

## Original Article

# Allicin improves endothelium-dependent relaxation in diabetic rats through maintaining eNOS protein stability

Yuan-Wan Yin<sup>1</sup>, Hong-Xia Ma<sup>2</sup>, Xiao-Hong Tian<sup>2</sup>, Yan-Cheng Xu<sup>1</sup>

<sup>1</sup>Department of Endocrinology, Zhongnan Hospital of Wuhan University, Wuhan 430071, Hubei Province, People's Republic of China; <sup>2</sup>Department of Endocrinology, Enshi Center Hospital, Enshi 445000, Hubei Province, People's Republic of China

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**Abstract:** Endothelial nitric oxide (NO) synthase (eNOS) dysfunction-mediated endothelium-dependent relaxation impairment has been suggested to be a principal feature of diabetes-related cardiovascular complications. Allicin is one of the most biologically active compounds in garlic and exerts therapeutic potentials in many pathologic conditions. However, the protective effects of Allicin on eNOS expression and endothelium-dependent relaxation in diabetes remain unknown. In the present study, diabetic rats were induced by intravenous injection of streptozotocin and then orally treated with various concentration of Allicin for 4 weeks. EVC-304 endothelial cells were pretreated with Allicin in prior to incubation of high glucose for 48 h. NO production was determined by biochemical and cellular approaches. eNOS expression was analyzed by immunohistochemistry, real-time quantitative PCR and western blot, respectively. We found that Allicin administration suppressed the elevation of blood pressure in diabetic rats. In addition, Allicin remarkably attenuated diabetes-induced the decrease of NO production and eNOS expression, with a concomitant improvement in endothelial-dependent relaxation. Furthermore, rather than affecting eNOS transcriptional activity and mRNA expression, Allicin prolonged eNOS protein half-life and decreased eNOS ubiquitination. Collectively, Allicin restored eNOS expression through inhibition of ubiquitin-mediated eNOS protein degradation in diabetes, suggesting a novel mechanism for Allicin in the treatment for diabetes-related cardiovascular complications.

**Keywords:** Allicin, diabetes, nitric oxide, endothelium-dependent relaxation, eNOS, degradation

## Introduction

Diabetes mellitus is one of the most common health problems worldwide, while diabetes-related vascular complications contribute the principal cause of morbidity and mortality among diabetics [1, 2]. Endothelial cells, the inner layer of the blood vessel wall, have been identified as the first target in hyperglycemia [3]. Many diabetes-related vascular diseases are caused by endothelial cells injury and dysfunction [4, 5]. Reduction of nitric oxide (NO) production and subsequent impairment of endothelium-dependent relaxation has been suggested to be a principal feature of endothelial dysfunction. It is well recognized that endothelial NO synthase (eNOS) is the key enzyme in regulating NO production and endothelium-dependent relaxation [6]. Therefore, improvement of endothelial dysfunction via regulating

eNOS-mediated endothelium-dependent relaxation may have great significance in the treatment of diabetic vascular complications.

Among the available medications for diabetes, a variety of natural compounds derived from traditional Chinese herbs have been known as valuable resources for diabetes treatment. Garlic is a traditional Chinese herbal medicine, which has been used for thousands of years to treat a range of diseases [7, 8]. Allicin, one of the most biologically active compounds in garlic, is produced in the crushed garlic cloves and responsible for the most of the functions of garlic [9]. Accumulating evidence suggests that Allicin processes a broad spectrum of health-promoting effects, including anti-inflammatory, anti-hypertensive, anti-microbial and anti-cancer activities [10-13]. However, the protective effects of Allicin on eNOS dysfunction in diabe-

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tes are not completely understood. In the current study, we aim to investigate the role of Allicin in diabetes-induced eNOS dysfunction and vascular reactivity impairment. Our data provide evidence that Allicin attenuates the impairment of endothelium-dependent relaxation via inhibition of eNOS protein degradation in diabetic rats.

## Materials and methods

### Materials and reagents

RPMI1640 medium, fetal calf serum (FCS), penicillin, streptomycin and L-glutamine were purchased from Life Technologies (NY, USA). pGL2-eNOS promoter and  $\beta$ -galactosidase plasmid were obtained from Addgene (MA, USA). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (MO, USA). Allicin (purity >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and dissolved in diethylether at a stock concentration of 40 mg/mL. Final concentration of diethylether in culture medium was no more than 0.1%.

### Animals

Sprague-Dawley males rats (n=100, 8 weeks old, 250-280 g) were purchased from Jackson Laboratories (Pennsylvania, USA). Animals were housed maintained at controlled temperature (22-25°C) under a 12-h light/12-h dark cycle, with free access to food and water. The experimental protocols for animals were approved by Institutional Animal Ethical Committee of Wuhan University and complied with the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health in China.

### Induction of diabetes

Following an overnight fast, experimental diabetes was induced by a single intravenous injection of streptozotocin (STZ) at a concentration of 60 mg/kg body weight in 10 mmol/L sodium citrate buffer (pH 4.0). Control rats were received with an equivalent amount of sodium citrate buffer alone. Blood glucose concentration was examined after 1 week using a OneTouch Diabetic Test Strips (Johnson & Johnson, WI, USA). Only those animals with fasting blood glucose concentration  $\geq 16.7$  mmol/L for

three consecutive tests were included in the study.

### Experimental design

Rats were randomly divided into following groups, with 16 in each group:

Control: non-diabetic rats with an equivalent amount of sodium citrate buffer were intraperitoneally administrated with same volume of diethylether.

Diabetes: diabetic rats were intraperitoneally administrated with same volume of diethylether.

Diabetes+Allicin (1 mg/kg/day): diabetic rats were orally administrated with 1 mg/kg/day Allicin for 4 weeks.

Diabetes+Allicin (10 mg/kg/day): diabetic rats were orally administrated with 10 mg/kg/day Allicin for 4 weeks.

Diabetes+Allicin (50 mg/kg/day): diabetic rats were orally administrated with 50 mg/kg/day Allicin for 4 weeks.

The concentrations of Allicin were selected on the basis of previous studies [14, 15]. After 4 weeks, blood samples were collected to determine blood glucose concentration using a OneTouch Diabetic Test Strips. Systolic blood pressure (SBP) was analyzed in unanesthetized rats via noninvasive tail cuff. Plasma insulin concentration was measured by an enzymatic immunoassay method (R&D systems, MN, USA).

### Cell culture

The human umbilical vein endothelial cells line EVC-304 were obtained from the cell line bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI1640 medium supplemented with 10% FCS, 100  $\mu$ g/ml penicillin, 100  $\mu$ g/mL streptomycin and 2 mmol/L L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> atmosphere. In this study, EVC-304 cells were pretreated with Allicin for 12 h followed by incubation of high glucose (35 mmol/L) for another 48 h.

### Enzyme immunoassay

The level of cGMP in serum was determined by immunoassay cGMP EIA kit (Cayman Chemical,

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MI, USA) and normalized to the protein content. The measurement was performed as recommended by the manufacturer's protocol.

### *Nitrite production measurement*

Nitrite production in serum was measured by a total nitric oxide assay kit (Beyotime, Jiangsu, China) according to the manufacturer's recommendations and was normalized to the protein content.

### *Direct NO production*

NO production in EVC-304 cells was detected using 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM, Molecular Probes, Invitrogen, CA, USA). Cells were incubated with DAF-FM (10  $\mu$ mol/L) for 1 h at 37°C in dark, and the supernatants were removed. The fluorescence images were captured using a laser-scanning confocal microscopy (LSM700, Zeiss, Munich, Germany) with excitation and emission wavelengths of 495 nm and 515 nm, respectively. The mean value of fluorescence intensity was calculated with Image-Pro Plus Version 6.0 image analysis system (Media Cybernetics, Inc., Silver Spring, MD, USA) and presented as the percentage of control.

### *Immunohistochemistry*

Thoracic aortas were isolated, and fixed in 4% paraformaldehyde and made into 10  $\mu$ m thick paraffin-embedded section. The slides were added with 5% normal goat serum (Zhongshan Jinqiao Bio-Technology Co. Ltd., Beijing, China) to block nonspecific binding, and then were incubated with eNOS antibody overnight. After 3 washes with PBS, the slides were incubated with biotinylated secondary antibody (Zhongshan Jinqiao Bio-Technology Co. Ltd.) and visualized with the streptavidin-peroxidase reaction using 3,3'-diaminobenzidine (DAB) Chromogen Solution (Zhongshan Jinqiao Bio-Technology Co. Ltd.) under a light microscope (magnification,  $\times$ 400) (IX70, Olympus, Tokyo, Japan).

### *Measurement of vascular reactivity*

Vascular reactivity of the isolated thoracic aortas was measured using the isolated artery techniques as previously described [5]. In brief, thoracic aortas were cut into rings (3-4 mm in length) and placed in Krebs solution, main-

tained at 37°C, and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4). Each ring was stretched to a resting tension of 10 mN and allowed to equilibrate for 2 h. During this period, the solution was changed every 15 min. For measuring the aortic contraction response, increases in tension due to cumulative additions of phenylephrine (PE, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L) were recorded by an isometric force transducer (model 610, DMT-USA, GA, USA). For measuring the endothelium-dependent relaxation, the aortic rings were first precontracted with maximal concentrations of PE (10<sup>-5</sup> mol/L) and then cumulative concentrations of acetylcholine (Ach, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L) were added to induce relaxation. The endothelium-independent relaxation was examined by the relaxant effects induced by sodium nitroprusside (SNP, 10<sup>-10</sup> mol/L to 10<sup>-6</sup> mol/L).

### *Western blot*

Western blot analysis was performed as previously described [10]. Thoracic aortas or cells were washed with ice-cold PBS, and harvested in RIPA Lysis Buffer (Beyotime) containing protease inhibitors. Protein concentrations were assayed with a BCA protein assay kit (Beyotime), and 40  $\mu$ g of protein from each sample were resolved on 8%-10% SDS-PAGE gels. The separated proteins were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked with 5% nonfat milk and then incubated overnight with the flowing primary antibodies: eNOS and  $\beta$ -actin (diluted 1:1000), ubiquitin (diluted 1:500) (Cell Signaling Technology, MA, USA). After 3 washes with PBS-T, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (diluted 1:1000, Cell Signaling Technology) for 1 h incubation at room temperature. Bands were visualized by ECL Kit (Beyotime) and quantified with Image-Pro Plus software.

### *Real-time quantitative PCR assay*

eNOS mRNA expression was determined by real-time quantitative PCR as previously described [5]. Briefly, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA of HAECs was isolated using RNeasy system (Qiagen, MD, USA) according to the manufacturer's instructions. 1  $\mu$ g RNA was reverse-trans-

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**Table 1.** Physiological characteristic of rats after Allicin administration

Parameter	Control	Diabetes	Diabetes+Allicin (1 mg/kg/day)	Diabetes+Allicin (10 mg/kg/day)	Diabetes+Allicin (50 mg/kg/day)
Body weight (g)	294.6±10.2	255.2±12.7**	259.8±11.4	277.5±9.6*	286.5±10.3##
Blood glucose (mmol/L)	9.4±1.7	25.3±2.8**	24.5±1.9**	23.8±2.2**	23.3±1.1**
Blood pressure (mmHg)	112.5±4.8	143.3±3.3**	139.2±2.7	130.3±2.1##	121.5±3.2##
Insulin (ng/mL)	1.3±0.11	0.58±0.07	0.54±0.06	0.61±0.05	0.57±0.04

Values are mean ± SEM. \*\*P<0.01 vs. control; \*P<0.01, ##P<0.01 vs. diabetes, n=16 in each group.

scribed with the SuperScriptIII First-Strand Synthesis system (Invitrogen) and real-time quantitative PCR was performed using Fast SYBR Green Master Mix Kit (Invitrogen). The specific primers were synthesized by Invitrogen: sense 5'-CCCTTCAGTGGCTGGTACAT-3' and antisense 5'-CACGATGGTGACTTTGGCTA-3' for eNOS; sense 5'-GGGCACGAAGGCTCATCATT-3' and antisense 5'-AGAAGGCTGGGGCTCATTG-3' for GAPDH. The amplification conditions were as follows: 10 s at 95°C, 30 s at 61°C, 20 s at 72°C, processed by 3 min at 95°C for polymerase activation. The mRNA expression for GAPDH was used an internal control.

### Transfection and reporter gene assays

eNOS luciferase activity was determined using a luciferase assay kit (Promega, WI, USA) according to the instructions of the manufacturer. Briefly, EVC-304 cells were cotransfected with pGL2-eNOS promoter and β-galactosidase using lipofactmin 2000 (Invitrogen). 24 h after transfection, the cells treated with Allicin (20 μg/mL) for 12 h followed by incubation of high glucose for 48 h. The luciferase activity was detected and normalized to by β-galactosidase activity.

### Immunoprecipitation

Cell lysates were precleared nonspecific binding with protein A/G agarose beads (Santa Cruz Biotechnology, CA, USA). The precleared supernatants were then incubated with eNOS antibody at 4°C overnight, followed by incubation with protein A/G agarose beads at 4°C for 2 h. The bound proteins were washed with PBS 3 times and boiled in SDS sample buffer and subjected to SDS-PAGE.

### Statistical analysis

All data were presented as the mean ± SEM. n represents the number of independent experi-

ments on different rats or different batches of cells. The statistical significance between samples was evaluated by the unpaired two-tailed Student's or the one-way analysis of variance (ANOVA). The level of P<0.05 was considered statistically significant.

## Results

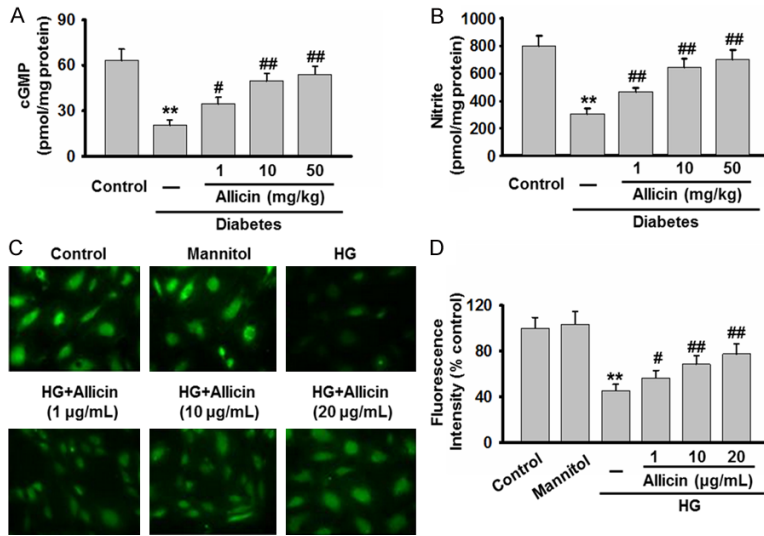
### Effects of Allicin on general physiological parameters in diabetic rats

As shown in **Table 1**, STZ-treated rats exhibited an obvious reduction in body weight and plasma insulin concentration and a significant increase in blood glucose and blood pressure. The oral administration of Allicin markedly increased body weight and suppressed the elevation of blood pressure, as compared with diabetes group. However, Allicin administration did not modify the blood glucose and plasma insulin concentration in diabetic rats.

### Allicin ameliorated diabetes-induced the decrease of NO production

To answer whether the suppression of blood pressure after Allicin treatment was associated with an enhancement of NO production, we assayed cGMP and nitrite concentrations in rat serum. STZ injection resulted in a marked decrease in cGMP and nitrite concentrations, respectively. However, oral administration of Allicin attenuated the decrease of cGMP and nitrite concentrations in a concentration-dependent manner (**Figure 1A** and **1B**). To further examine whether the in vivo findings were also operative in vitro, we treated endothelial cells with high glucose in the presence or absence of Allicin, and the direct NO production in EVC-304 cells was measured. Mannitol treatment, which acted as the osmotic control, had no effect on NO production, as compared with control group. Consistent with the results in cGMP and nitrite measurement, the direct NO

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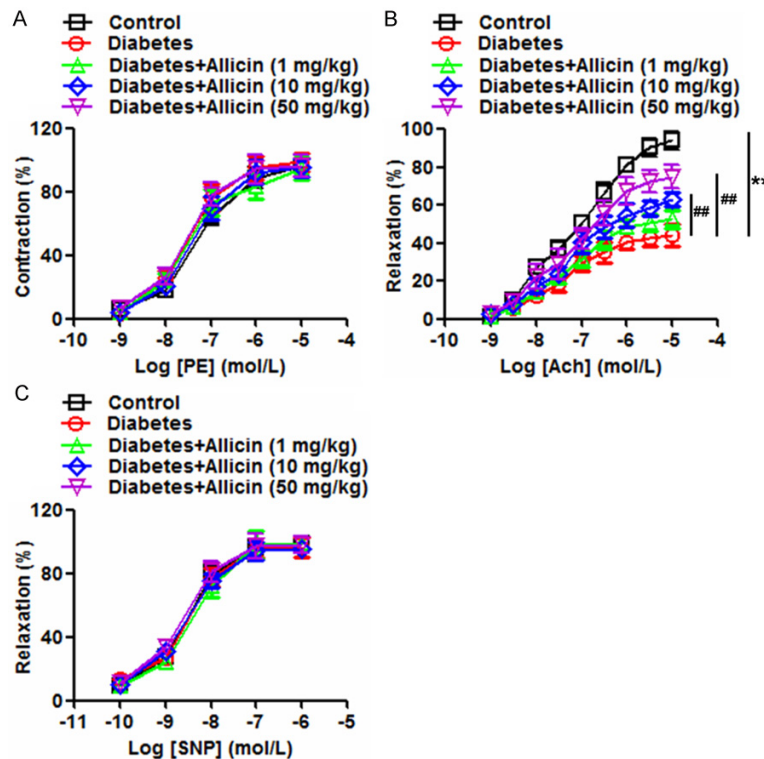
**Figure 1.** Allicin restored diabetes-induced the decrease of NO production. (A and B) Accumulation of cGMP (A) and level of nitrite (B) were determined in serum from control and diabetic rats treated with or without Allicin. (C) EVC-304 cells were treated with different concentrations of Allicin (1, 10, 20 µg/mL) for 12 h in prior to incubation of high glucose (35 mmol/L) for another 48 h. Cells were then incubated with DAF-FM (10 µmol/L) for 1 h. The representative images were captured by a laser-scanning confocal microscopy (200×). (D) DAF-FM fluorescence intensity was calculated using Image-Pro Plus software. All data are presented as mean ± SEM. \*\*P<0.01 vs. control; #P<0.05, ##P<0.01 vs. diabetes or high glucose, n=6.

dothelium-dependent relaxation was measured in response to acetylcholine (Ach, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L). (C) The endothelium-independent relaxation was examined by the relaxant effects in response to sodium nitroprusside (SNP, 10<sup>-10</sup> mol/L to 10<sup>-6</sup> mol/L). \*\*P<0.01 vs. control; ##P<0.01 vs. diabetes or high glucose, n=6.

production was significantly decreased after high glucose treatment, which was ameliorated by Allicin concentration-dependently (Figure 1C and 1D). The pharmacological results indicate that Allicin can restore NO production under high glucose conditions.

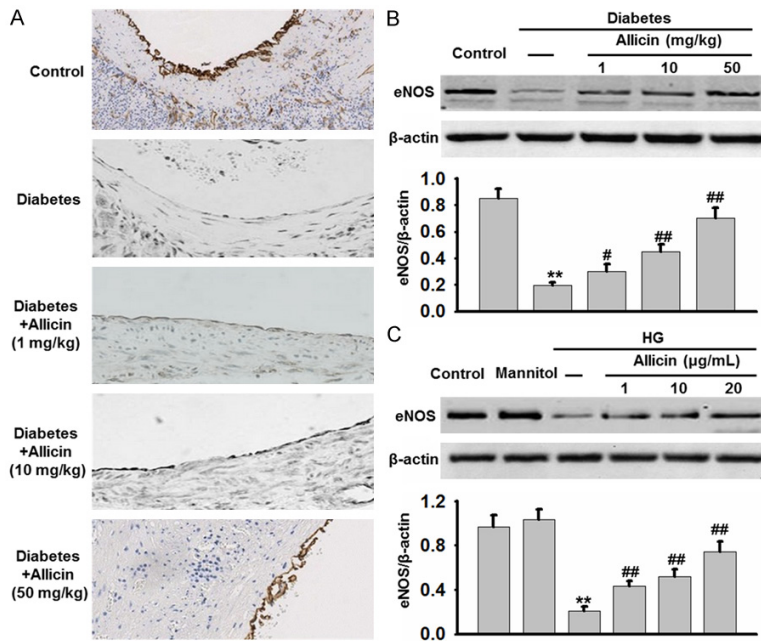
## Allicin improved impairment of endothelium-dependent relaxation in diabetic rats

To explore the functional effects of Allicin-mediated restored NO production, we measured the reactivity of thoracic aortas isolated from diabetic rats treated with or without Allicin. We first examined the response of thoracic aortas to the vasoconstrictor phenylephrine (PE, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L). Although Allicin decreased blood pressure in diabetic rats, PE-induced the contractions were similar among the five groups and no significant differences were observed (Figure 2A). Compared to control group, the endothelium-dependent relaxation induced by acetylcholine (Ach, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L) were decreased in thoracic aortas from diabetic rats. However, the impaired relaxation was improved by Allicin administration in a concentration-dependent manner (Figure 2B). In contrast, the endothelium-independent relaxation induced by the NO donor, sodium nitroprusside



**Figure 2.** Allicin treatment ameliorated the impairment of endothelium-dependent relaxation in diabetic rats. (A) Contraction in response to vasoconstrictor phenylephrine (PE, 10<sup>-9</sup> mol/L to 10<sup>-5</sup> mol/L) in thoracic aortas from normal and diabetic rats with or without Allicin treatment (B) En-

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**Figure 3.** Allicin treatment attenuated the decrease of eNOS expression in thoracic aortas from diabetic rats. (A and B) eNOS expression in thoracic aortas of diabetic rats before and after Allicin treatment was determined by immunohistochemistry (A) and western blot (B). (C) EVC-304 cells were pretreated with different concentrations of Allicin (1, 10, 20 µg/mL) for 12 h and then incubated with high glucose (35 mmol/L) for another 48 h. eNOS expression was analyzed by western blot using β-actin as an internal control. \*\*P<0.01 vs. control; #P<0.05, ##P<0.01 vs. diabetes or high glucose, n=6.

(SNP,  $10^{-10}$  mol/L to  $10^{-6}$  mol/L), were similar in all groups (**Figure 2C**). These results suggested that Allicin is involved in the improvement of endothelium-dependent relaxation via restoration of NO production.

### *Allicin attenuated diabetes/high glucose-induced the decrease of eNOS expression*

Because eNOS is a critical mediator of endothelium-dependent relaxation, we next investigated whether Allicin was involved in the regulation of relaxation due to eNOS expression. **Figure 3A** showed the slides from thoracic aortas, isolated from diabetic rats before and after Allicin treatment. Immunohistochemistry analysis showed that eNOS positive immunolabeling signal was found in the endothelium. STZ-injection resulted in a significant decrease in eNOS expression, as evidenced by a weak signal in the endothelia layer of thoracic aorta. However, oral administration of Allicin ameliorated the decreased eNOS expression in a concentration-dependent manner (**Figure 3A**). Consistently, western blot revealed that diabe-

tes-induced the decrease in eNOS expression was also restored after Allicin administration (**Figure 3B**). Similar to what was observed in diabetic rats, eNOS expression was significantly decreased in EVC-304 cells, which was attenuated by Allicin treatment in a concentration-dependent manner (**Figure 3C**).

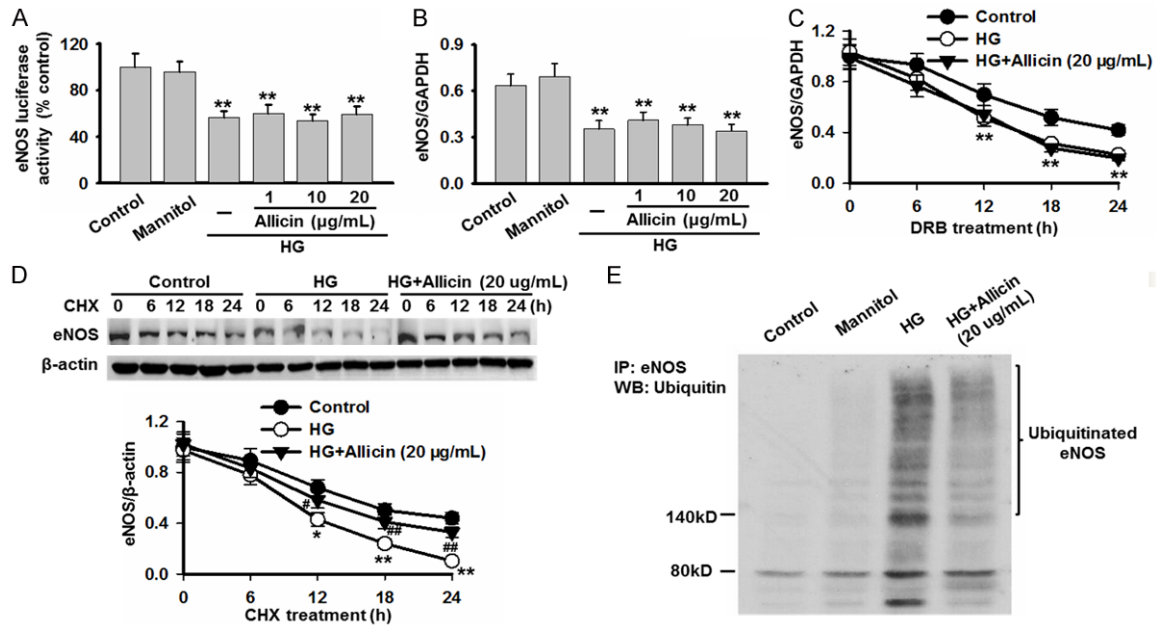
### *Allicin restored eNOS expression through increasing eNOS protein stability*

To further examine the mechanisms by which Allicin restored eNOS expression in diabetes, we first analyzed the eNOS transcriptional level. Reporter gene assay showed that eNOS transcriptional activity in EVC-304 cells was remarkably reduced following high glucose treatment. However, Allicin had no effects on high glucose-mediated inhibited eNOS transcriptional activity (**Figure 4A**).

We next determined if Allicin treatment restored eNOS expression at its post-transcriptional level. Real-time quantitative PCR showed that Allicin treatment for 48 h decreased eNOS mRNA expression more than 46% in EVC-304 cells but Allicin did not alter the observed effects of high glucose (**Figure 4B**). Furthermore, eNOS mRNA stability was examined in EVC-304 cells using a transcriptional inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, 20 µg/mL). As shown in **Figure 4C**, high glucose treatment decreased eNOS mRNA stability, as compared with control group. However, no significant differences were found in eNOS mRNA decay ratio between high glucose group and Allicin group. These results indicated that the transcriptional and post-transcriptional regulations are not involved in the protective effects of Allicin on eNOS expression.

We further examined eNOS protein stability by using a protein synthesis inhibitor, cycloheximide (CHX, 10 µg/mL). In agreement with previous study [5], CHX treatment resulted in a time-

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**Figure 4.** Allicin treatment increased eNOS expression via inhibition of eNOS protein degradation. A. EVC-304 cells were pretreated with various concentrations of Allicin (1, 10, 20  $\mu\text{g/mL}$ ) for 12 h in prior to incubation of high glucose (35 mmol/L) for another 48 h. eNOS transcriptional activity was analyzed by luciferase assay in EVC-304 cells co-transfected with pGL2-eNOS promotor and  $\beta$ -galactosidase. eNOS promotor luciferase activity was normalized by  $\beta$ -galactosidase activity. B. Real-time quantitative PCR analysis of eNOS mRNA expression. C. The cells were pretreated with Allicin (20  $\mu\text{g/mL}$ ) for 12 h and incubated with high glucose (35 mmol/L) for 48 h, and then, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB, 20  $\mu\text{g/mL}$ ) was added for the indicated times. eNOS mRNA stability was determined by real-time quantitative PCR. D. EVC-304 cells were treated with high glucose (35 mmol/L) in the presence or absence of Allicin (20  $\mu\text{g/mL}$ ). 48 h later, cycloheximide (CHX, 10  $\mu\text{g/mL}$ ) was added for the indicated times and the half-life of eNOS protein was determined. E. Cell lysates were immunoprecipitated with eNOS antibody and immunoprecipitated proteins were blotted with ubiquitin antibody to detect the level of ubiquitinated eNOS. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control; # $P < 0.05$ , ## $P < 0.01$  vs. high glucose,  $n = 4$ .

dependent decrease of eNOS protein expression in EVC-304 cells. High glucose and CHX treatment significantly decreased eNOS protein stability, shorting eNOS protein half-life more than 5 h. On the contrary, addition of Allicin markedly inhibited the rate of eNOS degradation, indicating Allicin regulates eNOS expression at post-translational level (Figure 4D). To test whether Allicin prevented eNOS protein degradation through ubiquitin pathway, the levels of ubiquitinated eNOS were detected with an anti-ubiquitin antibody after eNOS immunoprecipitation. High glucose dramatically increased the amount of ubiquitinated eNOS, whereas Allicin almost abolished this enhancement (Figure 4E). Taken together, Allicin increases eNOS expression mainly via inhibition of ubiquitin-mediated degradation.

### Discussion

Multiple epidemiological and preclinical studies evidence that long-term oral intake of garlic is

associated with reduced risks of many diseases such as cardiovascular diseases and some types of cancers [11-13, 16, 17]. As the most abundant organosulfur compound in freshly crushed garlic tissues, Allicin has been shown to relieve a number of cardiovascular diseases and improve endothelial function in human with coronary artery disease [11, 18, 19]. Previous study suggested that increased dietary intake of Allicin in rats was effective to prevent the development of hypoxia pulmonary hypertension [11]. It was also reported that Allicin can improve right heart hypertrophy in pulmonary hypertension rats [19]. Moreover, a number of in vitro studies showed that Allicin protected against endothelial cells injury and dysfunction [7, 10, 16, 20]. The protective effects were mediated via reducing oxidative stress [7], limiting inflammation [10] and decreasing apoptosis [20]. Together, these findings support the cardiovascular risk reducing effects of Allicin, and a better understanding of the therapeutic

potential of Allicin could facilitate the development of Allicin for diabetes-related cardiovascular complications prevention. In the present study, we found that Allicin increased eNOS expression and endothelium-dependent relaxation in STZ-induced diabetic rats, leading to a reduction of blood pressure. The inhibitory effect on blood pressure was consistent with the previous reports that Allicin can suppress the elevation of blood pressure in high cholesterol diet rats [21] and pulmonary hypertension rats [11].

Reduction of NO production and the subsequent impairment of endothelium-dependent relaxation under high glucose conditions play an important role in endothelial dysfunction [6]. Indeed, clinical studies have been indicated that diabetes patients invariably less NO production and relaxation impairment [22]. Here, we found that Allicin treatment improved endothelium-dependent relaxation in diabetes, but had no effects on vascular contraction. This protective effect was mediated via Allicin restoration of NO production in diabetes and was consistent with the results of a previous study [23]. By contrast, report by Kim et al. showed that the protective effects of Allicin on hypoxic pulmonary hypertension may be due to the inhibition of pulmonary contraction [17]. Presumably, the discrepancy is probably related to different vascular tissues in different pathological animal model.

It has been well documented that eNOS regulation occurs at the transcriptional, post-transcriptional and post-translational level [24]. We firstly aimed to investigate whether the restoration of eNOS expression after Allicin treatment simply occurs at eNOS transcriptional level. Therefore, eNOS promoter luciferase activity was determined. Nevertheless, our results revealed that Allicin treatment failed to attenuate high glucose-induced the decrease of eNOS transcriptional activity. Moreover, the post-transcriptional regulation seemed not to be taking place, because we found that Allicin treatment also produced no effects on high glucose-mediated decreased eNOS mRNA expression and mRNA half-life. Intriguingly, a study showed that high glucose did not decrease eNOS mRNA half-life in human umbilical vein endothelial cells [5]. Thus, cell type-specific response should be noted. Although the expression of

eNOS is regulated by multiple mechanisms, recent studies have suggested that post-translational regulation of protein stability plays an important role in control of eNOS [5, 24]. In the current study, we found that Allicin treatment inhibited high glucose-induced eNOS degradation via increasing eNOS protein half-life. Moreover, immunoprecipitation assay demonstrated that Allicin treatment decreased the amount of ubiquitinated eNOS induced by high glucose, suggesting the inhibition of ubiquitin-mediated degradation underlies, at least in part, the protective effect of Allicin on eNOS protein expression.

In summary, our data support a protective role for Allicin in diabetes-related endothelial dysfunction, which is critically linked to the impairment of NO production and endothelium-dependent relaxation. Allicin, acting at a post-translational level, increases eNOS protein stability via inhibition eNOS degradation, providing a novel mechanism that might help to better understand the biological effects and pharmacological activity of Allicin.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Yan-Cheng Xu, Department of Endocrinology, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuchang District, Wuhan 430071, Hubei Province, People's Republic of China. Tel: +86 027 67812888; Fax: +86 027 67812892; E-mail: yancheng\_x@163.com

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