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*Original Article*

# Blockade of Drp1 attenuates mitochondrial-mediated endothelial dysfunction under hyperglycaemic condition

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**Abstract:** Objective: Although high blood glucose is the most dominant feature of diabetes and mitochondrial function are closely linked to high glucose conditions, there is little information on whether and how mitochondrial dynamics participate in high glucose-induced ECs dysfunction. As the key regulatory protein of mitochondrial division, the question of whether and how Drp1 regulates high glucose-induced endothelial dysfunction has attracted our attention. Method: In our present study, we isolated ECs of mouse aortic and conducted a series of tests including protein determination, microscope scanning and fluorescent staining. We investigated oxidative stress alterations occurring in high glucose-treated ECs. We further determined whether and how Drp1 pharmaceutical inhibitor Mdivi, regulates ROS-induced ECs dysfunction in high glucose environment. Results: ECs underwent reduced tube length, increased paracellular permeability, increased ROS levels, and elevated Drp1 expression levels accompanied by increased p38 phosphorylation after high glucose treatment. Both protective effects of blocking Drp1 and antioxidant treatment could attenuate the high glucose-induced ECs damage and decreased p38 phosphorylation. Furthermore, blockade of p38 phosphorylation also reversed these impairments in high glucose-treated ECs. Conclusion: Blockade of Drp1 attenuates mitochondrial-mediated endothelial dysfunction under hyperglycaemic condition. Our research provides new insight into the role of expression of Drp1 in high glucose condition, highlighting the potential diagnostic and therapeutic application for T2DM.

**Keywords:** High glucose, endothelial function, oxidative stress, mitochondrial function, ROS, Drp1, p38

## Introduction

Type 2 diabetes mellitus (T2DM) is the most prevalent endocrine metabolic disease in the world, accounting for almost 90% of all diabetes mellitus, and is influenced by both genetic and environmental determinants [1]. Vascular disease is the most common complication of T2DM, and is characterized by impaired wound healing ability and reduced collateral circulation in ischemia, resulting in decreased vascular repair capacity [2]. Increasing evidence suggest that the excessive generation of reactive oxygen species (ROS) plays a crucial role in mitochondrial dysfunction and is involved in the process of ECs dysfunction and death [3, 4]. Although ROS-induced oxidative stress is considered to be the main pathological feature of T2DM, the detailed mechanisms underlying ROS-mediated ECs dysfunction under hyperglycaemic conditions have not been fully elucidated.

Mitochondria are dynamic organelles by several criteria. They are undergoing constant fission and fusion regulated by a machinery involving large dynamin-related GTPases [5]. Emerging evidences suggest that ROS production and accumulation are correlated with abnormal mitochondrial morphology and increased mitochondrial fission [6, 7]. Drp1 is a predominantly cytosolic protein that is recruited to mitochondria during fission. Regulation of Drp1 properties such as mitochondrial translocation, protein interactions, or GTPase activity might be expected to be important to the mitochondrial and cellular functions. In our previous study, we demonstrated that blockade of the mitochondrial fission protein Drp1 by a genetic manipulation or pharmacological inhibition effectively attenuates the effect of oxidative stress in AD (Alzheimer's disease) cybrid cells [8, 9]. These studies indicate the role of Drp1 in the oxidative stress-induced cellular perturbation and injury and preset Drp1 as a potential novel therapeu-

tic target for prevention or treatment of oxidative stress-related diseases. However, how Drp1 affect endothelial cell functions under hyperglycemic conditions remains further research.

Previously, it has been reported that increased phosphorylation of p38 signal pathway leads to cell death induced by oxidative stress in hyperglycaemia and are involved in cardiovascular complication in diabetes [10, 11]. However, it is unclear whether phosphorylation of p38 affects mitochondrial-mediated endothelial dysfunction in T2DM. In order to explore this question, we hypothesized that there is a direct link between p38 activation and high glucose-induced ECs dysfunction and related mitochondrial dysfunction.

In this study, we investigated oxidative stress alterations occurring in high glucose-treated ECs. We further determined whether and how Drp1 regulates ROS-induced ECsdysfunction in high glucose environment. Our research provides new insight into the role of expression of Drp1 in high glucose condition, highlighting the potential diagnostic and therapeutic application for T2DM.

## Materials and methods

### *Isolation of mouse aortic ECs*

Five-month-old C57Bl/6 mice were used for all experiments. All procedures were performed in accordance with the approval of the Ethical Committee on Animal Testing at the Sichuan University. ECs from the thoracic aorta were isolated using a modified explants technique previously described [12]. Briefly, after anesthetization with isoflurane and euthanasiaby cervical dislocation, the thoracic aorta was gently isolated. Aortas were cut into rings (approximately 1-2 mm long) and seeded on a Matrigel (Sigma, St Louis, MO, USA)-coated 12-well plates, and then incubated in the specific DMEM at 37°C in a 95% air/5% $CO_2$ incubator. Once cell outgrowth was observed, the aortic rings were removed from the medium. ECs primary cultures were observed by phase-contrast microscopy (10 × magnification) (OLYMPUS IX71; OLYMPUS, Tokyo, Japan). Approximately 4 days later, ECs were digested with Dispase (Sigma) and seeded on 0.1% gelatin (Sigma)-coated culture dishes with 10 cm diameter. After reaching confluence, ECs were digested with 0.25% trypsin (Sigma) and passaged at a 1:4 ratio.

### *Cell treatment*

Drugs were prepared as stock solutions and were diluted to a final concentration before use immediately. Final concentration and action time of the drugs were as follows: treatment with high glucose (D-glucose, 30 mM for 12 h; Sigma) *N*-acetyl cysteine (NAC; a common antioxidant) (1 mM for 24 h; Sigma), mdivi (a pharmacological inhibitor for Drp1) (10 μM for 1 h; Sigma), and SB203580 (a pharmacological inhibitor for p38) (10 μM for 24 h; Sigma).

### *Western blot analysis*

Equal amounts of total protein confirmed by BCA-protein assay kit (Beyotime, Biotechnology Institute, Haimen, China) were separated on 10% SDS-PAGE gels and subsequently transferred to PVDF membrane (Bio-rad, Hercules, USA). After blocking with 5% non-fat milk, the membranes were probed with primary antibodies raised against phospho-p38 (p-p38; 1:1000), total-p38 (t-p38; 1:1000), Drp1 (1:1000) and actin antibody (1:5000), followed by the addition of horseradish-peroxidase-conjugated secondary antibody (1:3000). All antibodies were purchased from Abcam (Cambridge, MA, USA). Protein bands were then visualized using chemiluminescence (ECL; GE Healthcare Bio-Sciences, Marlborough, MA, USA) and quantified using densitometry (Quantity One; Bio-Rad, Hercules, CA, USA).

### *Tube formation assay*

The tube formation assay was performed according to methods described in a previous study [13]. The tubular structure of endothelial cells was photographed using an inverted microscope (original magnification, X10; Olympus, Tokyo, Japan). Each well was scored for tube formation at 5 locations: center, north, south, east and west in each well. Scoring ranged from 0 (indicating no tube formation) to 3 (indicating an extensive tube network). Average scores for each well were determined. In addition, tube lengths were measured from representative pictures at 10X. Length was measured from the base of one cell projection to another. Average lengths were also determined for each well.

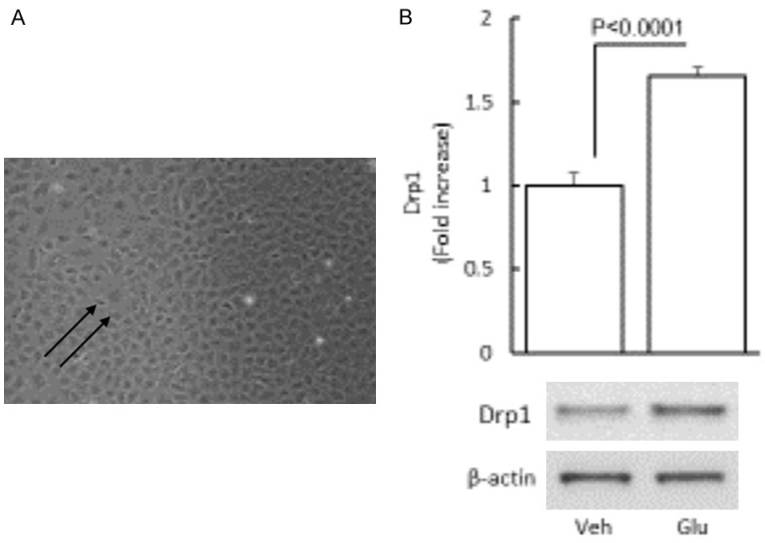


**Figure 1.** Alterations of Drp1 expression levels in high glucose-treated ECs. A. Endothelial cells (ECs) primary cultures were observed using phase-contrast microscopy (original magnification, × 10). ECs exhibited contact inhibition (black arrows). B. Quantification of immunoreactive bands of Drp1 by western blot analysis. Representative immunoblots were shown in lower panels. Image intensity was quantified using NIH Image J software. Data are expressed as fold increase relative to vehicle group. *P*-values < 0.05 versus vehicle group.

*Paracellular permeability assay*

The paracellular permeability was detected as described previously [14]. Briefly, cells in Transwell chambers were incubated on a polycarbonate membrane (diameter, 10 mm; pore size, 0.41 m) (NalgeNunc International, Rochester, NY) for 1 hour before adding Fluorescein isothiocyanate-dextran (Sigma) with two different molecular sizes (40 and 70 kDa). After incubation for 3 h, 50 μl of medium from lower chamber was aspirated and diluted to 500 μl with PBS. Fluorescence intensity was measured at an excitation wavelength of 492 nm by a fluorescent multi-mode microplate reader (Biotek, Winooski, VT, USA). The permeability coefficient was calculated based on methods previously reported [15].

*Oxidative stress, mitochondrial membrane potential analysis and measurement of cytochrome C oxidase activity*

Evaluation of intracellular ROS levels were accessed by election paramagnetic resonance (EPR) spectroscopy as previously described [9]. The EPR spectra were collected, stored, and analyzed with a BrukerEMXplus EPR spectrometer (Billerica, MA, USA) using the Bruker Software Xepr (Billerica). To estimate mitochondrial ROS production, ECs were co-stained with Mitosox Red (2.5 μM; Molecular Probes, Life Technologies Co., Grand Island, NY, USA) and Mitotracker Green (MT Green, 100 nM; Molecular Probes) for 30 min at 37°C. For mitochondrial membrane potential determination, cells were co-stained with tetramethylrodamine methyl ester (TMRM, 100 nM; Molecular Probes) and MT Green (100 nM) for 30 minutes at 37°C. Fluorescence from MTGreen is independent of membrane potential, whereas TMRM is sensitive to membrane potential. Images were captured under a fluorescent microscope (400 × magnification) (OLYMPUS IX71). NIH Image J software was used to quantify and measure fluorescent signals. More than 100 clearly identifiable mitochondria from 10-15 randomly selected cells per experiment were measured in 3 independent experiments.

Cytochrome C oxidase (CcO, or complex IV) is one of the key enzymes associated with the mitochondrial respiratorychain. CcO activity was measured in cell lysates according to our previous studies [16, 17].

*Statistical analysis*

Data are presented as mean ± SEM. Statistical analysis was performed using Statview software (Version 5.0.1; SAS Institute, Cary, NC, USA). Differences between means were assessed using a Student's t-test or one-way analysis of variance (ANOVA) with Fisher posthoc test. Differences with $P < 0.05$ were considered significant.

## Results

*Drp1 expression levels are elevated in aortic ECs treated with high glucose*

ECs isolated from the mouse aorta grew in monolayer and exhibited contact inhibition as shown in **Figure 1A**. In this study, whether and

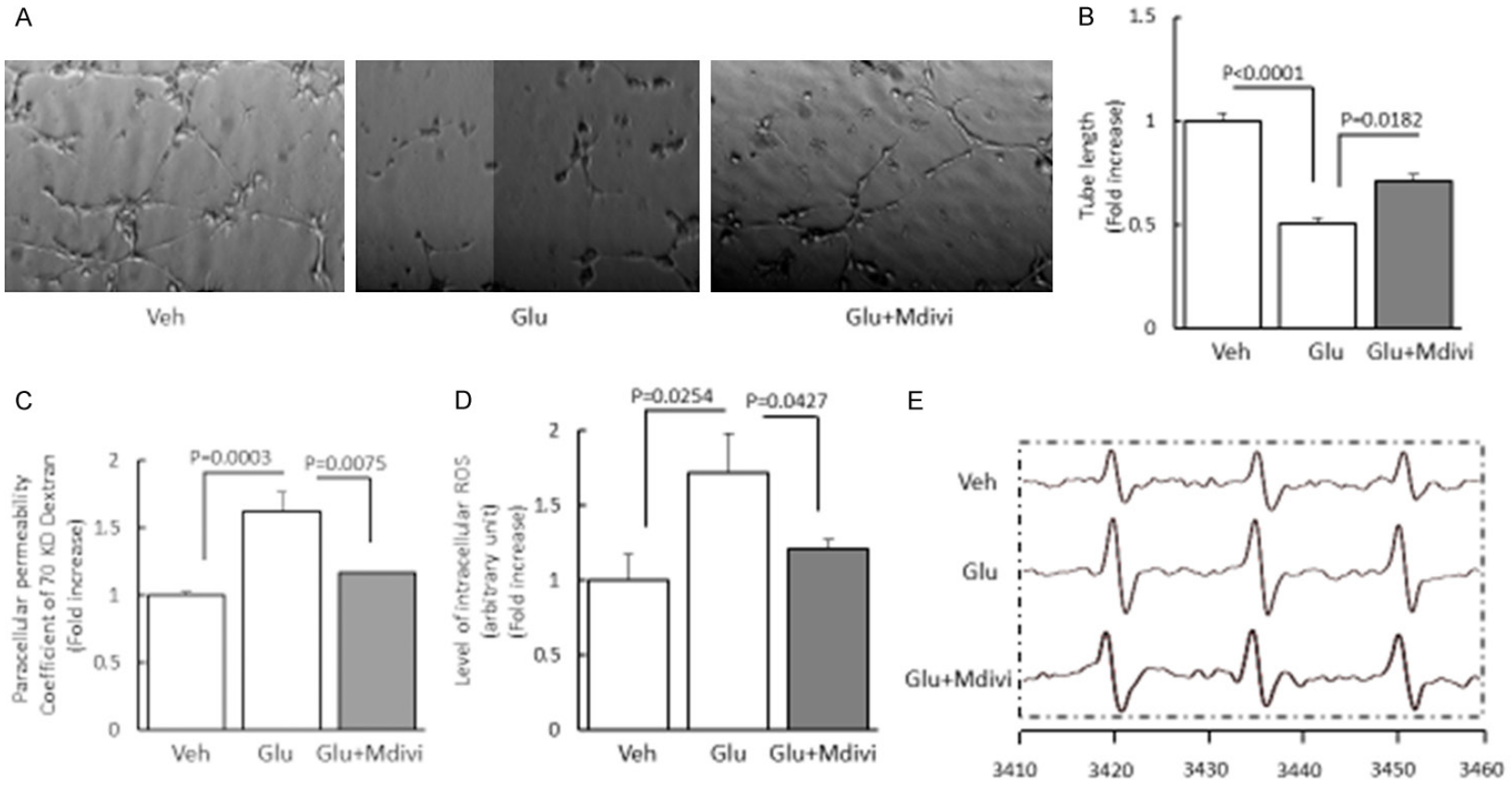


**Figure 2.** Effect of Drp1 inhibition on high glucose-induced ECs dysfunction. A. Tube formation of endothelial cells in Matrigel. After incubation, endothelial cells were fixed, and tubular structures were photographed (original magnification, × 100). B. Pooled data from three independent tube formation experiments, average tube length were measured. C. Paracellular permeability assay of endothelial cells. D. Levels of intracellular ROS were assessed using EPR values. The peak height in the spectrum represents the level of ROS. E. Representative EPR spectra. Data are expressed as fold increase relative to vehicle group. *P*-values < 0.05 versus high glucose-treated group.

how Drp1 modulates ECs dysfunction under hyperglycaemic condition has sparked our attention. Here, we determined the changes of protein levels of Drp1 in the glucose-treated group through western blot analysis. We found that Drp1 protein expression levels were significantly higher in high glucose-treated group (**Figure 1B**). These data indicate an increased expression of Drp1 in ECs treated with high glucose.

*Restorative effect of Drp1 inhibition on high glucose-induced ECs dysfunction*

To determine the contribution of Drp1 to ECs dysfunction and oxidative stress alteration resulting from high glucose stimulation, we validated whether blockade of Drp1 by using Mdivi can protect ECs against the impairment. Angiogenesis is a pretty complex process and tubulogenesis of ECs is the fundamental step for angiogenesis [2, 4]. Here, we used a two-dimensional Matrigel assay to examine the tubulogenetic ability of ECs *in vitro*. When seeded on Matrigel, ECs become elongated and form tube structures, mimicking the process of angiogenesis *in vivo* [2]. As shown in **Figure 2A**, 6 h after seeding, ECs organized into clusters and began forming projections to neighboring ECs, exhibiting a clear capillary-like network formation in the vehicle group. However, high glucose dramatically suppressed the formation of the capillary-like network. ECs treated with high glucose exhibited fewer projections and the arrangement into pre-tube clusters was not as distinct as in the vehicle group. When ECs were pretreated with Mdivi in the presence of glucose, the basic outline of the tube networks became more apparent as compared with the high glucose group. Average length was determined for each well, as shown in **Figure 2B**. A significant decrease in the average tube length ($P < 0.0001$) was observed in the high glucose-treated ECs, whereas pre-treatment of Mdivi could obviously increased the average tube length as compared with the ECs treated with high glucose alone. These data indicate that high glucose suppressed the angiogenetic ability of ECs, which could be altered by Drp1 pharmacological inhibition.

Next, we examined the paracellular permeability of ECs to dextran 40 kDa as described previously [14]. As shown in **Figure 2C**, high glucose caused an increase in endothelial paracellular permeability. And cells pre-treated with Mdivi under high glucose environment showed reversed paracellular permeability. These results presented above validate the involvement of Drp1 in high glucose-induced endothelial paracellular permeability.

Intracellular ROS level is a key factor affecting ECs function, and its excess accumulation can lead to oxidative damage and subsequent angiogenesis disorders [4, 18]. Therefore, it is of particular interest to investigate whether ROS generation is also altered when ECs were treated with high glucose. Here, we employed a highly specific EPR assay to quantitatively measure the intracellular ROS levels. We found an enhanced intracellular ROS production/accumulation in high glucose treated cells, and that Mdivi dramatically decreased ROS levels induced by high glucose (**Figure 2D**, **2E**). These data above validate the proposal that blockade of Drp1 could effectively protect ECs against high glucose damage, including restored vigorous angiogenesis potential, renovated paracellular permeability, and alleviated ROS accumulation.

*Restorative effect of Drp1 inhibition on high glucose-induced ECs mitochondrial dysfunction*

We next evaluated the effect of Drp1 inhibition on mitochondrial dysfunction by assessing mitochondrial membrane potential ($\Delta m\Psi m$), CcO activity, and mitochondrial ROS level. ECs were co-stained with TMRM and MT Green to monitor mitochondrial membrane potential. The intensity of TMRM staining was significantly decreased in high glucose-treated ECs by 50-60% compared to the vehicle group, whereas Mdivi treatment obviously restored the mitochondrial membrane potential (**Figure 3A**, **3B**). In parallel, the presence of high glucose significantly decreased the CcO activity as compared to the vehicle group, and consistently, and pre-treatment of Mdivi could largely reverse the CcO activity (**Figure 3C**). These results validate abnormalities found in high glucose-treated mitochondria.

Because ROS are generated as a by-product of electron transfer through various respiratory chain complexes and that ROS accumulation affects mitochondrial function, we examined whether abnormal mitochondrial ROS occurs in

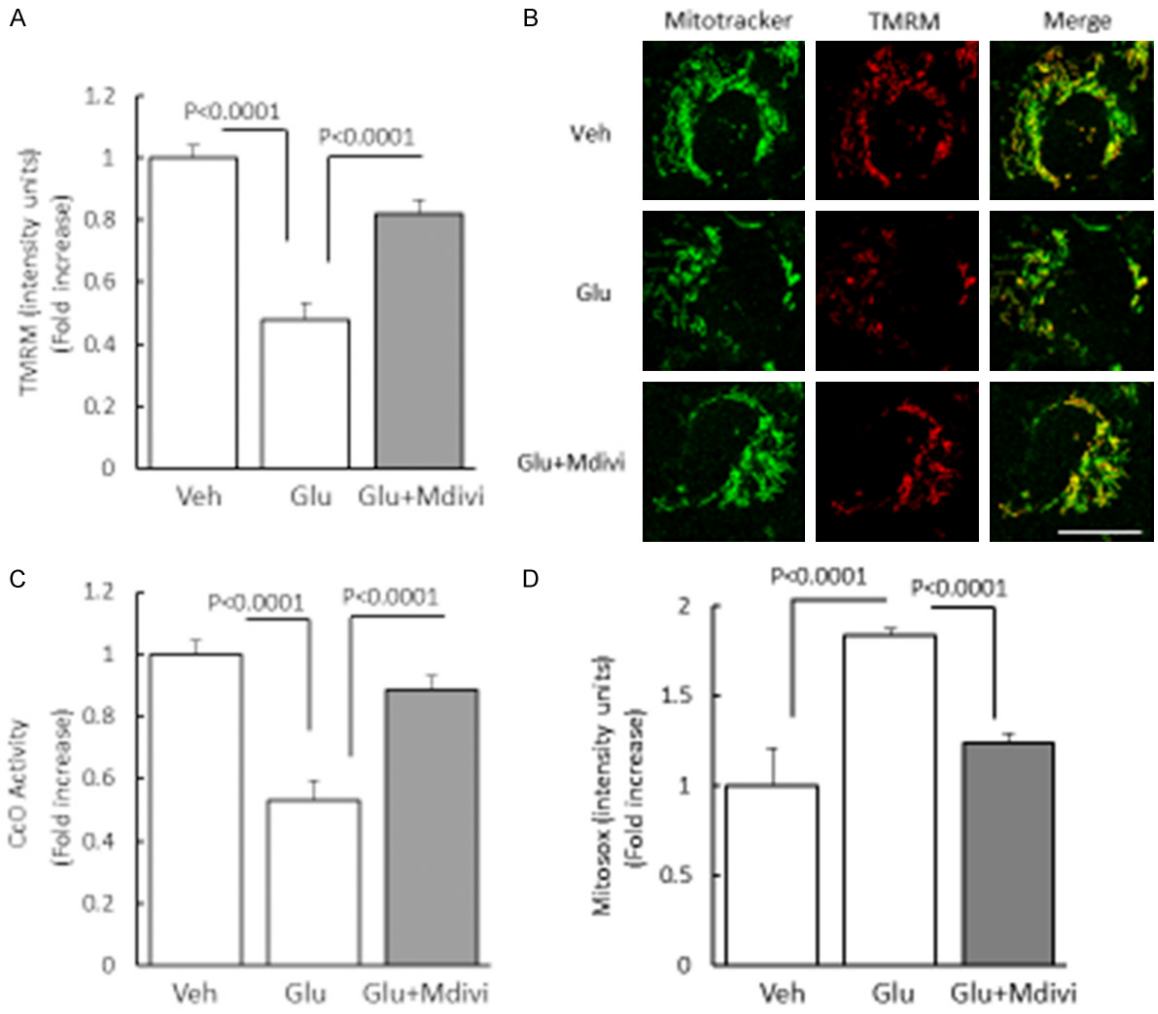


**Figure 3.** Effect of Drp1 inhibition on high glucose-induced ECs mitochondrial dysfunction. A. Level of mitochondrial membrane potential was assessed by TMRM staining. B. Representative images of TMRM staining. C. CcO activity was determined in cell lysate. D. Level of mitochondrial ROS were assessed by MitosoxRed staining intensity. Image intensity was quantified using NIH Image J software. Data are expressed as fold increase relative to vehicle group. Scale bar = 5 μm. *P*-values < 0.05 versus high glucose-treated group.

high glucose-treated ECs by using Mitosox Red staining. We observed that the intensity of Mitosox staining was significantly increased in ECs treated with high glucose (**Figure 3D**), suggesting that treatment induced high levels of ROS within the mitochondria of ECs, and the addition of Mdivi significantly suppressed the mitochondrial ROS production/accumulation induced by high glucose.

*Positive effect of antioxidant treatment and p38 inhibitor on mitochondrial function and EC function under high glucose treatment*

In light of our finding of increased ROS production/accumulation in high glucose-treated ECs, and the noticeable contribution of oxidative stress to mitochondrial dysfunction reported by previous studies [3, 18], we aimed to determine whether antioxidant NAC treatment could rescue mitochondrial and cellular dysfunction resulting from high glucose stimulation. We found that NAC treatment significantly enhanced capillary tube formation and average tube length compared to high glucose treatment alone (**Figure 4A**). Additionally, increased paracellular permeability induced by high glucose was alsosuppressed by NAC treatment (**Figure 4B**). Next, we evaluated the effect of NAC on mitochondrial dysfunction in high glucose-induced ECs. We found that deficits in mitochondrial membrane potential and CcO activity were

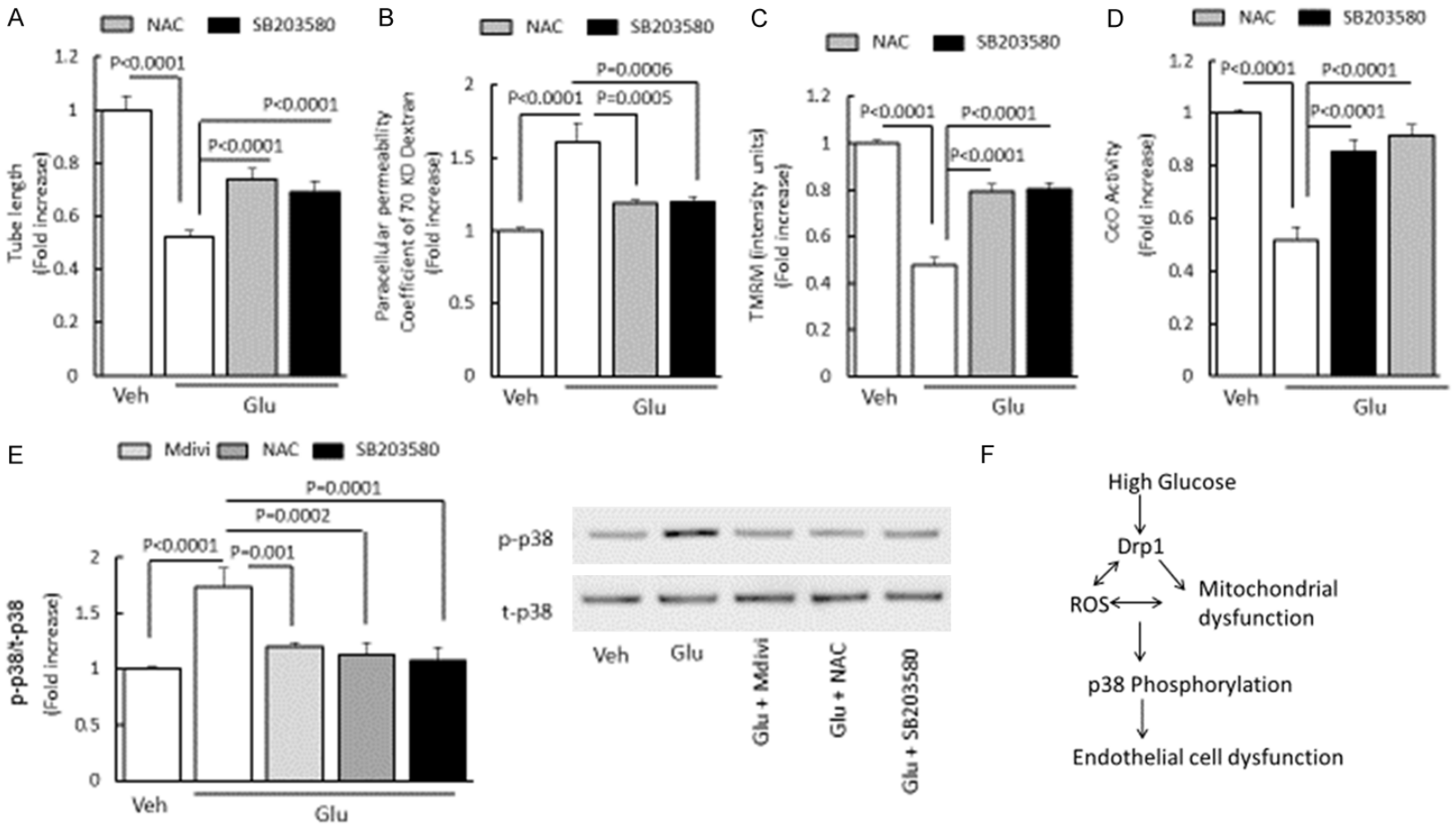


**Figure 4.** Effect of antioxidant treatment and p38 inhibitor on mitochondrial function and EC function under high glucose treatment. A. Average tube length assay. B. Paracellular permeability assay. C. Level of mitochondrial membrane potential was assessed by TMRM staining. D. CcO activity was determined in cell lysate. E. Quantification of immunoreactive bands of p-p38 normalized to t-p38 by western blot analysis. Representative immunoblots were shown in lower panels. Image intensity was quantified using NIH Image J software. Data are expressed as fold increase relative to vehicle group. *P*-values < 0.05 versus high glucose-treated group. F. Working hypothesis: excessive Drp1 expression and ROS-induced oxidative stress resulting from high glucose exposure lead to the activation of the p38 signal pathway, which subsequently accounts for endothelial dysfunction.

significantly decreased in the NAC-treated group compared to the high glucose-treated group (**Figure 4C**, **4D**).

The p38 signal pathway can be activated in response to hyperglycemia and is involved in cardiovascular complications associated with diabetes. To explore the potential role of the p38 signal pathway in this study, we detected alterations of p38 phosphorylation through western blot analysis. Compared to vehicle-treated group, high glucose treated group exhibited significantly increased p38 phosphorylation (p-p38), whereas total-p38 (t-p38) was not significantly changed. However, as compared to glucose treatment alone, NAC treatment significantly decreased p38 phosphorylation as well as Mdivi treatment (**Figure 4E**). These data suggested that the p38 signal pathway may be linked to high glucose-induced ECs dysfunction, and this impairment can be rescued by NAC or Mdivi treatment.

To further validate the potential role of the p38 signal pathway in high glucose-induced damage, a specific p38 phosphorylation inhibitor (SB203580) was adopted in this study. We found that SB203580 treatment significantly prompted tube formation and increased average tube length as compared to high glucose treatment alone (**Figure 4A**). In parallel, increased paracellular permeability in the high glucose-treated group was also significantly inhibited by SB203580 (**Figure 4B**). Furthermore, the deficits in the membrane potential (**Figure 4C**) and CcOactivity (**Figure 4D**) induced by high glucose treatment, were also reversed following treatment with SB203580. These data demonstrated that the inhibition of p38 activation effectively rescues high glucose-induced EC and mitochondrial dysfunction.

## Discussion

Although high blood glucose is the most dominant feature of diabetes and mitochondrial function are closely linked to high glucose conditions [19, 20], there is little information on whether and how mitochondrial dysfunction participates in high glucose-induced ECs dysfunction. In this study, protein level of Drp1in the high glucose-treated group were significantly increased compared to the vehicle group, suggesting its crucial role in high glucose-induced ECs dysfunction. However, blockade of Drp1 by pharmaceutical inhibitor Mdivi can attenuate the cell damage. The basic outline of the tube networks in the Mdivi-treated group became more apparent as compared to the high glucose group. Moreover, significantly increased tube length and decreased paracellular permeability were apparent after treatment with Mdivi. Indeed, obvious increased Drp1 levels in high glucose-treated ECs suggest that enhanced Drp1 expression might correlate with attenuated ECs function in the present study.

Imbalance of mitochondrial fission and fusion plays can lead to mitochondrial dysfunction, such asdecreased mitochondrial membrane potential, alleviated ATP generation, impaired mitochondrial respiratory function, and increased ROS generation/accumulation [21]. Therefore, we evaluated the alterations due to oxidative stress in high glucose-treated cells. Similar to previous findings [22], a significant increase in intracellular ROS generation as shown by an enhanced EPR spectrum occurred in the high glucose-treated group compared to the vehicle group. After high glucose treatment, we also found the obvious elevated mitochondrial ROS production evidenced by increased intensity of Mitosoxstaining, indicating an elevation of intracellular ROS generation/accumulation in high glucose-treated ECs. Conversely, treatment with high glucoselargely decreased mitochondrial membrane potential and CcO activity compared to the vehicle group. As expected, blockade of Drp1 by pharmaceutical inhibitor Mdivi can significantly attenuate intracellular ROS generation and mitochondrial dysfunctions. Taken together, we confirmed that blockade of Drp1 could attenuate the perturbed mitochondrial function and impaired ECs function in the high glucose-treated group.

ROS-induced oxidative stress plays a key role in the pathogenesis of various diseases including inflammation, osteoporosis, Alzheimer's diseaseanddiabetes [23, 24], and antioxidant treatment has been reported to protect against oxidative damage in these diseases. Similar to previous studies, NAC-treated ECs exhibited significantly increased tube length and reduced paracellular permeability compared to the high glucose-treated group. Furthermore, treatment with NAC largely restored the impaired mitochondrial membrane potential and CcO activity resulting from high glucose stimulation. In conjunction with the results of the Mdivi-treated

group, ROS-induced oxidative stress was appreciated as an indispensable factor of in Drp1 accounting for high glucose-induced ECs dysfunction.

Previous studies indicate that p38 MAPK acts as thedownstream of the bax-caspase-3 pathway, and it may play a key role in CVD [11]. Consist with this, we found that p38 phosphorylation was significantly elevated in high glucose-treated ECs, whereas Mdivi addition obviously inhibited p38 phosphorylation compared to high glucose treatment alone. These data indicate that Drp1 acts upstream of p38 in the present study. In parallel, p38 phosphorylation in the NAC-treated group was also largely decreased compared to high glucose-treated group, suggesting the emerging role of p38 in ROS-mediated ECs damage under high glucose condition. To further validate the role of p38 in this research, a specific p38 phosphorylation inhibitor (SB203580) was adopted. Indeed, p38 phosphorylation in the SB203580-treated group significantly fell to that of the vehicle group. Furthermore, we found that treatment with SB203580 obviously increased tube length and reduced paracelllular permeability compared to the high glucose group. Similar to the NAC group, mitochondrial membrane potential and CcO activity were also largely protected against high glucose damage. Acting downstream of Drp1, p38 was validated to participate in the high glucose-derived ECs dysfunction accompanied by ROS-induced oxidative stress.

Taken together, this research offers new insights into the mechanism of mitochondrial dysfunction in high glucose-induced ECs dysfunction, specifically the role of Drp1. Excessive Drp1 expression mediates perturbed mitochondrial function and subsequently inhibited angiogenesis in high glucose-treated ECs. Both blockade of Drp1 and antioxidant treatment could attenuate the mitochondrial-mediated endothelial dysfunction under high glucose condition. Furthermore, the p38 signal pathway was activated as the downstream of Drp1 in this process and p38 inhibition could protect ECs against high glucose damage. Hence, blockade of Drp1 may be a potential therapeutic strategy for preventing ECs dysfunction and CVD in diabetes.

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## Disclosure of conflict of interest

None.

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