apoptosis, ROS, and oxidative damage in cardiomyocytes [75]. Nrf2 was the key factor through which sAT exerted the protection, since the effects of sAT were markedly abolished in the presence of the *Nrf2* siRNA.

4.8. Magnolia Plant Extracts (BL153 or 4-O-Methylhonokiol). BL153 showed beneficial effects on high-fat diet-induced cardiac [76] and aortic damage [77], via inhibition of oxidative stress. In addition, it has been shown that 4-O-methylhonokiol (MH), a bioactive constituent of BL153, reduced high-fat diet- (HFD-) induced cardiac pathological changes, including increased heart weight and abnormal echocardiography parameters [78]. The observation of the enhanced Nrf2/HO-1 signaling in the hearts of the MH-treated mice [78] could be responsible for the decreased oxidative stress by MH. Both BL153 and MH had the capability to lower serum lipid level and improve insulin resistance in HFD animal models [79]. The effect of BL153 and MH should be tested in future studies on animal models of T1DM, since Nrf2 was shown to play a key role in protection against DCM in T1DM.

4.9. Abroma augusta L. Leaf. The effect of *Abroma augusta* L. leaf, a natural product that is traditionally used in treatment of DM in India and South Asia, was tested in a streptozotocin-nicotinamide-induced rat model of T2DM [80]. *Abroma augusta* L. leaf was found to reduce hyperglycemia, hyperlipidemia, membrane disintegration, oxidative stress, and oxidative stress-induced cell death in the kidneys and hearts of the diabetic rats [80]. Phytochemical screening revealed the presence of taraxerol, flavonoids, and phenolic compounds in *Abroma augusta* L. leaf [80]. Therefore, the specificity and off-target effects of this natural extract needs further investigation, although the doses provided in this study did not produce side effects in the diabetic rats.

4.10. Aegle marmelos Leaf Extract (AME). AME was studied in a rat model of alloxan-induced DM for its effect on early-stage DCM [81]. The results showed that AME evidently increased the antioxidants GSH, CAT, and SOD and rescued the DM-induced cardiac necrosis and inflammation [81].

4.11. Dendrobium officinale Extract (DOE). DOE, a traditional Chinese medicine, was shown to elevate the antioxidant SOD and decrease the production of malondialdehyde (MDA), the accumulation of lipid, and the expression of the fibrotic factors TGF-β, collagen-1, and fibronectin, as well as the inflammatory factors NF-κB, tumor necrosis factor alpha, and interleukin-1 beta, in the hearts of STZ-induced diabetic mice [82]. DOE at 300 mg/kg also had a significant inhibitory impact on hyperglycemia and cardiac hypertrophy [82]. The effects of DOE on DCM were evident. However, the severe cardiac remodeling, such as hypertrophy and fibrosis, during the 8-week period of DM [82], needs to be further confirmed in future studies.

4.12. Fermented Rooibos Extract (FRE). The protective effect of FRE, from the root of a South African plant containing the antioxidant aspalathin [83, 84], was evaluated on DCM in a STZ-induced rat model of DM [84]. The results showed that FRE preserved GSH activity in the cardiomyocytes isolated from the diabetic rats and prevented the cells from H_2O_2 or an ischemic solution-induced generation of ROS and apoptosis [84]. This protective effect was more prominent as compared with that of another antioxidant vitamin E [84].

4.13. Ficus racemosa Bark Extract. The Indian medicine *Ficus racemosa* bark extract, possessing antioxidant activity, was tested in STZ-induced diabetic rats, for its effect on DCM [85]. The extract was found to enhance the activity of SOD and reduce the level of MDA in the hearts of the diabetic rats [85].

4.14. Ginkgo biloba Extract. Ginkgo biloba has various uses in traditional medicine and as a source of food [86, 87]. Fitzl et al. treated STZ-induced diabetic rats with *Ginkgo biloba* extract and found that *Ginkgo biloba* extract could prevent the loss of myofibrils and the reduction of cardiomyocyte diameter [88]. *Ginkgo biloba* extract was able to increase cardiac SOD activity, without altering the mRNA of inducible nitric oxide synthase [88]. Therefore, the protective effect of this natural product on DCM could be due to its action in scavenging upon DM-induced free radicals, but not blocking the source of ROS.

4.15. Kalpaamruthaa. The effect of kalpaamruthaa on DCM was tested in a rat model of T2DM [89]. Kalpaamruthaa was found by Latha et al. to reduce the expression of NOX and endothelial nitric oxide synthase, the effects of which led to a decrease in DM-induced accumulation of cardiac lipid peroxides, proinflammatory cytokines, matrix metalloproteinase-2 and matrix metalloproteinase-9, and cardiac remodeling [89]. A subsequent study by the same group showed that kalpaamruthaa inhibited DM-induced cardiac lipid accumulation, chromatin condensation, and marginalization; increased hepatic antioxidants; improved insulin resistance; and lowered blood glucose level [90]. The ability to inhibit PKC-β and enhance Akt activity was suggested to account for the protective effect of kalpaamruthaa on DCM [90]. In a following study, the same group further observed that kalpaamruthaa could increase pancreatic antioxidants and reduce pancreatic lipid peroxides and carbonyl content, markers of injury in the plasma, heart, and liver [91]. The decreased cardiac expression of protease-activated receptor-1 by kalpaamruthaa was indicated for the cardiac protection by kalpaamruthaa [91]. These studies addressed the beneficial role of kalpaamruthaa in protection against DCM. It is needed to clarify the mechanism of this natural product, since it is still unclear whether the protection was due to kalpaamruthaa's alteration of DM or was as a result of other mechanisms. Moreover, several factors were suggested to be the key targets of kalpaamruthaa. It would be helpful to elucidate the exact key factor through

Table 2: Antioxidative natural products in clinical trials of diabetes.

Name	Disease	Dose	Effect	Ref.
Aloe vera inner leaf gel powder	Patients with impaired fasting glucose or glucose tolerance	UP780 or AC952 at 500 mg, twice a day, for 8 weeks	Fasting glucose ↓, glucose tolerance ↑, serum lipoprotein levels ↓ (both UP780 and AC952), urinary F2-isoprostanes ↓ (UP780)	[98]
Black tea	T2DM	2.5 g/200 ml or 7.5 g/600 ml/d, for 12 weeks	Serum glycosylated hemoglobin ↓, cholesterol ↓, markers of oxidative stress ↓, regulatory T cell secretion ↑, proinflammatory cells ↓	[99]
Chamomile tea	T2DM	3 g/150 ml, 3 times a day, for 8 weeks	Serum glycosylated hemoglobin ↓, malondialdehyde ↓, insulin ↓, insulin resistance ↓, total antioxidant capacity ↑, SOD ↑, GSH ↑ and CAT activity ↑	[100]
Nigella sativa	T2DM	2 g/day, for 1 year	Fasting blood glucose ↓, glycosylated hemoglobin ↓, glucose homeostasis ↑, total antioxidant capacity ↑, the levels of GSH ↑ and SOD ↑	[101]
Phyllanthus emblica	T2DM	500 mg, twice daily, for 10 days	Platelet aggregation ↓, bleeding and clotting time ↑	[102]
Aged garlic extract	Patients with T2DM and high cardiovascular risk	1200 mg/d, for 4 weeks	No significant beneficial effects on body weight, blood pressure, lipids, insulin resistance, and biomarkers of endothelial dysfunction, oxidative stress, and inflammation.	[108]
Salvia miltiorrhiza hydrophilic extract	Diabetic patients with chronic heart disease	5 g, twice per day, for 60 days	Serum MDA ↓, GSH ↑, SOD ↑, paraoxonase ↑, and glutathione reductase ↑	[109]

T1DM: type 1 diabetes; T2DM: type 2 diabetes; ↑: activation or improvement; ↓: inhibition or downregulation. Other abbreviations are the same as in Table 1.

which kalpaamruthaa exerts the function by using gene silencing or gene knockout models.

4.16. North American Ginseng (NAG). NAG (*Panax quinquefolius*) is known to have multiple pharmacological functions due to its diverse phytochemical constituents. NAG was studied for its effect on DCM in both STZ-induced type 1 diabetic mice and DB/DB spontaneous type 2 diabetic mice [92]. NAG had the capability to reduce the DM-induced expression of cardiac extracellular matrix proteins and vasoactive factors, as well as cardiac hypertrophy and dysfunction in both types of the mice [92]. The DM-induced cardiac oxidative stress was diminished by NAG, the effect of which might mediate NAG's protection against DCM [92]. Given the diverse phytochemical constituents within NAG, the outcome must be a combination of all the effects exerted by all the constituents. It would be interesting to clarify the key targets of the constituents that may play the major roles in the effect of NAG on DCM. As a result, the most effective constituent and the most potent target could be screened out. This may increase the bioavailability and specificity.

4.17. Pongamia pinnata. Pongamia pinnata is a traditional medicine used in the treatment of DM, and its effect on DCM was tested by using STZ/nicotinamide-induced type 2 diabetic rats [93]. *Pongamia pinnata* decreased blood glucose level, increased the antioxidants SOD and GSH in the hearts of the diabetic mice, and exhibited cardiac protection against DM-induced oxidative damage, biomarkers for cardiac injury, cardiac remodeling, and dysfunction [93]. The exact target of this natural product was not indicated in this study, which needs to be further elucidated in future studies.

4.18. Syzygium cumini. Syzygium cumini was evaluated in H9c2 cells for its protection against glucose stress-induced injury and was found to attenuate high glucose-induced cell hypertrophy and accumulation of extracellular matrix [94], which are hallmarks of long-term diabetic complications [95, 96], including DCM [97].

5. Clinical Trials

Despite the reports of animal and cell experiments on the effects of natural products on DCM (Table 1), the use of natural products in clinical trials, to date, has not been conducted. However, the clinical trials of antioxidative natural products in the management of DM should also benefit DCM and may provide clues for the future clinical trials of DCM. Therefore, the natural products used in clinical trials of DM, in terms of their efficacy to inhibit oxidative stress, are listed in Table 2.

5.1. Aloe vera Inner Leaf Gel Powder. Aloe vera inner leaf gel powders (UP780 and AC952) were used in patients with impaired fasting glucose or impaired glucose tolerance during an 8-week period in a double-blind, placebo-controlled study [98]. It was reported that both AC952 and UP780 could markedly reduce fasting glucose and improve glucose tolerance and lipoprotein levels in the plasma [98]. However, the reduction of oxidative stress marker urinary F2-isoprostanes was solely noted for UP780 compared with the placebo.

5.2. Black Tea. Mahmoud et al. tested the effects of black tea ingestion on the secretion of inflammatory cytokines and metabolic biomarkers in 30 patients with T2DM [99]. The

results indicated that treatment with black tea at 200 or 600 ml per day, for 12 weeks, resulted in significantly decreased glycosylated hemoglobin levels and decreased total serum cholesterol levels and the markers of oxidative stress. Furthermore, the supplementation of black tea could inhibit the inflammatory response including an increase in regulatory T cell secretion and a decrease in the production of proinflammatory cells [99].

5.3. Chamomile Tea. A single-blind randomized controlled clinical trial was conducted on 64 patients with T2DM to evaluate the effect of the antioxidative natural product chamomile tea [100]. Chamomile tea, at 3 g/150 ml, 3 times per day, was administered for 8 weeks and was shown to decrease the concentration of serum glycosylated hemoglobin, MDA, insulin, and improved insulin resistance [100]. Additionally, chamomile tea increased total antioxidant capacity, SOD, GSH, and CAT by 6.81%, 26.16%, 36.71%, and 45.06%, respectively [100]. This trial indicates that intake of chamomile tea could benefit glycemic control and antioxidant status in patients with T2DM.

5.4. Nigella sativa. The long-term effect of *Nigella sativa* on T2DM patients taking standard hypoglycemic drugs was evaluated [101]. The 1-year treatment with *Nigella sativa* led to elevated serum total antioxidant activity and the levels of GSH and SOD, as compared with the non-*Nigella sativa*-treated group [101]. Additionally, a significant decrease in fasting blood glucose and glycosylated hemoglobin and an improvement in insulin resistance and β-cell activity were observed in the *Nigella sativa*-treated group [101]. These findings suggest the potential of *Nigella sativa* supplementation as an alternative method to benefit patients with T2DM.

5.5. Phyllanthus emblica Extract (PEE). PEE has a rich source of vitamin C, which is an important antioxidant that prevents platelet aggregation in healthy individuals and patients with coronary artery disease [102]. The 10-day treatment with PEE, at a dose of 500 mg twice per day, was well tolerated in patients with T2DM, exhibited inhibitory effect on platelet aggregation, and prolonged the bleeding and clotting time, the effects of which were similar to those of the daily treatment with 75 mg clopidogrel or ecosprin [102].

5.6. Aged Garlic Extract. Aged garlic extract has antioxidative and antihyperglycemic effects [103–105]. Supplementation of aged garlic extract in the diet reduced oxidative stress and improved endothelial dysfunction in humans [106, 107]. In a double-blind randomized placebo-controlled crossover pilot trial in patients with T2DM and high cardiovascular risk (30% risk of a cardiovascular event in the next 10 years), the 4-week treatment with aged garlic extract, administered daily at 1200 mg, did not produce significant beneficial effects on body weight, blood pressure, lipids, insulin resistance, and biomarkers of endothelial dysfunction, oxidative stress, and inflammation [108]. Although the authors indicated that the recruitment of patients with higher cardiovascular risk or the supplementation of aged garlic extract for a longer period would produce more pronounced effects [108], the mechanism, dose, and safety of this natural product should be further explored in future studies.

5.7. Salvia miltiorrhiza Hydrophilic Extract (SMHE). A randomized controlled clinical study was conducted, using SMHE, to test the protective and antioxidative properties of the extract in diabetic patients with chronic heart disease [109]. SMHE was administrated at a dose of 5 g, twice per day, to the patients for 60 days in addition to their antihyperglycemic therapies. The serum MDA level was decreased by SMHE on day 30. On day 60, the serum GSH, SOD, paraoxonase, and glutathione reductase were significantly increased by SMHE. The lipid levels were not altered by SMHE [109]. This study indicates that SMHE has antioxidative activity and attenuates oxidative stress in diabetic patients with chronic heart disease. Determination of serum biomarkers for cardiac injury, cardiac remodeling, and cardiac dysfunction would be helpful in order to know the cardiac protective effect of SMHE under the diabetic condition.

6. Conclusions

Natural products with antioxidative properties have been shown to ameliorate DCM in animal models and cardiomyocytes. Although no natural product has been used in clinical trials specifically targeting DCM, several have been used in clinical trials in patients with DM, even as traditional medicines for the treatment of DM for many years. The capability of the natural products in enhancing serum antioxidative activity and reducing serum oxidative stress may also be beneficial for the amelioration of DCM. Given the antioxidative and even blood glucose-lowering effects of the natural products, they have a great potential for the future clinical use as alternative medicines for the management of DM and DCM.

Despite the bright future of the natural products in the treatment of DCM, a few challenges should be carefully considered before the use of natural products in clinical trials. Natural products have multiple targets. This fact may lead to off-target effects, including both the beneficial and the detrimental. Hence, more selective compounds should be developed. The development of SFN and curcumin, isolated from natural products, is a good example, although the two compounds have multiple targets. In-depth mechanisms need to be elucidated, the result of which can provide a solid basis for the development of novel high-selective compounds. In addition, the blood glucose-lowering effect of some natural products is beneficial. However, whether or not natural products lead to unstable blood glucose level in diabetic patients is a concern. Thus, attention should be paid to the fine control of blood glucose level using the combination of standard medicines and natural products as alternative medicines in the treatment of DM or DCM.

In summary, natural products with antioxidative profiles in the management of DCM on one hand have great potentials and face great challenges on the other. The success of natural products in DCM requires extensive studies on the mechanism, specificity, bioavailability, and drug-to-drug interactions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Bingdi Yan and Jin Ren contributed equally to this work.

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Review Article

SIRT1 Regulates Cognitive Performance and Ability of Learning and Memory in Diabetic and Nondiabetic Models

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Type 2 diabetes mellitus is a complex age-related metabolic disease. Cognitive dysfunction and learning and memory deficits are main characteristics of age-related metabolic diseases in the central nervous system. The underlying mechanisms contributing to cognitive decline are complex, especially cognitive dysfunction associated with type 2 diabetes mellitus. SIRT1, as one of the modulators in insulin resistance, is indispensable for learning and memory. In the present study, deacetylation, oxidative stress, mitochondrial dysfunction, inflammation, microRNA, and tau phosphorylation are considered in the context of mechanism and significance of SIRT1 in learning and memory in diabetic and nondiabetic murine models. In addition, future research directions in this field are discussed, including therapeutic potential of its activator, resveratrol, and application of other compounds in cognitive improvement. Our findings suggest that SIRT1 might be a potential therapeutic target for the treatment of cognitive impairment induced by type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of multiple age-related metabolic diseases [1]. Several latest studies have demonstrated severe and progressive abnormalities in brain structures and cognition during the early stage of T2DM [2]. T2DM is a risk factor for mild cognitive impairment (MCI) [3] and can accelerate the rate of functional decline in patients with mild dementia [4]. Cognitive dysfunction and learning and memory deficits have been considered one of the most prevalent and significant T2DM-related complications [4–8]. In recent years, silent information regulator 2 (Sir2), the highly conserved nicotinamide adenine dinucleotide- (NAD^+-) dependent histone deacetylase [9], was shown to extend lifespan and delay aging in numerous studies ranging from *Saccharomyces cerevisiae* to mammals [10, 11]. As the ortholog of the yeast Sir2, SIRT1 is the most evolutionally conserved member [12]. Accumulating evidence has suggested that SIRT1 is expressed in the liver, skeletal muscle, pancreas, adipose tissues, and brain [13, 14], but its levels in the brain are notably higher than those in the other tissues in mammals [12, 15, 16], especially in the hippocampus, a vital structure closely related to learning and memory of the central nervous system [17]. SIRT1 participates in apoptosis [18], autophagy [19], and development [20], as well as in metabolism [21, 22] and circadian rhythms [23, 24]; therefore, it is not surprising that SIRT1 affects more complex biological processes including aging [24–27], MCI [28], and cognitive decline [29–31].

Present opinion on SIRT1 in cognition, learning, and memory is inconclusive. Some scholars believe that SIRT1 is positive for memory conservation. The spontaneous senescence-accelerated P8 mouse strain (SAMP8) is widely used as an animal model of aging [32–34] due to learning and memory deficits and behavioral alterations of Alzheimer's disease (AD) [35–39]. It has been demonstrated that the expression of SIRT1 declines with age in the brain of SAMP8 and senescence-accelerated mouse resistant 1 (SAMR1) [40], which have been extensively used as a control model because of the same genetic background and normal aging characteristics [41]. However, SIRT1 was decreased in the cerebral cortex and hippocampus [42] of SAMP8 mice [43, 44] compared with those of age-matched SAMR1. In addition, SIRT1 was downregulated in diverse models of cognitive impairment

in vivo and *in vitro*, such as in juvenile C57BL/6J mice with dysmetabolism induced by high-caloric diet [45] and neurotoxic primary hippocampal neurons caused by toxins [44]. A study by Yokozawa et al. on antiaging effects of oligomeric proanthocyanidins found that SIRT1 was increased both in the cellular senescence model [46] and SAMP8 mouse model [47]. On the other hand, some researchers believed that SIRT1 has no effect on cognitive improvement and has a counterproductive effect. Most tellingly, earlier studies have proved that overexpression of SIRT1 may induce the memory deficit in transgenic (Tg) mice that overexpresses human SIRT1 in neurons [48]. Nicotinamide, an inhibitor of SIRT1, has been shown to attenuate cognitive deficits of 3xTg-AD mice via inhibition of SIRT1 and phosphorylation of tau [49]. Moreover, recent work has demonstrated that SIRT1 silencing could promote neuronal survival and protect neurons via the IGF-1 pathway [50].

Collectively, SIRT1 plays a significant role in learning and memory and provides enormous insights into T2DM-associated cognitive dysfunction. It is also rapidly emerging as a critical regulator of aging. However, positive or negative effects of SIRT1 on learning and memory have yet to be further discussed. In the ensuing paragraphs, we highlighted the involvement of SIRT1 in pathological processes of cognitive impairment in diabetic and nondiabetic models.

2. Role of SIRT1 on Cognition and Learning and Memory in Nondiabetic Models

2.1. Deacetylation of SIRT1. Many studies have confirmed that SIRT1 mediates chromatin silencing and chromatin remodeling through deacetylating histones, including H1, H3, and H4 [51] and modulates the activity of several protein targets that will be stated subsequently.

2.1.1. SIRT1-p53 Pathway. It has been claimed that SIRT1 directly bound to and deacetylated p53 with specificity for its C-terminal Lys382 residue, inhibited acetylation of p53, and reduced the activity of downstream target genes [52, 53].

Decreased level of SIRT1 and increased level of acetylated p53 were observed in the hippocampal tissue [54] and cortex [55] of SAMP8 and *in vitro* studies [56]. Coincidentally, in juvenile C57BL/6J mice, low-caloric intake increased learning and memory function through positively downregulating p53 and unremarkably upregulating SIRT1 [45]. In the following studies, although no difference in SIRT1 level was detected between the control and the resveratrol dietary groups, researchers found that resveratrol improved learning and memory through the SIRT1-p53 pathway [57].

2.1.2. SIRT1-AMPK Pathway. SIRT1 improves mitochondrial function by activating adenosine monophosphate-activated protein kinase (AMPK) through acetylating liver kinase B1 (LKB1) [58]. Conversely, AMPK improves SIRT1 activity by increasing cellular NAD^+ levels to trigger the deacetylation of SIRT1 [59]. In the SAMP8 model, the increases in phosphorylated AMPK (p-AMPK) that regulate energy expenditure and the decreases in the production of reactive oxygen species (ROS) paralleled to the rise in SIRT1 in the hippocampus [54] and cortex [55]. Although without detection of LKB1, we suggest that this process might be triggered by SIRT1 deacetylation. In addition, in a rat model of AD with intracerebroventricular injection of streptozotocin (ICV-STZ) [60], the level of p-AMPK and SIRT1 activity were decreased and the level of phosphorylated tau was increased, while AMPK-specific activator prevented cognitive impairment through rescuing SIRT1 activity, downregulating tau hyperphosphorylation, and repairing mitochondrial function reflected by increased ATP levels, mitochondrial membrane potential, complex I activity, and SOD activity, as well as decreased ROS generation.

2.1.3. Other Factors Deacetylated by SIRT1. In addition to the tumor suppressor factor p53 [61, 62] and serine-threonine protein kinase LKB1 [58, 63], SIRT1 deacetylated several transcriptional factors participated in transcriptional control of key genes in multiple cellular processes. These transcriptional factors regulate a wide range of metabolic activities, such as nuclear factor-kappa beta (NF$\kappa\beta$) [64], extracellular signal-regulated kinase (ERK) [65], the forkhead box subgroup O (FoxO) family [66, 67], peroxisome proliferator-activated receptors γ (PPARγ), and its transcriptional coactivator PPARγ coactivator 1-α (PGC-1α) [68, 69].

Direct *in vivo* evidence supported the link between SIRT1 and improvement of cognitive decline. The spatial memory deficit of ICV-STZ-treated rats was improved through ameliorating activation of SIRT1, which in turn attenuated tau phosphorylation by decreasing ERK1/2 phosphorylation [65]. In a study on neuroprotective role of intermittent fasting (IF) [70], upregulation of SIRT1 in the cortex and hippocampus of SAMP8 could possess neuroprotection via modulating downstream factors, including a decrease in phosphorylated Jun-terminal kinase (JNK), acetylated NF$\kappa\beta$ [71], and acetylated FoxO1, as well as an increase in phosphorylated FoxO1. Additionally, in the hippocampus and cortex of SAMP8 mice [71] and in the hippocampus of 3xTg-AD mice [72], SIRT1 upregulated a disintegrin and metalloprotease 10 (ADAM10) [73] and downregulated the phosphorylated form of glycogen synthesis kinase 3 beta (GSK3β) [55, 70] in order to reduce the production of amyloid beta (Aβ) peptides and tau phosphorylation, which have been widely accepted as vital causes of cognitive decline [74]. SIRT1 was also noted to increase the expression of heat shock protein 70 (HSP70), a biomarker of neuronal survival, in SAMP8 models [70] and 3xTg-AD mice [72].

Moreover, an indirect proof of the effect of SIRT1 on cognition was demonstrated *in vitro*. In a study on neurite outgrowth and cell survival, SIRT1 was shown to promote neuronal growth through negative modulation of the mammalian target of rapamycin (mTOR)/p70S6 kinase (p70S6K) pathway in wild-type mouse primary neurons and human SIRT1 transgenic mice [75]. Furthermore, Codocedo et al. have suggested that SIRT1 accelerated the development and maintenance of dendritic branching in Sprague-Dawley rat primary hippocampal neurons by inhibiting the RhoA/Rho-associated protein kinase (ROCK) pathway and activating the Rac1/JNK pathway [76].

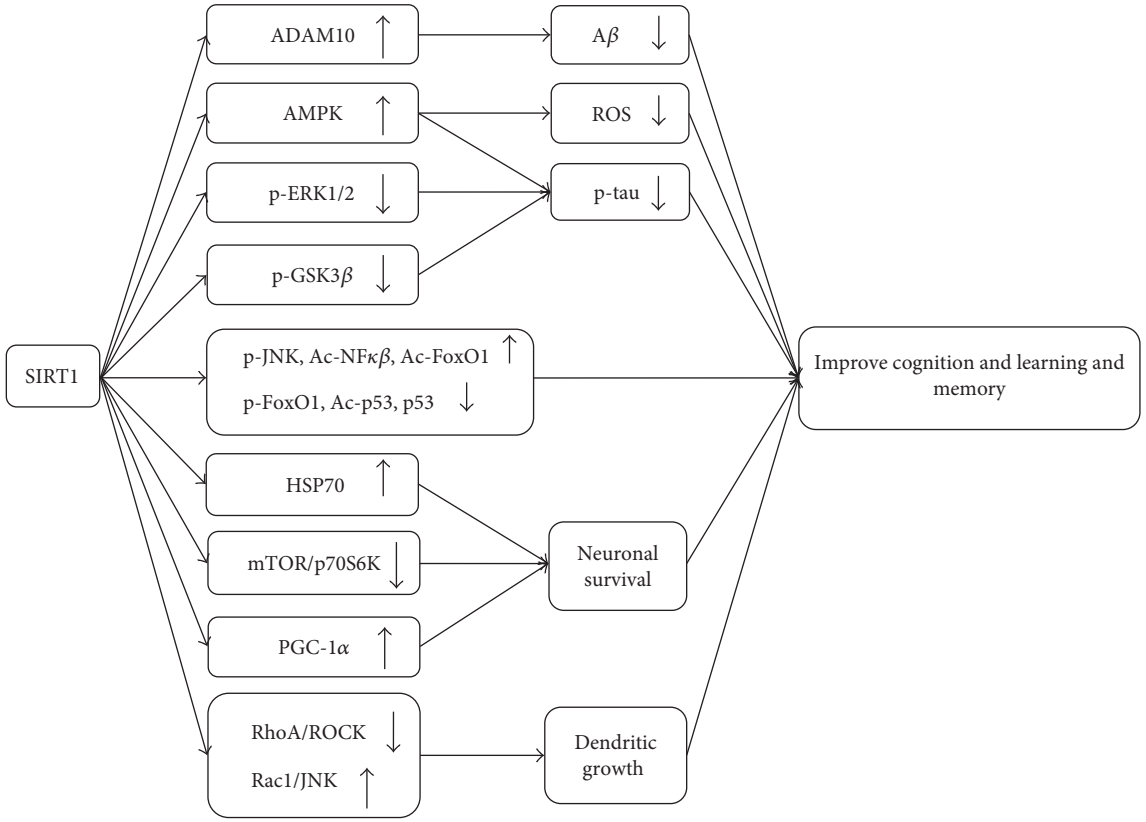

Figure 1: Deacetylation of SIRT1 in cognition and learning and memory. Increased SIRT1 level may reduce the production of ROS, Aβ, and p-tau, as well as promote neuronal survival and dendritic growth, contributing to improve learning and memory. ADAM10: a disintegrin and metalloprotease 10; AMPK: adenosine monophosphate-activated protein kinase; p-ERK: phosphorylated extracellular signal-regulated kinase; p-GSK3β: phosphorylated glycogen synthesis kinase 3 beta; p-JNK: phosphorylated Jun-terminal kinase; Ac-NF$\kappa\beta$: acetylated nuclear factor-kappa beta; Ac-FoxO1: acetylated the forkhead box subgroup O 1; Ac-p53: acetylated p53; HSP70: heat shock protein 70; mTOR: mammalian target of rapamycin; p70S6K: p70S6 kinase; PGC-1α: peroxisome proliferator-activated receptor γ transcriptional coactivator 1-α; ROCK: Rho-associated protein kinase; JNK: Jun N-terminal kinase; Aβ: amyloid beta; ROS: reactive oxygen species; p-tau: phosphorylated tau.

SIRT1was shown to attenuate glutamate-induced apoptosis in SH-SY5Y cells by upregulating PGC-1α [77].

Such a point is worthy of further confirmation since growing evidence has indicated the presence of relationship between the role of SIRT1 on learning and memory and histone H2A variant, H2A.Z, which has been considered a negative regulator of consolidation of recent and remote memory [78]. H2A.Z was negatively regulated by the expression and activity of SIRT1 in some tissues [79] (Figure 1).

2.2. Targeting Oxidative Stress (OS). As the basis of aging theories [80], OS can trigger the pathological processes of learning and memory deficits [81, 82]. A series of biomarkers represent the degree of OS, such as superoxide dismutase (SOD), reactive oxygen species (ROS), and malondialdehyde (MDA). Mitochondrial dysfunction is the central to oxidative damage and reflects the aging processes [83].

2.2.1. Amelioration of Mitochondrial Dysfunction by SIRT1. There is a growing body of evidence supporting that mitochondrial dysfunction is critical for synaptic aging induced by chronic OS [82]. Data gathered from diverse studies have confirmed that oxidative stress could cause damages in the brain of SAMP8 mice [84, 85]. SAMP8 primary neurons had poor mitochondrial function, lower mitochondrial membrane potential, and higher mitochondrial vulnerability, all of which was protected by increased SIRT1 expression [56]. In addition, electron transport chain (ETC) related to mitochondrial oxidative phosphorylation (OXPHOS) was changed *in vitro* [56]. It was remarkable to find that SIRT1 could enhance OXPHOS via increasing the electronic chain-specific components ranging from complex I to complex V in the hippocampus of SAMP8 [54]. Consistent with this notion, SIRT1 improved spatial learning and memory deficits via SIRT1-mediated antioxidant signaling pathways in the D-galactose-induced aging rats [86]. Manganese superoxide dismutase (Mn-SOD) is an important antioxidative enzyme present in mitochondria. Recent data have shown downregulation of Mn-SOD mRNA levels by increasing level of SIRT1 [86].

2.2.2. Interaction of ROS, Inflammatory Factor, and SIRT1. Lower levels of SOD, as well as higher levels of ROS [87], MDA [88], and some proinflammatory factors [87, 88], were

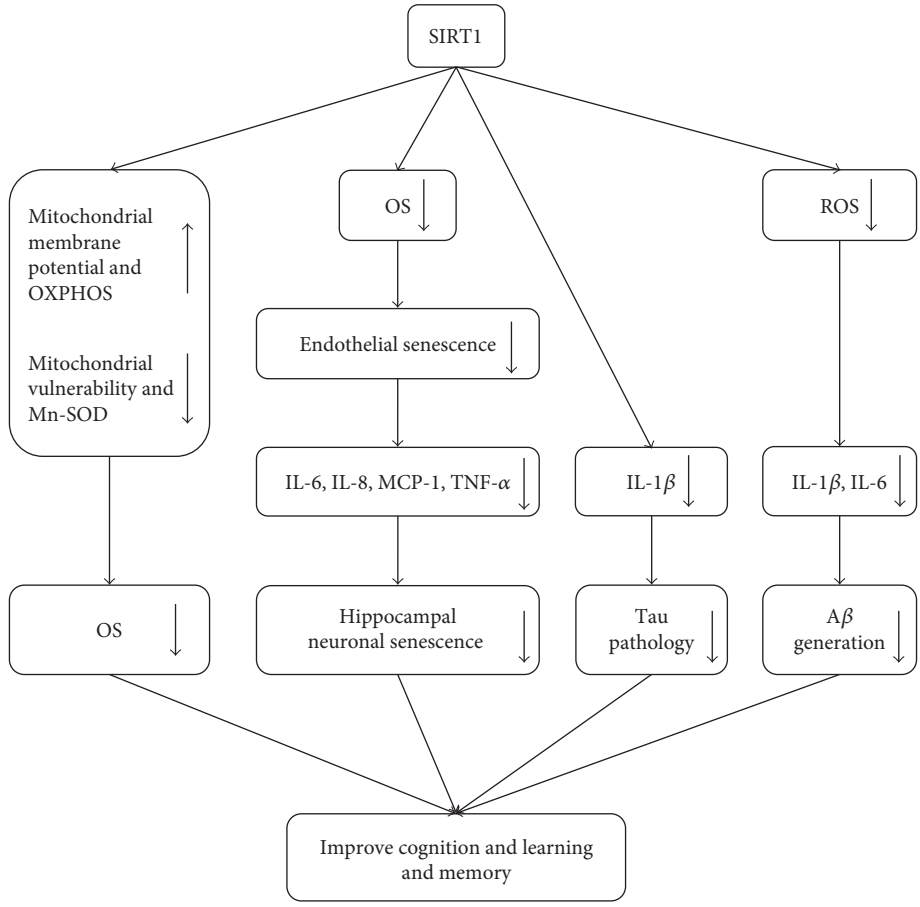

Figure 2: Oxidative stress (OS) is regulated by SIRT1 in cognition and learning and memory. SIRT1 may improve learning and memory by inhibiting OS, inhibiting the inflammatory response and hippocampal neuronal senescence, and decreasing the expression of tau and Aβ. OS: oxidative stress; OXPHOS: oxidative phosphorylation; Mn-SOD: manganese superoxide dismutase; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; ROS: reactive oxygen species; Aβ: amyloid beta.

found in SAMP8 compared with age-matched SAMR1. OS aggravated cognitive loss in SAMP8 models through either generating $A\beta_{1-40}$ and $A\beta_{1-42}$ by releasing interleukin-1β (IL-1β) and interleukin-6 (IL-6) [87] or enhancing neuroinflammatory activity by increasing IL-1β, tumor necrosis factor-α (TNF-α), and IL-6 [88]. In line with above evidence, *in vitro*, senescent endothelial cells induced by OS promoted the senescence of hippocampus neuronal cells through secretion of several inflammatory cytokines such as IL-6, interleukin-8 (IL-8), monocyte chemoattractant protein-1(MCP-1), and TNF-α [85]. Upregulation of SIRT1 could reverse inflammatory factors to rescue the production of Aβ and neuronal senescence [85, 87]. In addition, microglial SIRT1 deficiency elevated levels of IL-1β and exacerbated memory deficits in human P301S tau mice [29] exhibiting age-dependent synaptic loss and tau-mediated memory deficits [89]. All above have demonstrated that neuroinflammatory played a significant role in learning and memory modulated by SIRT1 (Figure 2).

2.3. SIRT1-microRNA Pathway. It is assumed that cyclic AMP response element-binding protein (CREB), a molecular switch of long-term memory that maintains cognitive function [90], binds to several promoters of brain-derived neurotrophic factor (BDNF) and regulates its expression. Recent studies have shown that SIRT1 promotes plasticity and memory in a direct manner via a miR-134-mediated posttranscriptional mechanism. The results suggested that SIRT1 cooperated with Yin Yang 1 (YY1) in binding to the upstream regulatory elements of miR-134 and then limited the expression of miR-134 resulting in overexpression of CREB and BDNF, thereby regulating synaptic plasticity and long-term memory formation in SIRT1-KO mice [91]. Additionally, resveratrol was shown to improve learning and memory in normally aged C57BL/6J mice through the SIRT1-microRNA pathway [92]. Furthermore, SIRT1 increased the expression of BDNF in SAMP8 models [70] and 3xTg-AD mice [72]. In the hippocampus of rats receiving lead exposure, SIRT1 and CREB phosphorylation were decreased in a dose-dependent manner, which could be reversed by resveratrol [93]. Resveratrol also ameliorated spatial learning memory impairment induced by $A\beta_{1-42}$ in rat hippocampus by elevating SIRT1 expression and CREB phosphorylation [31]. Although miR-134 was not detected

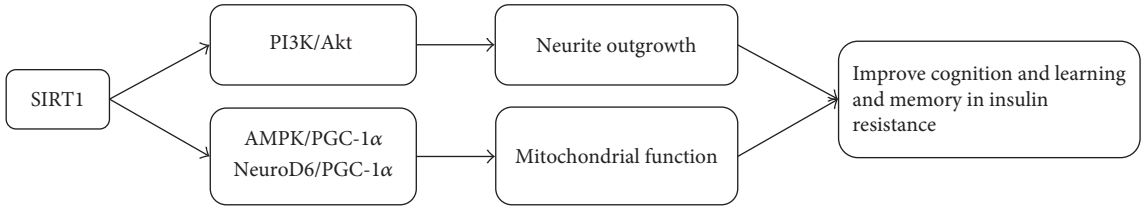

Figure 3: The role of SIRT1 in cognition and learning and memory under the condition of IR. SIRT1 has been shown to increase neurite outgrowth by activating the PI3K/Akt pathway and improve mitochondrial function through the AMPK/PGC-1α and NeuroD6/PGC-1α pathways. PI3K: phosphoinositide 3-kinase; AMPK: adenosine monophosphate-activated protein kinase; PGC-1α: PPARγ coactivator 1-α; NeuroD6: neuronal differentiation 6.

in the above three studies, we still suggested that SIRT1 protects learning and memory via the SIRT1-miR-134 pathway.

3. The Mechanism of SIRT1 on Cognition and Learning and Memory under the Condition of Insulin Resistance (IR)

In the above section, we have summarized the role of SIRT1 in cognitive dysfunction and learning and memory deficits under normal physiological condition. Next, we will discuss its role under the condition of insulin resistance. It is widely known that caloric restriction (CR) has benefits on cognition decline [94]. Emerging evidence has indicated a causal link between T2DM and cognition decline and learning and memory deficits [3–5, 95–97], such as MCI [98]. The mechanisms that trigger learning and memory deficits in diabetic models include inflammation [99], loss of neuronal plasticity [100, 101], alteration of mitochondrial structure and function [102, 103], elevation of cerebral Aβ, and tau phosphorylation [100]. Therefore, cognitive ability is distinctly affected by metabolic status.

Accumulating evidence has indicated the inhibition of SIRT1 protein expression and activity in T2DM or IR [104–107]. Data has shown that activated SIRT1 improves the insulin sensitivity of the liver, skeletal muscle, and adipose tissues, as well as protects the function and cell mass of pancreatic β-cells [13]. So, does SIRT1 involve in it? And whether SIRT1 regulates learning and memory directly or indirectly? Next, we set forth the role of SIRT1 in cognition and learning and memory under the condition of IR (Figure 3).

3.1. SIRT1 Promotes Neurite Outgrowth. Several studies have demonstrated that SIRT1 modulates neuronal viability [36, 37], neuronal differentiation [38–41], neuronal protection [42–44], and synaptic plasticity [21, 45–47], all of which are key factors largely linked to cognitive improvement. It is well established that insulin exerts its actions in a series of biological processes through binding to insulin receptors [108], as well as plays an essential role in IR and T2DM. Recently, researchers have demonstrated that insulin-induced neurite outgrowth is regulated by SIRT1, which is dependent on the PI3K/Akt signaling pathway in SH-SY5Y cells [109]. In accordance with the above views, we suggest that SIRT1 may be imbalanced when insulin signaling is impaired and cause an influence on cognition and neurodegeneration.

3.2. SIRT1 Improves Mitochondrial Function in the Brain. SIRT1 activation has a significant coordinating role in mitochondrial function. It is noteworthy that NeuroD6, as a regulator of ROS homeostasis [110], is related to learning and memory. As a marker of mitochondrial biogenesis, PGC-1α may take part in cognitive decline under metabolic stress. Moreover, AMPK is a sensor key that controls PGC-1α activity. In the aged C57BL/6J mouse model of IR induced by a high-fat diet, SIRT1 improved mitochondrial function via the SIRT1-AMPK-PGC-1α axis and the neuronal differentiation 6 (NeuroD6)-PGC-1α-SIRT1 axis to enhance cognitive decline [102]. Coincidentally, the SIRT1-AMPK-PGC-1α pathway was also verified in the SAMP8 model of IR induced by a high-fat diet, albeit the levels of SIRT1 were not significantly modified [103].

A study by Lennox et al. has demonstrated that increased SIRT1 enhanced cognitive function and synaptic plasticity via alleviating IR in high-fat-fed mice [111]. Similarly, upregulated SIRT1 simultaneously improved synaptic plasticity and insulin signaling in the hippocampus and cortex of high-fat-fed mice [112]. Although few studies have examined the association between IR and cognitive impairment, we concluded that SIRT1 might contribute indirectly to improve cognition, because many of SIRT1's downstream regulators are involved in memory processes.

4. The Effects of Resveratrol on Cognition and Learning and Memory

4.1. Resveratrol, Targeting SIRT1 or Not? Resveratrol has attracted considerable attention for its effects on the improvement of IR [113], cognitive decline [31, 65, 114–116], and cardiovascular diseases [117, 118]. Although resveratrol is widely accepted as a natural activator of SIRT1, there is also evidence showing that resveratrol may not be the direct agonist of SIRT1.

In vitro, resveratrol regulates brain function through increasing the biogenesis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), a glutamatergic receptor, mediated by AMPK and subsequent downstream PI3K/Akt signaling in rat primary neurons [119]. *In vivo*, resveratrol improves learning and memory through activating the IGF-1-PI3K-p-CREB signaling pathway in the hippocampal CA1 region of juvenile and healthy C57BL/6J mice [57], while maintaining the same expression level of SIRT1. In the same strain with isoflurane-induced cognitive impairment,

Li et al. have found that resveratrol exerts anti-inflammatory and antiapoptotic actions to recover cognition without alteration of SIRT1 [120]. In their work, the factors related to neuroapoptosis were changed, such as downregulation of cleaved caspase-3 and Bax and upregulation of Bcl-2. Meanwhile, NLRP3, an intracellular receptor of inflammatory responses, IL-1β, and TNF-α were decreased.

The effects of resveratrol on cognitive improvement are likely not to be fully dependent on SIRT1. After treatment with resveratrol, activation of the Wnt/β-catenin pathway by increasing GSK-3β might as well protect cognitive disturbances in diabetic C57BL/6J mice [102] and SAMP8 mice under the condition of metabolic stress induced by a high-fat diet [103]. In the latter study, Palomera-Avalos et al. have put forward that resveratrol improves mitochondrial morphology, dynamics, and OXPHOS via a decrease in mitofusin 2 (MFN2) and an increase in optic atrophy-1 protein (OPA1), I-NDUFB8, II-SDNB, III-UQCRC2, V-ATPase complexes, and voltage-dependent anion channel 1 (VDAC1)/porin [103].

4.2. Resveratrol, Improving Cognition and Learning and Memory or Not? According to above notions, resveratrol plays a significant role in cognitive enhancement. However, its effects on cognition and learning and memory are still controversial.

4.2.1. The Effects of Resveratrol on Animal Models. In several studies, the administration of oligomeric proanthocyanidins [47], pterostilbene [121], and rapamycin [122] displayed antiaging effects, whereas resveratrol did not show a marked effect. In the aspect of improvement of spatial learning and memory, five-week resveratrol administration to SAMP8 mice (90 μmol/kg body weight/day, about equal to 20 mg/kg body weight/day) showed no significant changes compared to oligomer administration with the same period (50 mg/kg body weight/day). Similarly, eight-week pterostilbene administration to SAMP8 mice (120 mg/kg body weight/day) exerted beneficial effects on learning and memory, but not resveratrol at an identical dose for 8 weeks. In the aspect of survival, resveratrol administration (50 mg/kg body weight/day and 200 mg/kg body weight/day) did not extend life span of genetically heterogeneous mice, while low-dose rapamycin-treated mice (2.24 mg/kg body weight/day) showed an increase in the life span.

4.2.2. The Effects of Resveratrol in Clinical Trials. Data of several clinical trials about resveratrol acting on cognition and learning and memory has indicated that resveratrol plays a protective role not only in diabetic patients but also in non-diabetic population except for patients with schizophrenia. In a randomized controlled trial on T2DM adults, a low dose of resveratrol (75 mg at weekly intervals) showed a positive but chronic effect on cerebrovascular function and cognitive function [123, 124]. In a 14-week randomized placebo-controlled intervention trial, resveratrol supplementation (75 mg twice daily) improved cognitive performance, mood, and cerebrovascular function in postmenopausal women [125]. However, resveratrol supplementation (200 mg/day for 1 month) did not improve memory and attention in 19 men with a diagnosis of schizophrenia [126].

Taken together, their findings have indicated that dosage and period of treatment may influence the effects of resveratrol. Compared to previous studies on antiaging and protection of cognitive decline, dosage of resveratrol and period of treatment are both insufficient. This idea suggests that resveratrol may not be the optimal choice for improvement of learning and memory in short-term treatment. So far, preclinical and clinical data in this area are limited, and an in-depth study of resveratrol on learning and memory needs to be further investigated.

5. Conclusion

SIRT1 may improve cognition and learning and memory through several pathways including deacetylation, OS, mitochondrial dysfunction, and inflammation and microRNA. However, the mechanisms of SIRT1 in cognitive decline under the condition of IR are inadequate and not in-depth and systematical, for example, a gap in the studies on mechanism of SIRT1 regulating neuronal energy metabolism and function. Meanwhile, the involvement of resveratrol, an activator of SIRT1, in the protection of cognitive deficits is still not completely clear. But in most cases, resveratrol can improve cognition and learning and memory. Thereby, the neuroprotection of SIRT1 and resveratrol and their interaction should be explored to act on preventing cognitive impairment in T2DM-associated cognitive dysfunction. In brief, SIRT1 may provide potential approaches to improve learning and memory. Long-term therapy of large-dose resveratrol may offer therapeutic possibilities for preventive strategies in T2DM-associated cognitive dysfunction.

Conflicts of Interest

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

Authors' Contributions

Yue Cao and Guixia Wang designed this review, Zi Yan and Tong Zhou participated in revising the article and polishing the language, and Yue Cao and Guixia Wang wrote the manuscript and were responsible for the final content of the manuscript. All authors read and approved the final manuscript.

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Review Article

Serotonin and Its Receptor as a New Antioxidant Therapeutic Target for Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is a widespread chronic microvascular complication of diabetes mellitus (DM), affects almost 30–50% of patients, and represents a leading cause of death of DM. Serotonin or 5-hydroxytryptamine (5-HT) is a multifunctional bioamine that has crucial roles in many physiological pathways. Recently, emerging evidence from experimental and clinical studies has demonstrated that 5-HT is involved in the pathogenesis of diabetic vascular complications. The 5-HT receptor (5-HTR) antagonists exert renoprotective effects by suppressing oxidative stress, suggesting that 5-HTR can be used as a potential target for treating DKD. In this review, therefore, we summarize the published information available for the involvement of 5-HT and 5-HTR antagonists in the pathogenesis of various diabetic complications with a particular focus of DKD. We conclude that 5-HTR is a potential therapeutic target for treating DKD, as it has been successfully applied in animal models and has currently being investigated in randomized and controlled clinical trials.

1. Introduction

Diabetic kidney disease (DKD) is one of the most epidemic chronic microvascular complications of diabetes mellitus (DM), and it is prevalent in approximately 30–50% of patients with diabetes [1–5]. DKD is the leading cause of chronic and end-stage renal diseases worldwide, and in the past few decades, it has been associated with high morbidity and mortality [6–11].

The pathogenesis of DKD remains not completely understood; however, there is strong experimental evidence that prolonged hyperglycemia leads to the mitochondrial production of reactive oxygen species (ROS), resulting in oxidative stress, which plays a key role in DKD [12–16]. Inflammation induced and exacerbated by oxidative stress is closely associated with the development and progression of DKD.

5-Hydroxytryptamine (5-HT) is a potent vasoactive amine that plays pivotal roles in insulin secretion [17–19], energy metabolism [20], mitochondrial biogenesis [21], the immune system [22, 23], and vascular inflammation [24–27]. However, the functions of 5-HT have not been elucidated yet. Recently, several studies have shown that 5-HT and 5-HT receptors (5-HTR) are involved in the pathogenesis of diabetic vascular complications [17, 28–31]. 5-HTR antagonists have a renoprotective effect by suppressing oxidative stress and inflammatory cytokines [32–35], suggesting that 5-HTR antagonists could be used to treat DKD. This review assesses and describes the current understanding of 5-HT's involvement in the pathogenesis of DKD and the potential use of 5-HTR antagonists in the clinical treatment of DKD.

2. 5-HT Synthesis and Metabolism and 5-HT Receptors

5-HT is a monoamine neurotransmitter and hormone mainly produced by enterochromaffin cells of the gastrointestinal tract [21]. 5-HT is derived from tryptophan and predominantly stored in circulating platelets, and it is distributed throughout the body to regulate the hormones of

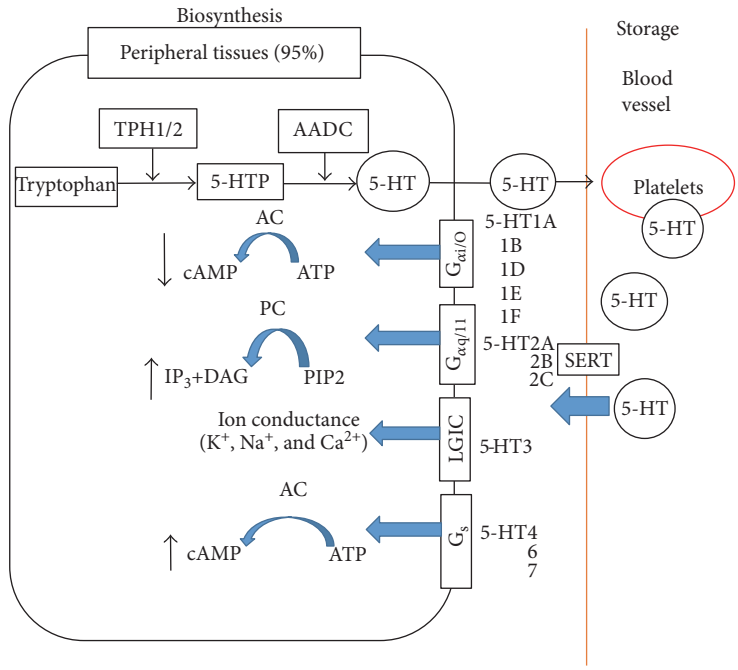

FIGURE 1: A model of 5-HT biosynthesis and metabolism in peripheral tissues. 5-HT synthesis is dependent on the enzyme tryptophan hydroxylase (TPH); the released 5-HT is controlled by the autonomous nervous system and released locally into the circulatory system, and most of them are stored in platelets. Reuptake of 5-HT is mediated by SERT. The effects of 5-HT are mediated through 14 serotonergic receptors that have been grouped into seven broad families. All 5-HTRs are G protein-coupled receptors (GPCRs), except 5-HT_3 that is a ligand-gated cationic channel. 5-HT GPCRs were coupled to all three canonical signaling pathways through $G_{\alpha i/O}$, $G_{\alpha q/11}$, and G_s that are involved in the cAMP pathway and allow this receptor family to modulate several biochemical signaling pathways.

several main physiological parameters, such as cardiovascular function [36], insulin secretion [17], energy homeostasis [20], and appetite [37].

5-HT synthesis is dependent on the enzyme tryptophan hydroxylase (TPH), which is encoded by two different genes: tryptophan hydroxylase 1 (Tph1) and Tph2, which are expressed in the peripheral tissues and brain, respectively. Peripheral 5-HT is presumed to be unable to cross the blood-brain barrier. The majority of the peripheral 5-HT is stored in platelets and also present in other tissues and many cells. The released 5-HT is controlled by the autonomous nervous system and released locally into the circulatory system, where it is used for the aggregation of platelets through various stimuli, including atherosclerosis [26, 38]. 5-HT is primarily inactivated by the reuptake of serotonergic neurons that secrete it; this reuptake is mediated by the highly selective plasmalemma 5-HT transporter (5-HTT), which is also known as the serotonin transporter (SERT) [39] (Figure 1).

5-HT produces a myriad of physiological and pathological effects in humans, which are mediated through 14 serotonergic (5-HTergic) receptors that have been grouped into seven broad families (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7). All 5-HTRs are G protein-coupled receptors (GPCRs), except 5-HT_3 that is a ligand-gated cationic channel. 5-HT GPCR was coupled to all three canonical signaling pathways through $G_{\alpha i}/_O$, $G_{\alpha q/11}$, and G_s that are involved in the cAMP pathway and allow this receptor family to modulate several biochemical signaling pathways [40].

3. 5-HT in Diabetes and Diabetic Complications

Pancreatic β-cells synthesize and store 5-HT, which is coreleased with insulin [41]. An increased plasma level of 5-HT is a biomarker for diabetic complications, and positive correlations have been established between the plasma 5-hydroxyindoleacetic acid (5-HIAA; the main 5-HT metabolite) level and coronary heart disease [36, 42–45]. Selective serotonergic functional alterations have shown therapeutic relevance in diabetic rats [29, 30, 46]. These studies and their findings have been summarized in the subsequent sections and suggest that 5-HT plays a role in DM.

3.1. 5-HT and Gestational Diabetes. In pregnant mice, prolactin (PRL) stimulates islet prolactin receptors (PRLRs) to trigger a strong upregulation of both isoforms of TPH. TPH upregulation activates 5-HT synthesis in some pancreatic β-cells, which in turn induce glucose-stimulated insulin secretion (GSIS) [47, 48]. The insulin secretion is upregulated by the 5-HT_{2B} receptor (5-HT_{2B}R) and downregulated by the 5-HT_{1D} receptor (5-HT_{1D}R) in β-cells, making 5-HT a paracrine regulator of β-cell proliferation. 5-HT_{3A}R channels in wild-type animals allow a 5-HT-mediated influx of

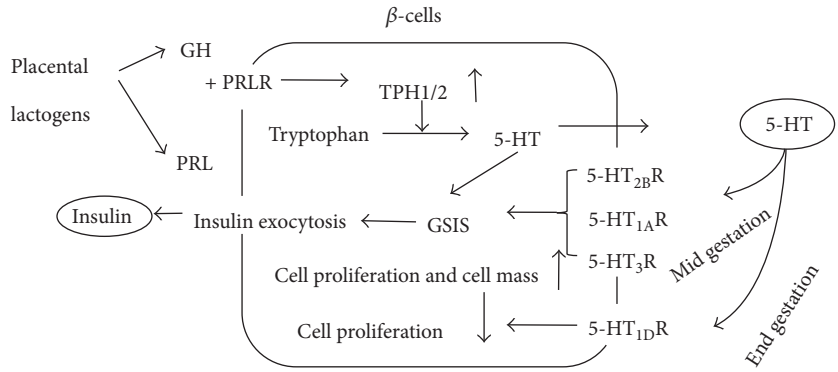

Figure 2: Mechanism of 5-HT in the mouse pancreatic beta-cells during pregnancy. In pregnant mice, prolactin (PRL) stimulates islet prolactin receptors (PRLRs) to trigger a strong upregulation of both isoforms of TPH. TPH upregulation activates 5-HT synthesis in some pancreatic β-cells, which in turn induce GSIS. The insulin secretion is upregulated by the 5-HT_{2B} receptor (5-HT_{2B}R) and downregulated by the 5-HT_{1D} receptor (5-HT_{1D}R) in β-cells, making 5-HT a paracrine regulator of β-cell proliferation. 5-HT_{3A}R channels in wild-type animals allow a 5-HT-mediated influx of cations, depolarizing the resting membrane potential and lowering the threshold for glucose-induced insulin exocytosis.

cations, depolarizing the resting membrane potential and lowering the threshold for glucose-induced insulin exocytosis [19, 49], as illustrated in Figure 2. Disrupting this balance can result in gestational diabetes.

3.2. 5-HTR and Type 2 DM. Type 2 DM (T2DM) describes a group of metabolic disorders characterized by defects in insulin secretion and insulin sensitivity. Impaired insulin secretion from pancreatic β-cells is an important factor in the etiology of T2DM. However, the complex regulation and mechanism of insulin secretion from β-cells have not been completely elucidated.

High plasma levels of 5-HT have been reported in patients with T2DM, although its potential effect on insulin secretion is unclear. The release of 5-HT from activated platelets is enhanced, decreasing intraplatelet 5-HT content and resulting in increased plasma levels of 5-HT in patients with diabetes [44].

3.2.1. 5-HT_{2C}R. 5-HT_{2C}R-deficient mice are overweight, exhibit an abnormal feeding behavior, show insulin resistance, and have significantly higher blood glucose concentrations, suggesting that 5-HT may affect glucose and lipid metabolism [17, 20, 50]. Insulin secretion is affected by 5-HT_{2C}R, which is indicative of the possibility that an aberrant 5-HT system could also affect the regulation of energy metabolism. Increased expression of 5-HT_{2C}R in both the hypothalamus and β-cells could mediate a protective strategy to prevent excess energy intake. As illustrated in Figure 3, 5-HT_{2C}R-expressing pro-opiomelanocortin neurons are required to control energy and glucose homeostasis [51].

Although, in human T2DM islet cells, the expression of 5-HT_{2C}R has not been observed [31], the 5-HT_{2C}R agonist Belviq (lorcaserin) is the first FDA-approved drug to treat obesity in 15 years [52], and central serotonin 2C receptors regulated glucose homeostasis and may represent a rational target for type 2 diabetes (T2DM) treatment [53, 54]. The 5-HT_{2C}R agonist m-chlorophenylpiperazine (mCPP) improves glucose homeostasis and insulin sensitivity, and antagonists or genetic loss of 5-HT_{2C}R impairs glucose homeostasis [55, 56].

3.2.2. 5-HT_{1D}R and 5-HT_{1A}R. Bennet et al. [31] reported that 5-HT_{1D}R and 5-HT_{1A}R messenger RNA expression was increased in human T2DM islets. 5-HT inhibits both basal- and glucose-induced insulin secretions, and the selective 5-HT_{1D}R agonist (PNU142633) inhibits GSIS in nondiabetic human islets, whereas the 5-HT_{1D}R antagonist (LY310762) stimulates GSIS. Interestingly, upon stimulation with 5-HT in isolated islets from patients with T2DM, the inhibitory effect of 5-HT was completely lost (both in basal and stimulatory conditions of glucose); instead, the stimulation of insulin secretion was observed. This indicated that 5-HT acts through increased signaling through the 5-HT_{2A}R in diabetic conditions. The 5-HT_{2A}R antagonist (sarpogrelate hydrochloride) markedly decreased the glycated hemoglobin A1c level. The expression of 5-HT_{1D}R had a negative correlation with somatostatin (SST) and SST receptors (SSTR) 1–5, whereas the expression of 5-HT_{2A}R did not have any correlation with either SST or any of the SSTRs; this suggests that increased expression of HT_{1D}R in human islet cells, as observed in T2DM islet cells, leads to decreased expression of SST and its receptors (Figure 4).

3.3. 5-HT as an Immunomodulator in DM. Although several physiological causes that lead to DM remain unknown, evidence suggests that autoimmunity plays an important role in DM and diabetic complications. There is an increasingly collective perspective regarding the association of 5-HT with the activation of immunoinflammatory pathways and the onset of autoimmune reactions. Almost all the circulating 5-HT are found in platelets and released following platelet activation, on contact with damaged endothelium or induced by ischemia, indicating that 5-HT also contributes to the innate and adaptive immune responses [22, 57]. 5-HT

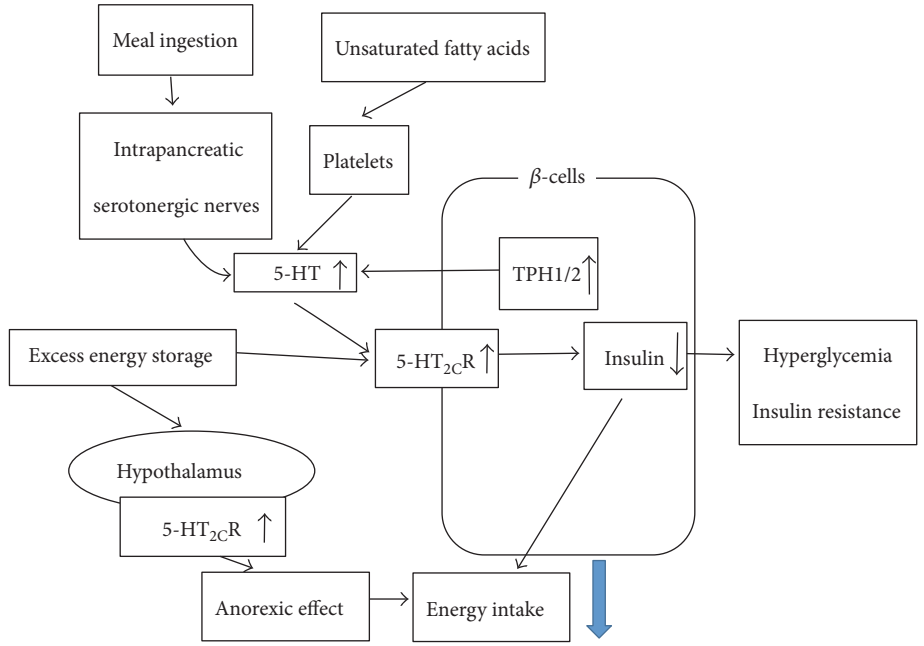

Figure 3: Model showing the modulation of 5-HT2cR in DM. 5-HT2CR-deficient mice showed that 5-HT may affect glucose and lipid metabolism. Insulin secretion is affected by 5-HT2CR, which is indicative of the possibility that an aberrant 5-HT system could also affect the regulation of energy metabolism. Increased expression of 5-HT2CR in both the hypothalamus and β-cells could mediate a protective strategy to prevent excess energy intake. 5-HT2CR-expressing pro-opiomelanocortin neurons are required to control energy and glucose homeostasis.

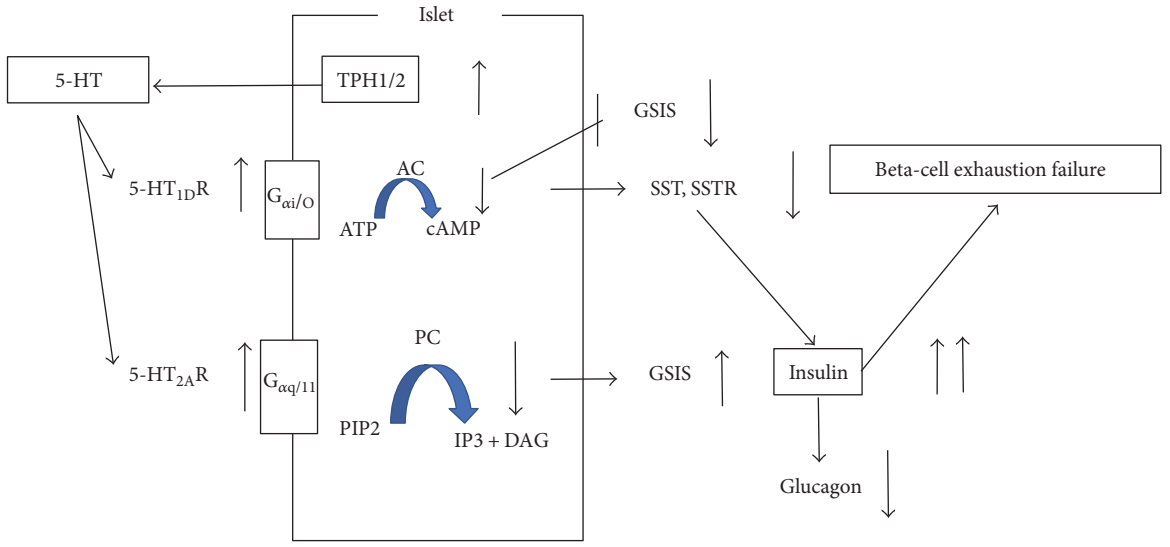

Figure 4: Illustration to show the mechanism of 5-HT1D and 5-HT2AR in human T2DM. 5-HT1DR and 5-HT1AR messenger RNA expression was increased in human T2DM islets. The 5-HT2AR antagonist (sarpogrelate hydrochloride) markedly decreased the glycated hemoglobin A1c level. The expression of 5-HT1DR had a negative correlation with somatostatin (SST) and SST receptors (SSTR), whereas the expression of 5-HT2AR did not have any correlation with either SST or any of the SSTRs; this suggests that increased expression of HT1DR in human islet cells, as observed in T2DM islet cells, leads to decreased expression of SST and its receptors.

stimulation increases murine peritoneal macrophage production of proinflammatory cytokines [25]. The expression of 5-HTRs has been identified in rodent and human innate immune cells, which include neutrophils, eosinophils, monocytes, macrophages, dendritic cells, mast cells, and natural killer cells [58].

5-HT was identified as an immunomodulator owing to its ability to stimulate or inhibit inflammation.

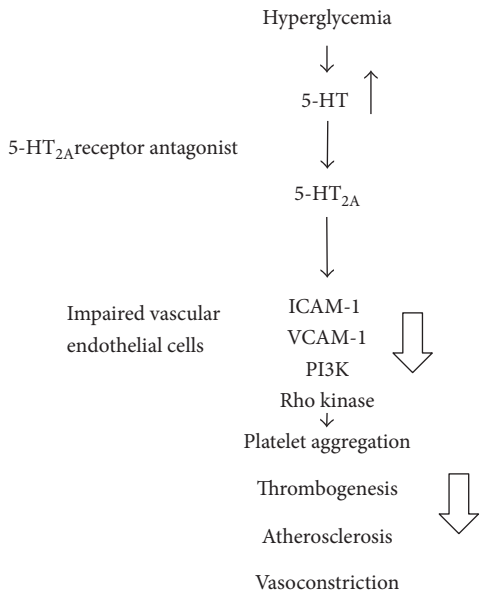

Figure 5: Mechanisms of 5-HT_{2A} receptor antagonist contributing to DM-induced cardiovascular complications. Sarpogrelate, a 5-HT2AR antagonist, has been shown to attenuate diabetes-induced cardiovascular complications, which decrease the blood glucose level, inhibit the release of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and reduce 5-HT-induced contraction in aortas through the PI3K and Rho kinase pathway.

Moreover, 5-HT has immunomodulatory effects that are induced by activating 5-HTR and SERT, which are differentially expressed in many leukocytes. Arthritis [59], systemic sclerosis [38, 60], lung fibrosis [61], and allergic asthma [62] are all associated with changes in the serotonergic system, which is associated with leukocytes.

3.4. 5-HT_{2A}R and DM-Induced Vascular Complications. 5-HT is a potent vasoactive amine in the cardiovascular system. Cardiovascular disorders of diabetes can be characterized by atherosclerosis [63]. There is strong evidence that impaired vascular endothelial and smooth muscle functions play important roles in the process of DM-induced cardiovascular complications [63, 64]. 5-HT, induced by impaired vascular endothelial cells, is involved in the pathological process of platelet aggregation [45], thrombogenesis [65], contraction of carotid arteries [66], and arteriogenesis [67] in DM-induced vascular complications through 5-HT_{2A}R.

Sarpogrelate, a 5-HT2AR antagonist, has been shown to attenuate diabetes-induced cardiovascular complications, which decrease the blood glucose level [66], inhibit the release of intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1(VCAM-1) [68], and reduce 5-HT-induced contraction in aortas through the PI3K [66] and Rho kinase [69] pathway, as illustrated in Figure 5.

4. Mechanism of the 5-HTR Antagonist for Treating DKD

DKD is a main microvascular complication of diabetes and the most common cause of end-stage renal disease strongly associated with cardiovascular morbidity and mortality, which cause an enormous burden on affected patients and health care systems [70]. Histopathological changes associated with DKD are characterized by thickening of the glomerular basement membrane; podocyte effacement and hypertrophy; accumulation of extracellular matrix and proteins, such as collagen and fibronectin; and the hyalinization of afferent and efferent glomerular arterioles [71, 72].

Studies have indicated that inflammation is an important mechanism in the pathogenesis of DKD that triggers a complex network of pathophysiological events that modulate intracellular signaling pathways involving protein kinase C [73–75] and ROS [76–78] and act in a concerted manner to induce transcription factors, cytokines, chemokines, and growth factors during hyperglycemia [13–15, 79–81]. Although many factors have been implicated in the pathogenesis of DKD, inflammation is believed to play a fundamental role in the early development and progression of DKD [14, 71, 78, 80, 82]. Drugs with anti-inflammatory effects have been used as a new clinical approach for treating DKD.

Previous reports have indicated that the increased plasma concentrations of 5-HT or its metabolite (5-HIAA) are valuable biomarkers for estimating the DKD-associated risk during the early stages of the disease [35, 83, 84]. 5-HT has been shown to enhance the production of type IV collagen by human mesangial cells, and its production is mediated by the activation of protein kinase C and a subsequent increase in active transforming growth factor-β (TGF-β) [85]. Stimulation of 5-HT_{2A}Rs by 5-HT induces the expression of TGF-β through extracellular signal-regulated kinases, a key mediator of proliferative and fibrotic signals in mesangial cells [86–89], as illustrated in Figure 6.

Studies have shown that 5-HTR antagonists are effective in preventing diabetic nephropathy. Sarpogrelate, a 5-HT2 subtype 2A antagonist [33, 34], reduced albuminuria in the early stages of DKD by improving glomerular endothelial function through the reduction in glomerular platelet activation and an increase in serum adiponectin concentrations in a diabetic animal model. Ogawa et al. [90] and Park et al. [91] found that sarpogrelate can reduce albuminuria and plasma and urinary monocyte chemoattractant protein-1 levels in patients with DKD. Tropisetron, a 5-HT_3 receptor antagonist, can attenuate early DM through calcineurin inhibition and by suppressing oxidative stress and some inflammatory cytokines in streptozotocin-induced diabetic rats [32].

5. Conclusions

There is an increasing repertoire of evidence supporting 5-HT as a causative agent for increased ROS generation in DM. Since 5-HT mediates accelerated atherosclerosis in diabetes, pharmacological inhibition of the 5-HT receptor presents an attractive therapeutic strategy for

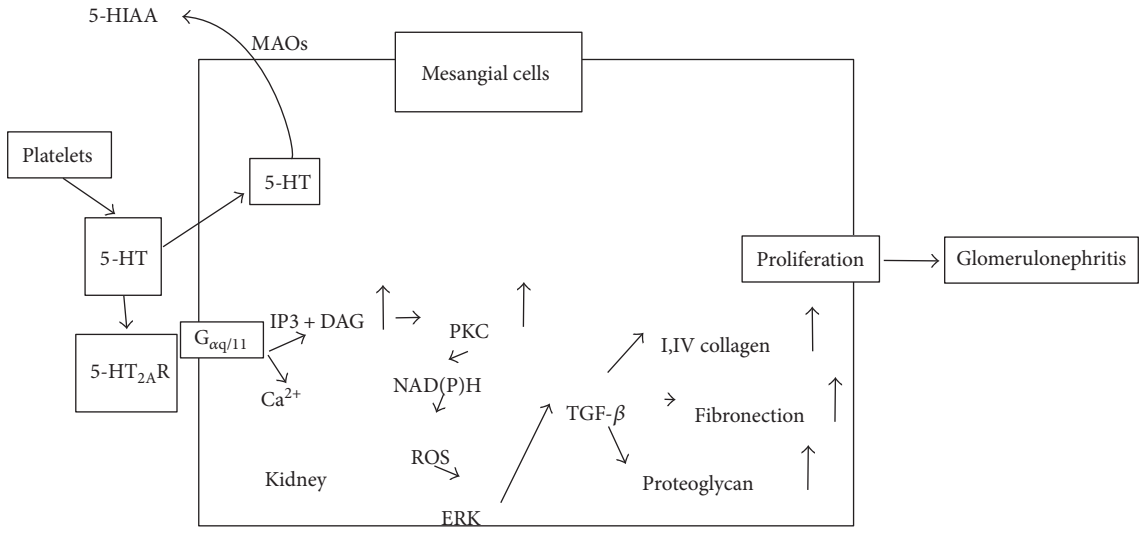

Figure 6: Illustration to show the mechanism of $5\text{-HT}_{2A}R$ in mesangial cells. 5-HT has been shown to enhance the production of type IV collagen by human mesangial cells, and its production is mediated by the activation of protein kinase C and a subsequent increase in active TGF-β. Stimulation of 5-HT2ARs by 5-HT induces the expression of TGF-β through extracellular signal-regulated kinases.

patients with diabetes to attenuate the development of nephropathy and macrovascular complications. A better understanding of the role of these new receptor targets in the context of DKD will facilitate the development of novel therapeutic strategies that can be successfully translated into clinical applications.

Abbreviations

5-HT:	5-Hydroxytryptamine
TPH:	Tryptophan hydroxylase
AADC:	Unbiquitous aromatic L-amino acid decarboxylase
5-HTT:	5-HT transporter
Cys-loop LGICs:	Cys-loop ligand-gated ion channels
5-HTT (SERT):	5-Hydroxytryptamine transporter
GPCRs:	G protein-coupled receptors
AC:	Adenylyl cyclase
cAMP:	Cyclic adenosine monophosphate
PIP2:	Phosphatidylinositol 4,5-bisphosphate
IP3:	Inositol trisphosphate
DAG:	Diacylglycerol
PRL:	Prolactin
PRLR:	Islet prolactin receptors
GSIS:	Glucose-stimulated insulin secretion
SST:	Somatostatin
SSTR:	SST receptors
Rho:	Ras homolog gene family member
ICAM-1:	Intercellular adhesion molecule-1
VCAM-1:	Vascular cell adhesion molecule-1
TGF-β:	Transforming growth factor-β
PKC:	Protein kinase C
ROS:	Reactive oxygen species
NADP:	Nicotinamide adenine dinucleotide phosphate
ERK:	Extracellular signal-regulated kinases.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Aldose Reductase Inhibitor Protects against Hyperglycemic Stress by Activating Nrf2-Dependent Antioxidant Proteins

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We have shown earlier that pretreatment of cultured cells with aldose reductase (AR) inhibitors prevents hyperglycemia-induced mitogenic and proinflammatory responses. However, the effects of AR inhibitors on Nrf2-mediated anti-inflammatory responses have not been elucidated yet. We have investigated how AR inhibitor fidarestat protects high glucose- (HG-) induced cell viability changes by increasing the expression of Nrf2 and its dependent phase II antioxidant enzymes. Fidarestat pretreatment prevents HG (25 mM)-induced Thp1 monocyte viability. Further, treatment of Thp1 monocytes with fidarestat caused a time-dependent increase in the expression as well as the DNA-binding activity of Nrf2. In addition, fidarestat augmented the HG-induced Nrf2 expression and activity and also upregulated the expression of Nrf2-dependent proteins such as hemeoxygenase-1 (HO1) and NQO1 in Thp1 cells. Similarly, treatment with AR inhibitor also induced the expression of Nrf2 and HO1 in STZ-induced diabetic mice heart and kidney tissues. Further, AR inhibition increased the HG-induced expression of antioxidant enzymes such as SOD and catalase and activation of AMPK-α1 in Thp1 cells. Our results thus suggest that pretreatment with AR inhibitor prepares the monocytes against hyperglycemic stress by overexpressing the Nrf2-dependent antioxidative proteins.

1. Introduction

Hyperglycemia is a major contributor to inflammation, apoptosis, profound vasodilation, tissue damage, and dysfunction in patients with diabetes mellitus [1]. The cytotoxicity of hyperglycemia is mediated by the increase in reactive oxygen species (ROS) which activate NF-κB and AP1 that results in the transcription of inflammatory cytokines [2]. Our recent studies indicate that inhibition of the polyol pathway enzyme aldose reductase (AR) prevents cytokine- and hyperglycemia-induced increase in inflammatory markers in macrophages, vascular cells, and diabetic mice [3, 4] by preventing the activation of NF-κB- and AP1-induced proinflammatory signals [3, 5]. We have shown that preincubation with AR inhibitor prevents hyperglycemia- and cytokine-induced proliferation of vascular cells and apoptosis of macrophages [6–8]. While these studies indicate that inhibition of AR could prevent oxidative stress-induced inflammatory response, the mechanism(s) by which inhibition of AR prepares the cells against oxidative stress is not known.

Previous studies indicate that transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) regulates a battery of cytoprotective genes which maintain cellular redox homeostasis. Nrf2 binds to the antioxidant response element (ARE) and transcriptionally regulates the gene expression of several antioxidant and phase II detoxifying enzymes including hemeoxygenase 1 (HO1), NAD(P)H-quinone dehydrogenase 1 (NQO1), γ-glutamylcysteine synthetase (GCS), glutathione *S*-transferases (GSTs), and AR [9]. Generally, under nonstress conditions, Nrf2 complexes with an adaptor protein, Keap1, which is a regulator of the proteasomal degradation of Nrf2. However, under stress conditions, Nrf2 dissociates from Keap1 and translocates to the nucleus and transcribes the genes responsible for defense against stress. Thus, it has been well established that activation of the Nrf2 pathway in response to oxidative stress protects the cells and tissues from oxidative injury [10].

Although our previous studies indicate that AR inhibition prevents hyperglycemia-induced NF-κB-dependent inflammatory signals, it is not known how AR inhibition increases the resistance of cells to withstand oxidative stress stimuli initiated by hyperglycemia. Therefore, in this study, we examined our hypothesis that AR inhibition promotes the activation of Nrf2-mediated cytoprotective pathways that protect cells against hyperglycemic stress. Our results suggest that AR inhibitor fidarestat increases the expression as well as the DNA-binding activity of Nrf2 in Thp1 monocytes. In addition, fidarestat increased the expression of Nrf2 downstream target proteins such as HO1 and NQO1 in Thp1 cells and heart and kidney tissues of STZ-induced diabetic mice. AR inhibitor also prevented the expression of antioxidant enzymes such as SOD and catalase. Collectively, our results demonstrate that AR inhibition protects the cells against hyperglycemia-induced changes in the cell viability by activating Nrf2/HO1-mediated antioxidative pathway, which could also account for the anti-inflammatory effects of AR inhibitors in diabetes.

2. Materials and Methods

2.1. Materials. RPMI-1640, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen. Antibodies against Nrf2 were obtained from Santa Cruz. Antibodies against KEAP1, HO1, NQO1, phospho-AMPK-α1, AMPK-α1, GAPDH, and histone H3 were purchased from Cell Signal Inc. Fidarestat was obtained from Livwel Therapeutics Inc., USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) and other reagents used in Western blot analysis were obtained from Sigma. HO1 assay kit was purchased from Abcam. Nrf2 transcription factor binding assay and superoxide dismutase activity assay kits were purchased from Cayman Chemicals. Catalase activity was determined spectrophotometrically using hydrogen peroxide (30%) (Sigma-Aldrich).

2.2. Cell Culture and Treatment. Human Thp1 monocytic cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Prior to treatment, cells were serum starved in the respective medium containing 0.1% FBS ± fidarestat (10 μM) for 14 h and further stimulated with high glucose (25 mM; 19.5 mM glucose was added to 5.5 mM glucose containing media) for different time intervals.

2.3. Cell Viability. Cell viability was determined using a standard MTT assay [11]. Briefly, Thp1 cells were growth arrested in 0.1% FBS containing RPMI medium. Cells were preincubated with fidarestat (10 μM) for overnight (14 h) at37°C followed by incubation with HG (25 mM) for another 48 h. At the end of incubation period, cells were incubated with 10 μl MTT reagent (5 mg/ml) for 4 h at 37°C. The formazan crystals formed by the viable cells were solubilized by the addition of 100 μl DMSO. Absorbance was measured at 570 nm using a microplate reader. Cell viability was also examined by counting the live and dead cells using a hemocytometer [6]. AR activity (mU/mg protein) was measured spectrophotometrically using glyceraldehyde as a substrate [6]. Nrf2 DNA binding activity was determined by Nrf2 transcription factor assay kit as per the manufacturer's instructions (Cayman Chemical).

2.4. Immunoblot Analysis. Nuclear and cytoplasmic proteins from the treated cells were isolated using a nuclear extraction kit (Cayman Chemicals). Protein concentration in the extracts was measured with Bradford reagent (Bio-Rad). Equal amount of proteins were subjected to 12% SDS-PAGE electrophoresis followed by Western blot analysis using specific antibodies against Nrf2, Keap1, HO1, NQO1, AMPK, histone H3, and GAPDH. The antigen-antibody complexes were detected by enhanced Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Membranes were stripped with Restore TM PLUS stripping buffer (Thermo Scientific) and used for reprobing with other antibodies or loading controls.

2.5. Ablation of Nrf2 by siRNA. Thp1 cells cultured in RPMI 1640 medium containing 10% FBS were incubated with Nrf2-siRNA (120 nM) or siRNA negative control with HiPerFect Transfection Reagent as per the manufacturer's instructions (Qiagen, USA). The cells were incubated in a humidified CO_2 incubator for 48 h at 37°C. Silencing of Nrf2 was determined by Western blotting.

2.6. Quantitative RT-PCR Analysis of HO1 mRNA. Total RNA was isolated from the treated cells using TRIzol reagent and was quantified by using a nanodrop spectrophotometer (NanoDrop Technologies). TaqMan reverse transcription reagents kit was used for the synthesis of cDNA from total RNA (Life Technologies). Q-PCR amplifications (performed in triplicate) were performed by using 1 μl of cDNA using the iTaq Universal SYBR Green Supermix (Bio-Rad). Housekeeping gene *GAPDH* was used as a normalizer. ABI Prism 7500 Sequence detection system using forward: 5′-C GGGCCAGCAACAAAGTG-3′, and reverse: 5′-CCAGAA AGCTGAGTGTAAGGACC-3′ was used for qPCR analysis of *HO1* gene.

2.7. Determination of HO1 and Nrf2 in STZ-Induced Diabetic Mice. Seven-week-old C57BL/6 male mice were purchased from Envigo. Diabetes was induced in mice by injecting a single dose of streptozotocin (STZ; 165 mg/kg, i.p.) and blood glucose levels were measured by a glucometer (True Metrix). The mice with blood glucose levels >400 mg/dl were selected and randomly divided into diabetic and diabetic + fidarestat groups. Fidarestat (10 mg/kg/day, i.p.) was administered to diabetic mice, and the animals were euthanized on day 3.

2.8. Statistical Analysis. Data are presented as mean ± SD. The p values were determined using the unpaired Student's t-test (GraphPad Prism software) and a p value of <0.05 considered as statistically significant.

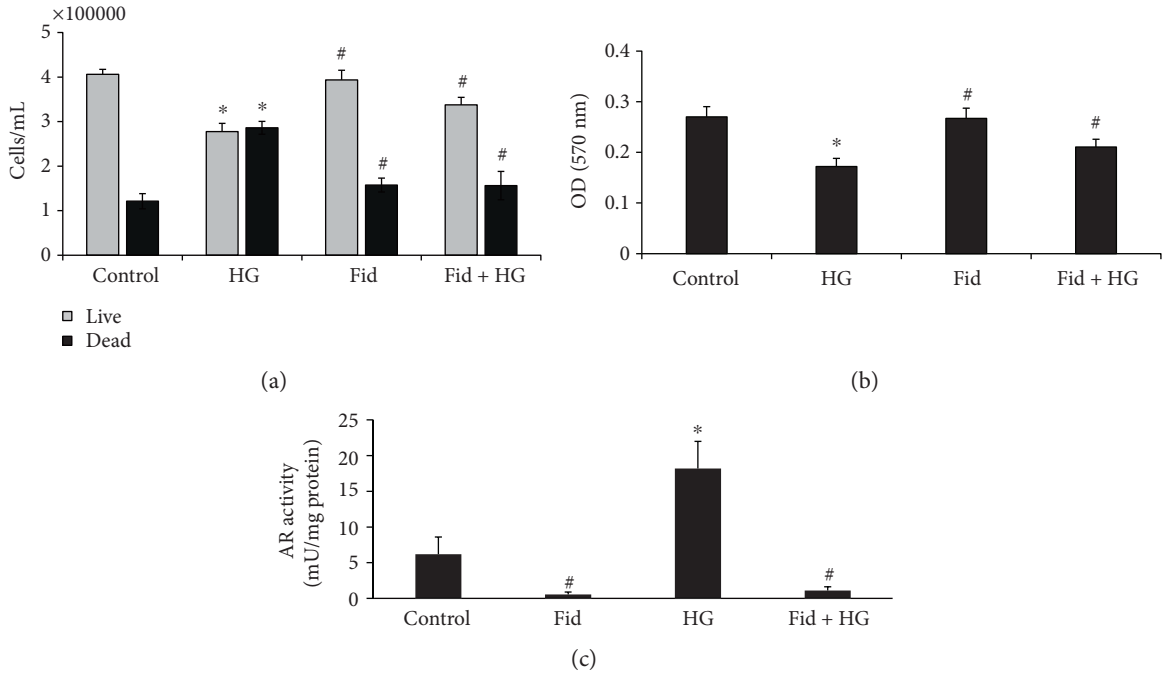

Figure 1: AR inhibition prevents HG-induced Thp1 cell viability. Thp1 cells (3000 cells/well) were pretreated with fidarestat for overnight followed by incubation with HG (25 mM) for another 48 h. (a) Cell viability was determined by MTT assay. (b) Live and dead cell counts were determined by staining with trypan blue using a hemocytometer. (c) AR activity was determined spectrophotometrically using glyceraldehyde as a substrate. Data represent mean ± SD ($n = 5$). $^{*}p < 0.01$ when compared with control, and $\#p < 0.05$ when compared with the HG-treated group.

3. Results

3.1. AR Inhibition Prevents HG-Induced Thp1 Cells Viability. The effect of AR inhibition on HG-induced Thp1 cells viability was examined by measuring the live and dead cell counts as well as MTT absorbance. The data shown in the Figure 1(a) indicates that HG treatment of Thp1 cells decreased the number of live cells and increased the number of dead cells indicating that HG decreases Thp1 cell viability. However, pretreatment of Thp1 cells with AR inhibitor prevented the HG-induced decrease in the Thp1 cell viability. Similar results were observed when we measured the cell viability by MTT assay (Figure 1(b)). The data shown in Figure 1(c) also indicates that AR activity was significantly increased in the HG-treated Thp1 cells and fidarestat prevented it. These results thus suggest that AR inhibition prevents HG-induced decrease in the cell viability of Thp1 cells.

3.2. AR Inhibitor Increases the Expression of Nrf2. To examine how pretreatment of cells with AR inhibitor prevents HG-induced decrease in Thp1 cell viability, we examined the effect of AR inhibitor on the expression of Nrf2. Pretreatment of Thp1 cells with fidarestat alone or HG alone induced Nrf2 expression in a time-dependent manner. Further, preincubation of cells with fidarestat followed by incubation with HG significantly augmented the HG-induced increase in the expression of Nrf2 (Figure 2(a)). Similarly, treatment of Thp1 cells with HG decreased the expression of Keap1, a negative regulator of Nrf2 and preincubation with fidarestat, followed by HG decreased the expression of the Keap1 protein (Figure 2(a)). We next examined the effect of AR inhibitor on Nrf2 DNA binding activity in Thp1 cells. Nrf2 transcriptional activity increased in the fidarestat-treated Thp1 cells in a time-dependent manner as compared to that in control cells (Figure 2(b)). Further, fidarestat augmented the HG-induced Nrf2 transcriptional activity in Thp1 cells. These results thus suggest that preincubation of cells with AR inhibitor prepares the cells against oxidative insult by inducing the expression of Nrf2.

3.3. AR Inhibition Increases the Antioxidative Protein Expressions in Thp1 Cells. We next examined the effect of AR inhibitor on the expression of various Nrf2-dependent antioxidative proteins. Results shown in Figure 3(a) indicate that fidarestat alone or HG alone increased the levels of antioxidant proteins such as HO1 and NQO1 in Thp1 cells. Further, pretreatment with fidarestat followed by HG synergistically increased the HO1 and NQO1 protein expressions in Thp1 cells (Figure 3(a)). Similarly, AR inhibitor also increased the levels of HO1 in Thp1 cell lysates (Figure 3(b)). Furthermore, mRNA expression of *HO1* increased significantly in cells treated with HG in the presence of fidarestat as compared to HG- or fidarestat-treated cells (Figure 3(c)). Furthermore, AR inhibitor also significantly increased the HG-induced SOD as well as catalase activities in Thp1 cells (Figures 3(d) and 3(e)). Thus, our results indicate that pretreatment of Thp1 cells with fidarestat enhances the antioxidant status of the cells as a defense against hyperglycemic stress.

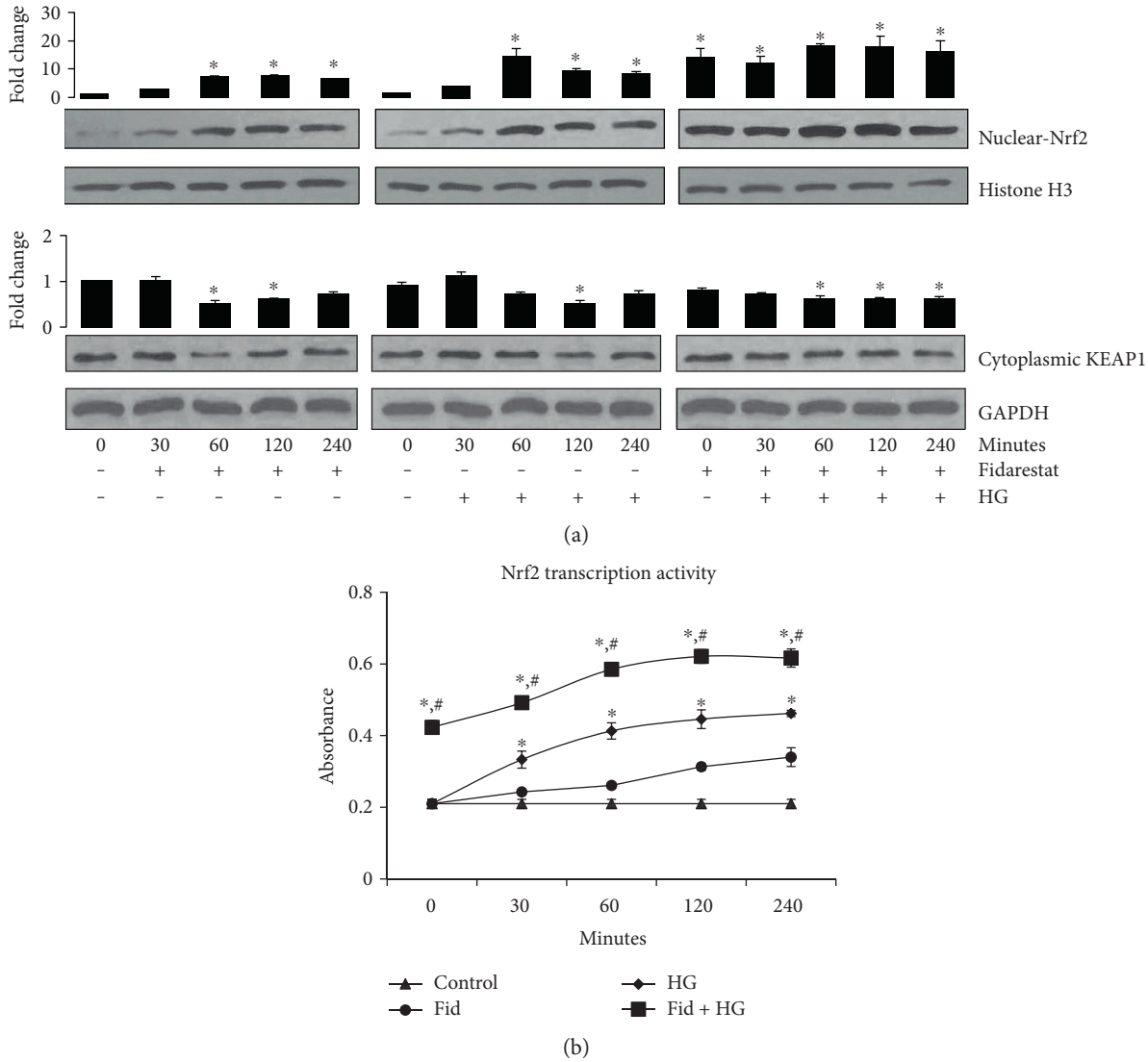

Figure 2: AR inhibition augments HG- induced Nrf2 activation in Thp1 cells. Thp1 cells were treated with fidarestat (10 μM) for indicated times. Subsequently, the cells were also pretreated with fidarestat for overnight followed by incubation with HG for 30, 60, 120, and 240 minutes. Equal amounts of nuclear and cytosolic proteins were subjected to Western blot analysis for the expression of Nrf2 and Keap1, respectively. Histone H3 and GAPDH served as loading controls for nuclear and cytosolic protein extract, respectively. A representative blot from three independent analyses is shown (a). Nrf2 transcription factor assay using the nuclear protein of treated Thp1 cells was carried out using an ELISA kit (b). Data represent mean ± SD ($n = 5$). $^*p < 0.05$ when compared with control, and $\#p < 0.05$ when compared with the HG-treated group.

3.4. Effect of AR Inhibitor on HG-Induced Cell Viability in Nrf-2-Knockdown Thp1 Cells. To examine the effect of Nrf2 on AR-regulated cell growth, we determined the Thp1 cell viability in the Nrf2-siRNA knockdown cells in the absence and presence of fidarestat. Incubation of Nrf2 knockdown cells with HG (25 mM) significantly increased cell death of Thp1 cells when compared to that of control cells (Figure 4). Further, fidarestat prevented the HG-induced Thp1 cell death in control siRNA-transfected cells but not in the Nrf2-siRNA-transfected cells, suggesting that fidarestat prevents Thp1 cell viability by increasing the Nrf2 expression.

3.5. AR Inhibitor Increases the Expression of Nrf2 and HO1 in Mouse Diabetic Heart and Kidney Tissues. We next examined the effect of fidarestat on the expression of antioxidant proteins, HO1, and Nrf2 in STZ-induced diabetic mouse heart and kidney tissues. Similar to data shown in the Thp1 cells, AR-inhibitor (fidarestat) also augmented the STZ-induced increase in the expression of Nrf2, HO1, and NQO1 in the heart and kidney homogenates of mice (Figure 5).

3.6. AR Inhibitor Regulates HG-Induced Phosphorylation of AMPK-α1 in Thp1 Cells. Since AMPK-α1 activation has been shown to activate Nrf2 signals, we next investigated the effect of fidarestat on HG-induced AMPK-α1 activation in Thp1 cells. Our results shown in Figure 6(a) indicate that fidarestat increased the HG-induced phosphorylation of AMPK-α1 in Thp1 cells. Further, to investigate the effect of Nrf2

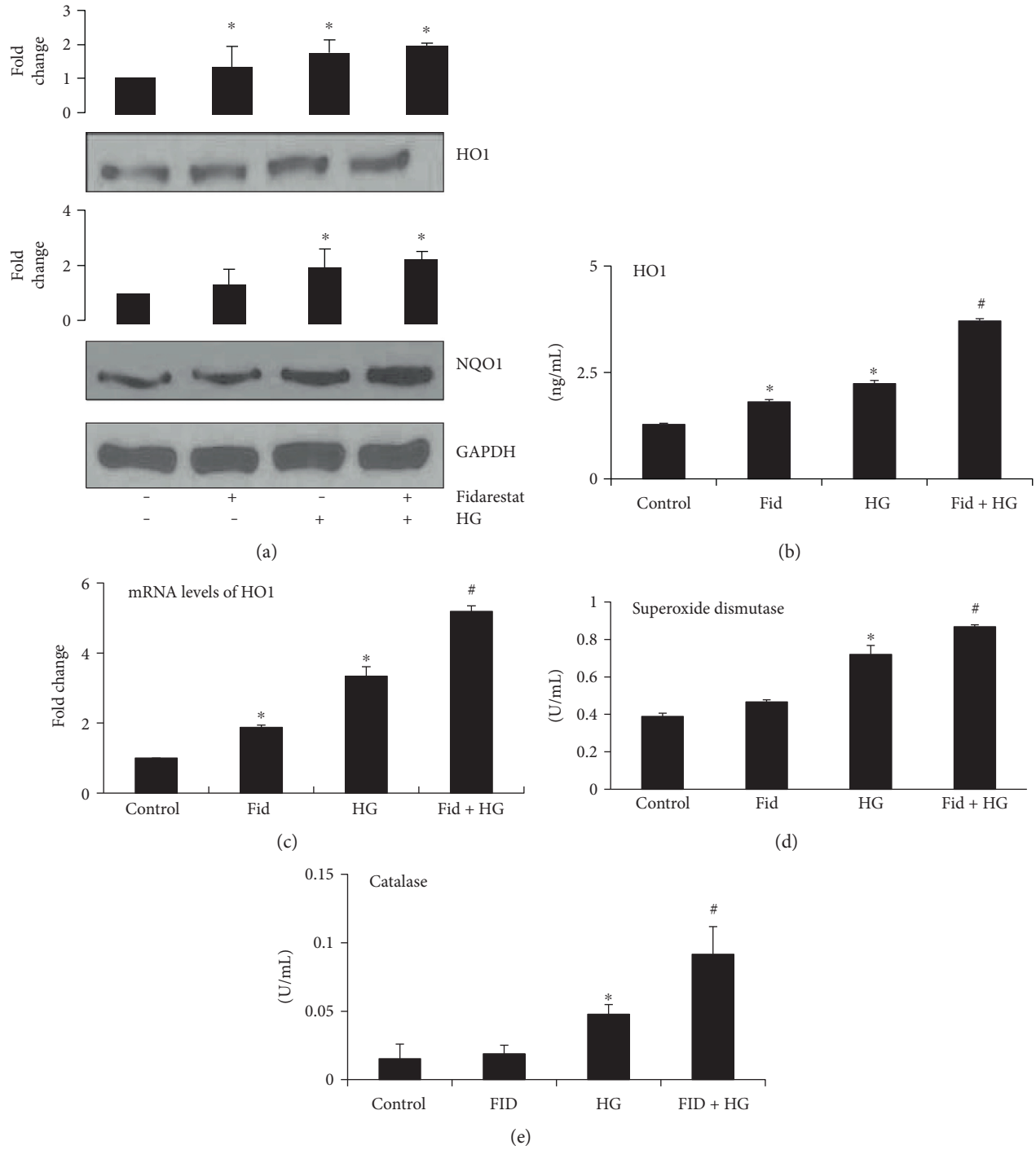

Figure 3: AR inhibitor induces the expression of Nrf2-dependent antioxidant enzymes in HG-treated Thp1 cells. Thp1 cells were pretreated with fidarestat for 14 h followed by incubation with HG (25 mM) for another 24 h. Equal amounts of proteins were subjected to Western blot analysis for the expression of HO1 and NQO1. Fold changes were determined after normalizing with loading control GAPDH. A representative blot from three independent analyses is shown (a). The HO1 levels in the cell lysates were determined by ELISA (b). The mRNA levels of the *HO1* gene in Thp1 cells were determined by RT-PCR as described in Section 2 (c). SOD and catalase activities were analyzed in Thp1 cell lysates by using specific kits as per the manufacturer's instructions (d and e). Data represent mean ± SD ($n = 5$). $^*p < 0.01$ versus control; $\#p < 0.05$ when compared with the HG-treated group or the Fidarestat alone-treated group.

on AMPK-α1 activation, Nrf2-siRNA-transfected Thp1 cells were stimulated with HG ± fidarestat and examined AMPK-α1 activation. The results shown in Figure 6 indicate that HG-induced increase in the phosphorylation of Nrf2 in the absence of fidarestat. However, pretreatment of fidarestat to the Nrf2 knockdown cells did not show any

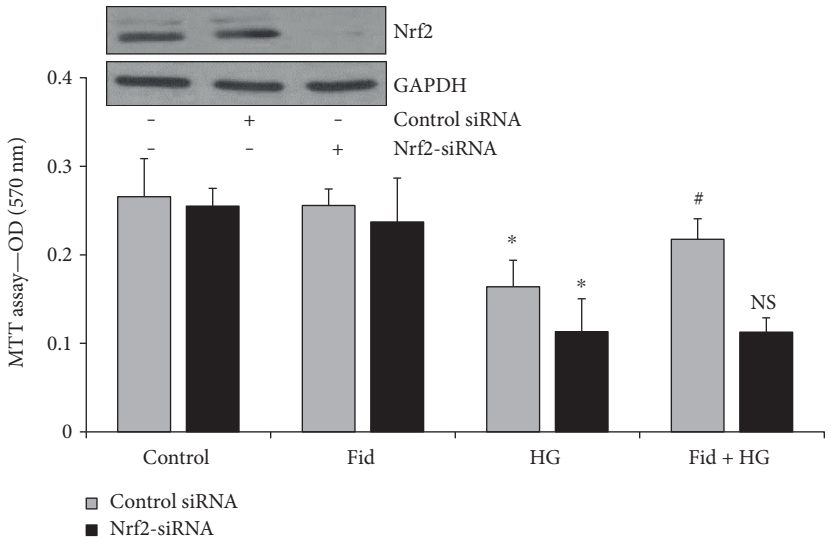

Figure 4: AR-inhibition regulates cell viability of Thp1 cells via Nrf2 activation. The control-siRNA and Nrf2-siRNA-transfected Thp1 cells were treated with HG ± fidarestat for 48 h, and the cell viability was determined by MTT assay. Data represent in mean ± SD ($n = 6$). $^*p < 0.01$ versus control; $\#p < 0.01$ versus HG; NS = nonsignificant versus HG-treated cells. The inset shows Western blots for Nrf2 in control and siRNA-transfected cells. GAPDH = loading control.

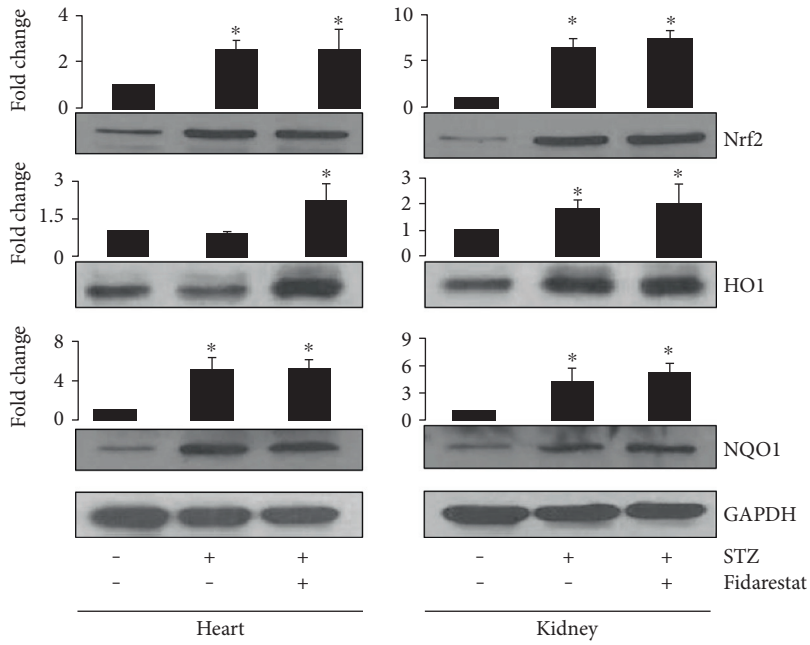

Figure 5: AR inhibitor induces the expression of Nrf2 and its dependent antioxidant enzymes in STZ-induced diabetic mice tissues. STZ-induced diabetic mice were treated without or with fidarestat as described in Section 2. An equal amount of proteins from the heart and kidney homogenates were subjected to Western blot analysis using specific antibodies against Nrf2, HO1, and NQO1. Fold changes were determined after normalizing with loading control, GAPDH. A representative blot from three independent analyses is shown. $^*p < 0.05$ when compared with control, and $\#p < 0.05$ when compared with the HG-treated group.

significant differences in the phosphorylation of AMPK-α1. These results suggest that by regulating the Nrf2-mediated AMPK-α1, AR inhibitor could modulate hyperglycemic stress in Thp1 cells.

4. Discussion

We have shown previously that pretreatment with AR inhibitors prevents cytokine-, chemokine-, HG-, and LPS-induced

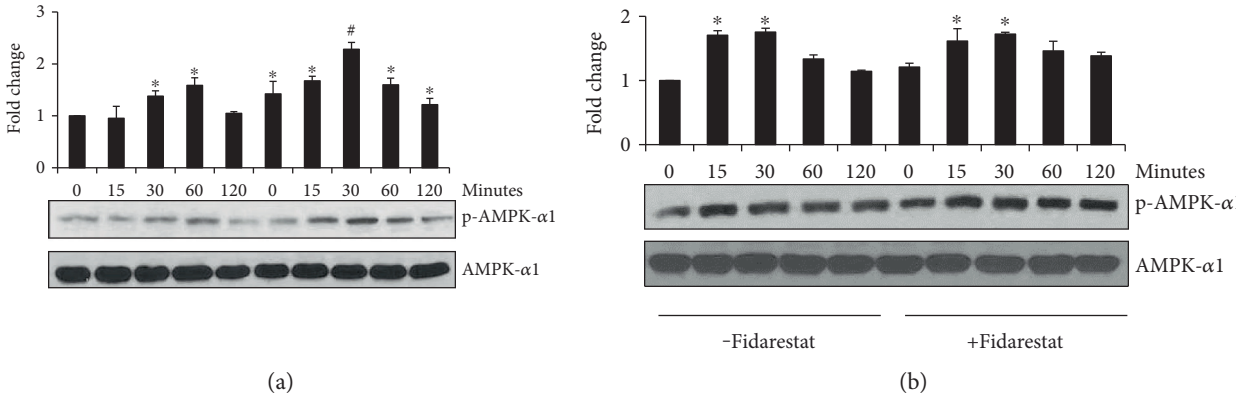

Figure 6: AR inhibition increases HG-induced phosphorylation of AMPK-α1 in Thp1 cells. (a) The Thp1 cells and (b) control and Nrf2-SiRNA-transfected cells were pretreated with fidarestat for overnight followed by incubation with HG for 15, 30, 60, and 120 min. Equal amounts of cytosolic proteins were subjected to Western blot analysis using antibodies against total and phospho-AMPK-α1. Fold changes were determined after normalizing with total AMPK-α1. A representative blot from three independent analyses is shown. $^{*}p < 0.05$ when compared with control, and $\#p < 0.05$ when compared with the HG-treated group.

inflammatory response mediated by NF-κB in various cellular studies [12–15]. Further, AR inhibitors prevent NF-κB-mediated proinflammatory pathways in vitro and in vivo models of hyperglycemia [12, 16]. However, it is not clear how pretreatment with AR inhibitors prepares cells against oxidative stress and activates Nrf2-mediated anti-inflammatory pathways. In this study, we have demonstrated that fidarestat induces Nrf2-mediated antioxidative and anti-inflammatory pathways in Thp1 monocytic cells. Further, fidarestat also augmented the HG-induced expression of Nrf2 and its downstream targets. These results suggest that preincubation with AR inhibitors prepares the cells to defend against pathological effects of hyperglycemia.

Nrf2 transcription factor regulates the expression of a number of cytoprotective antioxidative genes including *SOD*, catalase, *GSTs*, *AR*, *HO1*, *NQO1*, and so forth [9, 10]. Several studies indicate that antioxidants overexpress the Nrf2 pathway as a defense mechanism against various oxidative insults including hyperglycemia [17, 18]. Further, antioxidants such as flavonoids, triterpenoids, quinols, and tBHQ increase the activation of Nrf2 and protect against diabetes-induced nephropathy [19–23]. In addition, Nrf2 null mice are susceptible to the STZ-induced kidney injury [24]. Another study indicates that sulforaphane prevents metabolic dysfunction in hyperglycemia by increasing the expression of the Nrf2 pathway in human endothelial cells [25, 26]. Similarly, curcumin has been shown to decrease insulin resistance, improve pancreatic cell function, and reduce hyperglycemia-induced inflammatory response and complications by activating the Nrf2 pathway [27–29]. These studies suggest the significance of Nrf2 activation in diabetes complications. Consistent with these studies, our current data also suggest that treatment of Thp1 cells with fidarestat augmented the HG-induced Nrf2 activity indicating that fidarestat increases antioxidative balance in the cells and thereby regulates HG-induced cell viability. Furthermore, we have evaluated the effect of fidarestat on HG-induced Thp1 cell viability in Nrf2-ablated cells. Our results demonstrate that AR inhibitor prevented the HG-induced cell death in control cells but not in Nrf2-ablated cells suggesting that Nrf2-mediated antioxidative pathways are required for the actions of AR inhibitor.

Increased expression of Nrf2 leads to increased expression of enzymes linked to antioxidative (NQO1, GSTs, catalase, SOD) and anti-inflammatory (HO1, AR) functions that counteract against oxidative insults [10, 30]. HO1 is an anti-inflammatory protein, and its overexpression has been shown to prevent various inflammatory complications including diabetes [31]. Specifically, HO1 has been shown to protect against HG-induced retinal endothelial cells damage and also prevent vascular inflammatory response in hyperglycemia [32–34]. In this study, our results indicate that fidarestat increases the expression of HO1 and augments HG-induced HO1 in Thp1 cells as well as kidney and heart tissues of STZ-induced diabetic mice suggesting that anti-inflammatory activities of AR inhibition may be through activation of HO1. In addition, our studies also suggest that AR inhibitor increases the expression of SOD and catalase in Thp1 cells and HO1 and NQO1 proteins in STZ-induced diabetic mice heart and kidney tissues, indicating that AR inhibition prevents hyperglycemia-induced oxidative stress by overexpressing various antioxidative enzymes. Since activation of AMPK has been shown to be involved in the regulation of Nrf2 pathway [35], we have also examined the effect of AR inhibitor fidarestat on HG-induced changes in the phosphorylation of AMPK-α1 in Thp1 cells. Our results suggest that pretreatment with fidarestat stimulates phosphorylation of AMPK-α1 in HG-induced Thp1 cells. However, fidarestat pretreatment has no effect on the AMPK-α1 activation in Nrf2-ablated cells.

In conclusion, we have shown that AR inhibitor fidarestat prevents HG-induced Thp1 cell death by induction of Nrf2 expression; DNA binding activity; and expression of HO1, NQO1, SOD, and catalase via activation of AMPK-α1. This suggests that AR inhibition prevents hyperglycemia-induced complications by upregulating anti-inflammatory

Nrf2 pathway in addition to downregulating the proinflammatory NF-κB pathway.

Abbreviations

AR: Aldose reductase
FBS: Fetal bovine serum
Fid: Fidarestat
HG: High glucose
HO1: Hemeoxygenase 1
NQO1: NAD(P)H-quinone dehydrogenase 1
Nrf2: Nuclear factor erythroid 2- (NF-E2-) related factor 2
MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
SOD: Superoxide dismutase
STZ: Streptozotocin.

Conflicts of Interest

Authors declare no conflict of interest.

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