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Rescue of mesangial cells from high glucose-induced over-proliferation and extracellular matrix secretion by hydrogen sulfide

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Abstract

Background. Hydrogen sulfide (H_2S) is considered as the third gasotransmitter after nitric oxide and carbon monoxide. This gas molecule participates in the regulation of renal function. Diabetic nephropathy (DN) is one of the major chronic complications of diabetes. The present study aimed to explore the changes in H_2S metabolism in the early stage of DN and the effects of H_2S on cultured rat renal glomerular mesangial cells (MCs).

Methods. Cultured rat MCs and streptozotocin (STZ)-induced diabetic rats were used in this study. Expression levels of cystathionine γ-lyase (CSE), transforming growth factor-β1 (TGF-β1) and collagen IV in rat renal cortex and in cultured MCs were determined by quantitative real-time PCR and western blot. Reactive oxygen species (ROS) released from rat MCs was assessed by fluorescent probe assays. MCs proliferation was analyzed by 5′-bromo-2′-deoxyuridine incorporation assay.

Results. H_2S levels in the plasma and renal cortex and the levels of CSE messenger RNA (mRNA) and protein in renal cortex were significantly reduced, while the levels of TGF-β1 and collagen IV increased 3 weeks after STZ injection. Administration of NaHS, a H_2S donor, reversed the increases in TGF-β1 and collagen IV in diabetic rats. By contrast, NaHS did not alter the TGF-β1 and collagen IV levels in non-diabetic rats. But NaHS lowered the CSE mRNA level in renal cortex. Exposure to high glucose promoted ROS generation and cell proliferation, up-regulated the expression of TGF-β1 and collagen IV but decreased the CSE expression in cultured MCs. Treatment of cultured MCs with NaHS reversed the effect of high glucose. NaHS did not change ROS generation, cell proliferation, TGF-β1 and collagen IV expression in the cells cultured with normal glucose. Reduction of endogenous H_2S generation by DL-propargylglycine, a CSE inhibitor, produced similar cellular effects as high glucose, including increases in cell proliferation, TGF-β1 and collagen IV expressions and ROS generation.

Conclusion. Suppressed CSE-catalyzed endogenous H_2S production in the kidney by hyperglycemia may play an important role in the pathogenesis of DN.

Keywords: cystathionine γ-lyase; diabetic nephropathy; hydrogen sulfide; mesangial cell

Introduction

Diabetic nephropathy (DN) is one of the most common complications associated with diabetes and a major pathological cause of chronic renal dysfunction. DN is characterized by a progressive loss of glomerular filtration surface areas and capillary volume. The latter is largely due to an aberrant expansion of the mesangial matrix derived from excessive production and deposition of extracellular matrix [1,2]. Previous studies confirmed that high glucose induces excessive production of reactive oxygen species (ROS) and up-regulates the expression of transforming growth factor-β1 (TGF-β1) in renal mesangial cells (MCs) and tubular epithelial cells. Consequently, extracellular matrix accumulates excessively in the kidney, results in glomerular sclerosis and tubulointerstitial fibrosis [3,4]. However, the molecular mechanisms for the hyperglycaemia-induced DN have not been clear.

Hydrogen sulfide (H_2S), the third gasotransmitter after nitric oxide and carbon monoxide [5], is produced in significant amounts in almost all tissues or organs, including brain, cardiovascular system, pancreas [6], liver and kidney [7]. In Sprague–Dawley (SD) rats, the concentrations of H_2S in the brain and plasma have been reported to be 50–160 μM [5,8]. H_2S generation is mainly catalyzed by two pyridoxal

5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) [9]. CBS is the predominant H_2S-synthesizing enzyme in the central nervous system, while CSE is present in the cardiovascular system. In liver and kidney, both enzymes are distributed [9,10]. H_2S plays an important role in the regulation of both physiological and pathological functions of multiple organs. In the nervous system, H_2S is involved in regulating the learning and memory processes. In the cardiovascular system, H_2S relaxes blood vessels, inhibits smooth muscle cell proliferation and reduces oxidative damage [9]. In addition, H_2S participates in the functional regulation of the digestive system, urogenital system and metabolism [9,11]. Few previous studies focused on the role of H_2S in regulating renal function under both physiological and pathological conditions [12]. The present study was designed to determine the metabolism profile of H_2S in earlier stage of diabetes and the effect of H_2S in cultured renal MCs that are important cellular component of DN.

Materials and methods

Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, streptozotocin (STZ), D-glucose, mannitol, DL-propargylglycine (PPG) and NaHS were purchased from Sigma (St Louis, MO). NaHS was used as a H_2S donor as widely used in previous studies [9,13,14]. Antibodies against β-actin or collagen IV were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). CSE antibody was from Abnova Corp. (Taipei, Taiwan). M-MLV reverse transcriptase was from Promega Co. (Madison, WI). SYBR Green QRT-PCR Master Mixture was from Applied Biosystems (Tokyo, Japan). RNA extraction kit was from Sangon Co. (Shanghai, China). Polyvinylidene difluoride (PVDF) membrane was from Amersham (Piscataway, NJ). Kodak X-Omat K film was from Kodak Co. (Xiamen, China). Enhanced chemiluminescent detection kit (ECL detection kit) was from Pierce Biotechnology Inc. (Rockford, IL). 5′-Bromo-2′-deoxyuridine (BrdU) incorporation kit was from Roche (Mannheim, Germany). ROS assay kit was from Beyotime (Jiangsu, China). All other reagents used in this study were of analytical grade.

Animal model

Age-matched male SD rats, weighing 180–210 g, were provided by the Shanghai SLAC Laboratory Animal Center. All procedures followed the Criteria of the Medical Laboratory Animal administrative Committee of Shanghai and the Guide for Care and Use of Laboratory Animals of Fudan University. The rats were held for 5-day acclimatization in the animal care facility before use and had free access to water and standard chow.

Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (65 mg/kg) dissolved in 0.1 M sodium citrate buffer (pH 4.0). Only those rats with plasma glucose concentrations >16.7 mM 1 week after STZ injection were recruited in the study [15]. The rats were randomly divided into four groups ($n = 6$ for each group): (i) control (C) rats, injected with vehicle (0.1 M sodium citrate buffer, pH 4.0); (ii) diabetic (D) rat; (iii) diabetic rats with injection of NaHS (D + NaHS) and (iv) non-diabetic rats with injection of NaHS (C + NaHS) (50 μmol/kg/day, i.p.) during the third week [16]. At the end of the third week, rats were sacrificed and the plasma and renal tissues were harvested and stored at −80 °C until use.

Plasma glucose, creatinine, urea nitrogen and urea protein determination

Glucose, creatinine and urea nitrogen concentrations in plasma were determined by glucose detection kit, creatinine detection kit and urea nitrogen detection kit, respectively (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China) and urea protein was detected by urea protein detection kit (Jiancheng Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions.

Cell culture

The rat glomerular MC line (HBZY-1) was purchased from China Center for Type Culture Collection (Wuhan, China) and cultured in normal DMEM media (5.5 mM D-glucose) supplemented with 10% neonatal bovine serum in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. High glucose culture media was made by supplementing normal DMEM media with additional D-glucose for a final D-glucose concentration at 30 mM. The osmotic control media was made by supplementing normal media with 24.5 mM mannitol [17,18].

Cell proliferation assay

A total of 10^3 cells per well were cultured in 96-well plates. When the cells reached 60–70% confluence, they were serum starved for 24 h and then treated with high glucose media, NaHS or PPG. After 48 h, cell proliferation was assessed by BrdU incorporation assay as described previously [19]. Briefly, 10 μM BrdU labeling solution was added into the media and incubated at 37 °C for 4 h and then the cells were fixed, denatured and incubated in anti-BrdU-POD (peroxidase) antibody for another 90 min at room temperature. At the end of incubation, the cells were rinsed with phosphate-buffered solution (PBS), pH 7.0, three times to remove excessive antibody and then 100 μL of substrate solution was added into each well. After 30 min incubation at room temperature, the absorbance of the samples was measured on a TECAN Infinite M200 microplate reader (Salzburg Umgebung, Salzburg, Austria) at 370 nm, while the absorbance obtained at 492 nm served as a reference value.

Western blot analysis

Renal cortex or cultured renal MCs were lysed in 1× sodium dodecyl sulfate (SDS) supplemented with proteinase inhibitor at a dilution of 1:25. Protein concentrations were determined by bicinchoninic acid protein assay kit (Shenergy Biocolor BioScience and Technology, Shanghai, China). Thirty micrograms of protein lysate was electrophoresed on a 12% polyacrylamide SDS gel and transblotted onto a PVDF membrane at 270 mA for 90 min. The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) and 0.1% Tween (TBS/Tween) for 1 h at room temperature with gentle rocking and then incubated in mouse anti-rat CSE antibodies (1:150) or goat anti-rat collagen IV antibodies (1:2000) at 4 °C overnight. After three washes with TBS/Tween, the membranes were incubated with secondary anti-mouse/goat antibody (1:2000) for 1 h at room temperature. The hybridizing signals were developed using the ECL detection kit according to the manufacturer's instructions and exposed to X-ray film. Then the membranes were stripped and reprobed with mouse anti-rat β-actin antibody (1:10000) and developed as described above. The relative intensity of the bands exposed on the films was quantified using Smart viewer software (Furi Technology Co, Shanghai, China). The relative protein level was normalized by intensity of β-actin and the averaged relative protein level in control group is defined as 1.0.

Isolation of total RNA and synthesis of cDNA

Cultured rat MCs or renal cortex was lysed in TRIZOL reagent and total RNA was isolated. The amount of RNA isolated was determined by measuring the specific absorbance at 260 nm. One microgram of total RNA was used for cDNA synthesis in a 20 μL reaction mixture that contained 1 μg oligo dT, 10 mM dNTP, 20 U RNase inhibitor and 200 U M-MLV reverse transcriptase. A 1-μL aliquot of the resulting single-strand cDNA was used for polymerase chain reaction (PCR).

Quantitative real-time PCR

SYBR Green qRT-PCR was used to quantify the relative abundance of target messenger RNA (mRNA) in the samples. The accumulated fluorescence was detected using the iCycler iQ PCR detection system (Bio-Rad, Hercules, CA). The PCR amplification conditions were as follows: pre-denaturing at 95 °C for 3 min, followed by 40 cycles of amplifications by denaturing at 95 °C for 30 s, annealing at 62 °C (for TGF-β1), 60 °C [for collagen IV and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] or 58 °C (for CSE) for 1 min, extension at 72 °C for 1 min. After a final extension at 72 °C for 10 min, the amplified products were subjected to a stepwise increase in temperature from 55 to 95 °C to construct dissoci-

ation curves. The relative amount of each mRNA was normalized to the housekeeping gene, GAPDH. Each sample was run and analyzed in triplicate. The average of the relative amount of each mRNA in control group is defined as 1.0. All PCR primer sequence and product characteristics are listed in Table 1.

Measurement of H_2S in renal cortex homogenates and plasma

H_2S in renal cortex homogenates was measured by a method used before [20]. Briefly, renal cortex (~50 mg) was homogenized in 500 μL lyses buffer (100 mM potassium phosphate buffer, pH 7.4) containing 10 mM sodium orthovanadate. Protein concentration of homogenate was measured by a protein quantitative analysis kit (Shenergy Biocolor BioScience and Technology). Four hundred and thirty microliters of homogenate was added in a reaction mixture containing piridoxal-5′-phosphate (2 mM, 20 μL), L-cysteine (10 mM, 20 μL) and adjusted the final volume to 500 μL with ddH_2O. The reaction was initiated by transferring tube from ice to a water bath at 37 °C. After incubation for 30 min, 250 μL of 1% (wv-1) zinc acetate (ZnAC) was mixed into the reaction. Then, 250 μL of 10% (wv-1) trichloroacetic acid (TCA), 133 μL of 20 mM *N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl and 133 μL of 30 mM $FeCl_3$ in 1.2 M HCl were added in sequence. After reactions were terminated, the absorbance at 670 nm was measured on a spectrophotometer (TECAN Infinite M200 Systems Inc., Salzburg Umgebung, Salzburg, Austria). All samples were assayed in duplicate. The optimal density value obtained at 670 nm was normalized by protein concentration and extrapolated from the standard curve obtained from the same plate.

Plasma H_2S was detected by using 100 μL plasma sample in each reaction. The assay procedure was the same as to measuring H_2S in tissue. After reaction was terminated, the absorbance at 670 nm was measured on spectrophotometer and H_2S concentration was extrapolated from the standard curve obtained from the same plate [21].

Measurement of ROS production

Experiments were performed using the ROS assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Briefly, cells seeded in 96-well plates were incubated with 10 mM 2',7'-Dichlorofluorescein diacetate probes (100 μL/well) at 37 °C for 30 min and then washed with PBS three times in order to remove residual probes. The fluorescence intensity at 488 nm excitation wavelength and 525 nm emission wavelength was measured using a luminometer (Tecan, Salzburg, Austria).

Statistical analysis

Data were means ± standard error of the means and analyzed using one-way analysis of variance with the Bonferroni correction for all pairwise comparisons. A P-value of <0.05 was considered statistically significant.

Results

Changes in H_2S level and the expression of CSE and collagen IV in diabetic rat renal cortex

Three weeks after STZ injection, the plasma glucose concentration in both diabetic rats and NaHS-treated diabetic rats was significantly higher, while body weight was lower than that of control rats (Table 2). Blood creatinine, urea nitrogen and urea protein were elevated in diabetic rats and NaHS-treated diabetic rats. NaHS treatment did not change plasma glucose concentration in diabetic and non-diabetic rats nor affected the elevated blood creatinine

Table 1. The primer sets and PCR product characteristics[a]

Target	Oligonucleotide sequence	Tm	Product size (bp)
TGF-β1	F: 5′-TGGCGTTACCTTGGTAACC-3′ R: 5′-GGTGTTGAGCCCTTTCCAG-3′	62	277
Collagen IV	F: 5′-ATTCCTTTGTGATGCACACCAG-3′ R: 5′-AAGCTGTAAGCATTCGCGTAGTA-3′	60	151
CSE	F: 5′-GACGAGGAATTGCTTGGAAA-3′ R: 5′-GATGCCACCCTCCTGAAGTA-3′	58	180
GAPDH	F: 5′-CCTTCATTGACCTCAACTACATG-3′ R: 5′-CTTCTCCATGGTGGTGAAGAC-3′	60	216

[a]F, forward; R, reverse.

Table 2. Characterization of the experimental groups of rat

	Control	Diabetes (1–21 days)	Diabetes + NaHS (15–21 days)	Control + NaHS (15–21 days)
Weight (g)				
0 days	207.50 ± 4.23	209.29 ± 2.30	210.50 ± 3.83	205.00 ± 2.83
7 days	244.17 ± 5.23	218.57 ± 6.14**	220.50 ± 3.96	244.38 ± 3.95
14 days	296.67 ± 2.79	232.14 ± 6.80**	233.00 ± 5.12	295.00 ± 2.99
21 days	310.83 ± 9.87	242.86 ± 9.12**	250.50 ± 5.75	303.16 ± 4.72
Urea protein (mg)				
0 days	41.29 ± 2.59	42.14 ± 4.35	43.60 ± 3.61	40.95 ± 3.73
7 days	36.81 ± 3.35	52.70 ± 8.61	51.75 ± 3.99	37.42 ± 2.80
14 days	34.48 ± 3.96	58.62 ± 3.82**	56.52 ± 4.34	37.52 ± 4.03
21 days	35.71 ± 4.98	61.74 ± 4.12**	48.46 ± 3.50#	40.00 ± 5.28
Blood glucose (mmol/L) 21d	4.83 ± 0.43	19.56 ± 2.15***	22.66 ± 0.74	5.82 ± 0.67
Blood creatinine (μmol/L) 21 days	46.04 ± 2.12	70.89 ± 0.96**	69.18 ± 3.67	49.35 ± 1.64
Blood urea nitrogen (mmol/L) 21 days	8.87 ± 0.74	14.87 ± 1.32**	11.01 ± 0.59#	9.03 ± 1.12

**P < 0.01, ***P<0.001 versus control; #P < 0.05; ##P < 0.01 versus diabetes.

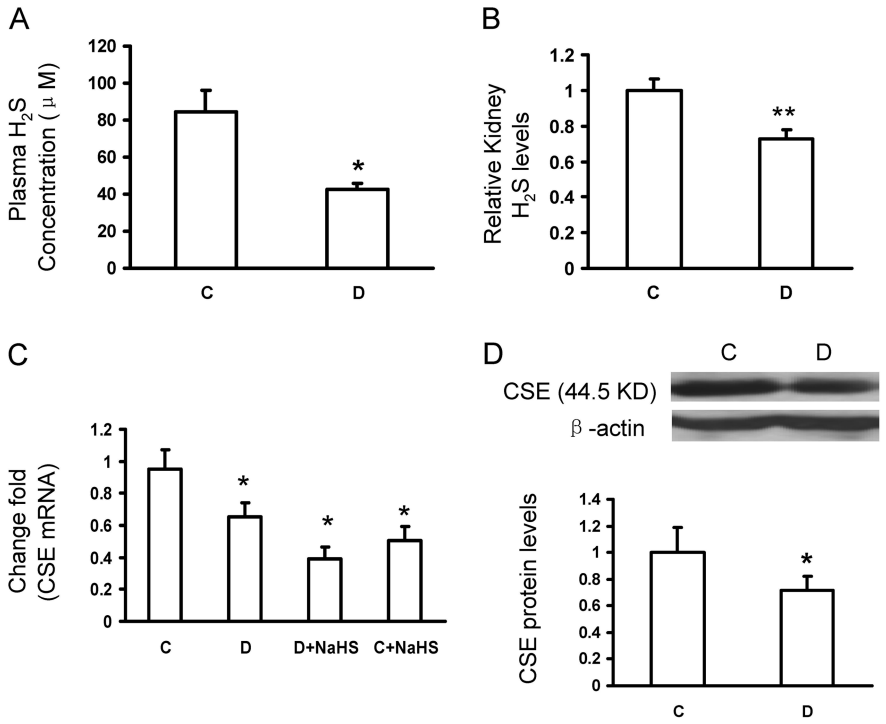

Fig. 1. Levels of H_2S in plasma and renal cortex, CSE mRNA and protein levels in renal cortex from control (C), diabetic (D), diabetic treated with NaHS (D + NaHS) and control treated with NaHS (C + NaHS) rats. (**A**) Plasma H_2S concentrations, (**B**) renal cortex H_2S levels, (**C**) real-time PCR assay for renal cortex CSE mRNA levels, (**D**) western blot analysis of renal cortex CSE protein levels (normalized by β-actin). $n = 6$ per group. Data are mean ± standard error of the mean. *$P < 0.05$ versus control rats.

in diabetic rats, but this treatment lowered blood urea nitrogen and urea protein in diabetic rats but not in non-diabetic control rats.

As shown in Figure 1A and B, H_2S in both plasma and renal cortex was decreased 3 weeks after STZ injection. Quantitative real-time-PCR data showed that the levels of both CSE mRNA (Figure 1C) and CSE protein (Figure 1D) in the renal cortex of STZ-induced diabetic rat was lower than that of control rats. Administration of NaHS, a H_2S donor, did not change the decreased CSE mRNA level in diabetic rat. However, NaHS treatment for a week significantly decreased the CSE mRNA level in nondiabetic rats. Compared with control rats, the collagen IV mRNA and protein levels were increased in the renal cortex of STZ-induced diabetic rats (Figure 2A and B). This increase was reversed by NaHS treatment in diabetic condition. By contrast, NaHS treatment did not alter the renal cortex collagen IV mRNA level in non-diabetic rats.

Effect of H_2S supplementation on TGF-β1 mRNA level in diabetic renal cortex

The TGF-β1 mRNA level in diabetic rat renal cortex was increased as compared with that of control rats. Administration of NaHS for 1 week reversed the elevation in TGF-β1 mRNA level in diabetic but not in control rats (Figure 3).

Effect of high glucose on CSE expression in cultured renal MCs

Incubation of rat renal MCs in high glucose media for 24 h resulted in a significant decrease of CSE mRNA and protein as compared with normal glucose group. NaHS treatment did not change the CSE mRNA level in both normal and high glucose groups. In addition, there was no significant change in CSE mRNA and protein levels in the osmotic control group (Figure 4A and B).

Effect of H_2S on MCs proliferation induced by high glucose stimulation

BrdU incorporation assay showed that treatment of the cultured renal MCs with high glucose media for 48 h increased cell proliferation. Treatment with NaHS suppressed high glucose-induced MC proliferation in a concentration-dependent manner. NaHS, as low as 30 μM, completely reversed high glucose-induced MCs proliferation. This concentration was, therefore, selected for all subsequent experiments. Thirty micromolars of NaHS had no effect on proliferation in normal glucose-treated cells. Treatment of MCs with CSE inhibitor PPG resulted in a significant increase in cell proliferation and this effect was similar to that induced by high glucose (Figure 5).

Effect of H_2S on collagen IV production by cultured MCs

Changes in collagen IV synthesis were shown in Figure 6. Collagen IV mRNA and protein expression levels were increased upon high glucose stimulation but unaffected by osmotic control media. Application of 30 μM NaHS reversed high glucose-induced elevations in both collagen IV mRNA and protein levels. By contrast, 30 μM NaHS did not change the collagen IV mRNA level in normal glucose-treated cells. On the other hand, PPG, a CSE inhibi-

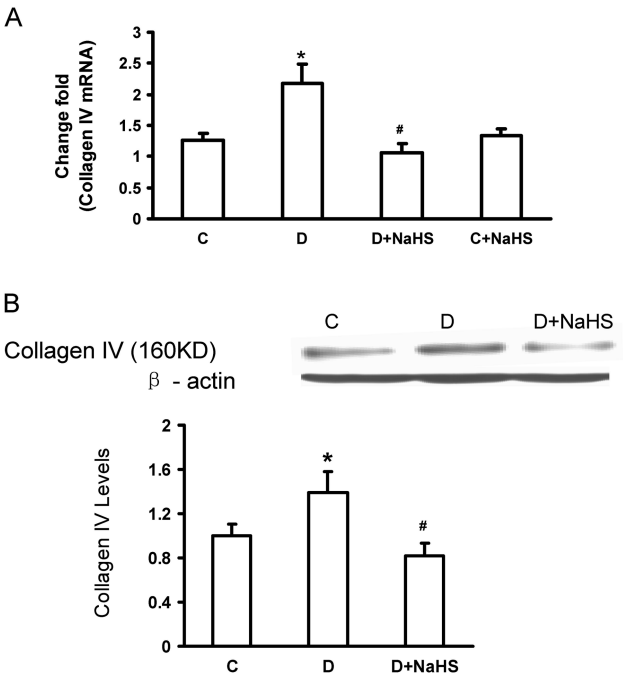

Fig. 2. Measurement of collagen IV mRNA and protein levels in renal cortex of control (C), diabetic (D), diabetic treated with NaHS (D + NaHS) and control treated with NaHS (C + NaHS) rats. (**A**) Real-time PCR assay for collagen IV mRNA levels, (**B**) western blot analysis for collagen IV protein levels (normalized by β-actin). $n = 6$ per group. Data are mean ± standard error of the mean. *P < 0.05 versus control rats; #P < 0.05 versus diabetic rats.

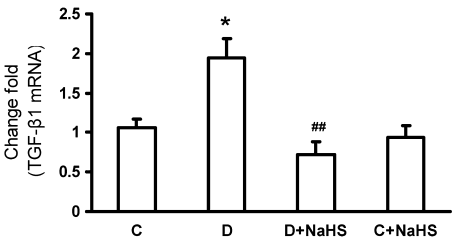

Fig. 3. Real-time PCR assay for TGF-β1 mRNA levels in renal cortex of control (C), diabetic (D), diabetic treated with NaHS (D + NaHS) and control treated with NaHS (C + NaHS) rats. $n = 6$ per group. Data are mean ± standard error of the mean. *P < 0.05 versus control rats; ##P < 0.01 versus diabetic rats.

tor, increased the expression of both collagen IV mRNA and protein.

Effect of H_2S on TGF-β1 mRNA level in cultured MCs

A significant elevation in TGF-β1 mRNA levels was detected in the MCs exposed to high glucose for 24 h but not to osmotic control media. Applications of 30 μM NaHS reversed high glucose-induced elevations in TGF-β1 mRNA levels. Again, PPG treatment resulted in a significant elevation in the TGF-β1 mRNA level (Figure 7).

Effect of H_2S on ROS generation in cultured MCs

The production of ROS in cultured MCs was enhanced after 24-h exposure to high glucose but not to osmotic control media. Application of 30 μM NaHS reversed high glucose-induced increase in ROS generation. PPG produced a similar effect to high glucose exposure on ROS generation by MCs (Figure 8).

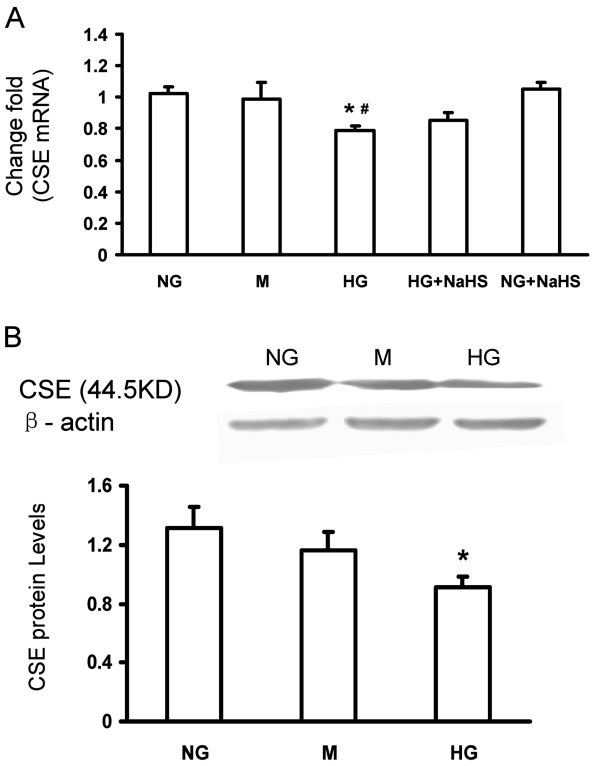

Fig. 4. Measurement of CSE mRNA and protein levels in MCs cultured in normal glucose (NG), mannitol osmotic control (M), high glucose (HG), normal glucose plus NaHS (NG + NaHS), high glucose plus NaHS (HG + NaHS) and normal glucose supplemented with 24.5 mM mannitol (M) for 24 h. (**A**) Real-time PCR assay for CSE mRNA levels and (**B**) western blot analysis for CSE protein levels (normalized by β-actin). $n = 7$ per group. Data are mean ± standard error of the mean. *P < 0.05 versus NG; #P < 0.05 versus M.

Discussion

H_2S has long been known as a colorless, flammable and toxic gas [5]. In recent years, H_2S is increasingly recognized as a gasotransmitter that exerts a wide spectrum of biological and physiological effects [22,23]. The role of H_2S in the regulation of renal function has also been recently reported [24]. H_2S participates in the control of renal function and increases urinary sodium excretion via both vascular and tubular actions in the kidney [25]. The synthesis of endogenous H_2S catalyzed by CSE is essential to protect the kidney against ischemia/reperfusion injury and facilitate the recovery [26]. A recent study shows that NaHS treatment inhibits renin activity elevation and blunted blood pressure elevation in 2-kidney 1-clip hypertensive rats [27].

DN, a long-term complication of diabetes associated with the highest mortality, is the leading cause of end-stage renal disease [28]. Although advanced glycation end products and dyslipidemia are all known to be associated with diabetic organ damages, hyperglycemia is likely to be the

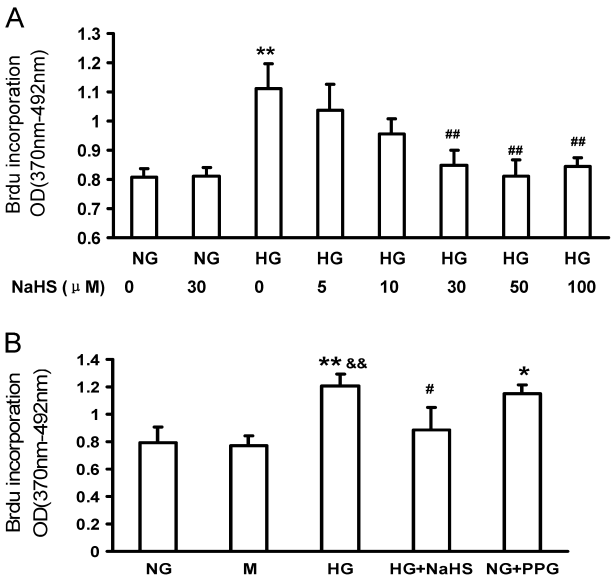

Fig. 5. Effect of NaHS on high glucose (HG)-induced MC proliferation. (**A**) NaHS inhibited cultured MC proliferation in a dose-dependent manner. (**B**) Cell proliferation increased significantly in HG-treated cells but not in osmotic control group, NaHS prevented HG-induced proliferation, CSE inhibitor PPG increased cell proliferation significantly. $n = 6$ per group. Data are means ± standard error of the means. n represents the number of separate experiments. $^{**}P < 0.01$ versus NG; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ versus HG; $^{\&\&}P < 0.01$ versus M. (NG represents normal DMEM media that has 5.5 mM D-glucose and supplemented with 10% neonatal bovine serum, HG represents high glucose culture media that supplements NG with additional D-glucose for a final D-glucose concentration at 30 mM, M represents the osmotic control media that supplements NG with 24.5 mM mannitol).

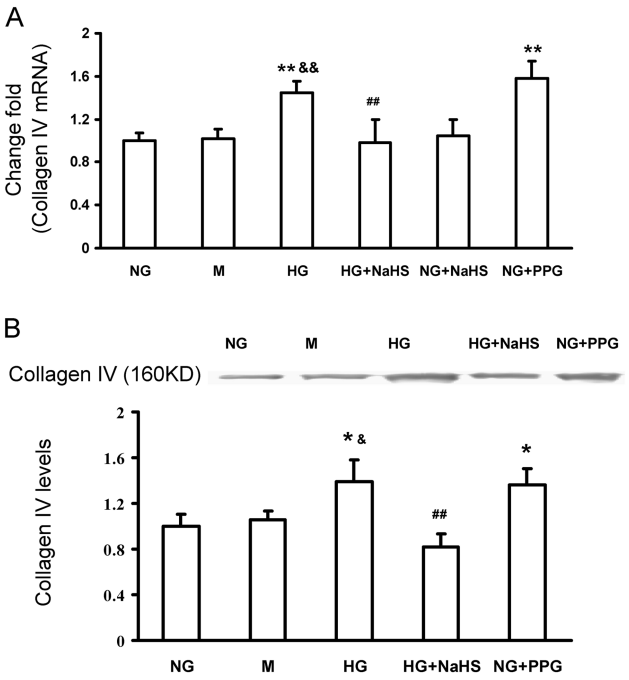

Fig. 6. Effect of NaHS on collagen IV mRNA and protein levels. (**A**) Real-time PCR assay for collagen IV mRNA levels and (**B**) western blot analysis of collagen IV protein levels (normalized by β-actin). $n = 6$ per group. Data are mean ± standard error of the mean. n represents the number of separate experiments. $^{*}P < 0.05$ versus NG; $^{\#\#}P < 0.01$ versus HG; $^{\&}P < 0.05$ versus M. (NG represents normal DMEM media that has 5.5 mM D-glucose and supplemented with 10% neonatal bovine serum, HG represents high glucose culture media that supplements NG with additional D-glucose for a final D-glucose concentration at 30 mM, M represents the osmotic control media that supplements NG with 24.5 mM mannitol).

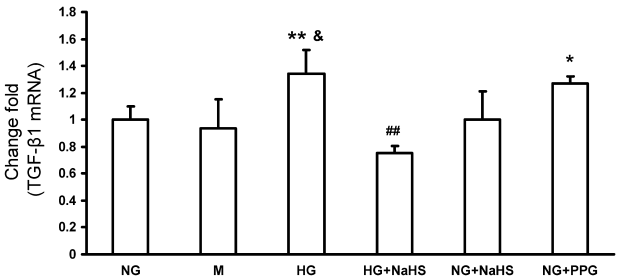

Fig. 7. Effect of NaHS on TGF-β1 mRNA levels in cultured MCs. $n = 7$ per group. Data are mean ± standard error of the mean. n represents the number of separate experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ versus NG; $^{\#\#}P < 0.01$ versus HG; $^{\&}P < 0.05$ versus M. (NG represents normal DMEM media that has 5.5 mM D-glucose and supplemented with 10% neonatal bovine serum, HG represents high glucose culture media that supplements NG with additional D-glucose for a final D-glucose concentration at 30 mM, M represents the osmotic control media that supplements NG with 24.5 mM mannitol).

primary pathological contributor to the development of DN [29]. The present result showed that both H_2S level and CSE expression in renal cortex decreased significantly in diabetic rats, which is consistent with a recent observation that blood H_2S levels are significantly lower in patients with type 2 diabetes compared with age-matched healthy subjects and in STZ-treated diabetic rats compared with control SD rats [30]. Besides, we showed that the reduced H_2S level and CSE expression were accompanied by up-regulation of TGF-β1 and collagen IV in the renal cortex. Administration of NaHS reversed the increased production of TGF-β1 and collagen IV. TGF-β1 is a key regulator of extracellular matrix synthesis and cell proliferation and considered to be a marker of renal fibrogenesis, while collagen IV is one of the major extracellular matrix synthesized and secreted by MCs, and overproduction of collagen IV is related to glomerular hypertrophy and sclerosis [31,32]. The present results suggest that the decreased generation of endogenous H_2S in diabetic rats might involve the development of DN and H_2S restoration could be a target in curtailing hyperglycemia-induced renal injury.

The present results suggest that the inhibitory effect of NaHS on TGF-β1 and collagen IV expression in diabetic rats is unlikely to be mediated by modulating plasma glucose level because the latter was not changed. Instead, H_2S might act directly on the renal cells. It is noted that these changes in CSE expression and H_2S production occurred at the very early stage of diabetes (3 weeks after STZ injection). It is thus possible that the altered H_2S level might be one of the triggering factors in the initiation of DN.

To determine the role of CSE/H_2S pathway, cultured MCs were used in the study. MCs are inherent vascular peripheral cells in renal glomeruli. Hyperglycemia-induced MCs over-proliferation and excessive synthesis and secretion of extracellular matrix have been considered as early

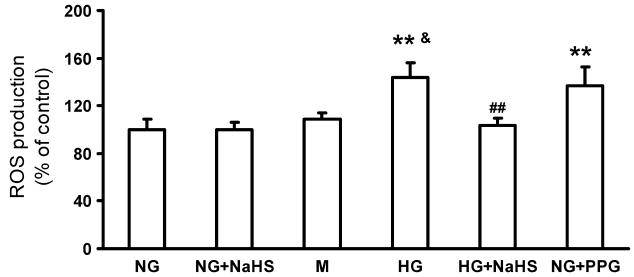

Fig. 8. Effect of NaHS on ROS generation. $n = 12$ per group. Data are mean ± standard error of the mean. $^{**}P < 0.01$ versus NG; $^{\#\#}P < 0.01$ versus HG; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus M. (NG represents normal DMEM media that has 5.5 mM D-glucose and supplemented with 10% neonatal bovine serum, HG represents high glucose culture media that supplements NG with additional D-glucose for a final D-glucose concentration at 30 mM, M represents the osmotic control media that supplements NG with 24.5 mM mannitol).

pathological events in DN [33,34]. *In vitro* observations were consistent with the result from diabetic animals in the present study. Both PCR and western blot results demonstrated that the CSE expression in renal MCs was decreased, while proliferation and extracellular matrix secretion were increased under high glucose condition. NaHS treatment suppressed high glucose-induced cell proliferation and reversed the elevated collagen IV synthesis. CSE inhibitor PPG evoked similar changes as high glucose in cultured MCs. In the kidney, CSE is the key enzyme in the trans-sulfuration pathway, which cleaves L-cysteine to release H_2S [9,10]. So, all results suggest that decreased endogenous H_2S generation due to down-regulating CSE in high glucose condition may account for high glucose-induced MC proliferation and extracellular matrix production. However, the mechanisms by which high glucose decrease the production of endogenous H_2S remains unclear, which deserves further investigation.

The present study also confirmed that TGF-β1 mRNA level was significantly elevated by high glucose in MCs. In agreement with the *in vivo* results, the elevation of TGF-β1 was reversed by NaHS treatment. Blockade of endogenous H_2S formation by PPG significantly elevated the TGF-β1 mRNA level. Based on these observations, we speculate that the decreased endogenous H_2S production caused by high glucose might be related to nephrotic fibrosis through increasing TGF-β1, then stimulates MCs proliferation and collagen IV over-production.

Over-production of ROS is involved in the pathophysiological process of DN. Hyperglycemia induces ROS generation [35] and ROS initiates up-regulation of TGF-β1, leading to MC proliferation and excessive extracellular matrix production [36–38]. The present study confirmed that high glucose increased ROS generation in MCs and provided evidence that NaHS is able to reduce high glucose-induced ROS generation. The antioxidant effect of low level of H_2S was previously observed in cultured vascular smooth muscle cells [39]. Homocysteine treatment of cultured vascular smooth muscle cells increased cellular levels of superoxide anion, hydrogen peroxide and peroxynitrite; this prooxidative effect was antagonized by low levels of NaHS. NaHS also potentiated the protective effects of other known antioxidants, such as *N*-acetyl-L-cysteine and superoxide dismutase against the cellular damage induced by homocysteine [39]. The exact mechanism underlying H_2S-decreased ROS generation in MCs is yet to be detailed. Whether or not reduced endogenous H_2S level under high glucose condition could disturb the redox balance or affect the activity of ROS generating-enzymes needs additional examination.

In summary, hyperglycemia or high glucose suppresses the expression of CSE in both renal cortex and cultured MCs and decreases endogenous synthesis of H_2S. Decreased H_2S renders elevation of ROS level, leading to the up-regulation of TGF-β1 that may mediate MC proliferation, and excessive collagen IV production/secretion. All those changes may participate in renal glomerular hypertrophy, sclerosis and interstitial fibrosis. Restoring H_2S level under diabetic conditions might represent a novel strategy in the management of DN. Nevertheless, there is limitation in the present study. For example, the treatment duration with NaHS was relatively short, which may explain the lack of apparent effects on key morphological parameters of DN, such as interstitial fibrosis and glomerular sclerosis, and functional parameters, such as glomerular filtration rate and proteinuria. The long-term impact of NaHS administration to diabetic rats on their renal function warrants careful examination.

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Conflict of interest statement. None declared.

References

1. Steffes MW, Osterby R, Chavers B *et al*. Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. *Diabetes* 1989; 38: 1077–1081
2. Davis LK, Rodgers BD, Kelley KM *et al*. Angiotensin II- and glucose-stimulated extracellular matrix production: mediation by the insulin-like growth factor (IGF) axis in a murine mesangial cell line. *Endocrine* 2008; 33: 32–39
3. Tan AL, Forbes JM, Cooper ME *et al*. AGE, RAGE and ROS in diabetic nephropathy. *Semin Nephrol* 2007; 27: 130–143
4. Nakajima T, Hasegawa G, Kamiuchi K *et al*. Differential regulation of intracellular redox state by extracellular matrix proteins in glomerular mesangial cells: potential role in diabetic nephropathy. *Redox Rep* 2006; 11: 223–230
5. Wang R. Two's company, three's a crowd—can H_2S be the third endogenous gaseous transmitter? *FASEB J* 2002; 16: 1792–1798
6. Wu L, Yang W, Jia X *et al*. Pancreatic islet overproduction of H_2S and suppressed insulin release in Zucker diabetic rats. *Lab Invest* 2009; 89: 59–67
7. Doeller JE, Isbell TS, Benavides G *et al*. Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. *Anal Biochem* 2005; 341: 40–51
8. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 1996; 16: 1066–1071
9. Lowicka E, Beltowski J. Hydrogen sulfide (H_2S)—the third gas of interest for pharmacologists. *Pharmacol Rep* 2007; 59: 4–24
10. Akahoshi N, Lzumi T, Ishizaki Y *et al*. Differential induction of cystathionine gamma-lyase in the livers and kidneys of mouse dams during gestation and lactation. *Bio Pharm Bull* 2006; 29: 1799–1802

11. Teague B, Asiedu S, Moore PK. The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* 2002; 137: 139–145
12. Wolf G. New insights into the pathophysiology of diabetic nephropathy: from hemodynamics to molecular pathology. *Eur J Clin Invest* 2004; 34: 785–796
13. Li L, Bhatia M, Zhu YZ *et al*. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J* 2005; 19: 1196–1198
14. Cai WJ, Wang MJ, Moore PK *et al*. The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. *Cardiovas Res* 2007; 76: 29–40
15. Soulis-Liparota T, Cooper M, Papazoglou D *et al*. Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes* 1991; 40: 1328–1334
16. Shi YX, Chen Y, Zhu YZ *et al*. Chronic sodium hydrosulfide treatment decreases medial thickening of intramyocardial coronary arterioles, interstitial fibrosis, and ROS production in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 2007; 293: 2093–2100
17. He M, Zang L, Shao Y *et al*. Inhibition of renin/prorenin receptor attenuated mesangial cell proliferation and reduced associated fibrotic factor release. *Eur J Pharmacol* 2009; 606: 151–161
18. Singh R, Sing AK, Alavi N *et al*. Mechanism of increased angiotensin II levels in glomerular mesangial cells cultured in high glucose. *J Am Soc Nephrol* 2003; 14: 873–880
19. Xue H, Yuan P, Zhou L *et al*. Angiotensin II mediates the cell proliferation effect of adrenotensin on cultured rat mesangial cells. *Acta Pharmacologica Sinica* 2009; 30: 1132–1137
20. Cai WJ, Wang MJ, Moore PK *et al*. The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. *Cardiovasc Res* 2007; 76: 29–40
21. Brancaleone V, Roviezzo F, Vellecco V *et al*. Biosynthesis of H_2S is impaired in non-obese diabetic (NOD) mice. *Br J Pharmacol* 2008; 155: 673–680
22. Wang R. Hydrogen sulfide: the third gasotransmitter in biology and medicine. *Antioxid Redox Signal* 2010; 12: 1061–1064
23. Wang R. Hydrogen sulfide: a new EDRF. *Kidney Int* 2009; 76: 700–704
24. Sen U, Basu P, Abe OA *et al*. Hydrogen sulfide ameliorates hyperhomocysteinemia-associated chronic renal failure. *Am J Physiol Renal Physiol* 2009; 297: F410–F419
25. Xia M, Chen L, Muh RW *et al*. Production and action of hydrogen sulfide, a novel gaseous bioactive substance, in the kidney. *J Pharmacol Exp Ther* 2009; 329: 1056–1062
26. Tripatara P, Patel NS, Collino M *et al*. Generation of endogenous hydrogen sulfide by cystathionine gamma-lyase limits renal ischemia/reperfusion injury and dysfunction. *Lab Invest* 2008; 88: 1038–1048
27. Lu M, Liu YH, Goh HS *et al*. Hydrogen sulfide inhibits plasma renin activity. *J Am Soc Nephrol* 2010; 21: 993–1002
28. Carey RM, Siragy HM. The intrarenal rennin-angiotensin system and diabetic nephropathy. *Trends Endocrinol Metab* 2003; 14: 274–281
29. Vidotti DB, Casarini DE, Cristovam PC *et al*. High glucose concentration stimulates intracellular rennin activity and angiotensin II generation in rat mesangial cells. *Am J Physiol Renal Physiol* 2004; 286: 1039–1045
30. Jain SK, Bull R, Rains JL *et al*. Low levels of hydrogen sulfide in the blood of diabetes patients and streptozotocin-treated rats causes vascular inflammation? *Antioxid Redox Signal* 2010; 12: 1333–1337
31. Jiang Z, Seo JY, Ha H *et al*. Reactive oxygen species mediate high glucose-induced plasminogen activator inhibitor-1 upregulation in mesangial cells and in diabetic kidney. *Biochem Biophys Res Commun* 2003; 309: 961–966
32. Ziyadeh FN, Hoffman BB, Han DC *et al*. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 2000; 97: 8015–8020
33. Fogo AB. Progression and potential regression of glomerulosclerosis. *Kidney Int* 2001; 59: 804–819
34. Suzaki Y, Yoshizumi M, Kagami S *et al*. BMK 1 is activated in glomeruli of diabetic rats and in mesangial cells by high glucose conditions. *Kidney Int* 2004; 65: 1749–1760
35. Rosen P, Nawroth PP, King G *et al*. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a congress series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 2001; 17: 189–212
36. Ha H, Lee HB. Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. *Kidney Int* 2000; 58: 19–25
37. Oh JH, Ha H, Yu MR *et al*. Sequential effects of high glucose on mesangial cell transforming growth factor-β1 and fibronectin synthesis. *Kidney Int* 1998; 54: 1872–1878
38. Hua H, Goldberg HJ, Fantus IG *et al*. High glucose-enhanced mesangial cell extracellular signal-regulated protein kinase activation and α1 (IV) collagen expression in response to endothelin-1 role of specific protein Kinase C isozymes. *Diabetes* 2001; 50: 2376–2383
39. Yan SK, Chang T, Wang H *et al*. Effects of hydrogen sulfide on homocysteine-induced oxidative stress in vascular smooth muscle cells. *Biochem Biophys Res Commun* 2006; 351: 485–491

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