

# Protective effects of hydrogen sulfide against angiotensin II-induced endoplasmic reticulum stress in HUVECs

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Received September 25, 2015; Accepted October 5, 2016

DOI: 10.3892/mmr.2017.6238

**Abstract.** The inhibitory effects of hydrogen sulfide (H<sub>2</sub>S) on angiotensin II (AngII)-stimulated human umbilical vein endothelial cell (HUVEC) dysfunction remain to be elucidated. Endoplasmic reticulum (ER) stress has been detected in endothelial dysfunction (ED). The present study aimed to determine whether H<sub>2</sub>S may exert an inhibitory effect on AngII-induced ER stress. Using HUVECs as a model system, the present study used western blotting to detect protein expression, intracellular reactive oxygen species (ROS) levels were determined by oxidative conversion of cell permeable DCFH-DA to fluorescent dichlorofluorescein, CCK-8 assay was used to investigate the cell viability, methylene blue was used to investigate the CSE activity, TUNEL was used to investigate the cells apoptosis. The present study demonstrated that AngII not only upregulated the expression levels of inducible nitric oxide synthase (iNOS), stimulated ROS production and increased cell apoptosis, but also down-regulated the expression levels of phosphorylated-endothelial nitric oxide synthase, decreased the expression and activity of cystathionine-c-lyase (CSE) and decreased cell viability. Furthermore, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; an exogenous ROS) downregulated the expression and activity of CSE, and had similar effects as AngII, whereas the inhibitory effects of AngII were completely suppressed by N-acetyl-L-cysteine (a ROS scavenger). In addition, AngII induced the expression of glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), which are markers of ER stress. Conversely, the stimulatory effects of AngII were completely inhibited by sodium hydrosulfide (NaHS; a H<sub>2</sub>S donor). Treatment with NaHS attenuated ROS production, inhibited

CHOP and GRP78 expression, and decreased cell apoptosis. The present study indicated that AngII induced ED via the activation of ER stress in HUVECs. In addition, the effects of AngII on ER stress could be suppressed by H<sub>2</sub>S.

## Introduction

The pathogenesis of cardiovascular disease (CVD) and cardiovascular complications, such as atherosclerosis, is associated with endothelial dysfunction (ED) (1,2). It is well established that ED contributes to the high rates of cardiovascular mortality (3). Excessive oxidative stress, particularly reactive oxygen species (ROS), exerts a diverse atherogenic effect that induces vascular and tissue injuries (4), which are considered to serve an important role in the development of ED.

The renin-angiotensin system (RAS) has long been known to have an important role in the maintenance of cardiovascular function, and electrolyte and fluid balance. Emerging evidence has suggested that angiotensin II (AngII) is a marker of the RAS system, and higher AngII levels correlate with a higher incidence of CVD (5). Furthermore, AngII has been shown to directly regulate vascular resistance and induce cardiac fibrosis-associated gene expression in a mouse model of cardiac dysfunction (6). It has also been hypothesized that overproduction of AngII participates in the progression of CVD via ROS (7). Furthermore, in model rats with hypertensive disease, AngII levels were much higher in endothelial cells compared with in plasma (8). Beyond vasoconstriction, previous studies have demonstrated that AngII exerts cytotoxic effects via the induction of apoptotic endothelial cell death, these effects were associated with phosphorylated-endothelial nitric oxide synthase (p-eNOS) and inducible nitric oxide synthase (iNOS) in a concentration-dependent manner (9-12). Notably, p-eNOS and iNOS have been indicated as biomarkers and novel therapeutic targets in oxidative stress-associated ED (12-15).

The endoplasmic reticulum (ER) is a subcellular organelle in which protein translation, folding and trafficking occur. In addition, inflammation generated by ER stress is cytotoxic to several cell types, and may facilitate the progression of ED. Previous studies have demonstrated that increased ER stress levels are a marker for cardiovascular risk, and are associated with higher levels of oxidative stress and hypoxia (16,17). In addition, numerous studies have reported that several

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**Key words:** hydrogen sulfide, angiotensin II, endoplasmic reticulum stress, endothelial dysfunction

conditions, such as diabetes mellitus and cardiovascular disorders, are correlated with increased ER stress levels (18,19). Glucose-regulated protein 78 (GRP78) and C/EBP-homologous protein (CHOP) are biological markers for ER stress (20,21). Furthermore, ER stress is associated with decreased p-eNOS expression (14,22).

A previous study demonstrated that AngII induces ED via ER stress (23). In addition, increased levels of GRP78 and CHOP have been reported to correlate with vascular ED, and decrease the activity of antioxidant enzymes, such as eNOS, in AngII-treated mice, thus suggesting that AngII-induced ED is associated with ER stress (23,24). Hydrogen sulfide (H<sub>2</sub>S) can attenuate ER stress by increasing superoxide dismutase expression and reducing ROS levels (25). Furthermore, H<sub>2</sub>S may attenuate RAS activation via reduced ROS overproduction (26). The present study hypothesized that H<sub>2</sub>S exerts cytoprotective effects against AngII-induced ED in human umbilical vein endothelial cells (HUVECs) via the inhibition of ER stress.

It has previously been demonstrated that H<sub>2</sub>S exerts cytoprotective effects against oxidative stress-induced endothelial cell injury via antioxidant defense mechanisms (27). However, to the best of our knowledge, no previous studies have verified whether H<sub>2</sub>S has any effect on AngII-induced ER stress and ED in HUVECs.

In the present study, HUVECs were treated with AngII to establish a cellular model of ED, and the effects of AngII on ER stress, which is considered a main cause of ED development, were detected. Furthermore, the protective effects of H<sub>2</sub>S on AngII-induced ER stress were investigated. The effects of H<sub>2</sub>S on AngII-induced apoptosis and ROS accumulation were also determined, which are important processes in the development of ED.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sodium hydrosulfide (NaHS), AngII, N-acetyl-L-cysteine (NAC), Hoechst 33258, deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and 2',7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Antibodies targeting iNOS (cat. no. ab15323), p-eNOS (cat. no. ab183046), GRP78 (cat. no. ab21685) and CHOP (cat. no. ab11419), p-eNOS (cat. no. ab183046), GAPDH (cat. no. ab8245) and caspase-12 (cat. no. ab62484) were provided by Abcam (Cambridge, UK). Cystathionine-c-lyase (CSE) antibody was purchased from by Proteintech Group Inc. (Rosemont, IL, USA; cat. no. 12032-1-AP). Cell Counting kit-8 (CCK-8) was obtained from Dojindo China Co., Ltd. (Shanghai, China). Horseradish peroxidase-conjugated IgG (H+L) secondary antibodies were purchased from Beyotime Institute of Biotechnology (Shanghai, China; cat. nos. A0192 and A0208). All other reagents, unless specified, were purchased from Beyotime Institute of Biotechnology.

**Cell culture.** HUVECs (Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai,

China) were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Prior to each experiment, the medium was replaced with fresh serum-containing medium unless otherwise indicated. Cells were divided into the following treatment groups: i) Untreated; ii) AngII, consisting of 1x10<sup>6</sup> HUVEC cells treated with 10<sup>-6</sup> M AngII for 0-24 h; iii) NaHS + AngII, consisting of 1x10<sup>6</sup> HUVEC cells exposed to 200 μM NaHS for 60 min prior to treatment with 10<sup>-6</sup> M AngII for 24 h; iv) NAC + AngII, consisting of 1x10<sup>6</sup> HUVEC cells exposed to 100 μM NAC for 60 min prior to treatment with 10<sup>-6</sup> M AngII for 24 h; v) NaHS, consisting of 1x10<sup>6</sup> HUVEC cells exposed to 200 μM NaHS for 24 h; and vi) NAC, consisting of 1x10<sup>6</sup> HUVEC cells exposed to 100 μM NAC for 24 h.

**Cell viability assay.** After the HUVECs were cultured in 96-well plates and had received various treatments, 100 μl CCK-8 was added to each well at a 1/10 dilution, and the eight plates were incubated for a further 2 h at 37°C. Absorbance was then measured at 450 nm using a microplate reader. The mean optical density (OD) of four wells in the indicated groups was used to calculate the percentage of cell viability, according to the following formula: Cell viability (%) = OD treatment group / OD control group x 100%.

**Detection of intracellular ROS generation.** After the HUVECs were cultured in 96-well plates and had received various treatments, 10 μmol/l DCFH-DA in serum-free DMEM was added to each well and incubated for a further 30 min at 37°C. The cells were then washed three times with serum-free DMEM prior to the detection of dichlorofluorescein fluorescence. A fluorescent microscope connected to an imaging system was used to observe the entire field of vision. Mean fluorescence intensity (MFI) from three random fields was analyzed using ImageJ 1.41o software (National Institutes of Health, Bethesda, MD, USA); MFI was used to represent the amount of ROS.

**Measurement of CSE activity.** After receiving the various treatments, HUVECs were collected and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture contained 100 mmol/l potassium phosphate buffer (pH 7.4), 10 mmol/l L-cysteine, 2 mmol/l pyridoxal 5'-phosphate and 10% (w/v) homogenate. Cryovial test tubes (2 ml) were used as the center wells, into which 0.5 ml 1% zinc acetate was added as the trapping solution and 2x2.5 cm filter paper was used to increase air:liquid contact surface. The reactions were performed in Erlenmeyer flasks. The flasks containing the reaction mixture and the center wells were flushed with N<sub>2</sub>, before being sealed with a double layer of parafilm. The reactions were initiated by transferring the flasks from ice to a 37°C shaking water bath. Following a 90 min incubation at 37°C, 0.5 ml of 50% trichloroacetic acid was added to terminate the reaction. The flasks were then resealed and incubated at 37°C for a further 60 min to ensure the complete trapping of H<sub>2</sub>S released from the mixture. The contents of the center wells were transferred to test tubes, each containing 3.5 ml water. Subsequently, 0.5 ml 20 mM N,N-dimethyl-p-phenylenediamine sulphate in 7.2 M HCl was added, immediately followed by the addition of 0.5 ml

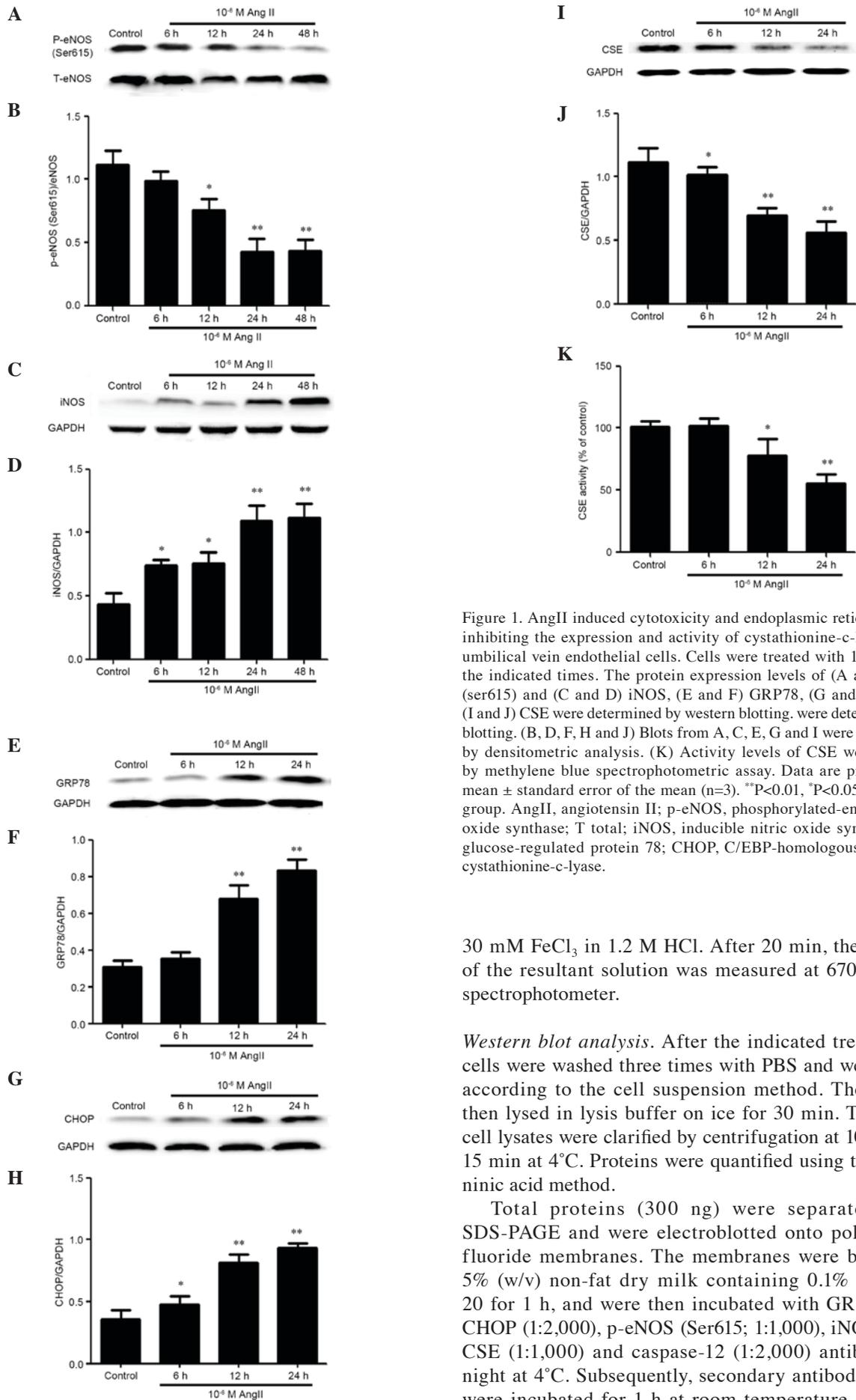


Figure 1. AngII induced cytotoxicity and endoplasmic reticulum stress by inhibiting the expression and activity of cystathionine-c-lyase in human umbilical vein endothelial cells. Cells were treated with  $10^{-6}$ M AngII for the indicated times. The protein expression levels of (A and B) p-eNOS (ser615) and (C and D) iNOS, (E and F) GRP78, (G and H) CHOP and (I and J) CSE were determined by western blotting. (B, D, F, H and J) Blots from A, C, E, G and I were semi-quantified by densitometric analysis. (K) Activity levels of CSE were determined by methylene blue spectrophotometric assay. Data are presented as the mean  $\pm$  standard error of the mean (n=3). \*\*P<0.01, \*P<0.05 vs. the control group. AngII, angiotensin II; p-eNOS, phosphorylated-endothelial nitric oxide synthase; T total; iNOS, inducible nitric oxide synthase; GRP78, glucose-regulated protein 78; CHOP, C/EBP-homologous protein; CSE, cystathionine-c-lyase.

30 mM  $\text{FeCl}_3$  in 1.2 M HCl. After 20 min, the absorbance of the resultant solution was measured at 670 nm using a spectrophotometer.

**Western blot analysis.** After the indicated treatments, the cells were washed three times with PBS and were collected according to the cell suspension method. The cells were then lysed in lysis buffer on ice for 30 min. The resulting cell lysates were clarified by centrifugation at  $10,943 \times g$  for 15 min at  $4^\circ\text{C}$ . Proteins were quantified using the bicinchoninic acid method.

Total proteins (300 ng) were separated by 10% SDS-PAGE and were electroblotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% (w/v) non-fat dry milk containing 0.1% (v/v) Tween 20 for 1 h, and were then incubated with GRP78 (1:800), CHOP (1:2,000), p-eNOS (Ser615; 1:1,000), iNOS (1:1,000), CSE (1:1,000) and caspase-12 (1:2,000) antibodies overnight at  $4^\circ\text{C}$ . Subsequently, secondary antibodies (1:1,000) were incubated for 1 h at room temperature. Membranes were visualized using an enhanced chemiluminescence

kit according to the manufacturer's protocols (Beyotime Institute of Biotechnology) and relative protein expression levels were semi-quantitatively analyzed by densitometry using QuantityOne software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Determination of HUVEC apoptosis.** The apoptosis of HUVECs was detected using TUNEL and Hoechst 33258 staining. TUNEL staining was performed using an *In Situ* Cell Death Detection kit, according to the manufacturer's protocol. Briefly, after the indicated treatments, cells were fixed with 4% paraformaldehyde in PBS for 10 min. After three washes in PBS, the cells were stained with 50  $\mu$ l TUNEL dye for 1 h, after which the cells were rinsed briefly with PBS and were air-dried. Subsequently, the cells were visualized under a fluorescence microscope. Apoptotic cells with condensed nuclei exhibited green fluorescence, whereas viable cells displayed normal nuclear size and uniform fluorescence.

Hoechst 33258 staining was also conducted. Briefly, after the indicated treatments, cells were fixed with 4% paraformaldehyde in PBS for 10 min. After three washes in PBS, cells were stained with 5 mg/l Hoechst 33258 dye for 10 min before being rinsed briefly with PBS and air-dried. The cells were then visualized under a fluorescence microscope. Apoptotic cells with condensed nuclei exhibited blue fluorescence, whereas viable cells displayed normal nuclear size and uniform fluorescence. The ratios of different fluorescent densities from 4 random fields was analyzed using ImageJ (version 1.41o; imagej.nih.gov/ij/). The experiment was repeated 3 times.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean. Differences between groups were analyzed by one-way analysis of variance followed by Bonferroni's correction. Data were analyzed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**AngII induces cytotoxicity and ER stress by inhibiting the expression and activity of CSE in HUVECs.** iNOS and phosphorylation of eNOS (ser615) (an essential step in eNOS activation) have been reported to be involved in AngII cytotoxicity (28,29) and ED (30). Therefore, the present study investigated whether AngII could induce ED through alterations in the expression levels of p-eNOS (ser615) and iNOS in HUVECs. As presented in Fig. 1, untreated HUVECs failed to produce detectable levels of iNOS, whereas AngII-treated cells produced high levels of iNOS. Conversely, the other ED marker, p-eNOS (ser615), was reduced in AngII-treated cells. ER stress has been reported to be associated with AngII in mice (31,32); therefore, the present study further observed the effects of AngII on the expression of ER stress-associated molecules, such as GRP78 and CHOP. Similar to iNOS, AngII was able to induce GRP78 and CHOP expression. Conversely, CSE expression and activity was reduced in a time-dependent manner following AngII treatment.

**Stimulation of ROS production inhibits the expression and activity of CSE in AngII-treated HUVECs.** Oxidative stress, which is generated by AngII, can induce endothelial cell

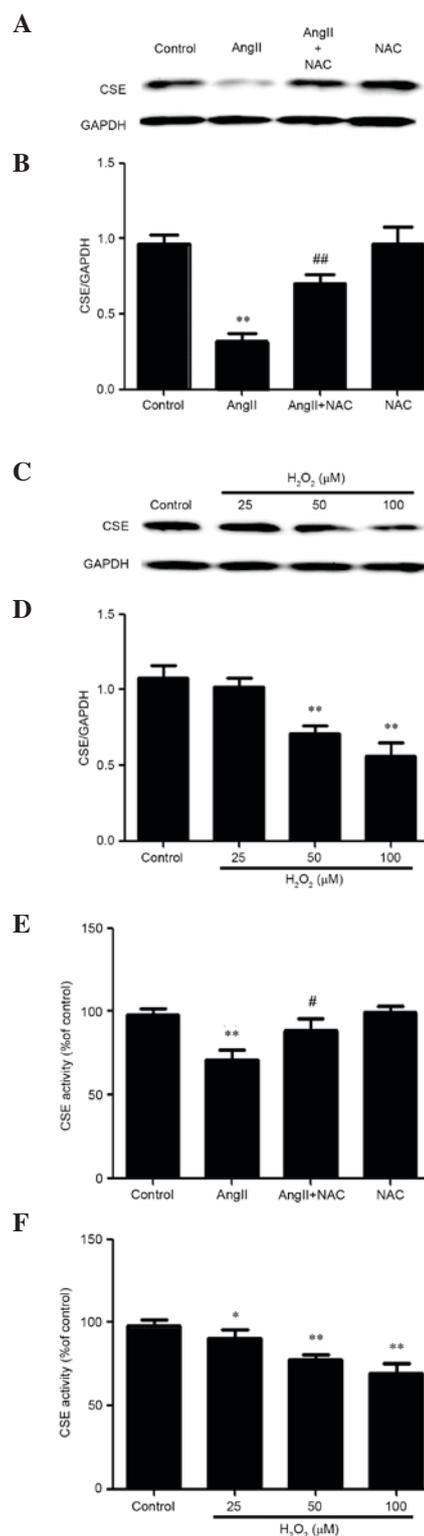


Figure 2. Stimulation of reactive oxygen species production suppressed the expression and activity of CSE in AngII-treated human umbilical vein endothelial cells. (A, B and E) Cells were treated with  $10^{-6}$ M AngII for 24 h, or were pretreated with 1 mM NAC for 1 h prior to AngII treatment for 24 h. (A and B) Protein expression levels of CSE were determined by western blotting, and (E) the activity levels of CSE were determined by methylene blue spectrophotometric assay. (C, D and F) Cells were treated with the indicated concentrations of  $H_2O_2$  for 24 h. (C and D) Protein levels of CSE were determined by western blotting, and (F) the activity levels of CSE were determined by methylene blue spectrophotometric assay. (B and D) Blots in A and C were determined by densitometric analysis. Data are presented as the means  $\pm$  standard error of the mean ( $n=3$ ). \*\* $P < 0.01$ , \* $P < 0.05$  vs. the control group, ## $P < 0.01$ , # $P < 0.05$  vs. the AngII treatment group. AngII, angiotensin II; NAC, N-acetyl-L-cysteine; CSE, cystathionine- $\gamma$ -lyase;  $H_2O_2$ , hydrogen peroxide.

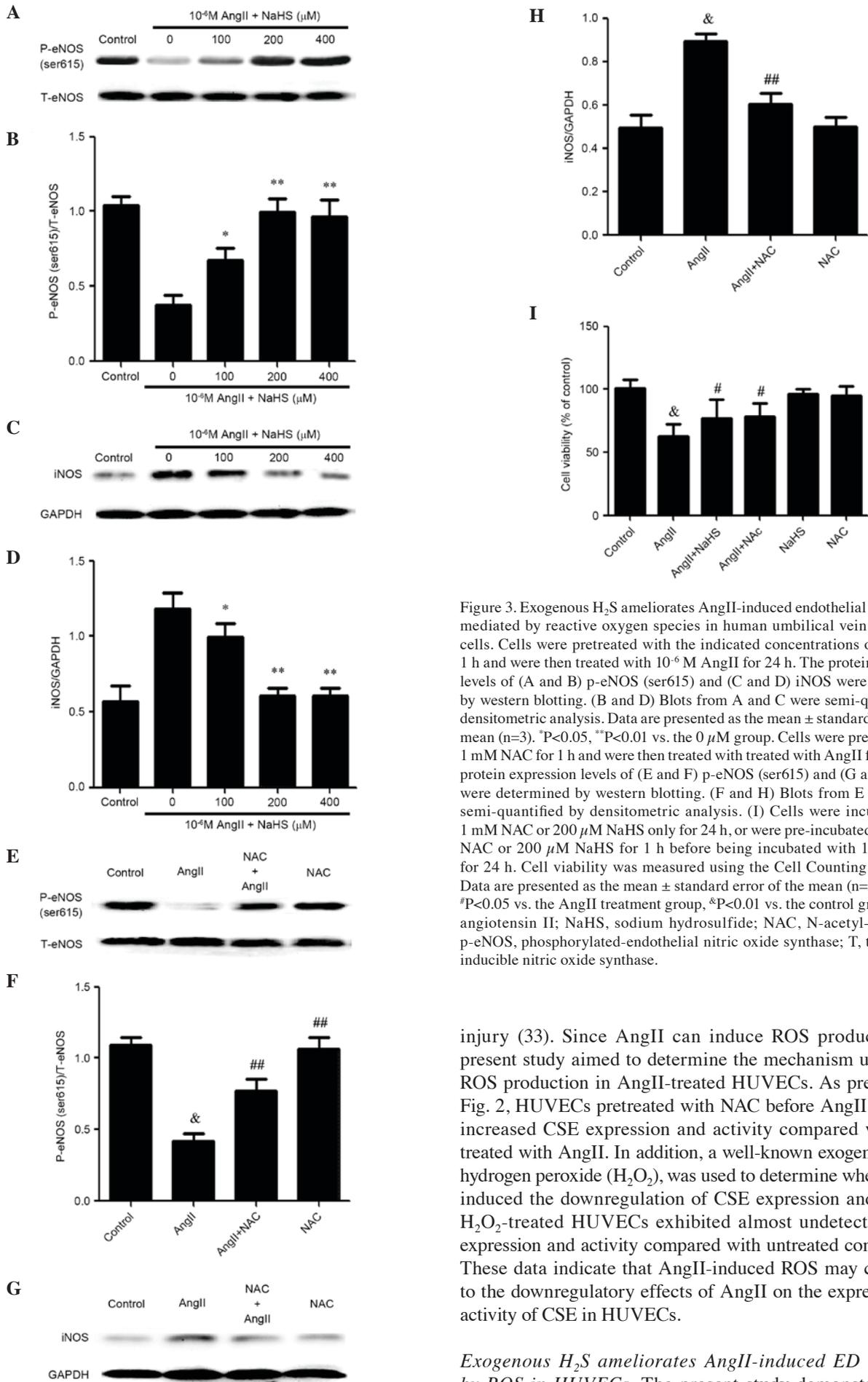
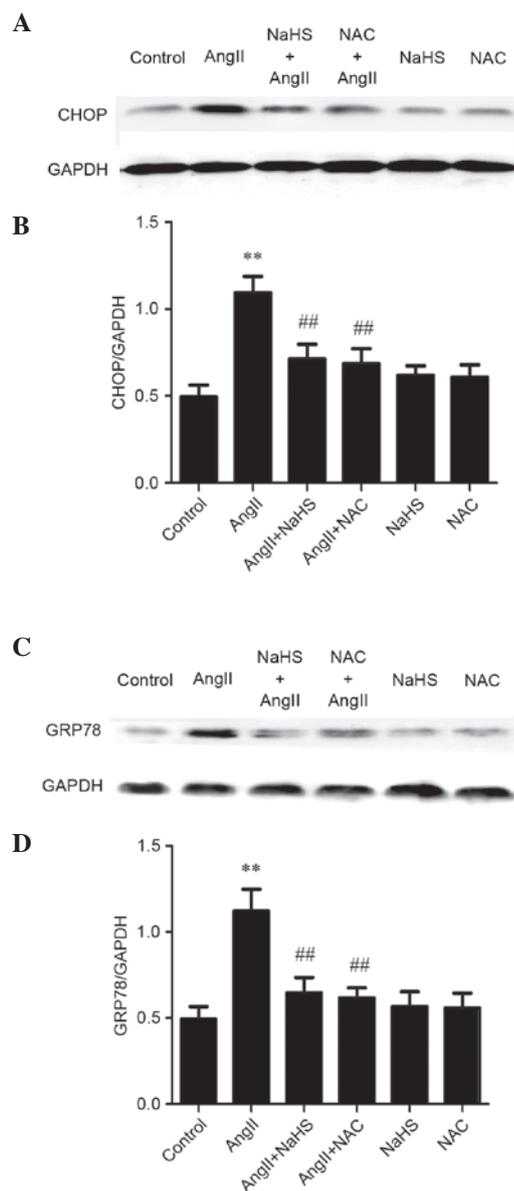


Figure 3. Exogenous H<sub>2</sub>S ameliorates AngII-induced endothelial dysfunction mediated by reactive oxygen species in human umbilical vein endothelial cells. Cells were pretreated with the indicated concentrations of NaHS for 1 h and were then treated with 10<sup>-6</sup> M AngII for 24 h. The protein expression levels of (A and B) p-eNOS (ser615) and (C and D) iNOS were determined by western blotting. (B and D) Blots from A and C were semi-quantified by densitometric analysis. Data are presented as the mean ± standard error of the mean (n=3). \*P<0.05, \*\*P<0.01 vs. the 0 μM group. Cells were pretreated with 1 mM NAC for 1 h and were then treated with treated with 10<sup>-6</sup> M AngII for 24 h. The protein expression levels of (E and F) p-eNOS (ser615) and (G and H) iNOS were determined by western blotting. (F and H) Blots from E and G were semi-quantified by densitometric analysis. (I) Cells were incubated with 1 mM NAC or 200 μM NaHS only for 24 h, or were pre-incubated with 1 mM NAC or 200 μM NaHS for 1 h before being incubated with 10<sup>-6</sup> M AngII for 24 h. Cell viability was measured using the Cell Counting kit-8 assay. Data are presented as the mean ± standard error of the mean (n=3). ##P<0.01, \*P<0.05 vs. the AngII treatment group, &P<0.01 vs. the control group. AngII, angiotensin II; NaHS, sodium hydrosulfide; NAC, N-acetyl-L-cysteine; p-eNOS, phosphorylated-endothelial nitric oxide synthase; T, total; iNOS, inducible nitric oxide synthase.

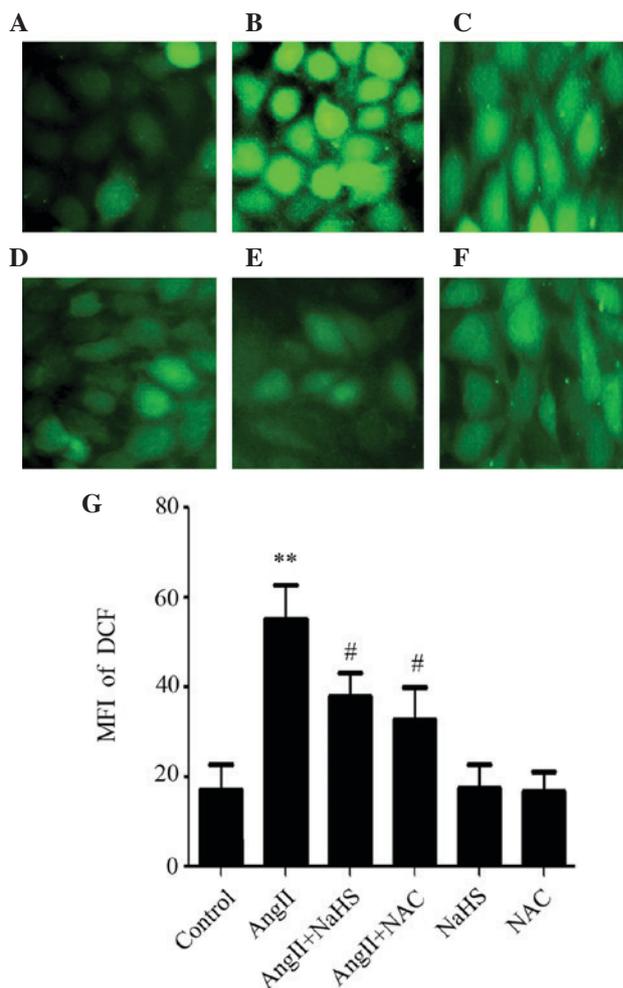
injury (33). Since AngII can induce ROS production, the present study aimed to determine the mechanism underlying ROS production in AngII-treated HUVECs. As presented in Fig. 2, HUVECs pretreated with NAC before AngII exhibited increased CSE expression and activity compared with cells treated with AngII. In addition, a well-known exogenous ROS, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), was used to determine whether ROS induced the downregulation of CSE expression and activity. H<sub>2</sub>O<sub>2</sub>-treated HUVECs exhibited almost undetectable CSE expression and activity compared with untreated control cells. These data indicate that AngII-induced ROS may contribute to the downregulatory effects of AngII on the expression and activity of CSE in HUVECs.

*Exogenous H<sub>2</sub>S ameliorates AngII-induced ED mediated by ROS in HUVECs.* The present study demonstrated that



**Figure 4.** Hydrogen sulfide supplementation or NAC ameliorates AngII-induced GRP78 and CHOP overexpression in human umbilical vein endothelial cells. Cells were incubated with  $10^{-6}$ M AngII,  $200 \mu\text{M}$  NaHS or  $1 \text{ mM}$  NAC for 24 h, or were pretreated with  $200 \mu\text{M}$  NaHS or  $1 \text{ mM}$  NAC for 1 h before being treated with  $10^{-6}$ M AngII for 24 h. The protein expression levels of (A and B) CHOP and (C and D) GRP78 were determined by western blot analysis. (B and D) Blots from A and C were semi-quantified by densitometric analysis. Data are presented as the mean  $\pm$  standard error of the mean ( $n=3$ ). \*\* $P<0.01$  vs. the control group, ## $P<0.01$  vs. the AngII treatment group. AngII, angiotensin II; NaHS, sodium hydrosulfide; NAC, N-acetyl-L-cysteine; CHOP, C/EBP-homologous protein; GRP78, glucose-regulated protein 78.

AngII can inhibit activation of the CSE/ $\text{H}_2\text{S}$  pathway in AngII-treated HUVECs; therefore, the present study used a well-known endothelial protective agent, NaHS, to determine whether NaHS could reduce AngII-induced ED. As presented in Fig. 3, cells treated with NaHS and AngII had lower levels of iNOS, compared with NaHS-unstimulated AngII-treated cells. Conversely, NaHS markedly ameliorated AngII-reduced p-eNOS (ser615) expression. The results suggest that treatment with  $200$  or  $400 \mu\text{M}$  NaHS may inhibit AngII-induced ED. Similarly, treatment with NAC significantly ameliorated



**Figure 5.** Hydrogen sulfide supplementation or NAC ameliorates AngII-induced reactive oxygen species generation in human umbilical vein endothelial cells. Cells were incubated with  $10^{-6}$ M AngII,  $200 \mu\text{M}$  NaHS or  $1 \text{ mM}$  NAC for 24 h, or were pretreated with  $200 \mu\text{M}$  NaHS or  $1 \text{ mM}$  NAC for 1 h before being treated with  $10^{-6}$ M AngII for 24 h. Cellular ROS accumulation was observed by microscopy following 2',7'-dichlorodihydrofluorescein diacetate staining. (A) Control; (B) AngII; (C) NaHS + AngII; (D) NAC + AngII; (E) NaHS and (F) NAC groups. Magnification,  $\times 400$ . (G) MFI was determined using ImageJ 1.41o software with quantitative analysis. Data are presented as the mean  $\pm$  standard error of the mean ( $n=5$ ). \*\* $P<0.01$  vs. the control group, # $P<0.05$  vs. the AngII treatment group. AngII, angiotensin II; NaHS, sodium hydrosulfide; NAC, N-acetyl-L-cysteine; MFI, mean fluorescent intensity; DCF, dichlorofluorescein.

the expression levels of AngII-suppressed p-eNOS (ser615) (Fig. 3E and F) and AngII-enhanced iNOS (Fig. 3G and H). Notably, cells treated with either NaHS or NAC alongside AngII exhibited improved cell viability compared with AngII-treated cells, whereas treatment with either NaHS or NAC alone did not alter cell viability in HUVECs (Fig. 3I). These data indicate that antioxidants may mediate the effects of  $\text{H}_2\text{S}$  on AngII-induced ED.

*H<sub>2</sub>S supplementation or NAC ameliorates AngII-stimulated GRP78 and CHOP overexpression in HUVECs.* The present study detected the potential mechanism underlying the protective effects of  $\text{H}_2\text{S}$  against AngII-induced ED in HUVECs. It has previously been demonstrated that ROS may induce ER stress. The present results indicated that treatment with the antioxidants NaHS or NAC could suppress AngII-induced

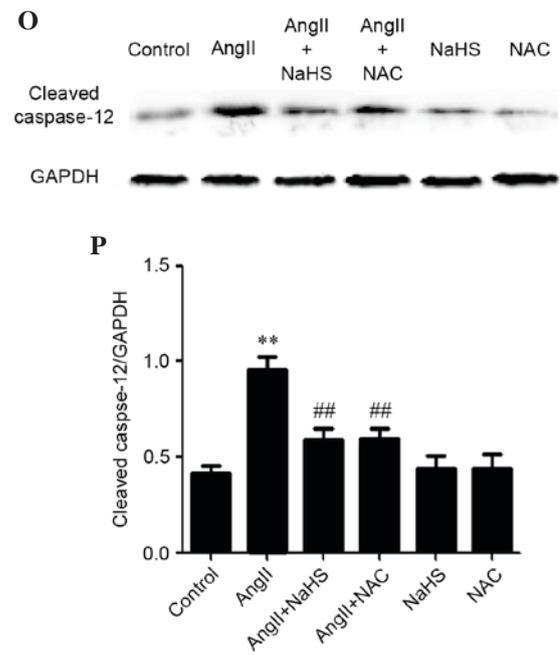
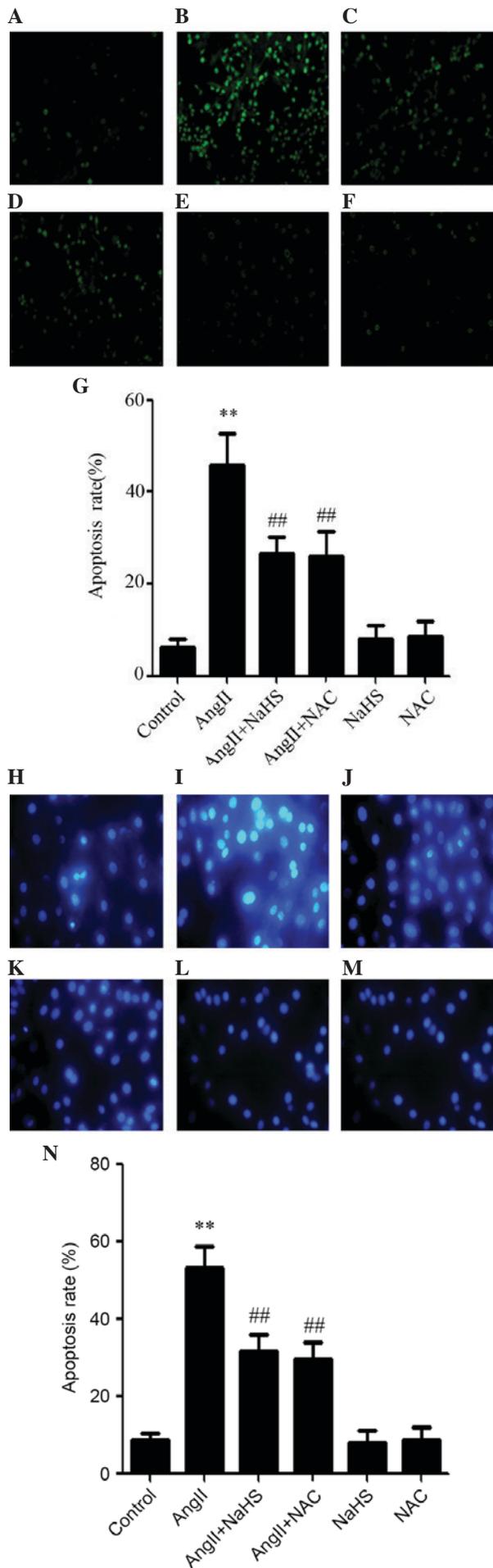


Figure 6. Exogenous hydrogen sulfide and NAC reduce AngII-induced apoptosis in HUVECs. Cells were incubated with  $10^{-6}$ M AngII, 200  $\mu$ M NaHS or 1 mM NAC for 24 h, or were pretreated with 200  $\mu$ M NaHS or 1 mM NAC for 1 h before being treated with  $10^{-6}$ M AngII for 24 h. (A-F) HUVECs apoptosis was observed under a microscope after Hoechst 33258 staining. Magnification,  $\times 200$ . (G) Mean fluorescent intensity was detected using ImageJ 1.41o software with quantitative analysis. (H-M) HUVECs apoptosis was observed under a microscope after terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling staining. Magnification,  $\times 100$ . (A and H) Control (untreated HUVECs); (B and I) AngII; (C and J) NaHS + AngII; (D and K) NAC + AngII; (E and L) NaHS and (F and M) NAC groups. (N) Mean fluorescent intensity was detected using ImageJ 1.41o software with quantitative analysis. (O and P) The protein levels of caspase-12 were determined by western blot analysis. (P) Blot presented in O was semi-quantified by densitometric analysis. Data are presented as the mean  $\pm$  standard error of the mean (n=5). \*\*P<0.01 vs. the control group, ##P<0.05 vs. the AngII treatment group. AngII, angiotensin II; NaHS, sodium hydrosulfide; NAC, N-acetyl-L-cysteine; HUVECs, human umbilical vein endothelial cells.

GRP78 and CHOP expression, whereas either NaHS or NAC alone did not alter the expression levels of GRP78 and CHOP in HUVECs (Fig. 4). These data suggest that the inhibitory effects of  $H_2S$  may protect against AngII-induced dysfunction in HUVECs via suppressing ER stress.

*H<sub>2</sub>S supplementation or NAC ameliorates AngII-induced ROS generation in HUVECs.* To determine whether the cytoprotective effects of  $H_2S$  against AngII-induced ED were due to its antioxidative activity, NaHS and NAC were used to treat HUVECs. AngII exposure markedly enhanced intracellular ROS generation in HUVECs (Fig. 5B). However, pretreatment with either NaHS or NAC for 60 min markedly attenuated AngII-induced ROS accumulation in HUVECs (Fig. 5C and D). Pretreatment with NAC had a similar antioxidant effect to NaHS against AngII-induced ROS accumulation, whereas NaHS or NAC treatment alone did not alter base intracellular ROS levels (Fig. 5E and F).

*Exogenous H<sub>2</sub>S and NAC reduce AngII-induced apoptosis in HUVECs.* The present study also determined the effects

of NaHS and NAC on AngII-induced apoptosis. As shown in Fig. 6, AngII-stimulated HUVECs exhibited typical characteristics of apoptosis, including chromatin condensation, nuclear shrinkage, and the presence of apoptotic bodies. However, treatment with either NaHS or NAC, alongside AngII, resulted in amelioration of the typical characteristics of apoptosis, including chromatin condensation, nuclear shrinkage, and the presence of apoptotic bodies compared with in cells treated with AngII only. Treatment with NaHS or NAC alone did not markedly alter cell morphology or the percentage of apoptotic HUVECs. Consistent with these results, the protein expression levels of cleaved caspase-12 were examined by western blot analysis, and were reduced in AngII-treated cells following NaHS or NAC treatment. However, treatment with either NaHS or NAC alone did not alter the expression of cleaved caspase-12. These findings indicate that exogenous H<sub>2</sub>S protects HUVECs against AngII-induced ER stress.

## Discussion

RAS activity and oxidative stress are two major interacting pathogenic factors associated with ED, which is the initiating factor and pathological basis of CVD (34,35). As an important mediator of endothelial function maintenance, reduced levels of nitric oxide (NO) could accelerate the ED process (36). Cellular and animal studies have demonstrated that NO, via constitutive NOS isoforms, modulates endothelial cell dilation and blood flow distribution, and low levels of p-eNOS activity are associated with injured endothelial function (37,38). However, high levels of iNOS also exert a similar effect (39). Nakao *et al* (40) demonstrated that suppressed p-eNOS and enhanced iNOS expression are associated with AngII cytotoxicity; together with the present findings, these results suggested that AngII may result in HUVECs dysfunction.

Emerging evidence from experimental and clinical research has indicated that ER stress is involved in CVD with ED (41) and RAS activity (42). ER stress modulates various factors, including GRP78 and CHOP. Oxidative stress (43) or AngII stimulation (44) have been suggested to trigger GRP78 and CHOP overexpression. Furthermore, AngII can upregulate the expression of inflammatory mediators, including GRP78 and CHOP, leading to cytotoxicity in HUVECs. The present findings are in agreement with those of a previous study, which demonstrated that high levels of AngII led to cardiac damage and vascular ED via ER stress (23). CSE serves atherogenic and endothelial cytoprotective roles in CVD (27,45). Conversely, AngII exerts cytotoxic effects, and in the present study AngII was shown to downregulate the expression and activity of CSE in HUVECs, which was accompanied by decreased cell viability. In addition, insufficient production of endogenous H<sub>2</sub>S or inhibition of its synthesis have been reported to enhance the overproduction of ROS in HUVECs (46). Taken together, it may be hypothesized that AngII-induced ER stress leads to ED in HUVECs via the inhibition of endogenous H<sub>2</sub>S production.

Insufficient production of endogenous H<sub>2</sub>S has been reported to be associated with AngII-induced cytotoxicity via oxidative stress (47). The present study, alongside a previous study, confirmed that ROS, such as H<sub>2</sub>O<sub>2</sub>, serve a primary

role in ER stress, are regulated by AngII and are responsible for the consequent generation of ED (48). Furthermore, the present study demonstrated that treatment with the exogenous ROS, H<sub>2</sub>O<sub>2</sub>, downregulated the expression and activity of CSE; the inhibitory effects of H<sub>2</sub>O<sub>2</sub> were similar to those of AngII in HUVECs. Subsequently, HUVECs were treated with NAC prior to AngII exposure; the results demonstrated that NAC suppressed overproduction of ROS and insufficient productions of endogenous H<sub>2</sub>S. These findings suggested that ROS serves a key role in the inhibition of CSE induced by AngII in HUVECs.

The present study also demonstrated that the effects of exogenous H<sub>2</sub>S on AngII-induced ED were due to antioxidative mechanisms. The findings indicated that H<sub>2</sub>S supplementation prior to AngII treatment in HUVECs resulted in a reduction in the expression levels of iNOS, an increase in p-eNOS (Ser615) expression and increased cell viability. Furthermore, treatment with NAC before AngII exposure not only exerted inhibitory effects on iNOS expression, but also enhanced p-eNOS (Ser615) expression and cell viability, whereas supplementation of H<sub>2</sub>S (NaHS) prior to AngII-treated HUVECs had similar effects. The present study further explored the potential mechanism underlying the protective effects of exogenous H<sub>2</sub>S, by treating cells with either NaHS or NAC prior to AngII exposure. Since NaHS or NAC exert similar antioxidative effects, NAC, similar to NaHS, was able to reduce ER stress, including GRP78 and CHOP expression. In addition, DCFH-DA was used to detect cellular ROS accumulation. The results indicated that the antioxidative effects of H<sub>2</sub>S may exert protection against AngII-induced ED by suppressing ER stress. ROS are associated with accelerated ER malfunction (49); the present study demonstrated that in HUVECs pretreated with NaHS or NAC prior to AngII exposure, ROS overproduction was inhibited.

ER stress is particularly associated with apoptosis. Song *et al* (50) demonstrated that GRP78 and CHOP are markedly activated in apoptosis. The present study pretreated HUVECs with NaHS or NAC, prior to AngII exposure, and TUNEL and Hoechst 33258 staining were used to detect apoptosis. The results indicated that pretreatment with either NaHS or NAC significantly decreased AngII-induced apoptosis and cleaved caspase-12 expression. In addition, Qabazard *et al* (51) reported that H<sub>2</sub>S significantly suppressed H<sub>2</sub>O<sub>2</sub>-induced ER stress in bovine aortic endothelial cells, and ameliorated cell apoptosis.

In conclusion, using AngII-treated HUVECs, the present study demonstrated that ROS participates in AngII-induced ER stress-mediated ED, by inhibiting the expression and activity of CSE. In addition, H<sub>2</sub>S supplementation may be considered a beneficial antioxidative therapy in AngII-induced ED via the suppression of ER stress. The results of the present study provide novel evidence to suggest that exogenous H<sub>2</sub>S may be considered a novel therapeutic strategy for the treatment of CVD.

## Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81100106), Aid Programme for Science and Technology Innovative Research Team.

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