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# Mechanisms of protection against diabetes-induced impairment of endothelium-dependent vasorelaxation by Tanshinone IIA

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ABSTRACT

*Background:* Impairment of endothelium-dependent vasorelaxation has been suggested to play a principle role of endothelial dysfunction in the development of cardiovascular complications of diabetes. Recent studies have demonstrated a protective effect of Tanshinone IIA (Tan) on endothelial nitric oxide synthase (eNOS)–NO pathway. However, its role in endothelium-dependent vasorelaxation in diabetes and precise mechanisms remain elusive.

*Methods:* Sprague–Dawley rats were injected intraperitoneally with streptozotocin (STZ) to induce diabetes and then administered orally with Tan for 2 weeks. For the in vitro study, human umbilical vein endothelial cells (HUVECs) were co-incubated with Tan and high glucose for 48 h.

*Results:* eNOS expression and NO generation were significantly decreased in diabetic rats. These decreases were accompanied by an impairment of endothelium-dependent relaxation. Administration of Tan ameliorated the aberrant changes in eNOS expression, NO generation and endothelium-dependent relaxation in diabetic rats. Expectedly, Tan also inhibited high glucose-induced decrease of eNOS expression and NO generation in a concentration-dependent manner in HUVECs. Mechanistically, high glucose attenuated eNOS transcriptional activity through inhibiting the binding activity and nuclear translocation of Sp1 and AP-1. However, Tan did not prevent these effects. At post-transcriptional level, Tan increased eNOS expression and activity through multiple mechanisms including regulation of mRNA and protein half-life, degradation, coupling and serine 1177 phosphorylation. Rather than affecting protein phosphatase 2A (PP2A) expression and activity, Tan markedly inhibited the translocation of PP2A-A from cytosol to membrane and subsequently impaired PP2A-A/eNOS interaction, leading to prevent eNOS dephosphorylation. All these alterations underlie the protective role of Tan on eNOS expression following high glucose stimulation.

*Conclusions:* Our data demonstrate that high glucose decreases eNOS expression initiating at a transcriptional level, whereas Tan prevents such effect through multiple ways of post-transcriptional mechanism.

*General significance:* Our work provided novel mechanisms for Tan in regulating vasorelaxation and may help to better understand the cardiovascular protective action of Tan.



## 1. Introduction

Endothelial cell dysfunction underlies the development of various cardiovascular complications of diabetes [1]. Endothelial nitric oxide synthase (eNOS) is the key enzyme in maintaining vascular endothelium homeostasis. The activity of eNOS is regulated by multiple mechanisms that include transcriptional regulation, post-transcriptional regulation of mRNA stability, and post-translational regulation of protein stability by degradation, reversible coupling and reversible phosphorylation of serine and threonine residues [2,3]. At transcriptional level, although lacking with a typical TATA box, there are a number of transcriptional factors that bind to eNOS promoter, including activator protein-1 (AP-1), Sp1, NF-κB and shear stress-response elements [4,5]. On the other hand, mRNA stability is necessary to maintain eNOS normal expression. In a number of pathological conditions such as inflammation and hypoxia, eNOS mRNA stability is regulated by modulating the binding of some cytoplasmic proteins with eNOS mRNA 3′ untranslated regions (3′-UTR) [3,6]. At post-translational level, phosphorylation of eNOS serine 1179/1177 has been suggested as a central mechanism of

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eNOS regulation. Study revealed that serine 1179/1177 of eNOS was distinctively dephosphorylated by protein phosphatase 2A (PP2A), which is a highly conserved and ubiquitous serine/threonine phosphatase [7].

Tanshinone IIA (Tan) is a main active constituent extracted from the rhizome of the *Salvia miltiorrhiza* (Danshen), a traditional Chinese medicinal plant used for preventing and treating cardiovascular disorders [8]. It has functional roles of improving microcirculatory, vasodilatory, anti-thrombotic, anti-inflammatory, free radical scavenging, and mitochondria-protective effects [9,10]. Current research showed that Tan induced vasorelaxation and subsequently reduced blood pressure in hypertension rats, mainly through increasing eNOS expression [11]. On the other hand, it also has been reported that Tan increased eNOS phosphorylation and NO generation in HUVECs [12]. Moreover, Zhou et al. reported that Tan could reverse high glucose-induced eNOS uncoupling, leading to reduce intracellular oxidative stress and increase NO generation [13]. All these findings indicate that Tan plays an important role in maintaining eNOS/NO pathway homeostasis. However, the detailed mechanism by which Tan increases eNOS activity is not well understood, and whether Tan could improve endothelial relaxing dysfunction in diabetes remains enigmatic. In the present study, we investigated the effects of Tan on eNOS expression, NO generation and endothelium-dependent vasorelaxation in diabetic rats.

## 2. Material and methods

### 2.1. Materials and reagents

Medium 199 and fetal calf serum were purchased from Gibco (Carlsbad, USA). All other reagents utilized were purchased from Sigma Chemical Co. (St. Louis, USA) unless otherwise specified. Antibodies targeting eNOS, p-eNOS (serine 1177), p-eNOS (threonine 495), Sp1 and c-Jun were purchased from Cell Signaling Technology (Danvers, USA). Antibodies targeting eNOS (serine 615) were obtained from Abcam (Cambridge, USA). C2-ceramide, protein A/G agarose, antibodies targeting PP2A-Aα/β, PP2AB56-α, and PP2A-C, CD31, Hsp90, $Na^+/K^+$ ATPase, Lamin B and GAPDH were purchased from Santa Cruz Biotechnology (Dallas, USA). Tanshinone IIA (Tan, 98% purity assayed by HPLC) was purchased from the Chinese Institute for Drug and Biological Product Control (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L. Final concentration of DMSO in culture media was ≤0.1%.

### 2.2. Animals and induction of diabetes

The experimental protocols for rats were approved by the Institutional Animal Care and Use Committee at People's Liberation Army General Hospital, and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Institute of Health in China.

8-Week (230–250 g) male Sprague–Dawley (SD) rats were provided by Jackson Laboratories (Pennsylvania, USA). Rats were fasted for 16 h before the induction of diabetes with streptozotocin (STZ). A freshly prepared solution of STZ (65 mg/kg body weight) in 0.01 mol/L sodium citrate buffer (pH 4.0) was injected intraperitoneally (i.p.) [14], and the controls were injected with an equivalent amount of sodium citrate buffer alone. The hyperglycemia was confirmed (1 week later) by measuring blood levels using a OneTouch Test Strips. Only those rats with blood glucose concentrations ≥16.7 mmol/L after STZ injection were selected as diabetic rats. Rats were randomly divided into 4 groups, with 8 in each group, as follows:

1. Control group: rats were non-diabetic rats with equal amount of sodium citrate buffer, received the same volume of DMSO.
2. Control + Tan group: rats were non-diabetic rats with equal amount of sodium citrate buffer, received Tan (0.5 mg/kg) via oral gavage once daily for 2 weeks.
3. Diabetes: rats were injected with STZ as described above, received the same volume of DMSO.
4. Diabetes + Tan: rats were injected with STZ as described above, received Tan (0.5 mg/kg) via oral gavage once daily for 2 weeks.

The concentration of Tan was selected on the basis of previous publications [15,16], and has been proved to be safe for SD rats. After 1 week following a period of Tan treatment (2 weeks), blood samples were collected to determine the level of blood glucose, blood pressure, triglyceride and total cholesterol through biochemical means or radioimmunoassay. The thoracic aortas were harvested for quantitative real-time PCR, western blot or immunofluorescent staining.

### 2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were extracted and cultured as previously described [12]. In brief, HUVECs were removed from human umbilical veins by collagenase digestion and cultured in medium 199 supplemented with 20% fetal calf serum, and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). HUVECs were trypsinized and subcultured in 35-mm culture dishes, and grown to 80% confluent before treatment. Passage 3–6 HUVECs were used for experiments. In this study, cells were co-incubated with different concentrations (1, 5, 10 μmol/L) of Tan and high glucose (35 mmol/L) for 48 h unless otherwise stated.

### 2.4. Quantitative real-time PCR

Total RNA (1 μg) from HUVECs and aortas was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was reverse-transcribed using the ReverTra ACE qPCR RT Kit (Toyobo, Osaka, Japan). RT-PCR was performed using SYBR Green PCR master mix (Invitrogen, Grand Island, USA) with an ABI Prism 7300 Fast Real-Time PCR system (Applied Biosystems, Grand Island, USA). The eNOS mRNA expression was normalized to the mRNA levels of GAPDH. The specific primer sequences used for the amplification were as follows: eNOS (human), 5′-CCCTTC AGTGGCTGGTACAT-3′ and 5′-CACGATGGTGACTTTGGCTA-3′; eNOS (rat), 5′-TCCGATTCAACAGTGTCTCCT-3′ and 5′-ACAGAAGTGCGGGTAT GCTC-3′; GAPDH (human), 5′-GGGCACGAAGGCTCATCATT-3′ and 5′-AGAAGGCTGGGGCTCATTTG-3′; and GAPDH (rat), 5′-TGCTGGTGCTGA GTATGTCGTG-3′ and 5′-CGGAGATGATGACCCTTTTGG-3′. Reactions were carried out by 30 (eNOS) and 19 (GAPDH) cycles (95 °C for 10 s and 61 °C for 30 s) after an initial 3 min at 95 °C.

### 2.5. Western blot analysis

Aorta homogenates and cell extracts were subjected to western blot analysis as previously described [12]. Equal amounts of protein were separated with SDS-PAGE and then transferred onto a Polyvinylidene Fluoride (PVDF) membrane (Millipore, Billerica, USA). Blots were incubated with appropriate antibodies. Bands were visualized by ECL system (Thermo Scientific, Pittsburgh, USA) and quantified by Gel-ProAnalyzer 6.0 (Media Cybernetics Corporation, Warrendale, USA).

### 2.6. Immunohistochemistry

At the age of 12 weeks, rat thoracic aorta was carefully excised, and embedded and frozen using optimal cutting temperature compound (Tissue-Tek, Sakura, Japan) for immunohistochemistry. 3% hydrogen peroxide aqueous solution was applied to block endogenous peroxidase activity. The sections were blocked by 10% goat serum and incubated with rabbit-anti-eNOS (1:100 dilution) overnight at 4 °C, followed by incubation with secondary antibody for 1 h at room temperature and