DJ-1 alleviates high glucose-induced endothelial cells injury via PI3K/Akt-eNOS signaling pathway

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Received July 2, 2017; Accepted October 13, 2017

DOI: 10.3892/mmr.2017.7975

Abstract. Hyperglycemia mediated endothelial cells (ECs) injury is closely associated with diabetic vascular complications. It was revealed that DJ-1 possesses cellular protective effects by suppressing oxidative stress. The present study aimed to investigate the beneficial effects of DJ-1 on high glucose (HG)-induced human umbilical vein endothelial cell (HUVEC) injury and to elucidate its underlying mechanisms. HUVECs were incubated under 5.5 mM (control group) or 25 mM D-glucose (HG group) and then transfected with recombinant adenoviral vectors to overexpression of DJ-1. Cell proliferation and apoptosis were measured using the EdU incorporation assay and flow cytometry with Annexin V-FITC/propidium iodide double staining, respectively. Apoptotic-related proteins were determined using western blot analysis. Reactive oxygen species (ROS) production, lactate dehydrogenase (LDH) and nitric oxide (NO) levels, the content of malondialdehyde (MDA), and the activities of superoxide dismutase (SOD) were measured. Results demonstrated that overexpression of DJ-1 promoted cell proliferation and inhibited HUVECs apoptosis stimulated by HG. DJ-1 also suppressed the HG-induced reduction in the Bcl-2/Bax ratio and HG activated ROS generation in HUVECs. Furthermore, HG significantly increased the levels of LDH and MDA, and reduced the level of SOD; however, these effects were reversed by Ad-DJ-1 transfection. Furthermore, the cellular protective effect of overexpression of DJ-1 enhanced p-Akt/Akt ratio, eNOS activation and NO production, and these trends were partially reversed by a phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor (LY294002). Taken together, the present study highlighted the involvement of DJ-1 in HG-related EC injury and identified that DJ-1 exerts a cellular protective effect in HUVECs exposed to HG induced oxidative stress via activation of the PI3K/Akt-eNOS signaling pathway.

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Abbreviations: T2DM, type 2 diabetes mellitus; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; HG, high glucose; ROS, reactive oxygen species; NO, nitric oxide

Key words: diabetes mellitus, endothelial cells, DJ-1, hyperglycemia, oxidative stress

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic syndrome that has an increasing prevalence, especially in China (1). T2DM is characterized by high blood glucose levels, relative insufficiency of insulin secretion from pancreatic beta cells and insulin resistance, worse still, persistent increased blood glucose will result in vascular complications including microvascular complications (2,3). The life-threatening T2DM associated microvascular complications include long-term damage, dysfunction and failure of the vital organs such as retinopathy, nephropathy neuropathy and cardiovascular diseases (4,5). Of note, the metabolic changes of diabetes induce endothelial cells (ECs) dysfunction, which is critical to the initiation and progression of vascular complications (6). Accumulating evidence indicates that hyperglycemia induced by T2DM could increase cell apoptosis, which has emerged as one of the key mechanisms leading to ECs damage (7). Thus, there is an urgent need to identify the therapeutic strategies against ECs damage, which could be useful for prevention and treatment of diabetic vascular lesions.

DJ-1 was first identified as a novel oncogene (8), and subsequent studies have identified its role in the pathogenesis of neurodegenerative disorders, such as Parkinson's disease (9) and Alzheimer's disease (10). Indeed, DJ-1 exerts ubiquitously in variety of EC types, containing human umbilical vein endothelial cells (HUVECs) (11) and corneal ECs (12). Mutations in the gene encoding DJ-1 can cause familial Parkinsonism and overexpression of DJ-1 protects neurons against oxidative stress-induced cell death (13,14). Besides, various studies have shown that DJ-1 could decrease oxidative damage and increase antioxidant gene levels, which contributing to its pro-survival activity (15-17). The evidence described above suggests DJ-1 possesses potential therapeutic activities for oxidative stress associated ECs dysfunction. Although a recent study showed that overexpression of DJ-1 protects endothelial progenitor cells against angiotensin II-induced dysfunction by reducing reactive oxygen species (ROS) production (18), it is

still unclear whether DJ-1 could also play an antioxidant role in ECs injuries.

It has been well established that high glucose (HG) could induce endothelial apoptosis, dysfunction and inflammation, resulting in ECs injury (19-21). Given above findings, we therefore studied the potential protective effects of DJ-1 on HG-induced ECs damage and investigated the relationship between its effect and the modulation of PI3K/Akt-eNOS signaling pathway.

Materials and methods

Cell culture and treatment. HUVECs were purchased from AllCells Biotechnology Co., Ltd. (Shanghai, China). The culture medium was DMEM with either 5.5 mM (control group) or 25 mM (HG group) D-glucose, containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated in a humidified incubator with 5% CO_2 at 37˚C. Recombinant adenoviral vectors, including green fluorescent protein expression (GFP) vectors pAdEasy-1 pShuttle-bSYN and pGEM-3ZF (+) and carrying a human DJ-1 (PARK7) gene were constructed utilizing the AdEasy Vector system (22). pAdEasy-DJ-1 was linearized with PacI and transfected into HUVECs to generate adenovirus that encoded DJ-1 (Ad-DJ-1). The viral titers of adenoviral Ad-DJ-1 and Ad-GFP used for transfection were $1.0x10^9$ and $2.5x10^9$ pfu/ml.

EdU incorporation assay. Cell proliferation was determined with EdU incorporation assay. In brief, cells were seeded into 96-well plates at $1x10^4$ cells/well and then 50 μM of EdU was added to each well with the incubation for 4 h. HUVECs were fixed with 4% formaldehyde and permeated with 0.5% Triton X-100 for 20 min. After washing with PBS, 100 μl of 1X Apollo reaction cocktail was added for an additional 30 min. Then HUVECs were stained with 100 μl of Hoechst 33342 for 30 min and the EdU positive cells (red cells) was counted under an inverted Nikon microscope (Nikon Corporation, Tokyo, Japan) at magnification, x200.

Determination of cellular apoptosis. Cell apoptotic rates were measured by flow cytometric analysis using Annexin V-FITC/propidium iodide (PI) staining (Beyotime, Shanghai, China). In brief, HUVECs in groups were trypsinized and rinsed with PBS. Subsequently, cells were resuspended in Annexin V binding buffer and stained with 10 μl Annexin V-FITC for 15 min under dark. Then, 5 μl of PI was also added for an additional 5 min. Stained cells were analyzed by flow cytometry (FACS Calibur; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of ROS production. For ROS detection, an Image-iT LIVE Green ROS Detection kit (Invitrogen, Carlsbad, CA, USA) was used. HUVECs were incubated with DMEM containing 10 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 30 min and then washed with PBS. The results were obtained using flow cytometry analysis.

Biochemical assay. HUVECs ($1x10^5$ cells/ml) were plated in 6-well plates for 18 h and treated with the method described above. The appropriate volume of supernatant was collected to determine the release of lactate dehydrogenase (LDH) and nitric oxide (NO), the content of malondialdehyde (MDA), and the activities of superoxide dismutase (SOD) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Western blot analysis. Total protein extracts were obtained with RIPA lysis buffer (Shanghai Biyuntian Bio-Technology Co., Ltd., Shanghai, China) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein lysates were then separated by 8-15% SDS-PAGE, transferred to polyvinylidene fluoride membranes (PVDF; MA, USA) and blocked with 1% bovine serum albumin (BSA). Then the membranes were probed with specific primary antibodies against PARK7/DJ-1 (1:10,000), Bcl-2 (1:500), Bax (1 μg/ml), caspase-3 (1:500; all from Abcam, Cambridge, MA, USA), and Akt (1:1,000), p-Akt (1:2,000), eNOS (1:1,000), p-eNOS (1:1,000; all from Cell Signaling Technology, Shanghai, China), and GAPDH (Sigma-Aldrich). Subsequently, after washing with TBST for three times, the blots were incubated in peroxidase conjugated immunoglobulin G anti-rabbit secondary antibody (1:5,000; Sigma-Aldrich) for 2 h. The immune complexes were visualized using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) and quantified with the Quantity One v5.0 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were undertaken using the SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation. Comparisons among groups were carried out with a two-tailed Student t-test or one-way ANOVA. The value of $P<0.05$ was considered to be statistically significant.

Results

Overexpression of DJ-1 promotes proliferation in HG-induced HUVECs. To determine whether DJ-1 affects the proliferation of HG-induced HUVECs, the adenoviral vector Ad-DJ-1 or Ad-GFP was used to overexpression of DJ-1 in HUVECs and the results were illustrated in Fig. 1A. The western blot analysis showed that DJ-1 protein expression was markedly increased in Ad-DJ-1 group, compared to that in Ad-GFP group ($P<0.05$; Fig. 1B). HUVECs exposed to HG showed significant decrease in proliferation ability comparing with control group, whereas overexpression of DJ-1 reversed the inhibitory effect caused by HG ($P<0.05$; Fig. 1C and D).

DJ-1 inhibits HG-induced apoptosis in HUVECs. Flow cytometry analysis with Annexin V-FITC and PI double staining was then used to determine the effect of DJ-1 on HG-induced apoptosis. In comparison with the control group, the results of flow cytometry displayed that HG caused an obvious increase on the cell apoptosis rates of HUVECs. Unexpectedly, this injury was restored by overexpression of DJ-1 (Fig. 2A). In addition, the expressions of pro-apoptotic proteins, including Bax (Fig. 2B) and caspase-3 (Fig. 2C), were elevated, whereas anti-apoptosis protein Bcl-2 (Fig. 2B) was decreased in HG-induced HUVECs. Overexpression of DJ-1 significantly reversed these effects.

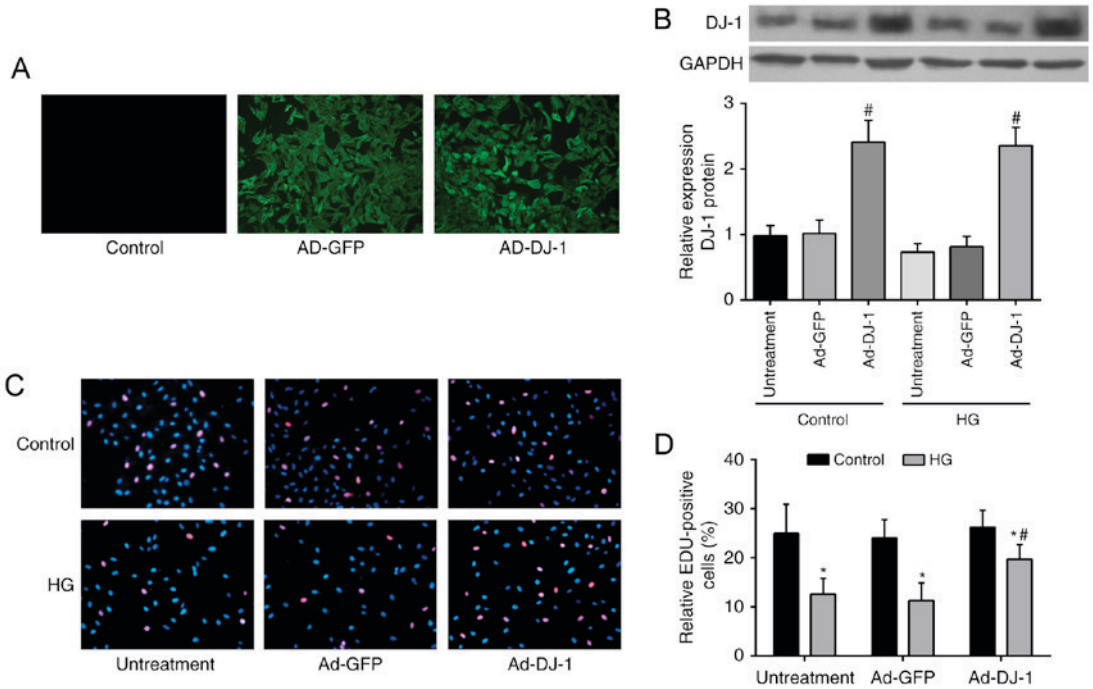

Figure 1. Effects of DJ-1 on proliferation ability of HUVECs induced by HG. (A) Fluorescence showed that recombinant adenoviral vectors were successfully transfected into HUVECs. (B) Expression of DJ-1 protein was detected by western blot analysis in HUVECs in different groups. (C and D) Cell proliferation ability of HUVECs in different groups were measured using EdU incorporation assay. *P<0.05 compared with control group; #P<0.05 compared with Ad-GFP group. HUVECs, human umbilical vein endothelial cells; HG, high glucose.

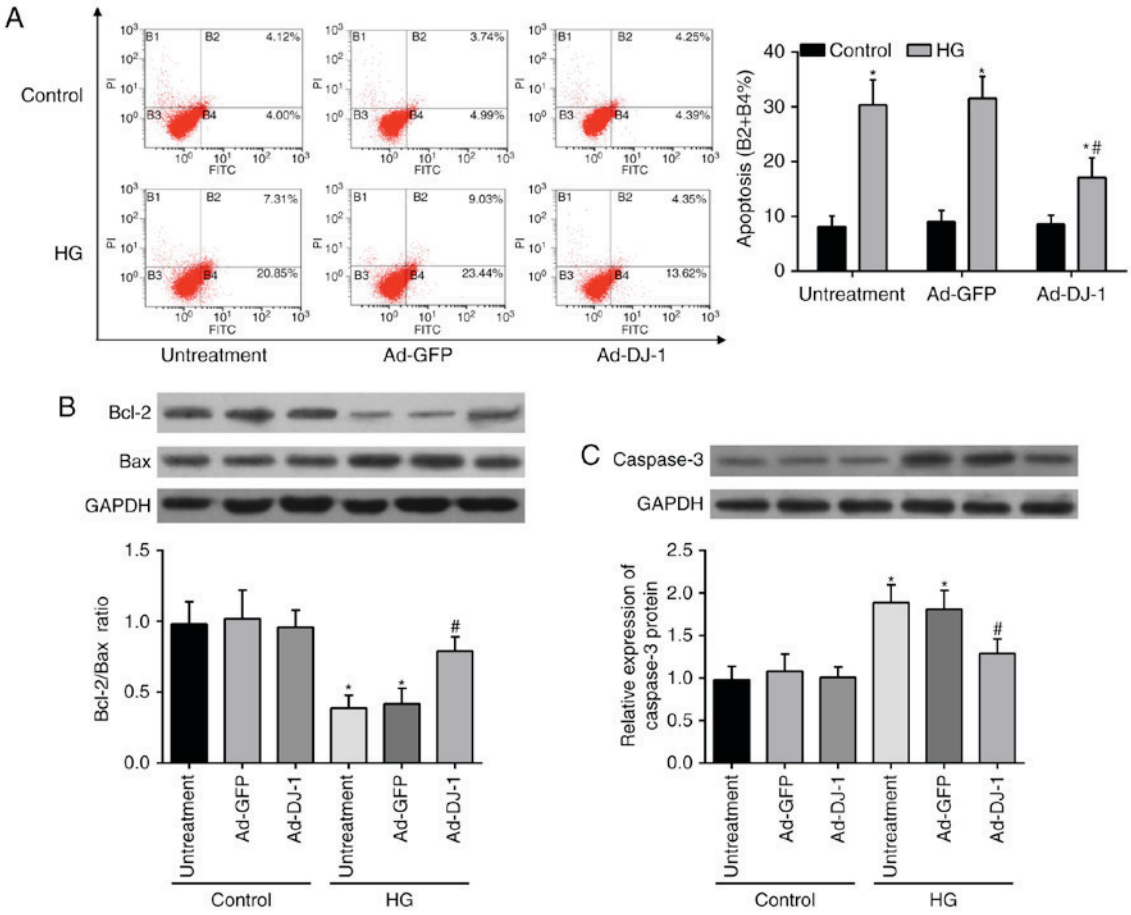

Figure 2. Overexpression of DJ-1 alleviates HG-induced apoptosis in HUVECs. (A) Cell apoptosis was measured by flow cytometry and apoptotic cells represent the percentage of Annexin V single positive and Annexin V/PI double positive cells (region B2+B4). (B) Western blot analysis showed the levels of Bcl-2 and Bax proteins in different groups. (C) Western blots of caspase-3 protein expression. GAPDH was used as an internal control. ICAM-1. #P<0.05 compared with control, *P<0.01 compared with Ad-GFP group. HG, high glucose; HUVECs, human umbilical vein endothelial cells; PI, propidium iodide.

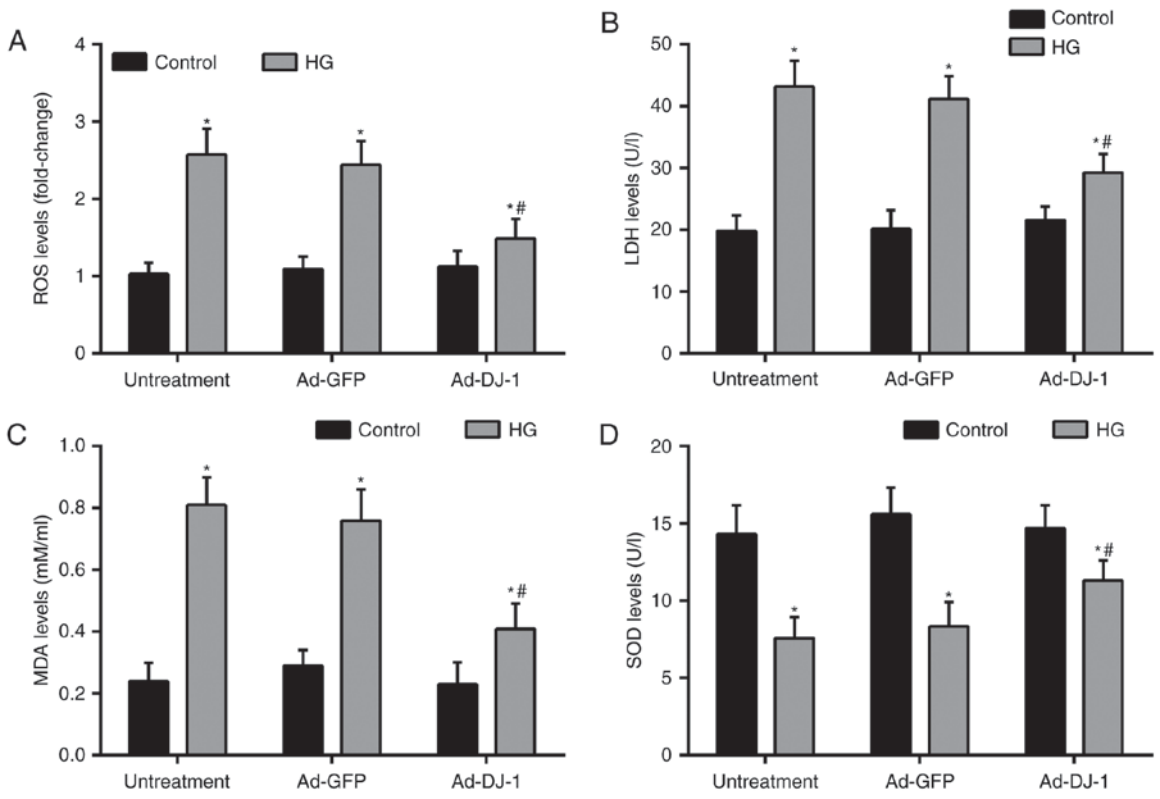

Figure 3. Suppressive effects of DJ-1 on HG-induced HUVECs ROS production. (A) The ROS levels in HUVECs were detected by flow cytometry. (B) LDH, (C) MDA, and (D) SOD levels in the culture medium of HUVECs were measured in different groups. *P<0.05 compared with control group; #P<0.05 compared with Ad-GFP group. HG, high glucose; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase.

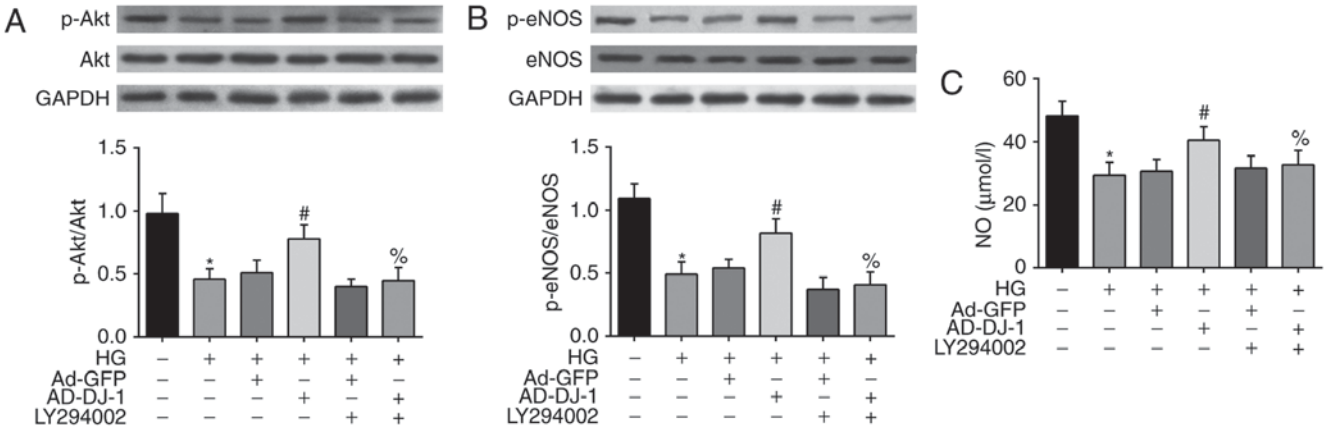

Figure 4. Overexpression of DJ-1 restored the impairment of the PI3K/Akt-eNOS pathway in HG-stimulated HUVECs. (A) The expression of phosphorylated Akt with or without an PI3K inhibitor LY294002 detected by western blot analysis. The Akt phosphorylation was calculated as the ratio of normalized arbitrary units of p-Akt over total Akt. (B) Representative western blots of total and phosphorylated eNOS. (C) The production of NO was measured in cultural medium of HUVECs in groups. *P<0.05 compared with control group; #P<0.05 compared with Ad-GFP group; %P<0.05 compared with HG + Ad-DJ-1 group. HG, high glucose; HUVECs, human umbilical vein endothelial cells; NO, nitric oxide.

DJ-1 possesses antioxidative property in HG-induced HUVECs. Oxidative stress plays a critical role in ECs apoptosis. Thereby, we investigated the levels of markers of oxidative stress, including ROS, LDH, MDA, and SOD in HUVECs. Treatment of cells with HG dramatically caused ROS generation compared with the control group. While, overexpression of DJ-1 suppressed ROS production in HUVECs exposed to HG (Fig. 3A). Besides, the subsequent tests showed that compared with the control group, HG significantly increased the levels of LDH, MDA, and reduced the level of SOD in the supernatant. Similarly, overexpression of DJ-1 effectively reduced above elevated oxidative stress markers (Fig. 3B-D).

DJ-1 activates PI3K/Akt-eNOS pathway in HG induced HUVECs. To reveal the mechanism underlying the above protective effects of DJ-1 on HG-induced HUVECs, we investigated whether PI3K/Akt-eNOS signaling pathway was involved. As shown in Fig. 4A, the treatment of cells

with HG inhibited the phosphorylation of Akt. Interestingly, overexpression of DJ-1 significantly increased p-Akt level. However, LY294002, the PI3K inhibitor of Akt pathway, markedly suppressed the effect of DJ-1 on Akt phosphorylation level. As eNOS is an important downstream target of Akt, we then examined the alteration of eNOS and p-eNOS protein in HG-induced HUVECs. As a result, HG also inhibited the phosphorylation of eNOS in HUVECs, and this effect was reversed by overexpression of DJ-1. Similarly, the effects of DJ-1 on p-eNOS protein expression was blocked by LY294002 (Fig. 4B). Besides, we found that HG treatment significantly decreased the NO production in the culture medium, while overexpression of DJ-1 reversed this tendency (Fig. 4C).

Discussion

In the present studies, we found that DJ-1 significantly promoted HUVECs proliferation and protected it from HG-induced cell apoptosis through suppressing the oxidative stress. Moreover, we demonstrated that the protective effects of DJ-1 on ECs function rely heavily on the PI3K/Akt-eNOS signaling pathway. These findings provided new information about the role of DJ-1 in protecting ECs from HG mediated injury, representing a novel therapeutic strategy in the treatment of ECs damaged associated diabetic vascular lesions.

A variety of reports demonstrated that diabetes-associated hyperglycemia could induce apoptosis in pancreatic islet ECs (23), pancreatic beta-cells (24), and HUVECs (25), via an intrinsic apoptotic pathway. Similarly, we used (25) mM D-glucose to simulate hyperglycemia in this work and found cultured HUVECs showing reduction in cell proliferation ability and possessed of high cell apoptotic rates after treatment with HG. Of note, emerging evidence indicates a link between HG-induced apoptosis of ECs and ROS production (26). And as expected. We also showed HG triggered oxidative stress in HUVECs, which was detected via ROS production, levels of LDH, MDA, and SOD in cell supernatant.

The growing body of evidences demonstrated that DJ-1 is involved in various regulatory functions, including transcriptional regulation and anti-oxidative stress regulation (27). DJ-1 is a homodimeric protein, belonging to the Thi/Pfp1 superfamily and is abundant in most living things from humans to bacteria (28). With the increase in blood glucose levels, the levels of DJ-1 increase in pancreatic β-cells, to inhibit oxidative stress induced ROS (29). However, decreased expression of DJ1 has been detected in the islets of elderly T2DM patients in a gender dependent manner (29). These showed conflicting results concerning DJ-1 expression in this metabolic disease. Here, we focused on the function of DJ-1 on HG-induced cell dysfunction rather than the expression levels of DJ-1 in ECs. It has been shown that DJ-1 protects the morphology and function of the mitochondria and protects against cell injury (30), and in the present study, we revealed that DJ-1 reversed HG-induced HUVECs apoptosis. In accordance with our findings, Wang and Gao found that DJ-1 silencing in HeLa cells increased cell apoptotic rates while DJ-1 overexpression significantly inhibited cell apoptosis (31). In addition, DJ-1 transgene protects cortical neurons from H_2O_2-induced apoptosis and re-expression of DJ-1 into the cortical neurons from DJ-1-knockout mice could reduce H_2O_2-induced cell death via Akt1 signaling pathway (32). The Bcl-2 family proteins, consisting pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) molecules, are known to participate in the regulation of the apoptotic pathway (33). DJ-1 increased the reduction of Bcl-2/Bax ratio and reduced the upregulation of caspase-3, caused by HG in the current study.

Furthermore, it was recognized that loss of DJ-1 increases ROS production (34). In corneal ECs, downregulation of DJ-1 increases caspase-3 activation and phospho-p53 under ultraviolet A oxidative stress and the decline in DJ-1 levels results to increased oxidative damage (12). Excessive DJ-1 expression also inhibited oxidative stress-induced HepG2 cell death (35) and pancreatic β-cell death (36). Thereby, the effects of DJ-1 against oxidative stress induced intracellular ROS production, were then explored in HUVECs. We showed that DJ-1 alleviates the HUVECs damage induced by HG by suppressing oxidative stress through detecting ROS, LDH, MDA and SOD levels. In accordance with our results, Shen *et al* demonstrated that overexpression of DJ-1 exerts protective effects against HG-induced tubular epithelial cells injury, as evidenced by increased SOD activity, the decreased release of LDH and the decreased MDA content (37).

Nevertheless, how DJ-1 regulates ROS is still not completely clear. DJ-1 is oxidized on its cysteine residues which are also critical for the ability of DJ-1 to manage ROS (38). Studies showed that DJ-1 exerts its antioxidant ability through interaction with nuclear factor erythroid 2-related factor2 (Nrf2) (12), paraoxonase-2 (39), receptor of activated C kinase 1, or activation of signaling pathway such as PI3K/Akt/mTOR (40), NF-κB and MAPK pathway (36). Of note, the PI3K/Akt signaling pathway is widely present in cells playing a regulatory role for eNOS, and is also involved in cell proliferation and apoptosis (41). As a vascular endothelial protective factor, eNOS exerts its role by adjusting the biosynthesis of NO. So far, there remains a lack of investigation on the effects of DJ-1 on PI3K/Akt-eNOS signaling pathway in HG induced HUVECs. Once activated, the phosphorylation of Akt can directly phosphorylate eNOS and induce the subsequent production of NO (42). We found that, accompanied with the inhibition of oxidative stress induced HUVECs apoptosis, DJ-1 also attenuated the decrease in the phosphorylation of Akt and eNOS when exposed to HG, as well as increased NO levels. It is known that impaired ECs function arises from decreased production and/or bioactivity of NO induced by eNOS phosphorylation (43,44). In addition, deficiencies in generation of eNOS-derived NO have been proposed as mechanisms responsible for ECs dysfunction in diabetes (45). Our subsequent chemical stressors analysis demonstrated that PI3K specific inhibitors, LY294002, significantly abolished the activation of this pathway induced by overexpression of DJ-1, suggesting that DJ-1 might exert its antiapoptotic effect by activating the PI3K/Akt-eNOS pathways.

Collectively, our preliminary study showed that DJ-1 could antagonize endothelial dysfunction by attenuating oxidative stress via activation of the PI3K/Akt-eNOS signaling pathway. As increasing evidence have validated the important role of oxidative stress in the pathological process of diabetic vascular complications (46-48), the results of the present study highlights DJ-1 as a potential therapeutic target. Accordingly, further studies should focus on the function of DJ-1 in the hyperglycemia related ECs dysfunction in the future.

Acknowledgements

Financial support for this study was provided by Xianning Central Hospital, the First Affiliated Hospital of Hubei University of Science and Technology (no. 2016XYA004).

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