Hydrogen sulfide reduced renal tissue fibrosis by regulating autophagy in diabetic rats

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Received February 28, 2016; Accepted March 9, 2017

DOI: 10.3892/mmr.2017.6813

Abstract. The present study aimed to explore the effect of hydrogen sulfide (H2S) on renal tissue fibrosis and its mechanism in diabetic rats. Rats were randomly divided into four groups (n=13/group): Control group; induced diabetes mellitus group (STZ); induced diabetes mellitus treated with H2S group (STZ + H2S); normal rats treated with H2S group (H2S). The diabetic model was induced by intraperitoneal (i.p.) injections of 40 mg/kg body weight streptozotocin (STZ); the control group was treated with saline every day (i.p); NaHS (100 µmol/kg i.p) was administered to rats of STZ + H2S group and H2S group. After 8 weeks, rat body weight and 24 h proteinuria levels were determined in each group, renal pathological morphology was analyzed by Masson's trichrome staining, collagen IV content was detected by immunohistochemistry, and periodic acid-Schiff (PAS) staining was performed on renal glomerular and tubular basement membranes. The expression levels of matrix metalloproteinase 9 (MMP9), MMP7, tissue inhibitor of metalloproteinase 1 (TIMP1), superoxide dismutase (SOD), serine/threonine kinase AKT, transforming growth factor (TGF)-β1, nuclear factor (NF)-κB and several autophagy related proteins were assessed by western blot analysis. Compared with the control group, renal tissue fibrosis was observed, collagen IV expression and the 24 h proteinuria quantity was markedly increased and the amount of PAS positive material in renal glomerular and tubular basement membranes was notably increased in STZ-treated rats. Furthermore, the expression levels of MMP9, MMP7, TIMP1, autophagy-associated proteins, AKT, TGF-β1 and NF-κB protein were significantly increased, and SOD expression levels were significantly decreased in the STZ group compared with the control (P<0.05). In the H2S+STZ group, renal tissue fibrosis and the expression of collagen IV were improved, 24 h proteinuria was decreased, the amount of PAS positive material in renal glomerular and tubular basement membranes was decreased, the expression levels MMP9, MMP7, TIMP1, autophagy-associated proteins, AKT, TGF-β1 and NF-κB protein were significantly decreased, and the expression levels of SOD were significantly increased compared with the STZ group (P<0.05). In conclusion, H2S may improve renal tissue fibrosis by inhibiting autophagy, upregulating SOD and downregulating AKT, TGF-β1 and NF-κB.

Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD). The worldwide occurrence of DN is increasing, and this disease is associated with increased morbidity and mortality in patients with type 1 and type 2 diabetes (1). DN is characterized by the accumulation of extracellular matrix (ECM), resulting in progressive kidney fibrosis that leads to kidney function decline and irreversible loss of tissue (2). The increasing prevalence of DN has resulted in a growing research focus on this disease. Therefore, the identification of a novel therapeutic target in the management of DN is required.

Autophagy is emerging as a key cellular stress response that is involved in a variety of disease states, including DN (3). Autophagy is a highly conserved ‘self-feeding’ pathway involved in degrading and recycling macromolecules and damaged organelles, to maintain intracellular homeostasis (4). This cellular function serves important roles in human health and disease. Generally, autophagy serves a dual purpose; it may perform a cytoprotective or deleterious role in the body, depending on the type and severity of the inducing injury (5). A certain degree of autophagic activity serves a critical role in promoting tissue homeostasis and cell survival. However,
excessive autophagic activity may also contribute to type II programmed cell death and, in certain circumstances, promote the development of DN (6).

Hydrogen sulfide (H₂S) is an endogenously produced gaseous molecule with important roles in cellular signaling. This chemical has been implicated in the regulation of inflammatory responses, cardiovascular functions, renal functioning and the gastrointestinal system. Furthermore, H₂S has been shown to exert potent cytoprotective abilities against tissue injury, including organ fibroses such as myocardial and renal fibrosis (7). However, the role of H₂S and autophagy in the pathogenesis of DN, and the relationship between H₂S and the dysregulation of autophagy remains unclear. Therefore, further studies are required to clarify these mechanisms in detail. The present study established a streptozotocin (STZ)-induced diabetic rat model to investigate the role of H₂S and autophagy in the pathogenesis of DN, and the protective effects of H₂S against DN.

Materials and methods

Animals and reagents. Adult male Sprague-Dawley rats (n=52; weight, 280±40 g) were obtained from the animal experiment center of South China University (Henyang, China). The study was approved by the Ethics Committee of the Department of Laboratory Animals of South China University [animal qualified number: SYXX (Hunan), 2015-0006]. All rats were maintained in a climate-controlled room with a 12-h light/dark cycle, at constant temperature (23±1°C) and humidity (40‑50%) with free access to food and water. Antibodies against collagen IV (cat. no. BA3858-2), GAPDH (cat. no. PB0141), matrix metalloproteinase 9 (MMP9; cat. no. BA0573), MMP7 (cat. no. BA2110), tissue inhibitor of metalloproteinase 1 (TIMP1; cat. no. BA3836-2), AKT (cat. no. BA0631), transforming growth factor (TGF)-β1 (cat. no. BA0290) and nuclear factor (NF)-κB (cat. no. BA1872-2) were purchased from Wuhan Boster Biological Technology Co., Ltd. The antibody against DN. This chemical has been implicated in the regulation of inflammatory responses, cardiovascular functions, renal functioning and the gastrointestinal system. Furthermore, H₂S has been shown to exert potent cytoprotective abilities against tissue injury, including organ fibroses such as myocardial and renal fibrosis (7). However, the role of H₂S and autophagy in the pathogenesis of DN, and the relationship between H₂S and the dysregulation of autophagy remains unclear. Therefore, further studies are required to clarify these mechanisms in detail. The present study established a streptozotocin (STZ)-induced diabetic rat model to investigate the role of H₂S and autophagy in the pathogenesis of DN, and the protective effects of H₂S against DN.

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Animal model and grouping. All rats had free access to food and water and, following 7 days of acclimatization, the rats were randomly divided into four equal groups (n=13/group): Control group, STZ group, STZ + H₂S group and H₂S group. The STZ and STZ + H₂S groups received intraperitoneal injections of 40 mg/kg body weight STZ, 5% glucose water was provided for STZ-treated rats within 24 h to prevent hypoglycemic shock, and blood sugar levels were tested 72 h after injection. The diabetic animal model was considered successful if blood glucose levels rose to >16.7 mmol/l. Following animal model set-up, the H₂S and STZ + H₂S groups were intraperitoneally administered the H₂S donor NaHS after the models were established successfully, at a dose of 100 µmol/kg/day for a further 8 weeks. The first dose of NaHS was administered 24 h after models were successfully established. Control and STZ groups were treated with an equivalent volume of intraperitoneal 0.9% saline for 8 weeks. During this 8-week period a total of 12 rats died (control, n=2; STZ, n=4; STZ + H₂S, n=3; H₂S, n=3); the overall survival rate was 77%. Diabetic rats were observed to be unresponsive and lackluster, and displayed various degrees of polydipsia, polyphagia, diuresis and moved slowly.

Specimen collection and processing. At the end of the 8 weeks, the 24 h proteinuria levels were determined by using a UP ELISA kit (cat. no. SBJ-H1384; Nanjing Senbeijia Biological Technology Co., Ltd. Nanjing, China), and the rats were sacrificed following chloral hydrate anesthesia. Both kidneys were removed; the right kidney was immersed in 10% formalin and embedded in paraffin. The left kidney was frozen (-70°C) and later used for western blot analysis.

Histopathological examinations. Paraffin-embedded specimens were cut into 5 µm thick sections and stained with Masson's trichrome. Five random microscopic fields per well were quantified at a magnification of x400 using a BH-2 light microscope (Olympus Corporation, Tokyo, Japan).

Immunohistochemistry. Paraffin-embedded specimens were cut into 4 µm thick sections, dewaxed, rehydrated and boiled. Sections were washed three times for 5 min each in PBST [PBS (pH 7.4), 0.05% Tween-20], blocked with 5% bovine serum albumin (Wuhan Boster Biological Technology, Ltd.) for 10 min at room temperature and then incubated with the collagen IV primary antibody (diluted 1:50) overnight at 4°C. The following day, sections were washed three times for 5 min each in PBST, incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 30 min at 37°C, washed in PBST and visualized using a DAB staining kit (Nanjing Senbeijia Biological Technology Co., Ltd.). Sections were counterstained with hematoxylin and collagen IV deposition was observed at a magnification of x400 using a digital microscope (Olympus Corporation).

Periodic acid-Schiff (PAS) staining. Paraffin-embedded kidney specimens were cut into 2-3 µm thick sections, dewaxed, rehydrated, and oxidized with 5% periodate for 6-8 min at 15°C. Sections were subsequently washed with distilled water for 2 min, placed into colorless Schiff's reagent and incubated in the dark for 20 min at 15°C. The samples were then droplet washed twice for 1 min with sodium sulfite solution (0.5%), rinsed with running water for 2 min, counterstained with hematoxylin for 1 min at 18°C, washed in running water and differentiated with 1% hydrochloric acid alcohol at 18°C. Samples were then repeatedly rinsed with running water, the blue stain was developed with warm water and sections were dehydrated with graded ethanol and sealed with
neutral balsam, and the PAS-positive material was observed in the renal glomerular and tubular basement membranes under high-power magnification (x400) using a BH-2 light microscope.

**Western blot analysis.** Proteins were extracted in ice-cold radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (100X; Beyotime Institute of Biotechnology), and quantified using the BCA colorimetric method. Proteins (50 µg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk powder in TBST buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween-20] for 2 h and incubated with anti-MMP9, anti-MMP7, anti-TIMP1, anti-SOD, anti-AKT, anti-TGF-β1, anti-NF-κB (all 1:400) and anti-LC3, anti-Atg3, anti-Atg5, anti-Atg7, anti-Atg12, anti-Atg16 (all 1:1,000) antibodies for 1 h at 37°C, and then overnight at 4°C. GAPDH served as the endogenous control. The membranes were washed three times for 10 min each and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 2 h at room temperature. The membranes were detected using Western Blotting Luminol reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), protein bands were visualized using the FluorChem® FC2 Imaging System (ProteinSimple; Bio-Techne, Minneapolis, MN, USA) and the band density was semi-quantified using AlphaView v3.2.2 software (ProteinSimple; Bio-Techne).

**Statistical analysis.** Data were analyzed using SPSS v18.0 software (SPSS, Inc., Chicago, IL, USA), and expressed as the mean ± standard deviation of the mean. Comparisons between groups were performed using one-way analysis of variance followed by Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Changes in proteinuria.** The 24 h proteinuria was significantly increased in the STZ group (P<0.05), compared with the untreated control group. Notably, the 24 h proteinuria level was significantly decreased in the STZ + H$_2$S group, following H$_2$S intervention (P<0.05; Table I).

| Group          | 24 h proteinuria (mg/24 h) |
|----------------|---------------------------|
| Control        | 11.8±3.3                  |
| STZ            | 44.5±6.4*                 |
| STZ + H$_2$S   | 26.6±6.1*                 |
| H$_2$S         | 12.6±4.0                  |

Values are expressed as the mean ± standard deviation. *P<0.05 vs. control group, †P<0.05 vs. STZ group. STZ, streptozotocin; H$_2$S, hydrogen sulfide.

**Immunohistochemistry.** Immunohistochemical analysis was performed to observe collagen IV deposition and brown staining indicated the presence of collagen IV in the tissues. In the control group, collagen IV was lightly observed in the glomerulus, tubules and interstitial areas. In the STZ group, collagen IV positivity was observed in the glomerulus, tubules and interstitial areas. However, in the STZ + H$_2$S group, collagen IV positive staining was decreased in these areas, compared with the STZ group. In the H$_2$S group, collagen IV was not observed in the renal tubules, the glomerulus or the renal interstitium (Fig. 2).

**PAS staining.** Fuchsia staining represented PAS positive material. PAS staining was lowest in the renal glomerular and tubular basement membranes of the control group. Marked PAS staining of the renal glomerular and tubular basement membranes was observed in the STZ group. Compared with the STZ group, the presence of PAS positive material in renal glomerular and tubular basement membranes was markedly improved in the STZ + H$_2$S group. PAS positive staining of the renal glomerular and tubular basement membranes was low in the H$_2$S group (Fig. 3).

**Effects of STZ and H$_2$S on CBS protein expression.** CBS is an enzyme that can endogenously produce H$_2$S in kidney tissue. The present study observed that CBS expression was significantly decreased in the STZ group (P<0.05). Compared with the STZ group, however, the expression levels of CBS protein were significantly increased in the STZ + H$_2$S group (P<0.05; Fig. 4). These results indicated that renal fibrosis was elevated following STZ treatment, and was markedly improved with H$_2$S treatment; H$_2$S may therefore improve renal tissue fibrosis.

**Effects of STZ and H$_2$S on MMP9, MMP7, TIMP1 and SOD expression.** The expression of MMP9, MMP7 and TIMP1 protein were significantly increased in the STZ group compared with the control group (P<0.05), whereas SOD protein was significantly decreased in this group (P<0.05). However, the expression of MMP9, MMP7 and TIMP1 protein were significantly decreased and SOD protein was significantly increased in the STZ + H$_2$S group, compared with the STZ group (P<0.05). There was no difference in MMP9, MMP7, TIMP1 and SOD protein expression between the control and H$_2$S-only groups (Fig. 5).
Effects of STZ and H₂S on LC3, Atg3, Atg5, Atg7, Atg12 and Atg16 expression. The expression of LC3, Atg3, Atg5, Atg7, Atg12 and Atg16 was significantly increased in the STZ group, compared with the control group (P<0.05). However, the expression levels of these proteins were significantly decreased in the STZ + H₂S group, compared with the STZ group (P<0.05). There was no difference in LC3, Atg3, Atg5, Atg7, Atg12 or Atg16 protein expression between the control group and the H₂S-only group (Fig. 6).

Effects of STZ and H₂S on TGF-β1, NF-κB and AKT expression. The expression levels of TGF-β1, NF-κB and AKT were significantly increased in the STZ group compared with the control group (P<0.05). The expression levels of TGF-β1, NF-κB and AKT were significantly decreased in the STZ + H₂S group, compared with the STZ group (P<0.05). There was no difference in TGF-β1, NF-κB or AKT expression between the control group and the H₂S group (Fig. 7).

Discussion

The results of the present study demonstrated a significant increase in urinary protein content in diabetic rats.
Immunohistochemical examination discovered prominent renal tissue fibrosis and significant upregulation of renal tissue collagen IV expression amongst STZ-induced diabetic rats. The results of Masson’s staining indicated that collagen fibers...
were present in the renal interstitium in the STZ group and the presence of PAS-positive material in renal glomerular and tubular basement membranes was markedly increased in the STZ group compared with the control group. These results also indicated that there was dysregulation of MMPs and TIMP expression in the renal tissue of diabetic rats. Collectively, these results suggested the presence of renal tissue fibrosis in diabetic rats. Furthermore, the present study observed an elevation of activated autophagy biomarkers, including LC3, Atg3, Atg5, Atg7, Atg12 and Atg16. These biomarkers were upregulated in the renal tissue of diabetic rats, suggesting that autophagic activation may be associated with renal tissue fibrosis in these rats. Previous research has indicated that renal fibrosis is accompanied by an upregulation of autophagy (6), whereas a different study suggested that a downregulation of autophagy occurs in diabetic rats (7). Such a difference may be associated with disease duration and stage of diabetic nephropathy; the majority of the autophagic downregulation is observed in early stages of diabetes and is associated with diabetic kidney hypertrophy, whereas enhanced autophagy is often observed in the late stages of diabetes and is associated with diabetic kidney fibrosis (8). Evidence of cellular autophagy inhibition was observed in the proximal and distal tubules of early STZ-induced diabetic rats by electron microscopy analysis, three days following STZ administration (8). Whereas some studies have demonstrated the presence of autophagy in renal tissue fibrosis (9,10). Therefore, it is speculated that the level of autophagy may be upregulated when significant renal tissue fibrosis occurs in DN. This phenomenon was also observed in another experimental report (11). Such time-dependent alterations also conform to the characteristics of autophagy: Early mild damage leads to protective autophagy, whereas sustained serious damage may result in traumatic autophagy. However, whether protective autophagy or traumatic autophagy occurred in the present study remains unclear.

Excessive oxidative stress and autophagy has previously been demonstrated in DN. Reactive oxygen species (ROS) are mediators of autophagic activation, furthermore, ROS-induced activation of autophagy may result in alternative outcomes that promote either cell death or survival (12). The activation of autophagy is frequently associated with ROS and oxidative stress. The downregulation of SOD expression in the STZ-induced DN tissues observed in the present study suggests an associated increase in oxidative stress, and this may be associated with the activation of autophagy.

The regulatory mechanism of autophagy is closely associated with the AKT signaling pathway (13). Inhibition of AKT may activate autophagy, however the opposite result has also been reported (14). Furthermore, suppression of the phosphatidylinositol-3-kinase/AKT pathway may attenuate renal fibrosis; previous findings indicated that phospho-AKT expression was upregulated in rat kidneys with chronic allograft nephropathy, and these rats presented with severe interstitial fibrosis and tubular atrophy (15,16). A previous study indicated that AKT may have a bidirectional regulation role in autophagy and produce time-dependent effects, however this is also likely to be associated with the feedback regulation of AKT by Atg5 (17). In the present study, the level of autophagy was significantly increased in the renal tissue of diabetic rats, however the expression of AKT was also markedly increased. These results suggest that AKT upregulation may be associated with feedback regulation by Atg5, and other regulatory pathways of autophagy may be involved in the development of renal fibrosis associated with DN.

Similar to AKT, TGF-β and NF-κB also have critical roles in the pathogenesis of DN (18,19), and are common signaling pathways involved in autophagic regulation. TGF-β has a central role in the pathogenesis of tissue fibrosis, and overexpression of this protein in renal tubular epithelial cells resulted in widespread peritubular fibrosis and induction of autophagy (20). Furthermore, cell culture studies indicated that TGF-β may activate autophagy in tubular cells (21). Therefore, it seems that TGF-β may regulate autophagy, which in turn may regulate several critical aspects of kidney fibrosis, such as tubulointerstitial fibrosis and diabetic nephropathy. Regarding NF-κB, Haar et al (22) reported that acute high-fat feeding mediates cardioprotection against ischemia-reperfusion injury, and this was associated with a NF-κB-dependent increase in autophagy. The results of the present study indicate that the mechanism of renal interstitial fibrosis in diabetic rats may be associated with the activation of TGF-β and the NF-κB pathways, with a subsequent increase in the level of autophagy.

Previous evidence has demonstrated that H2S has acritical role in the pathogenesis of DN (23,24). H2S is an endogenous signaling gas, which possesses potent anti-inflammatory, anti-oxidant and other regulatory functions (7). Together with nitric oxide and carbon monoxide, H2S has important regulatory roles in different physiological and pathological situations. Accumulating evidence has indicated that H2S may have an
antifibrotic effect in the development of fibrosis in the heart, liver and kidneys (25-27). One study demonstrated a decrease in H$_2$S-producing enzymes and subsequently a decrease in the endogenous H$_2$S level in plasma and tissues (28), whereas administration of exogenous H$_2$S was able to inhibit the development of fibrosis. The production of H$_2$S from L-cysteine is catalyzed primarily by two enzymes, cystathionine γ-lyase and CBS (29). The results of the present study demonstrated that CBS expression was significantly decreased in the STZ group. Compared with the control group, and the expression level of CBS protein was significantly increased in the STZ + H$_2$S group compared with the STZ group. In a rat model of unilateral ureteral obstruction, H$_2$S inhibited renal fibrosis by attenuating excessive collagen production and ECM protein expression (30). The results of the present study indicated that H$_2$S may attenuate mesangial matrix deposition and renal tissue fibrosis, and may inhibit excessive autophagy in the diabetic rat kidney. The protective mechanism of H$_2$S against diabetic nephropathy may be associated with the downregulation of autophagy.

The present study aimed to investigate whether exogenous H$_2$S was able to protect against the development of diabetic nephropathy. The results indicated that H$_2$S was able to improve proteinuria, to reduce the presence of PAS-positive zones in the renal glomerular and tubular basement membranes, and to reduce renal tissue fibrosis in STZ-induced diabetic rats. Furthermore, administration of H$_2$S increased the expression of SOD and decreased the production of collagen IV and expression of AKT, TGF-β1 and NF-κB. TGF-β1 is an essential regulator of extracellular matrix synthesis and cell proliferation, and is considered to be a marker of renal fibrogenesis, whereas collagen IV and collagen II are major extracellular matrix components (31). NF-κB activation has been documented to be associated with renal inflammation and renal fibrogenesis (32). In neonatal rat cardiomyocytes exposed to hypoxia/reoxygenation, H$_2$S was able to significantly reverse the reduced cell viability and augmented cell injury, via the inhibition of autophagy (25). Jung et al (33) revealed that treatment with H$_2$S Sattenuated unilateral ureteral obstruction-induced oxidative stress and kidney fibrosis, and activated TGF-β1 and NF-κB. The antifibrotic mechanisms of H$_2$S may involve anti-oxidative stress and autophagy-suppressing roles, together with blockade of TGF-β1 and NF-κB signaling. In the present study, H$_2$S was observed to alleviate renal fibrogenesis and autophagy activation in diabetic kidney tissue, and this possibly occurred via regulation of SOD, AKT, TGF-β1 and NF-κB signaling.

The results of the present study demonstrated that H$_2$S may improve renal tissue fibrosis of STZ-induced diabetic rats, and may reverse the dysregulation of MMPs/TIMPs and the activation of autophagy. Furthermore, H$_2$S intervention may inhibit oxidative stress and activation of the AKT, TGF-β1 and NF-κB pathways, indicating that these signaling pathways may be involved in the pathogenesis of renal tissue fibrosis of diabetic rats, and may be associated with the regulation of autophagy.

In conclusion, the present study demonstrated that autophagic suppression may represent a protective mechanism of H$_2$S in the development of DN, by attenuating the imbalance of MMPs/TIMPs and the excessive deposition of collagen. H$_2$S may alleviate renal tissue fibrosis and activation of autophagy in STZ-induced diabetes, and this may occur via anti-oxidative stress mechanisms, together with blockade of AKT, TGF-β1 and NF-κB signaling. Further potential mechanisms of autophagic suppression by H$_2$S remain poorly defined, and should be explored in the future.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81202830).

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