Original Article

MDG-1 prevents high glucose-induced cytotoxicity and inflammation in rat brain microvessel endothelial cells

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Abstract: Hyperglycemia-triggered accelerated endothelial cell injury is a critical event in the process of brain-associated microvascular disease. Ophiopogon japonicus is a traditional Chinese medicine used to treat cerebrovascular and cardiovascular disease. Recent studies have confirmed its beneficial properties, but not the mechanism of action. In the current study, rat brain microvessel endothelial cells (BMECs) were treated with 10 mM D-glucose to establish a high-glucose (HG) induced cell injury model. Herein, pretreatment of BMECs with MDG-1 for 24 h significantly attenuated HG-induced injuries and inflammatory responses, evidenced by increased in cell viability and decreased in cell apoptosis and ROS generation and secretion of IL-8. In addition, pretreatment with MDG-1 markedly reduced HG-induced Cox-2 up-regulation and intranuclear activated NF-κB accumulation. Similar to the protective effect of MDG-1, PDTC depressed not only HG-induced cytotoxicity, but also the secretion of IL-8. Importantly, PDTC obviously attenuated up-regulation of Cox-2 induced by HG. Taken together, these data suggest that MDG-1 protects BMECs against HG-induced injuries and inflammatory responses through inhibition of NF-κB/Cox-2 pathway.

Keywords: Brain microvessel endothelial cells, high glucose, MDG-1, apoptosis, NF-κB/Cox-2

Introduction

Hyperglycemia is a key factor in the development of vascular complications in patients with diabetes [1]. A growing body of evidence indicates the close correlation between hyperglycemia and the abnormalities in endothelial function. In vitro, high glucose has been reported to be toxic for endothelial cells, as represented by lengthened cell proliferation [2], disturbed cell cycle [3], and increased the production of reactive oxygen species (ROS) [4, 5] and DNA damage [6], and slightly accelerated cell death [7]. Moreover, systemic and vascular ROS production lead to modulate inflammatory that contribute to microvascular and macrovascular damage [8]. In vivo, hyperglycemia in diabetes has been found to be responsible for endothelial abnormalities, including accelerated disappearance of capillary endothelium and weaken intracellular junctions [9, 10].

Ophiopogon japonicus (O. japonicus) is a traditional Chinese medicine widely distributed in Southeast Asia, and treatment of cerebrovascular and cardiovascular disease for thousands of years [11, 12]. Recent evidence demonstrated that certain compounds can be used therapeutically to stimulate vessel growth in ischemic tissues [13]. MDG-1, a water-soluble polysaccharide extracted from O. japonicus has an interesting role in anti-myocardial ischemia, and protects cardiomyocyte from hypoxia/reoxygenation-induced damage [14] and relieves the symptom of type 2 diabetes mellitus by decreasing blood glucose [15]. But the possible contribution of MDG-1 to the HG-induced cerebrovascular injury has not been explored.

In the present study, we investigated the cytoprotection of MDG-1 in BMECs treated with HG. Endothelial cells are widely understood to play a crucial role in the development of vascular diseases in addition to serving a broad range of physiological functions in the cerebrovascular system. Our findings showed that MDG-1 protected BMECs against HG-induced injury and inflammatory response by inhibiting the NF-κB/Cox-2 pathway.
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway

Materials and methods

Isolation of rat brain microvessel endothelial cells (BMECs)

Care of laboratory animals and animal experimentation were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were approved by the animal ethics committee of First People’s Hospital of Huzhou. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

This procedure was performed using a method introduced by Deli et al. [16]. Briefly, fresh rat brains were obtained from 3-week-old Sprague Dawley rats, stored in Dulbecco’s modified Eagle’s medium (DMEM) on ice, and the cerebellum, brain stem, choroid plexus, and the meninges were carefully removed. The cortices were dissected away from the surrounding tissue and much of the brain white matter was subsequently removed. The cortices were mashed with forceps and thoroughly triturated, followed by a digestion at 37°C with 0.7 mg/ml type 2 collagenase and 20 U/ml Dnase I in DMEM, and then was centrifuged at 1000×g for 8 min at 4°C. The pellet was re-suspended in a 20% bovine serum albumin (BSA) to separate microvessel from other components. Then, the pellet was further digested in 1 mg/ml collagenase/dispase and 20 U/ml Dnase I in DMEM for 1 h at 37°C. The digested microvessel solution was diluted with DMEM and centrifuged at 700×g and 4°C for 6 min. The pellet was re-suspended and layered over a 50% continuous Percoll gradient and centrifuged at 1000×g for 10 min at 4°C. Subsequently, the microvessel was re-suspended in DMEM (20% fetal calf serum, 100 μg/ml heparin sodium) and seeded on either collagen IV/fibronectin coated plastic 24-well tissue culture plates for 24 h at 37°C. Culture medium was added 1 ng/ml basic fibroblast growth factor (bFGF) and changed within 24 h of initial plating to remove unattached cells or vessel fragments. Cultures were maintained in a 37°C incubator under humidified 5% CO₂/95% air and culture medium was changed every 2-3 days.

High D-glucose (HG) induced BMECs injury

BMECs were cultured in DMEM for 24 h or co-cultured with NF-κB inhibitor (PDTC) for last 1 h, then treated with high D-glucose (HG, 10 mM) for another 24 h.

Cell viability assay

The Cell Count Kit-8 (CCK-8, Dojindo, Rockville, MD, USA) was used to assess the effects of MDG-1 on the BMECs viability [17]. In brief, BMECs in logarithmic growth-phase were collected, and 5×10^4 cells/well was dispensed into 96-well culture plates with 100 μl culture medium. After 24 h culture different concentrations of MDG-1 (1, 2, 5, 10, 50 and 100 mM) were put in different wells. Each of the concentrations above was regarded as one treated group while there was no MDG-1 in the control group. Culture plates were then incubated for 24 and 48 h. Subsequently the cell viability was evaluated by CCK-8 following the manufacturer’s instructions. The absorbance at wavelength 450 nm was measured for the supernatant of each well using the plate reader Multiskan EX (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell apoptosis assay

BMECs were collected after treatment with MDG-1 (5, 10 and 50 mM) for 24 h. Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double stain assays (Biovision Inc, Mountain View, CA, USA) were performed following the manufacturer’s protocol. BMECs were stained with annexin V-fluorescein isothiocyanate and apoptotic rates were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Detection of reactive oxygen species (ROS)

Detection of ROS was performed by flow cytometry analysis as described previously [18]. In brief, 5×10^5 cells/well were cultured in 24-well plate, after treatment, cells were washed with PBS and re-suspended in complete medium followed by incubation with 0.5 μM dihydrodorodamine 123 (Sigma, St. Louis, MO, USA) for 30 min at 37°C. ROS fluorescence intensity was determined by flow cytometry with excitation at 490 nm and emission at 520 nm.

Reverse transcription and Real-time PCR

Total RNA was isolated using Trizol reagent (Gibco@life technology, Carlsbad, CA, USA). Reverse transcription reactions were per-
formed as described. Real-time PCR was performed on ABI 7500 (Applied Biosystem, Foster City, CA, USA) thermal cycler using a standard SYBR Green PCR kit (Thermo Fisher Scientific). The relative mRNA expression of target gene compared with GAPDH was calculated using the $2^{-\Delta\Delta C_t}$ method. The primers for each gene were listed in Table 1.

### Western blot analysis

BMECs were harvested and washed twice with PBS and cell lysates were collected with radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. Cell lysates were centrifuged at 1000×g for 10 min at 4°C. The supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, MS, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against NF-κB, NF-κBp65, IL-8, Cox-2, H3 and GAPDH were purchased from Santa. Blots were then incubated with goat anti-mouse secondary antibody (Beyotime, Shanghai, China) or goat anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Millipore).

### Statistical analysis

The GraphPad Prism 5.0 software system was employed for statistical analysis. Data are expressed as the mean ± standard error. Student’s t test was used to compare the differences between two groups, while one-way analysis of variance was used when more than two groups were compared. $P < 0.05$ was taken as statistical significance.

### Results

#### Effect of MDG-1 on the viability of BMECs

To identify whether high D-glucose (HG) may cause cytotoxicity in vitro, the effect of HG on BMECs viability was determined using the CCK-8 assay. BMECs were treated with HG for 24 h and showed significantly decreased cell viability (Figure 1A). However, HG-induced BMECs were treated with increasing concentrations of MDG-1 for 24 and 48 h markedly increased cell viability compared with BMECs with HG treatment alone. After 48 h MDG-1 has no significant effect on BMECs viability at lower concentrations less than 5 mM. Only treatment with high concentrations of MDG-1 (5, 10, 50 and 100 mM) reduced cell viability. Therefore, MDG-1 was only used at concentrations of 5, 10 and 50 mM in all subsequent experiments.

#### Effect of MDG-1 on the apoptosis of HG-induced BMECs

Oxidative stress is one of the main damage for the BMECs. We used flow cytometry assay to assess the effect of MDG-1 on HG-induced BMECs apoptosis. BMECs were treated with increasing concentrations of MDG-1 for 24 h. For BMECs, without MDG-1 treatment, under HG conditions, the apoptotic rates was significantly increased compared with the control BMECs. However, BMECs with MDG-1 treatment reduced HG-induced cell apoptosis significantly in a dose-dependent manner (Figure 1B). The apoptotic rate was reduced from 34.9 ± 4.36% to 17.9 ± 2.1%.

#### Effect of MDG-1 on the ROS level of HG-induced BMECs

Destruction of mitochondrial membrane potential was the initial process of mitochondria apoptosis. To elucidate the possible mechanisms by which MDG-1 prevented HG-induced BMECs apoptosis, we measured intracellular ROS after BMECs were incubated with HG in the presence or absence of MDG-1. The ROS level of HG-induced BMECs was significantly increased compared with that of control BMECs without MDG-1 treatment. However, BMECs with MDG-1 treatment reduced HG-induced increase of ROS level significantly in a dose-dependent manner (Figure 1C). The ROS level was reduced by 80.0 ± 4.16%, 91.9 ± 5.16% and 97.0 ± 5.71%, compared with that of HG-induced BMECs.

### Table 1. Primers sequences used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>NF-κBp65-forward</td>
<td>5'-AGACCTGGAGACAGCACTCATAG-3'</td>
</tr>
<tr>
<td>NF-κBp65-reverse</td>
<td>5'-CGGACCGCATTCACTCAGTAG-3'</td>
</tr>
<tr>
<td>IL-8-forward</td>
<td>5'-CTCCACGAACTACACAGAG-3'</td>
</tr>
<tr>
<td>IL-8-reverse</td>
<td>5'-CACCCCTAAGCAAAACACAT-3'</td>
</tr>
<tr>
<td>Cox-2-forward</td>
<td>5'-CAACCAGCTGTTCCAGATCAG-3'</td>
</tr>
<tr>
<td>Cox-2-reverse</td>
<td>5'-TGAGCAAGTCTGGTTCCAAG-3'</td>
</tr>
<tr>
<td>GAPDH-forward</td>
<td>5'-GTGGTTGGAACGGATTGAC-3'</td>
</tr>
<tr>
<td>GAPDH-reverse</td>
<td>5'-TCCATTCTAGGCCTGAC-3'</td>
</tr>
</tbody>
</table>
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway.
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway

Figure 1. Effects of MDG-1 on the viability, apoptosis and ROS level of BMECs. The effects of various concentrations of MDG-1 on the viability (A), cell apoptosis (B) and ROS level (C) of BMECs were assessed by CCK-8 and flow cytometry assay. Data are presented as mean ± SD, n=3, *P < 0.01, compared with the control group, #P < 0.01, compared with the HG treatment group.

Effect of MDG-1 on the NF-κB, IL-8 and Cox-2 expression

To determine whether NF-κB downstream signaling was affected by MDG-1, we measured NF-κBp65, IL-8 and Cox-2 expression after BMECs were incubated with HG in the presence or absence of MDG-1. The protein expression of NF-κBp65, IL-8 and Cox-2 was significantly increased in HG-induced BMECs. After MDG-1
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway

treatment, the BMECs showed a 94.4 ± 5.11%, 66.4 ± 3.16% and 64.9 ± 2.96% decrease in expression of NF-κBp65, IL-8 and Cox-2 association compared with HG-induced BMECs without MDG-1 treatment as determined by Western blot analysis (Figure 2A and 2B), which was consistent with the results of mRNA expression of indicated proteins (Figure 2C). These data suggest that HG induces BMECs injury through activated NF-κB and increased the expression of NF-κB downstream signaling, IL-8 and Cox-2.

Effect of combination of MDG-1 and PDTC on the apoptosis and ROS level of BMECs

PDTC was the specific inhibitor of NF-κB and the effects of combination of MDG-1 and PDTC on the apoptosis and ROS level of BMECs were performed in this study. As shown in Figure 3A, MDG-1 at a dose of 10 mM and PDTC at a dose of 10 mM prevented HG-induced apoptosis of BMECs in a synergistic manner, which was significantly reduced to 5.20 ± 1.14% compared with HG treatment alone. Figure 3B showed that the ROS level of BMECs also inhibited by combination of MDG-1 and PDTC treatment (91.9 ± 4.14% reduction compared with HG treatment alone).

Effect of combination of MDG-1 and PDTC on the NF-κB, IL-8 and Cox-2 expression

Both MDG-1 and PDTC can effectively decrease the expression of NF-κBp65 and the downstream signaling, Cox-2 and IL-8 in a synergistic manner. Combination of MDG-1 and PDTC showed a 90.2 ± 5.41%, 53.1 ± 2.61% and 67.1 ± 3.46% decrease in expression of NF-κBp65, IL-8 and Cox-2 association compared with HG-induced BMECs without MDG-1 and PDTC combined treatment as determined by Western blot analysis (Figure 4A and 4B).

Discussion

High glucose-induced injury of oxidative stress occurs in many diseases, including causes apoptosis in proximal tubular epithelial cells [19], mitochondrial dysfunction in neurons [20] and has a primary role in the pathogenesis of diabetic nephropathy [21]. Oxidative stress and inflammatory response are two key risk factors of these diseases. MDG-1, a drug extracted from Ophiopogon japonicas, exerts various physiological and physiopathological effects in vivo, including anti-ischemic properties [22], anti-diabetic activity [23], cytoprotective and proangiogenic effects [24]. We therefore hypothesize that MDG-1 may confer protective effects against HG-induced injury.

In the present study, high glucose was induced in the rat brain microvessel endothelial cells (BMECs) cultured with 10 mM high D-glucose (HG). Our results showed that BMECs cultured with HG led to cytotoxicity, evidenced by the decreased cell viability. To investigate whether MDG-1 can protect BMECs against HG-induced cytotoxicity, BMECs were treated with MDG-1 at concentrations ranging from 1 to 100 mM for 24 h after cultured with HG. Interestingly, we found that treatment with MDG-1 significantly attenuated HG-induced cytotoxicity and apoptosis in BMECs (Figure 1A and 1B). This anticytotoxic and anti-apoptotic effect of MDG-1 is similar to previous results in cardiomyocyte and HMEC-1 [22, 24].

Another important finding of this study was that MDG-1 inhibited oxidative stress induced by HG in BMECs. Cultured with HG elicited a marked increased in ROS generation in BMECs and the increased ROS production was significantly abrogated by treatment with MDG-1 (Figure 1C). We speculated that one of the mechanisms underlying HG-induced ROS elimination may be associated with an inhibition of autoxidation of glucose, formation of intermediate products of cyclooxygenase catalysis, or mitochondrial respiration [25]. Another mechanism for the inhibition of oxidative stress by MDG-1 may be associated with a radical scavenger function [26].

Inflammatory response is an important injury factor in high glucose-induced injury. In this study, besides cytotoxicity and oxidative stress, HG-induced inflammatory response was evidenced by increase in IL-8 secretions. Importantly, we observed that treatment with MDG-1 significantly attenuated HG-stimulated IL-8 secretions from BMECs (Figure 2B), suggesting that MDG-1 can protect BMECs against HG-induced inflammatory response. Cox-2 is a potent pro-inflammation mediator, which can promote the production of many inflammation factors in various experiments. Our current study showed that BMECs cultured with HG elevated expression of Cox-2 and BMECs treat-
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway.
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Figure 3. Effect of MDG-1 and PTDC on the apoptosis and ROS level of BMECs. The effect of BMECs treated with MDG-1 (10 mM) and PTDC (10 mM) on apoptosis (A) and ROS level (B) were detected by flow cytometry assay. Data were presented as mean ± SD, n=3, *P < 0.01, compared with the control group, #P < 0.01, compared with the HG treatment group.

Figure 4. Effects of MDG-1 and PTDC on NF-κB, IL-8 and Cox-2 expression. BMECs were treated with MDG-1 and PTDC, and lysed for Western blot analysis using antibodies against the NF-κBp65 (A), and IL-8 and Cox-2 proteins (B). H3 and GAPDH were detected as the control of sample loading. Data were presented as mean ± SD, n=3, *P < 0.01, compared with the control group, #P < 0.01, compared with the HG treatment group.

ment with MDG-1 for 24 h suppressed HG-stimulated Cox-2 up-regulation (Figure 2B). Similarly, a traditional Chinese medicine Huang Qi can reduce I/R-induced dermatic injury partly by inhibition of Cox-2 [27]. In addition, Cox-2 inhibition by NS-398 can confer anti-inflammatory effects, decreasing IL-6 secretion and increasing IL-10 secretion in rat liver damage model [28].

NF-κB is an inducible transcription factor and can potently augment Cox-2 expression. The p65 protein is one of the most abundant sub-units of NF-κB and indicates the activation of NF-κB. In the study, we showed that BMECs cultured with HG led to the accumulation of intranuclear NF-κBp65 subunit, which was significantly repressed by treatment with MDG-1 (Figure 2A). By inhibiting NF-κB, both MDG-1 and PDTC attenuated HG-induced cell apoptosis (Figure 3A), ROS generation (Figure 3B), and up-regulation of NF-κBp65, Cox-2 and IL-8 in BMECs (Figure 4). These results are comparable with the previous findings that salvianolic acid B protects against HG-induced mesangial proliferation through inhibiting activation of NF-κB [29]. Collectively, we provide new evidence that activation of NF-κB regulates Cox-2-mediated inflammation and cytotoxicity, and that MDG-1 protects against HG-induced inflammation and cytotoxicity by inhibition of NF-κB/Cox-2 pathway in BMECs.

In summary, the present study has for the first time demonstrated that MDG-1 confers a cytoprotective effect against HG-induced cytotoxicity and inflammation through inhibition of the NF-κB/Cox-2 pathway in BMECs. Our study provides new insights into the roles of MDG-1 in attenuating HG-induced injury and administration of MDG-1 may be a novel therapeutic strategy for microvascular injury induced by HG.
MDG-1 exerts protective effects through the NF-κB/Cox-2 pathway

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Disclosure of conflict of interest

None.

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