

High glucose induces the release of endothelin-1 through the inhibition of hydrogen sulfide production in HUVECs

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Abstract. Hydrogen sulfide (H₂S) has recently been identified as an endogenous gaseous signaling molecule. In the vascular system, the formation of H₂S is catalyzed by cystathionine γ -lyase (CSE). Previous studies have demonstrated the protective effects of H₂S on ischemic injury in various types of tissue. However, little is known about the role of H₂S in diabetes-associated vascular diseases. Thus, the aim of the present study was to examine the possible role of H₂S in high glucose-induced vascular dysfunction, and to explore the underlying mechanisms. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins. The levels of H₂S following treatment with various levels of glucose were determined and the secretion of endothelin-1 (ET-1) was measured by ELISA. The mRNA and protein expression of CSE in the HUVECs was determined by real-time RT-PCR and western blot analysis, respectively. Treatment with high glucose (25 mmol/l) for 48 h significantly increased the secretion of ET-1 by HUVECs, with the concomitant suppression of H₂S production and CSE protein expression. The increase in exogenous H₂S levels through the administration of sodium hydrosulfide (NaHS) attenuated the high glucose-induced downregulation of CSE protein expression, and significantly inhibited the secretion of ET-1. These results suggest that the downregulation of CSE protein expression and the subsequent decrease in H₂S production play a role in high glucose-induced vascular dysfunction possibly by increasing the secretion of ET-1 by endothelial cells.

Introduction

Vascular diseases involving atherosclerosis are the major chronic complications of diabetes mellitus (DM) and the primary prognostic determinants of diabetic patients. It has been estimated that 75% of all deaths among diabetic patients are caused by cardiovascular complications (1). The mechanisms underlying diabetic vascular injuries, however, remain unclear.

Endothelial dysfunction, defined as an imbalance of endothelium-derived vasoconstrictor and vasodilator substances, precedes and dominates the pathogenesis and progression of both macro- and microvascular complications associated with diabetes (2). Nitric oxide (NO) is one of the most well characterized vasodilators, and is also the first gaseous molecule identified as a smooth muscle relaxer (3). Following the discovery of NO, carbon monoxide (CO) was found to have similar functions (4,5). Hydrogen sulfide (H₂S) was the third endogenous gasotransmitter identified following NO and CO (6). H₂S was first discovered in the brain as an endogenous neuromodulator (7,8). Shortly after, it was found that H₂S is also present in the endothelium and plays an important role in the regulation of vascular tone (9). To date, 3 enzymes that produce H₂S have been identified: cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE) and 3-mercaptopyruvate sulfur transferase (3MST). Both CBS and 3MST are predominantly expressed in the brain, whereas CSE is primarily localized in the vascular system (6,10). Numerous studies have demonstrated that H₂S is able to relax blood vessels and lower blood pressure by opening adenosine triphosphate (ATP)-sensitive potassium (K⁺) channels in vascular smooth muscle (9,11-13). In a previous study, the targeted deletion of the CSE gene in mice markedly reduced H₂S levels in serum and these mice had elevated blood pressure and reduced endothelium-dependent vasorelaxation (14). Moreover, H₂S has been shown to exert cytoprotective effects against ischemic injury in various animal models of acute ischemia (15-19).

Recently, there has been evidence suggesting that H₂S metabolism is dysregulated in diabetes. In non-obese diabetic (NOD) mice, it has been reported that endogenous H₂S production in the vascular system is significantly impaired, and that this is associated with marked endothelial dysfunction.

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tion (20). Similarly, it has been shown that plasma H₂S levels are markedly reduced in diabetic patients (21). Hyperglycemia is the hallmark of diabetes and has been recognized as an initiator of diabetic endothelial dysfunction. In the present study, the effects of high glucose on the production of H₂S in human umbilical vein endothelial cells (HUVECs) were investigated. Furthermore, the effects of H₂S on the secretion of endothelin-1 (ET-1) by HUVECs were also determined. The aim of the present study was to address the role and mechanisms of action of H₂S in endothelial dysfunction and vascular complications associated with diabetes.

Materials and methods

Cell culture. Human umbilical cords were collected from healthy full-term pregnant mothers during delivery, following the approval of the Shandong University Research Ethics Committee (Jinan, China). Signed informed consent was provided by all donors. The umbilical cords were collected from the Department of Obstetrics, Shandong Provincial Hospital from March to December 2010.

HUVECs were isolated from human umbilical veins and were identified by their cobblestone morphology under a microscope (Zeiss Axioplan; Zeiss, Weimar, Germany) and the strong positive immunoreactivity to von Willebrand factor (data not shown). The cells were grown in 5% CO₂ at 37°C in M199 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine and 20 ng/ml vascular endothelial growth factor (VEGF). For all the experiments, cells of passage 2-3 were used. When the cells were 80% confluent, they were grown in medium containing 2% FBS and treated with various concentrations of glucose. To maintain an equal osmotic pressure, 24.5 mmol/l D-mannitol was used to adjust the osmotic concentration.

Measurement of H₂S. The levels of H₂S were measured as previously described (22,23). Briefly, the cells were collected in 500 µl ice-cold 100 mmol/l potassium phosphate buffer (pH 7.4) and homogenized. The assay mixture containing homogenized cell lysates (430 µl), L-cysteine (10 mmol/l; 20 µl), pyridoxal 5'-phosphate (2 mmol/l, 20 µl) and phosphate-buffered saline (PBS; 30 µl) was incubated at 37°C for 30 min in tightly sealed Eppendorf vials. Zinc acetate [1% (w/v), 250 µl] was then injected to trap the generated H₂S followed by trichloroacetic acid (TCA) [10% (w/v), 250 µl] to precipitate the protein and thus terminate the reaction. Subsequently, *N,N*-dimethyl-*p*-phenylenediamine sulfate (NNDPD) (20 mmol/l; 133 µl) in 7.2 mol/l hydrochloric acid (HCl) was added followed by FeCl₃ (30 mmol/l; 133 µl) in 1.2 mol/l HCl and the absorbance (670 nm) of the aliquots of the resulting solution was determined. The H₂S concentration of each sample was calculated against a calibration curve of sodium hydrosulfide (NaHS; 0-250 µmol/l) and expressed as nanomoles of H₂S per milligram soluble protein.

Assay for the secretion of ET-1. The HUVECs were seeded in 60-mm cell dishes at 3×10⁵ cells/ml, followed by treatment with various concentrations of glucose. Cell supernatants were collected and the content of ET-1 was detected by ELISA

(R&D Systems, Minneapolis, MN, USA). The results were normalized to the cellular protein content in all experiments.

Real-time RT-PCR for CSE. Total RNA was isolated using TRIzol reagent (Takara Bio, Beijing, China) from the treated cells. For real-time RT-PCR, 160 ng template was used in a 10-µl reaction containing 8 pmol of each primer pair and 10 µl of SYBR-Green Premix Ex TaqII (Takara Bio). Reactions were performed using the following cycling conditions: 95°C for 15 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec. The value of each sample was calculated and expressed as the cycle threshold. The amount of gene expression for each sample was calculated as the difference (ΔCT) between the CT value of the target gene and the CT value of the endogenous control (β-actin). Relative expression was calculated as the difference (ΔΔCT) between the ΔCT values of the test and the control samples for the target gene. The relative level of expression was measured as 2^{-ΔΔCT}. The human primers used were as follows: CSE, forward, CAC TGTCACCACGTTCAAG and reverse, GTGGCTGCTAA ACCTGAAGC; β-actin, forward, ACAGAGCCTCGCCTT TGCCG and reverse, ACATGCCGGAGCCGTTGTCTG.

Western blot analysis for CSE. The cells were homogenized in RIPA lysis buffer with 1% proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF solution). The protein concentration was measured using the Bradford method. A total of 40 µg of total protein was separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked in 5% BSA in TBST solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 30 min at room temperature, followed by incubation for 1 h at room temperature in 1% BSA in TBST solution containing monoclonal anti-CSE antibody (1:4,000, Cat. no. H00001434-M01; Abnova, Taipei, Taiwan). Following incubation with horseradish peroxidase-conjugated secondary antibodies (1:5,000, Cat. no. DkxMu-003-DHRPX), the membranes were washed and developed using an enhanced chemiluminescence kit. Anti-β-actin was routinely blotted and used as a protein loading control. The quantification of band intensity upon western blot analysis was conducted using NIH Image software (ProteinSimple, Santa Clara, CA, USA).

Statistical analysis. All data are presented as the means ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA. The Student-Newman-Keuls test was used for comparisons between groups. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

High glucose reduces the production of H₂S in HUVECs. The effects of high glucose on the production of H₂S in HUVECs were investigated. As shown in Fig. 1, treatment with high glucose concentrations (25 mmol/l) for 48 h significantly reduced the production of H₂S compared with treatment with low glucose (5.5 mmol/l).

High glucose suppresses the expression of CSE in HUVECs. To elucidate the mechanisms responsible for the inhibition of H₂S production by high glucose, further experiments were

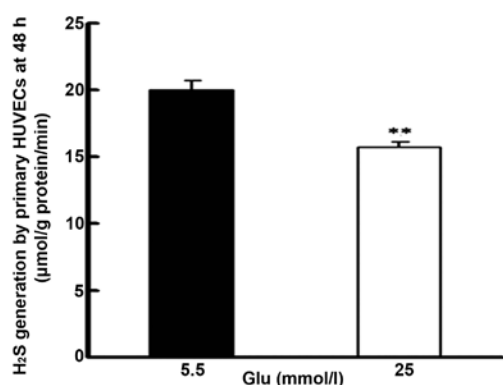


Figure 1. Effect of high glucose on L-cysteine-induced hydrogen sulfide (H₂S) production in human umbilical vein endothelial cells (HUVECs). Cells were cultured in the presence of 5.5 or 25 mmol/l glucose for 48 h and the production of H₂S was determined with L-cysteine as a substrate. Data represent the means \pm SD of 3 independent experiments. **P<0.01.

conducted to determine the effects of high glucose on the expression of CSE. As shown in Fig. 2B, treatment of the HUVECs with high glucose (25 mmol/l) did not significantly alter the CSE mRNA levels compared to treatment with low glucose (5 mmol/l). By contrast, compared to treatment with 5.5 mmol/l glucose, treatment with 25 mmol/l glucose significantly inhibited the CSE protein expression in a time-dependent manner (Fig. 2A and C).

Exogenous H₂S inhibits the high glucose-induced secretion of ET-1 by HUVECs. As shown in Fig. 3, treatment with 25 mmol/l glucose significantly increased the level of ET-1 following 24 and 48 h of treatment. Pre-treatment for 30 min with 50 μ mol/l NaHS inhibited the high glucose-induced secretion of ET-1 at each time point.

Effects of NaHS on CSE protein expression in HUVECs. To determine whether NaHS has an effect on CSE expression, the HUVECs were cultured in 5.5 mmol/l or 25 mmol/l glucose, and treated with 50 or 100 μ mol/l NaHS for 48 h. The CSE protein levels were then determined by western blot analysis. As shown in Fig. 4, treatment with 25 mmol/l glucose for 48 h significantly reduced CSE protein expression. This response was significantly attenuated by NaHS at both concentrations examined (50 and 100 μ mol/l). However, NaHS at these concentrations showed no evident effect on the basal CSE protein expression in the cells cultured under normal glucose conditions (5.5 mmol/l).

Discussion

As a novel gasotransmitter, H₂S has been demonstrated to play important roles in the pathophysiology of several biological systems. In particular, H₂S has been investigated extensively in the cardiovascular system. The association between H₂S and hypertension was firstly investigated in a study on hypertensive rats (24). In that study, the authors demonstrated that in the hypertensive rats, the level of endogenous H₂S was reduced and that exogenous H₂S effectively prevented the development of hypertension (24). In the nervous system, H₂S has been demonstrated to protect neurons from apoptosis by increasing the production of the antioxidant, glutathione, thus reducing the toxic effects induced by glutamic acid (25). In addition, H₂S has been shown to play important roles in the digestive system (26,27) and the respiratory system (28-30). To date, little is known however about the role of H₂S in diabetes-associated vascular complications. In the present study, the effects of high glucose on the production of H₂S in HUVECs were investigated, as well as the effects of exogenous H₂S on the secretion of ET-1.

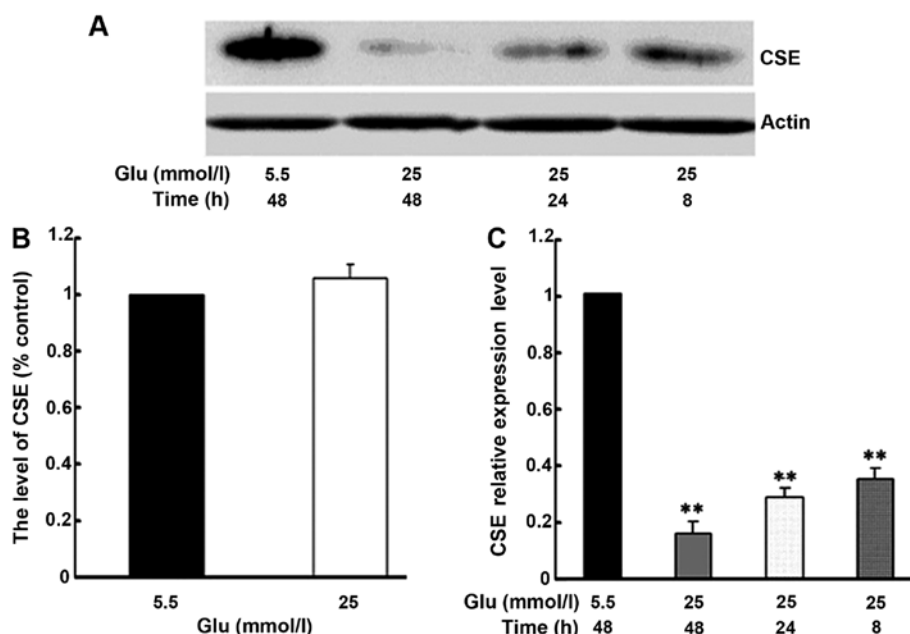


Figure 2. High glucose suppresses cystathionine γ -lyase (CSE) protein expression but not mRNA expression in human umbilical vein endothelial cells (HUVECs). Real-time RT-PCR and western blot analysis were performed to determine CSE expression at the indicated time points upon glucose challenge. (A) CSE protein expression; (B) CSE mRNA expression following glucose challenge for 48 h; (C) quantitative analysis of CSE protein expression (determined as ratio of band densities for CSE vs. β -actin). **P<0.01, vs. 5.5 mmol/l glucose group for 48 h. Data represent the means \pm SD of 3 different experiments.

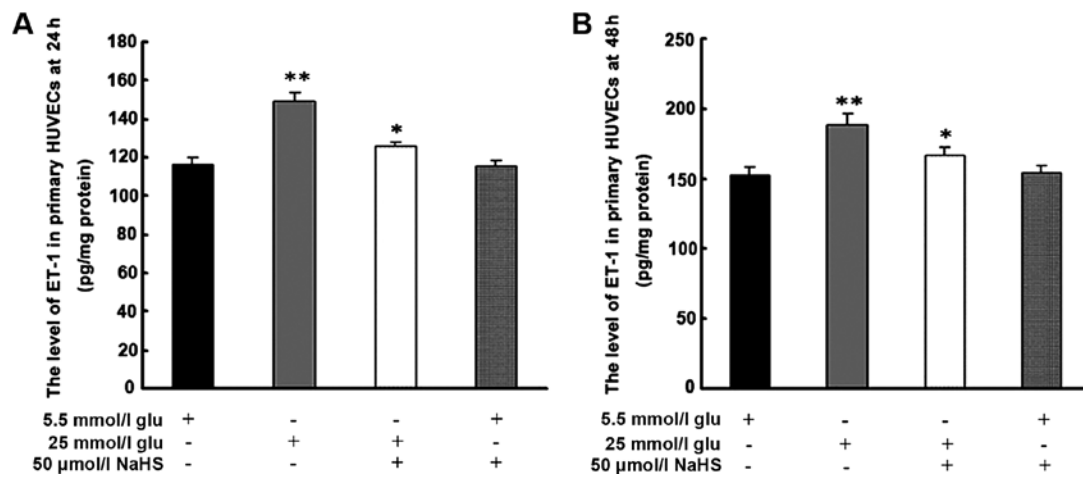


Figure 3. Exogenous hydrogen sulfide (H_2S) inhibits the high glucose-induced secretion of endothelin-1 (ET-1) in human umbilical vein endothelial cells (HUVECs). Cells were pre-treated or not with 50 μ mol/l sodium hydrosulfide (NaHS) for 30 min, followed by treatment with 5.5 or 25 mmol/l of glucose for the indicated periods of time. The ET-1 level was determined by ELISA. (A) Twenty-four hours post-treatment. * $P < 0.05$ vs. 25 mmol/l glucose group, ** $P < 0.01$ vs. 25 mmol/l glucose group; (B) Forty-eight hours post-treatment. * $P < 0.05$ vs. 5.5 mmol/l glucose group, ** $P < 0.01$ vs. 25 mmol/l glucose group. Data represent the means \pm SD of 3 different experiments.

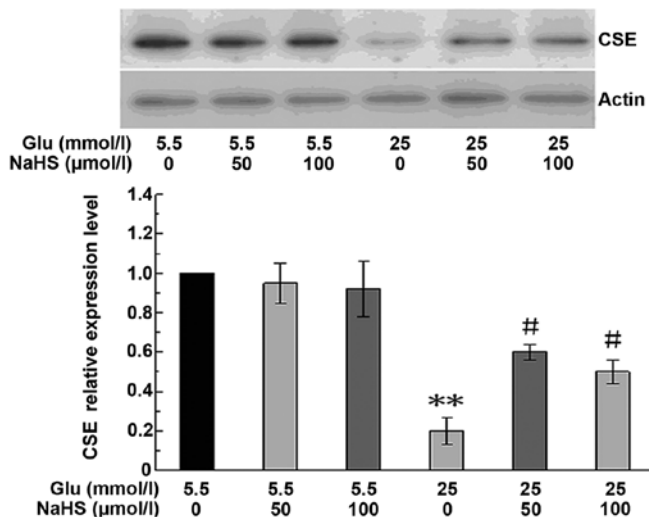


Figure 4. Effects of sodium hydrosulfide (NaHS) on cystathionine γ -lyase (CSE) protein expression in human umbilical vein endothelial cells (HUVECs). HUVECs were cultured in 5.5 mmol/l glucose or 25 mmol/l glucose in the presence or absence of sodium hydrosulfide (NaHS) for 48 h. CSE protein expression was determined by western blot analysis. ** $P < 0.01$, vs. 5.5 mmol/l glucose group, # $P < 0.05$, vs. 25 mmol/l glucose group. Data represents the results of 3 independent experiments.

A previous study demonstrated that a decrease in the plasma levels of H_2S correlated with coronary heart disease (CHD) risk factors, such as smoking, hypertension and high blood glucose levels (31). The direct association between H_2S and high blood glucose levels, however, remains unclear. In the present study, treatment with high glucose reduced the production of H_2S in the HUVECs. In the vascular system, CSE is the primary enzyme responsible for H_2S production (6). To investigate the mechanisms through which high glucose reduces the production of H_2S , CSE protein expression and mRNA levels were analyzed by western blot analysis and real-time RT-PCR, respectively in the current model. The results revealed that high glucose significantly reduced CSE protein expression

in the HUVECs, whereas the CSE mRNA expression was not affected. The precise mechanisms involved however are unclear and require further investigation.

ET-1 is one of the most potent endogenous vasoconstrictors released by endothelial cells (32). The balance between NO and ET-1 plays an essential role in the maintenance of vascular tone. It is known that the effects of ET-1 are mediated by a G-protein coupled receptor (33). In response to various endothelial injuries, the release of ET-1 is increased. Consequently, the level of ET-1 is recognized as an indicator of endothelial dysfunction (34). In addition, it has been shown that ET-1 expression is upregulated by high blood glucose levels (35,36). In accordance with the above observations, the present study demonstrated that high glucose increased the secretion of ET-1 from primary HUVECs.

In the present study, the high glucose-induced secretion of ET-1 coincided with the reduced CSE protein expression and the reduced generation of H_2S . Therefore, the effects of H_2S on the release of ET-1 in HUVECs were investigated. NaHS is a widely used source of exogenous H_2S . When dissolved in solution, NaHS rapidly dissociates to Na^+ and HS^- . Following this, HS^- associates with H^+ to produce H_2S (37). Our results demonstrated that NaHS, as a donor of exogenous H_2S , significantly inhibited the high glucose-induced release of ET-1 by HUVECs, consistent with the results of previous studies (38,39). Taken together, these data suggest that H_2S may be an upstream regulator of ET-1. In addition to direct conversion into H_2S , it has been suggested that NaHS may also indirectly influence the endogenous generation of H_2S . In guinea pigs with allergic rhinitis (AR), NaHS was shown to increase CSE expression and H_2S production (40). In cultured rat vascular smooth muscle cells, methylglyoxal (MG), an intermediate of glucose metabolism, decreased cellular H_2S levels by downregulating CSE protein expression (41). At the same time, NaHS prevented the reduction in CSE protein expression by reducing the cellular MG levels (41). In the present study, NaHS prevented the high glucose-induced downregulation of CSE expression, but did not affect CSE protein expression under normal glucose condi-

tions. These results suggest that NaHS may indirectly influence the production of H₂S by regulating CSE protein expression in a high glucose environment.

In conclusion, the results of the present study suggest that the induction of ET-1 secretion by high glucose may be partially mediated through the downregulation of CSE protein expression and thereby, the reduction of the production of H₂S. This study also raises the possibility of the use of NaHS as a potential therapeutic agent for diabetic vascular complications. Additional studies are required to confirm these findings *in vivo*.

Acknowledgements

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