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Astragaloside IV protects against hyperglycemia-induced vascular endothelial dysfunction by inhibiting oxidative stress and Calpain-1 activation

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ABSTRACT

Aims: Vascular endothelial cells act as a selective barrier between circulating blood and vessel wall and play an important role in the occurrence and development of cardiovascular diseases. Astragaloside IV (As-IV) has a protective effect on vascular endothelial cells, but its underlying mechanism remains unclear. This study is aimed at investigating the effect of As-IV on endothelial dysfunction (ED).

Methods: Male Sprague-Dawley (SD) were injected intraperitoneally with 65 mg/kg streptozotocin (STZ) to induce diabetes and then administered orally with As-IV (40, 80 mg/kg) for 8 weeks. Vascular function was evaluated by vascular reactivity *in vivo* and *in vitro*. The expression of calpain-1 and eNOS in the aorta of diabetic rats was examined by western blot. NO production was measured using nitrate reductase method. Oxidative stress was determined by measuring SOD, GSH-px and ROS.

Results: Our results showed that As-IV administration significantly improved diabetes associated ED *in vivo*, and both NAC (an antioxidant) and MDL-28170 (calpain-1 inhibitor) significantly attenuated hyperglycemia-induced ED *in vitro*. Meanwhile, pretreatment with the inhibitor l-NAME nearly abolished vasodilation to ACh in all groups of rats. Furthermore, As-IV increased NO production and the expression of eNOS in the thoracic aorta of diabetic rats. In addition, the levels of ROS were significantly increased, and the activity of SOD and GSH-px were decreased in diabetic rats, while As-IV administration reversed this change in a concentration-dependent manner.

Conclusion: These results suggest that As-IV improves endothelial dysfunction in thoracic aortas from diabetic rats by reducing oxidative stress and calpain-1.

1. Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia, which is a major global health problem with increasing prevalence, especially in China [1]. According to the most recent data from International Diabetes Federation diabetes atlas estimates that the prevalence of diabetes in the age groups between 18 and 99 years worldwide will rise from 8.4% in 2017 to 9.9% in 2045 [2]. As a result, vascular complications including macroangiopathy and microangiopathy are expected to occur more frequently. There are many differences in the pathogenesis of diabetic macroangiopathy and microangiopathy, there are some common features, such as vascular endothelial dysfunction (VED) [3–5]. Therefore, preventing the development of VED is essential to delay the progression of vascular complications. Several downstream biochemical signaling pathways affected by hyperglycemia, such as oxidative stress [6] and calpain [7], are considered to be the key factors causing endothelial dysfunction [7–9]. Although many factors are involved in the development of endothelial dysfunction, oxidative damage caused by reactive oxygen species (ROS) is critical in this disease in various proposed mechanisms [10]. The excessive ROS generation also induces a dysfunctional endothelial nitric oxide synthase (eNOS) activity, which is responsible for producing nitric oxide (NO) thereby leads to impaired vasorelaxation [11,12].

Astragalus membranaceus, the main ingredient of most Chinese herbal anti-diabetic formulas [13], has been used in the treatment and prevention of cardiovascular disease in clinical practice, such as hemorrhagic stroke [14], asymptomatic left ventricular diastolic dysfunction [15] and viral myocarditis [16]. Astragaloside IV (As-IV), the main natural small molecule compound extracted and purified from

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Astragalus membranaceus, its pharmacokinetics in rats and humans of As-IV have been studied [17,18]. As-IV has a variety of cardiovascular pharmacological activities, such as anti-cardiac hypertrophy [19], anti-inflammatory [20] and anti-oxidation [21]. Our previous studies have proved that As-IV has protective effect on endothelial dysfunction of diabetic rats [22]. However, the mechanism of As-IV on endothelial dysfunction remains unclear. In the present study, we further studied the protective mechanism of As-IV on vascular endothelial dysfunction through *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials and reagents

Astragaloside IV (HPLC ≥ 98.0%, Cat No. JZ16042403) were purchased from Nanjing Jingzhu Biotechnology (Nanjing, China). Superoxide Dismutase (SOD, Cat No. A001-1) assay kit was purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). GSH-px ELISA Kits (Cat No. ml097316) was purchased from Mlbio (Shanghai, China). Dihydroethidium (DHE, Cat No. S0063) was purchased from Beyotime Biotechnology (Nantong, China). Streptozotocin (STZ, Cat No. S0130), phenylephrine (PE, Cat No. P1240000) and acetylcholine (Ach, Cat No. A6625) were purchased from Sigma-Aldrich (Shanghai, China). *N*-acetyl-L-cysteine (NAC, Cat No. S1623) was purchased from Selleck (Houston, USA). MDL-28170 (Cat No. M7934) was purchased from Abmole Bioscience (Houston, USA). Calpain-1 (Cat No. 10538) and β-actin (Cat No. 66009) were purchased from Proteintech (Wuhan, China). eNOS (Cat No. A1548) was purchased from Abclonal (Wuhan, China).

2.2. Animal models and drug treatments

Male Sprague Dawley rats weighing 280–300 g (Experimental Animal Center, Jinzhou Medical University, Jinzhou, China) were housed in cages with free access to food and water, and exposed to a 12 h light/dark cycle at a controlled temperature (25 ± 2 °C). Briefly, after 7 days of adaptive feeding, diabetes mellitus was induced by intraperitoneal injection of STZ at a dose of 65 mg/kg, whereas non-DM rats (control) received only citrate buffer. Three days after the STZ injection, rats were considered diabetic when blood glucose levels were ≥ 16.7 mmol/L for two or more consecutive tests. After the successful establishment of diabetic rats, treatment of diabetic rats with As-IV (40, 80 mg/kg) for 8 weeks. After 8 weeks of treatment, rats were anesthetized with 20% urethane, and then blood samples were collected from the apex of the left ventricle, and thoracic aortas were obtained for subsequent experiments.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured and maintained in 10% FBS-DMEM supplemented with 1% penicillin/streptomycin at 37 °C in a CO2 incubator. HUVECs were grown in low (Con, 5.5 mM glucose + 27.5 mM mannitol) conditions in the presence or absence of As-IV (100 μM), NAC (a ROS scavenger, 2 mM), MDL-28170 (calpain inhibitor, 20 μM) for 48 h. HUVECs were pretreated with As-IV, NAC, MDL-28170 for 2 h prior to treatment with high glucose (HG, 33 mM glucose).

2.4. Vascular reactivity study

Rats were anesthetized with 20% urethane (5 mL/kg, intraperitoneally), and the thorax was opened, then thoracic aortas were rapidly removed, and cut into approximately 2 mm rings in ice-cold physiological salt solution (PSS, pH 7.4) containing 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.16 mM $CaCl_2$, 14.9 mM $NaHCO_3$, 0.026 mM EDTA, and 11.1 mM glucose. The prepared aortic rings were then placed in PSS at a resting tension of 15 mN for 60 min before starting measurements. After an equilibration period of 60 min, ring were contracted with K-PSS (60 mM KCl in PSS solution; equimolar substitution of KCl for NaCl) to check their functional integrity. Rat aortic rings were precontracted with PE (10^{-6} M), and when a stable plateau was reached, increasing concentrations of ACh (10^{-9} to 10^{-5} M) were added. The vasorelaxation response to ACh was expressed as the percentage of maximal response to PE. To confirm As-IV treatment restored vascular endothelial dysfunction in diabetic rats *via* enhanced NO vasodilator production, the nitric oxide synthase inhibitor NG-nitro-l-arginine (L-NAME, 100 μM) was incubated 30 min before PE-induced contraction.

In order to explore the role of ROS generation and calpain-1 in endothelial dysfunction related to hyperglycemia, vascular reactivity was detected using an established *in vitro* experiment that have been described previously [23,24] with the following modifications. Briefly, the isolated thoracic aortas were removed and cleaned under sterile conditions from control rats. To mimic diabetes, aortic rings were placed in DMEM-F/12 supplemented with low glucose (5.5 mM glucose + 27.5 mM mannitol) or high glucose (33 mM glucose) with or without NAC (an antioxidant, 2 mM), MDL-28170 (calpain-1 inhibitor, 20 μM) for 24 h. Following incubation, aortic rings were used for vascular reactivity.

2.5. Determination of SOD and NO

The SOD activity and NO level were determined using the assay kits according to the manufacturer's protocols. NO is converted to nitrite plus nitrate (NO^{3-} + NO^{2-}), and NO^{3-} plus NO^{2-} could be used to evaluate NO level.

2.6. Western blot

Sample proteins were extracted in lysis buffer (RIPA + 1% PMSF) and total protein concentration was quantified by the BCA protein assay kit. 40 μg of each sample was separated on 8–12% SDS-PAGE gel and subsequently transferred to a PVDF membrane for 21 min at 15 V. Membranes were blocked with 1% BSA in TBST for 90 min at room temperature, and then incubated with calpain-1 (1:1000), eNOS (1:1000) and β-actin (1:5000) overnight at 4 °C. Membranes were washed 3 times with TBST and then incubated with HRP conjugated secondary antibody for 2 h at room temperature. Densitometric analysis of band was analyzed with the ImageJ software and normalized to β-actin.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations of GSH-px in aortas and supernatant were analyzed by ELISA according to the manufacturer's instructions.

2.8. Measurement of ROS production

The production of ROS in aorta was detected by Dihydroethidium (DHE) as described previously [25] with small modifications. Briefly, OCT embedded frozen sections (5 μm thick) of aorta tissues were incubated with 5 μM of DHE for 30 min at 37 °C in the dark. After incubation, the sections were incubated in DAPI for 10 min. After that, sections were coversliped and fluorescent images were viewed under a fluorescence microscope (Leica DMI3000B, Germany) and the relative fluorescence intensity measured by image J software.

2.9. Statistical analysis

All statistical analyses were analyzed using SPSS version 23.0 statistical software (IBM, USA). Data were presented as means and standard deviation (SD). One-way analysis of variance (ANOVA) was used for statistical analysis of the data. Differences were accepted as a

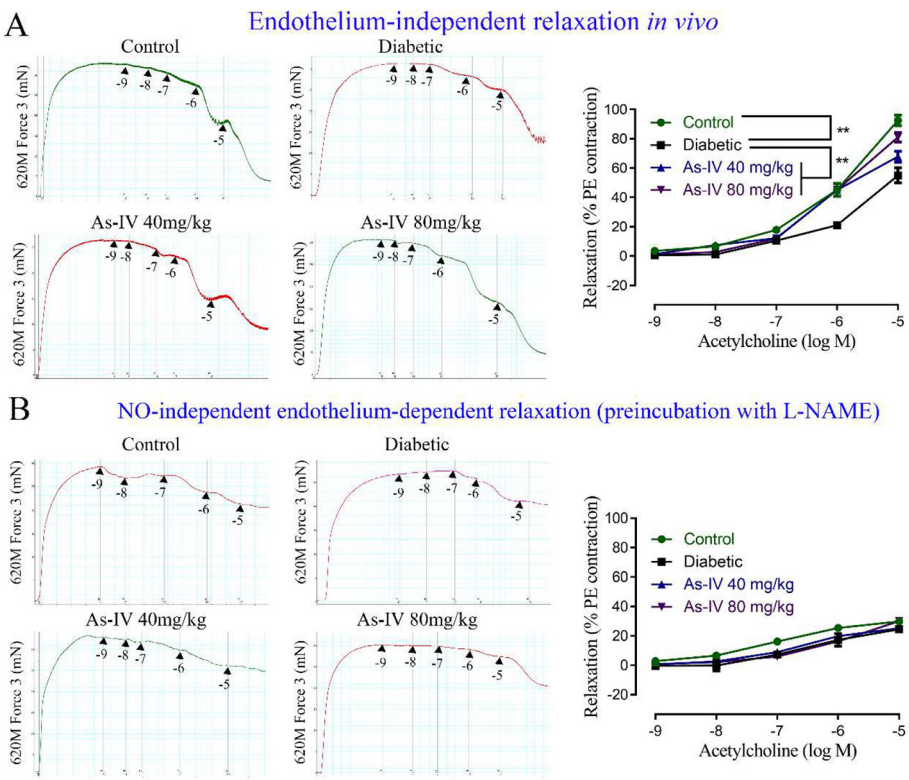

Fig. 1. As-IV ameliorates the impairment of endothelium-dependent relaxation in isolated aortic rings of diabetic rats *in vivo*. Rings from control, diabetic or As-IV-treated diabetic were incubated without (A) and with (B) L-NAME (an eNOS inhibitor, 100 μM) for 30 min, Rings were then evoked by acetylcholine(ACh, 10^{-9} to 10^{-5} M) after precontraction phenylephrine (PE, 10^{-6} M). Data are means ± SD, n = 4. **P < 0.01.

criterion of significance if the P-values < 0.05.

3. Results

3.1. Effect of As-IV on endothelium-dependent relaxation (EDR) in thoracic aorta in vivo

Vascular reactivity was evaluated in the isolated thoracic aortic rings obtained from control and 8 weeks diabetic rats treated with As-IV *in vivo*. EDR in response to ACh was significantly impaired in diabetic rats compared to the control rats, and As-IV dose-dependently reversed the impaired ACh-mediated EDR in isolated aortic rings of diabetic rats (Fig. 1A). Meanwhile, the ACh-induced relaxation was abolished by L-NAME in all groups (Fig. 1B).

3.2. Effect of NAC and MDL-28170 on endothelium-dependent relaxation in thoracic aorta in vitro

To investigate the effect of oxidative stress and calpain-1 on vascular dysfunction in diabetic rats, rings from control rats were preincubated with high glucose (HG) in the presence or absence of the antioxidant NAC and calpain-1 inhibitor MDL-28170 for 24 h *in vitro*, and then rings were incubated with L-NAME for 30 min before precontracted with PE and dilated with Ach. *In vitro* studies showed that the EDR was significantly impaired in HG group after high glucose treatment. Importantly, preincubation with NAC and MDL-28170, restored relaxation to ACh in high glucose-treated aortic rings (Fig. 2A). Moreover, after rings incubated with L-NAME, the ACh-induced vasodilatory response was almost completely eliminated in all groups (Fig. 2B).

3.3. Effect of As-IV administration on calpain-1 in STZ-induced diabetic rats

To test the effect of As-IV on calpain-1, protein levels of calpain-1 was assessed by western blot. Compared with the control group, the expression of calpain-1 was increased in STZ-induced diabetic rats, while protein expression of calpain-1 was markedly reduced in aortas from As-IV-treated diabetic rats (Fig. 3).

3.4. Effect of As-IV administration on oxidative stress biomarkers in STZ-induced diabetic rats

In order to elucidate the impact of oxidative stress in the progression of vascular endothelial dysfunction and the effect of As-IV on oxidative stress in STZ-induced diabetic rats, the levels of SOD, GSH-Px and ROS were detected. Compared with the control group, the levels of the SOD and GSH-px activity (Fig. 4A and B) were inhibited, and ROS (Fig. 4C) was significantly increased in diabetic group. In contrast, after diabetic rats were treated with As-IV (40 or 80 mg/kg) for 8 weeks, the activity of SOD and GSH-px were increased, and the levels of ROS was significantly decreased.

3.5. Effect of As-IV administration on eNOS/NO in STZ-induced diabetic rats

To further investigate the underlying mechanism of As-IV on vascular endothelial dysfunction, we evaluated the expression of eNOS and NO in aortas. Western blot demonstrated that the levels of eNOS were markedly lower in aorta from diabetic rats compared with controls, and the expression of eNOS was reversed after treatment with As-IV (Fig. 5A and B). Meanwhile, to determine the effect of As-IV on the bioavailability of NO, NO production was assessed by nitrate reductase method. The production of NO in diabetic group was greater than the As-IV, or no-treatment aortic sections (Fig. 5C).

3.6. Effects of As-IV administration on calpain-1 and oxidative stress biomarkers in HUVECs

In order to investigate the key role of calpain-1 and oxidative stress, HUVECs were cultured with As-IV (100 μM), NAC (2 mM), MDL-28170 (20 μM) 2 h prior to addition of high glucose (33 mM glucose) for 48 h.

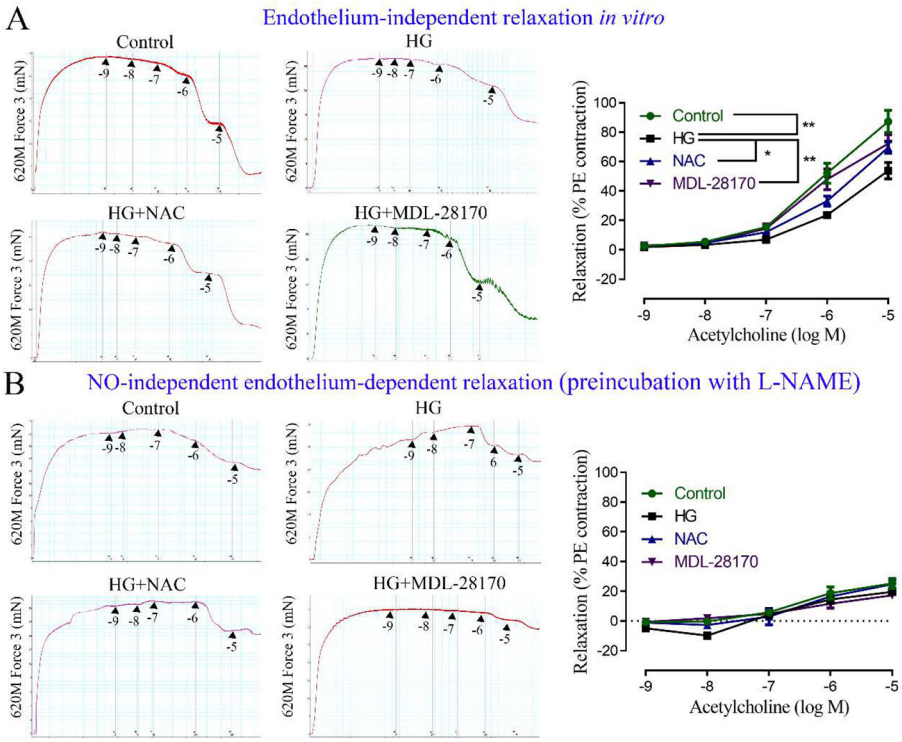

Fig. 2. NAC and MDL-28170 attenuates high glucose (HG)-induced impairment of endothelium-dependent relaxation in rat thoracic aorta mainly through NO. The rings were preincubated with 33 mM HG in the absence or presence of NAC (an antioxidant) and MDL-28170 (calpain-1 inhibitor) for 24 h (A), and after incubation with L-NAME (an eNOS inhibitor, 100 μM; B) for 30 min, followed by precontraction with phenylephrine (PE, 10^{-6} M) and cumulative administration of acetylcholine (ACh, 10^{-9} to 10^{-5} M). Data are means ± SD, n = 4. *P < 0.05; **P < 0.01.

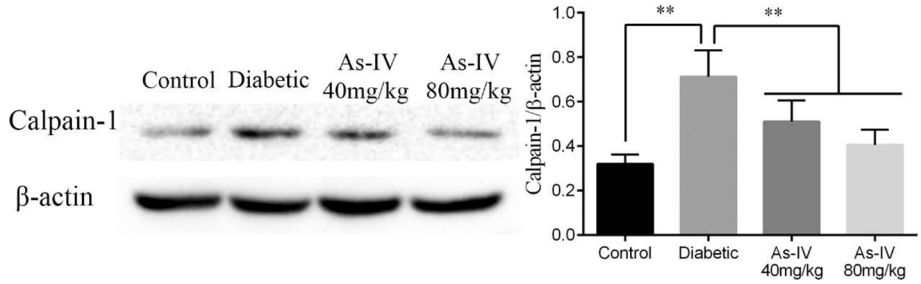

Fig. 3. As-IV reduces expression of calpain-1 in aorta from diabetic rats. After diabetic rats were treated with As-IV (40 or 80 mg/kg) for 8 weeks, the expression of calpain-1 was determined by western blot analysis. Data are means ± SD, n = 3. **P < 0.01.

The results showed that As-IV inhibited calpain-1 expression, reduced ROS generation and increased the activity of SOD and GSH-Px. Meanwhile, the above mentioned effects of As-IV on HUVECs were similar to that of NAC and MDL-28170 (Fig. 6).

3.7. Effects of As-IV administration on eNOS and NO in HUVECs

In order to study the mechanism of As-IV, As-IV (100 μM), NAC (2 mM) and MDL-28170 (20 μM) were added 2 h prior to the addition of high glucose (33 mM glucose) for 48 h. As shown in Fig. 7, high glucose treatment decreased the expression of eNOS and the production of NO, and these changed was reversed in the presence of As-IV, NAC, MDL-28170.

4. Discussion

The endothelium is the innermost layer of the inner vascular system and the same basic structure of all blood vessels [26]. The vascular endothelium is a metabolically active organ that maintains vascular homeostasis through a variety of complex physiological functions, and thus to prevent the occurrence of vascular disease. Endothelial dysfunction can be defined as the impaired synthesis, release, and/or activity of endothelium-derived NO or the reduced vascular vasorelaxation in response to ACh [27,28]. Endothelial dysfunction is not only an early marker of vascular disease, but also a bridge between cardiovascular disease and diabetes [29,30]. Extensive research suggests that chronic hyperglycemia is prone to cause endothelial dysfunction, and there is a common change, that is, the bioavailability of NO is decreased [31]. Endothelial NO synthase (eNOS) is a constitutively expressed enzyme responsible for the physiological production of NO in the vasculature, and is expressed mainly in the endothelium of large arteries [32]. In the vascular endothelium cells, NO is synthesized by eNOS from its precursor L-arginine in the presence of several cofactors such as tetrahydrobiopterin (BH4), NADPH, FAD and FMN [33]. Once formed, NO passes the endothelial cell membrane, and then diffuses into adjacent smooth muscle cells and promoting vascular relaxation and decreased vascular tone. Our data from STZ-induced diabetic rats demonstrated that after 8 weeks of STZ treatment, EDR in response to ACh was impaired, and treatment of diabetic rats with As-IV significantly improved the endothelium-dependent relaxation which is consistent with previous research [22]. Meanwhile, the production of NO and the expression of eNOS were decreased in diabetic rats, and treatment with As-IV increased the release levels of NO and expression of eNOS. In addition, we also observed that As-IV, NAC and MDL-28170 could increase the levels of eNOS and NO in high glucose-treated HUVECs. To determine the protective effect of As-IV on endothelial

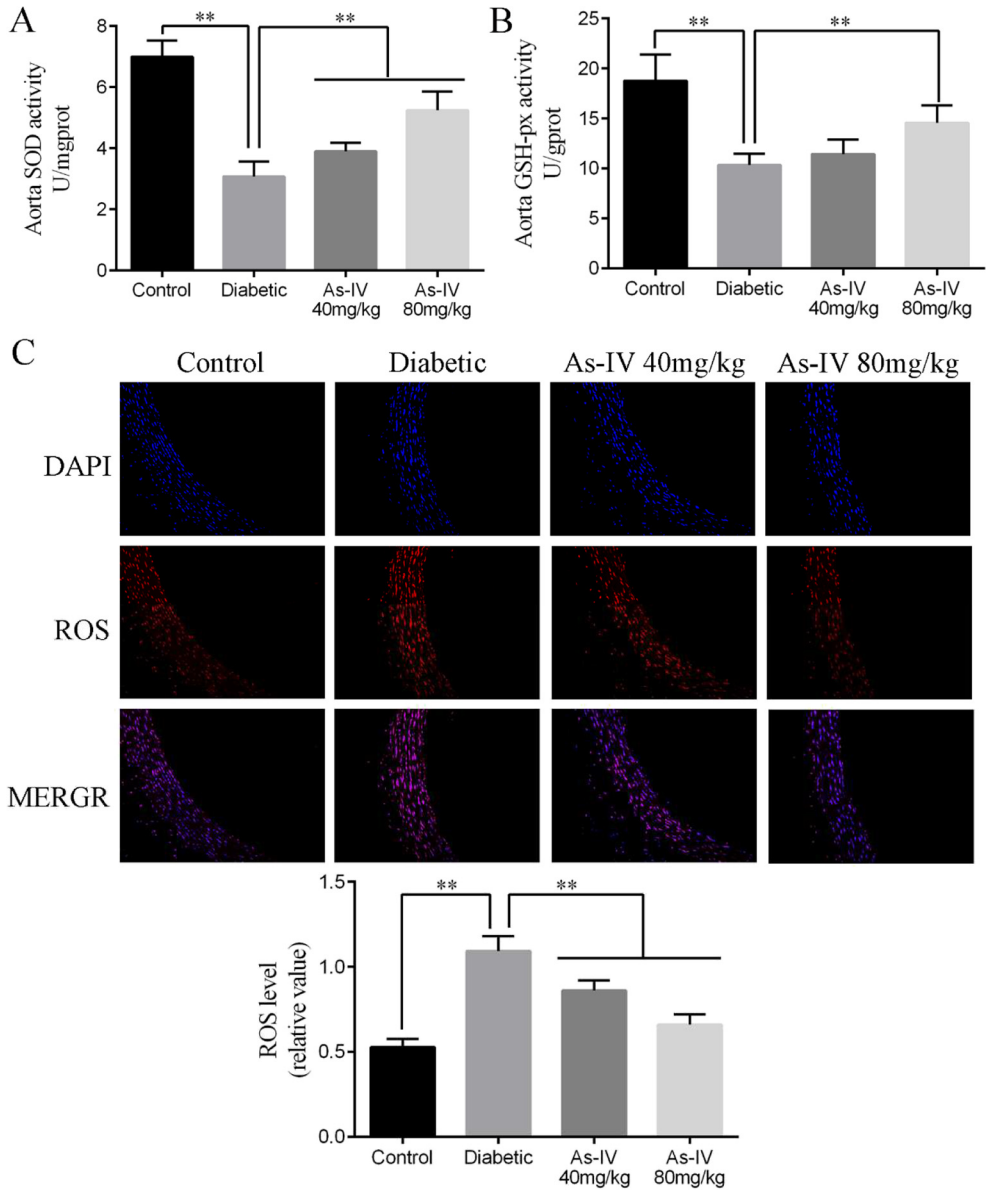

Fig. 4. As-IV treated STZ-induced diabetic rats by resisting oxidative stress. The levels of SOD (A) and GSH-px (B) in aortas were detected with an SOD assay kit and a GSH-px ELISA kit, and the generation of ROS (C) in aortas was evaluated by DHE staining. Data are means ± SD, n = 3. **P < 0.01.

dysfunction in diabetic rats, isolated aortas from all groups were treated with the nitric oxide synthase inhibitor L-NAME *in vivo*. We found that administration of L-NAME almost completely abolished vasodilations either in diabetic group or As-IV group. These results indicate that As-IV improves vascular endothelial dysfunction through NO pathway.

Oxidative stress reflects an imbalance between the production of oxidants and antioxidants in favor of the oxidants [34], with potential damage such as endothelial dysfunction [35]. Experimental evidence suggests that diabetes-mediated ROS production and accumulation appears to be the major driver of endothelial dysfunction [36]. In the process of body defense, cells have several antioxidant defense mechanisms against ROS, including enzymes (SOD and GSH-px) and nonenzymatic antioxidants [37,38]. In the process of oxidative stress these defense mechanisms are not sufficient to resist the exaggerated generation of ROS, NO reacts with Superoxide to form peroxynitrite ($ONOO^-$), leading to reduced bioavailability of NO, and thus leads to endothelial dysfunction [39]. The calpains belong to a family of calcium-dependent proteases that involved in various biological processes including endothelial dysfunction [40]. The calpain-1 (or μ-calpain) and calpain-2 (or m-calpain) are major isoforms of the calpain system, are ubiquitously expressed, and found in the vascular endothelial cells [41]. Earlier studies suggest that calpain-1 was activated by diabetes or hyperglycemia in endothelial cells and inhibition of calpain-1 could improve diabetes or hyperglycemia-induced endothelial dysfunction [40]. Our data clearly indicate that As-IV significantly increased the levels of SOD and GSH-Px, while decreased the levels of ROS with increased dosage of As-IV in diabetic rats and high glucose treated HUVECs. Moreover, the elevated expression of calpain-1 was reversed by As-IV and NAC and MDL-28170. Based upon these observations, control rings were incubated with antioxidant NAC and calpain-1 inhibitor MDL-28170 in a hyperglycemic microenvironment to study the effects of oxidative stress and calpain-1 on vascular endothelial dysfunction. We found that suppression of oxidative stress and calpain-1 protects endothelial-dependent vasodilation after high glucose treatment. Moreover, EDR in response to ACh was almost completely abrogated by an eNOS inhibitor L-NAME in a high glucose microenvironment treated with NAC and MDL-28170, indicating that treatment with the antioxidant NAC and calpain-1 inhibitor MDL-28170 increased NO

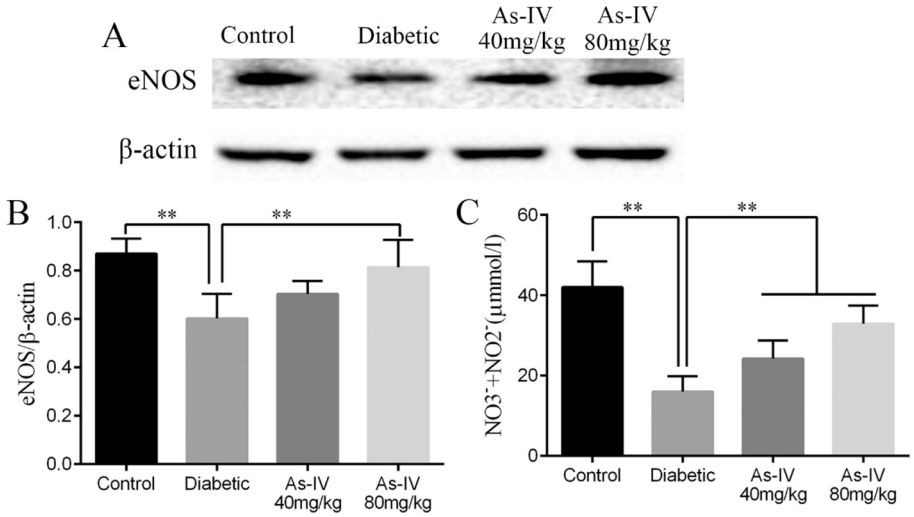

Fig. 5. As-IV increases eNOS and NO in aortas from diabetic rats. After diabetic rats were treated with As-IV (40 or 80 mg/kg) for 8 weeks, western bolting was used for the detection of eNOS (A and B), and NO production was assessed by nitrate reductase method (C). Data are means ± SD, n = 3. **P < 0.01.

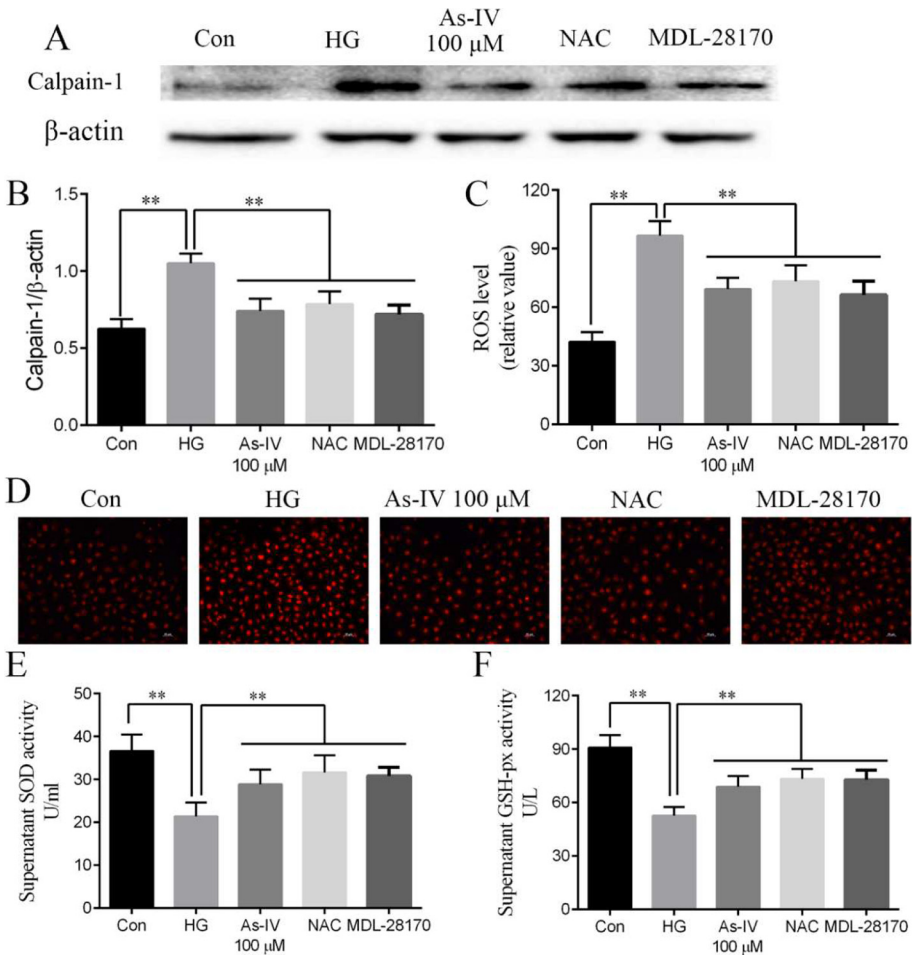

Fig. 6. As-IV administration inhibited calpain-1 expression and oxidative stress in HUVECs. After HUVECs were cultured with As-IV (100 μM), NAC (a ROS scavenger, 2 mM), MDL-28170 (calpain inhibitor, 20 μM) under high glucose (HG, 33 mM glucose) conditions, the expression of calpain-1 was detected by western blot, and the intracellular ROS level was measured by DHE staining, and the levels of SOD and GSH-px in HUVECs were assessed by an SOD assay kit and a GSH-px ELISA kit. Data are means ± SD, n = 3. **P < 0.01.

bioavailability. Thus, NAC and MDL-28170 therapy has been shown to improve vascular endothelial dysfunction by increasing production of NO.

In conclusion, the present study, demonstrated that As-IV improves vascular endothelial dysfunction by reducing oxidative stress, down-regulating calpain-1 and improving eNOS/NO signaling. These findings suggest that As-IV become a potential therapeutic approach for diabetes-associated endothelial dysfunction (Fig. 8).

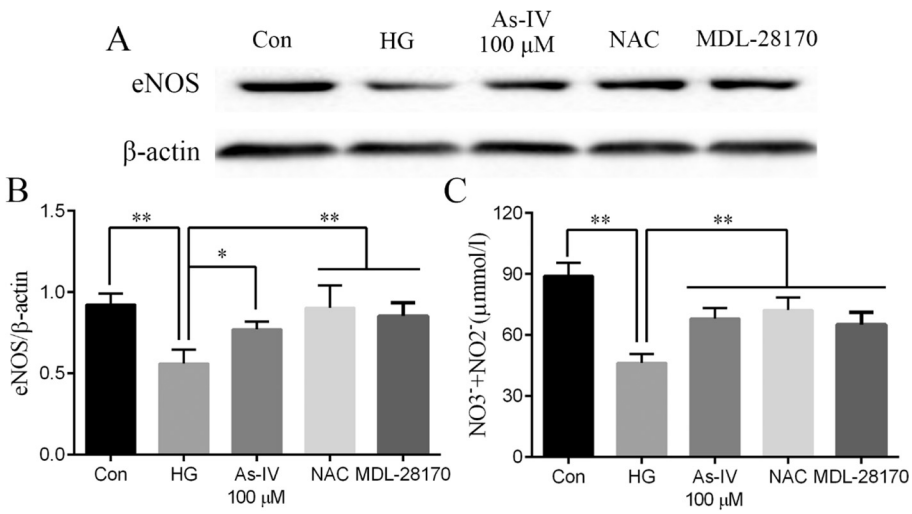

Fig. 7. As-IV administration increased eNOS expression and NO bioavailability in HUVECs. After HUVECs were cultured with As-IV (100 μM), NAC (a ROS scavenger, 2 mM), MDL-28170 (calpain inhibitor, 20 μM) under high glucose (HG, 33 mM glucose) conditions, the expression of eNOS was determined by western blotting and NO production was determined by nitrate reductase method. Data are means ± SD, n = 3. *P < 0.05; **P < 0.01.

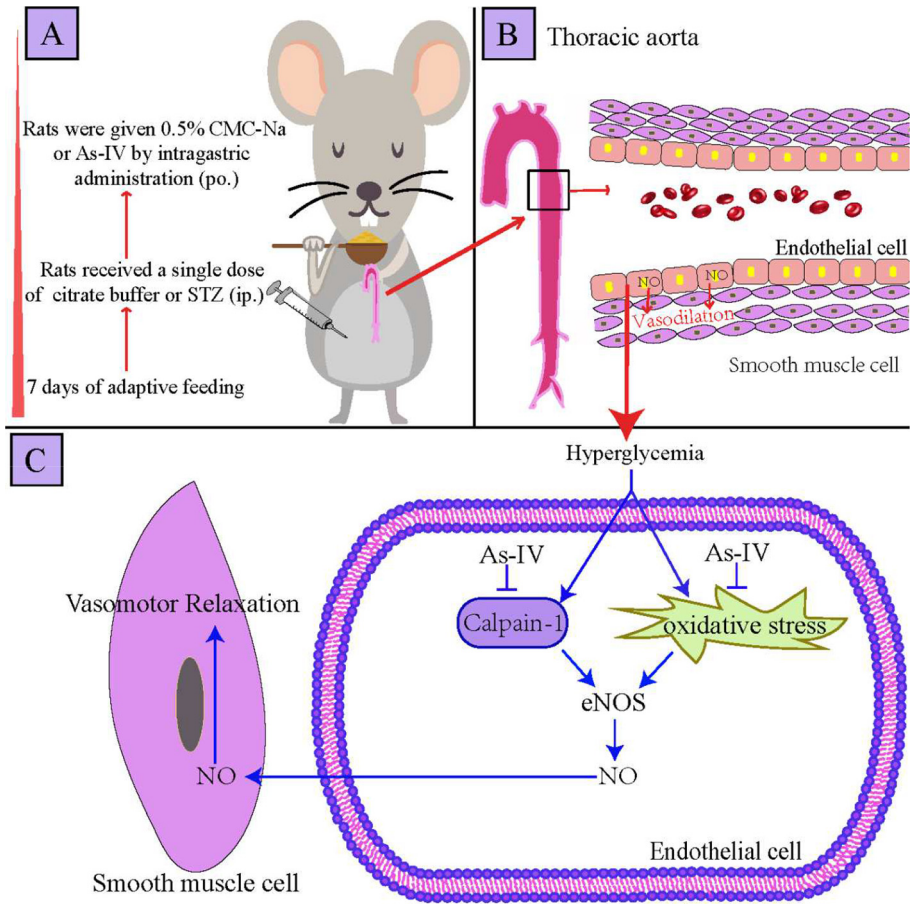

Fig. 8. Possible mechanism of protective effect of As-IV on endothelial dysfunction.

Declaration of Competing Interest

The authors report no declaration of interest.

Acknowledgments

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Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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