NOS activity mediates some pathways in protective effects of H$_2$S in a model of diabetic nephropathy

Aliaa F. Anter$^{*1}$, Ashraf Taye$^1$, Mohamed A. El-Moselhy$^{1,2}$

$^1$Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, Minia, Egypt.
$^2$Department of Clinical Pharmacy and Pharmacology, Ibn Sina National College for Medical Studies, Jeddah, Saudi Arabia.

Received: April 22, 2018; revised: May 4, 2018; accepted: May 6, 2018

Abstract

Hydrogen sulfide (H$_2$S) is considered as one of the gaseous transmitters participating in different physiological and pathophysiological conditions. H$_2$S possesses vasoprotective, anti-inflammatory and anti-apoptotic properties so this study aimed to investigate the role of H$_2$S in diabetic nephropathy and to examine the role of nitric oxide on H$_2$S induced effects. The protective effects of H$_2$S were tested by administration of sodium hydrogen sulfide (NaHS) as an exogenous source of H$_2$S.To study the role of nitric oxide, N-Nitro L-arginine methyl ester (L-NAME) was administrated with NaHS. After four weeks of type 2 diabetes induction, diabetic rats showed signs of nephropathy evident in increased serum urea and creatinine levels. On the other hand, treatment with an H$_2$S donor (NaHS) exhibited protective effects against nephropathy induced by diabetes. NaHS diminished the elevated TNF-κ, NF-κB and caspase 3 expression induced by diabetes. Treatment with NaHS resulted in decreased MDA and H$_2$O$_2$ production with subsequent increase of SOD and CAT activity measured in kidney tissues. Chronic administration of L-NAME in combination with NaHS diminished the protective role of H$_2$S on inflammatory and oxidative status. In conclusion, H$_2$S has a protective role in diabetic nephropathy through its antioxidant, and anti-inflammatory on kidney tissue. This study provides evidence that nitric oxide is important in mediating some of the protective effects of H$_2$S.

Key words

Cystathionine gamma lyase, high fat diet, NF-κB, streptozotoxin

1. Introduction

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes [1, 2]. Diabetic nephropathy is considered among important factors for reducing the lifespan in diabetic patients [3]. Uncontrolled diabetic patients with DN have a higher mortality rate and are more susceptible to cardiovascular diseases than diabetic patients without DN [4]. Inflammation and oxidative stress are among the molecular pathways involved in DN pathophysiology [5]. In DN there is an imbalance between afferent and efferent arteriole resistance which leads to activation of renin secretion and increased level of angiotensin II resulting in efferent vasoconstriction with proinflammatory cytokines production [6]. Nitric oxide plays an important role through endothelium dependent vasodilatation [7]. Hydrogen sulfide is a gaseous signaling molecule. It is produced in many organs including heart, brain and kidney [8]. H$_2$S is synthesized by the effect of three enzymes: desulphhydration of L-cysteine by cystathionine gamma lyase (CSE) or L-cystathionine β synthase (CBS),and transamination followed by desulphhydration of L-cysteine by 3-mercaptopropionate [9]. Similar to other gasotransmitters, nitric oxide and carbon monoxide, recent studies has reported that H$_2$S has a role in a wide range of pathological conditions such as cardiovascular and cerebral dysfunction, fatty liver disease and ischemia reperfusion injuries[8].

Recently, H$_2$S has been known for its antioxidative, anti-inflammatory, and anti-apoptotic properties[10]. Studies suggested that its deficiency may participate in the progression of chronic kidney diseases [11, 12]. The present study was designed to investigate the effect of H$_2$S supplementation on diabetes induced nephropathy and to evaluate the role of nitric oxide synthase (NOS) activity on H$_2$S protective effects in a diabetic nephropathy model.

2. Materials and methods

Male wistar rats weighing 90 -120 g were purchased from the Experimental Animal Center of Al-Azhar University of Medical Science (Cairo, Egypt). Rats were acclimatized to lab conditions for one week and kept under constant environmental conditions throughout the period of the experiment. All experiments were approved by the Research Ethics Committee for the ethical principles and guidelines of the care and use of laboratory animals, Faculty of Pharmacy, Minia University ( Permit Number: MPH-12-015).

2.1. Induction of diabetes

Type II diabetes in rats was induced by HFD/STZ as previously described [13]. Rats were fed either normal chow (control non diabetic group) or a high fat diet (HFD) composed of 600 g powdered normal pellets diet + 400 g beef fat (suet) per kilogram for 12 weeks followed by a single injection of STZ (35
mg/kg, Sigma Aldrich, USA) dissolved in citrate buffer pH 4. Three days after STZ injection, blood glucose level was measured. Rats were considered diabetic when fasting blood glucose exceeded 200 mg/dL. Then, they were allowed free access to HFD diet for additional 4 weeks.

### 2.2. Experimental design

Diabetic rats were randomly divided into three groups; 1: diabetic group (DM), 2: diabetic group treated with sodium hydrogen sulfide (NaHS; 56mmol/kg [14, 15]-Sigma USA, cat. No.13590), 3: diabetic group treated with NaHS+ N-Nitro L-arginine methyl ester (L-NAME; 25mg/kg [16]-Sigma USA , cat. No. N5751). All drugs were dissolved in saline and given by intraperitoneal injection daily for four weeks after induction of diabetes.

At the end of the treatment period, rats were scarificated, animals were fasted for 12 hours, blood samples were collected and centrifuged and kidneys were rapidly dissected and cut into pieces, one was flash frozen in liquid nitrogen for further biochemical analyses and another one was prepared for histopathological examination.

### 2.3. Assessment of fasting BGL, insulin resistance and lipid profile

Animals were fasted for 12 hours before the measurement. Blood samples were obtained by tail puncture and measured by a commercial glucometer (Accu-check® glucometer, Roche, USA). Serum insulin was assayed using an ELISA kit (Bioscience, USA) and insulin resistance was calculated based on Homeostasis Model Assessment of Insulin Resistance (HOMA IR) [17]. Cholesterol, triglycerides and low density lipoprotein (LDL) levels in serum samples were determined colorimetrically using the available commercial kits purchased from Biodiagnostic, Egypt.

### 2.4. Measurement of CSE activity and its mRNA expression in kidney tissue:

CSE activity was measured by ELISA (Mybiosource, USA) according to the manufacturer’s instructions. CSE mRNA expression was measured in kidney tissue using RT-PCR.

Total RNA was isolated using Trizol reagent (Life Technologies, Rockville, MD, USA), treated with DNase I (Roche Applied Science Mannheim, Germany), and purified using RNA clean-up kit (CW biotech, Beijing, China). One microgram of total RNA was applied for cDNA reverse transcription kit. Primer used was 5'-CATGGATGAAGTGTATGGAGGC-3' and the reverse primer sequence was 5'-CGGCAGCAGAGGTAACAATCG-3'.

The PCR reactions were performed in a total volume of 20 µL using the thermal cycling parameters as follow: The PCR condition for β-actin was 95 °C for 5 min., 95 °C for 15 s, and 60 °C for 1 min. for 40 cycles. The amount of β-actin cDNA in the sample was used to calibrate the amount of sample needed for quantification.

### 2.5. Determination of urea and creatinine serum levels

Creatinine and urea nitrogen concentrations were measured in serum samples by an enzymatic colorimetric method using commercial kits purchased from Biomed, Egypt for urea and Spinreact, Spain for creatinine.

### 2.6. Evaluation of oxidative stress parameters

Determination of Malondialdehyde MDA contents in kidney homogenate was colorimetrically measured according to Buege and Aust method [18]. Hydrogen peroxide level was measured in tissue homogenate using colorimetric assay using a commercial kit according to the manufacturer instructions (Sigma Aldrich, USA).

Activities of the antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT) were measured in kidney homogenate by colorimetric assay according to the instructions of commercial kits provided from Biodiagnostics, Egypt for catalase and Abcam, United states for SOD.

### 2.7. Western blot analysis

The kidney homogenate protein concentration was determined using Bradford assay. Protein preparations in equal amounts (20 mg in 10 µl) were run on SDS polyacrylamide gels. Then, electro transferred to polyvinylidinedifluoride membranes, and blotted with a primary antibody against NF-κB (Invitrogen, Thermofischer, USA) overnight at 4 °C using a slow rocking shaker. The membranes were blotted with HRP-conjugated secondary antibody (Sigma, USA) and HRP-conjugated monoclonal antibody against β-actin (Sigma, USA). The chemiluminescent signals were captured using a CCD camera-based imager. The results were calculated as the mean ratio of the target protein density to the β-actin density.

### 2.8. Evaluation of tumor necrosis factor alpha, transforming growth factor beta- and caspase-3 concentrations:

Renal TNF-α, TGF-β and caspase-3 concentrations were determined using ELISA technique according to instructions of the manufacturer; Thermo Fisher, USA for TNF-α, Invitrogen, USA for TGF-β measurement and Biovision, USA for determination of caspase-3.

### 2.9. Histopathological Examination

Kidney samples from different groups were fixed in 10% neutral buffered formalin for 24 h, and formic acid was used for decalcification. Then they were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for a day. Paraffin bees wax blocks were sliced by a microtome. The tissue sections prepared were put on glass slides, deparaffinized and stained with hematoxylin and eosin, tissue slides were examined using a light microscope (Olympus CX41).
2.10. Statistical analysis

All data were expressed as mean ± SEM (standard error of the mean) and were analyzed using one-way ANOVA followed by the Tukey-Kramer post analysis test to compare all groups. p values < 0.05 were considered significant. GraphPad Prism version 6.00 (San Diego, CA, USA) was used.

3. Results

3.1. Effect of NaHS treatment alone or in combination with L-NAME on fasting BGL, serum insulin level and insulin resistance (HOMA-IR)

Induction of diabetes showed a significant higher fasting BGL (212.70 ± 7.14 mg/dl) compared to control rats, while chronic treatment with NaHS or L-NAME + NaHS prevented the increased BGL caused by diabetes to be 120.00 ± 5.70 mg/dl for NaHS receiving group and 147.70 ± 10.99 mg/dl in NaHS + L-NAME group. NaSH with L-NAME treatment showed non-significant decrease in fasting BGL compared to NaHS treatment alone. These results are shown in (Figure 1A).

Figure 1 (B and C) showed that diabetes caused a significant increase in insulin level and insulin resistance when compared to control group. NaHS treatment significantly attenuated the insulin level and resistance compared to diabetic rats. Administration of L-NAME with NaHS showed a significant decrease in insulin resistance compared to diabetic animals. Addition of L-NAME to NaHS treatment caused a non-significant change in insulin level and resistance compared to animals received NaHS alone.

3.2. Effect of NaHS treatment alone or in combination with L-NAME on lipid profile:

Induction of diabetes by this model caused a significant elevation in serum cholesterol, triglycerides and low density lipoprotein (LDL) as compared to normal control group. Administration of NaHS alone and in combination with L-NAME to diabetic induced animals showed a significant (p<0.05) reduction in serum cholesterol, triglycerides and LDL level when compared to diabetic untreated group. Results are listed in (Table 1).

3.3. Effect of NaHS treatment alone or in combination with L-NAME on urea and creatinine level:

Figure 2 (A and B) showed that induction of diabetes resulted in a significant increase in serum urea and creatinine level when compared with control animals (1.31 ± 0.06 mg/dl in diabetic rats vs 0.31 ± 0.02 for control group) for creatinine and (145.30 ± 2.36 mg/dl for diabetic animals vs 79.53 ± 0.99 mg/dl for control group) for urea level. NaHS chronic treatment prevented the elevation of serum urea and creatinine level induced by diabetes to be 0.53 ± 0.03 mg/dl creatinine and 71.78 ± 1.12 mg/dl urea. Administration of L-NAME with NaHS significantly diminished NaHS effects on serum urea and creatinine.

3.4. Effect of NaHS treatment alone or in combination with L-NAME on relative CSE mRNA expression and its activity in kidney tissue:

Diabetic rats showed a significant lower relative CSE mRNA expression when compared to normal rats as shown in Figure 3 (A and B). Treatment with NaHS significantly prevented
diabetes induced reduction in CSE mRNA expression. Rats treated with a combination of NaHS and L-NAME significantly decreased CSE expression when compared to NaHS treated rats. Also, compared to control rats, the CSE activity in kidney was significantly lower in diabetic group (15.93 ± 1.16 vs. 55.98 ± 1.69). Treatment with NaHS for 4 weeks prevented the reduced effect of diabetes on activity of CSE enzyme to be 40.43 ± 3.05. L-NAME + NaHS showed a significant decrease in enzyme activity when compared to diabetic group treated with NaHS alone (20.18 ± 1.11; Figure 3c).

3.5. Effect of NaHS treatment alone or in combination with L-NAME on NF-κb protein expression in kidney tissue:

Figure (3): A) RT PCR blots and B) bar chart representing the CSE mRNA expression in different groups. C) CSE activity in kidney tissue represented by a bar chart. Diabetes model showed a significant decrease in CSE mRNA expression and activity. Exogenous H2S significantly prevented effects of diabetes on these two parameters. CSE: cystathionine gamma layase, NaHS: sodium hydrogen sulfide, L-NAME: N-Nitro L-arginine methyl ester.

Data represent the mean ± SEM of 6 observations; # significant difference from the control group at p<0.05, * Significant difference from diabetic group at p<0.05, ○: significant difference from diabetes + NaHS group at p<0.05.

3.6. Effect of NaHS treatment alone or in combination with L-NAME on caspase-3, TGF-β and TNF-α levels in kidney tissue:

Induction of T2DM led to a significant elevation of caspase-3, TGF-β and TNF-α concentrations in kidney homogenate when compared to control normal rats. However, NaHS treatment significantly suppressed diabetes induced apoptotic enzyme and inflammation signaling. However, NaHS combination with L-NAME reversed the effect of NaHS alone and resulted in a significant increase in caspase-3, TGF-β and TNF-α levels.

Figure 4 (A, D and E).

Figure (4): A) Bar chart showing the effect of diabetes and H2S donor and nitric oxide synthase inhibitor on caspase-3 activity in kidney tissue. B) representative western blots and C) bar chart. Both represent the protein expression of NF-kB in kidney tissue and its alteration by diabetes induction and chronic administration of different drugs. D & E) the two bar charts showing the level of renal TGF-β and serum level of TNF-α in different animal groups respectively. Diabetes significantly increases levels of C and TNF-α. While treatment with NaHS diminishes this increase, animals received L-NAME in combination with NaHS showed a significant elevation of TGF-β level in renal tissue and TNF-α level in serum.


Data represent the mean ± SEM of 6 observations; # significant difference from the control group at p<0.05, * Significant difference from diabetic group at p<0.05, ○: significant difference from diabetes + NaHS group at p<0.05.

3.7. Effect of NaHS treatment alone or in combination with L-NAME on malondialdehyde and hydrogen peroxide production and the activity of antioxidant enzymes in kidney tissue:

Diabetic untreated rats significantly showed an elevated level of MDA and H2O2 production in kidney tissues compared to control rats. Rats treated with NaHS showed a significant attenuation of MDA level and H2O2 production induced by diabetes when compared to untreated HFD/STZ group. On the other hand, MDA level and H2O2 production were significantly
increased in animals receiving both L-NAME and NaHS. Figure 5 (A and B).

CAT and SOD levels were measured in kidney tissue as a measure for antioxidant capacity. Diabetic rats had significantly lower CAT and SOD levels when compared to control. Chronic treatment for 4 weeks with NaHS abolished the effect of diabetes on enzyme levels in the kidney tissue. Administration of NaHS in combination with L-NAME showed a significant reduction in antioxidant levels when compared to NaHS administered alone. Results are shown in Figure 5 (C and D).

**Figure 5:** Bar charts represent A) MDA level, B) H2O2 production C) catalase activity and D) SOD activity in kidney tissue. Model of diabetes showed elevated levels of MDA and H2O2 and has a reduced effect on antioxidant enzyme activity (CAT and SOD). H2S donor diminished the effects observed in model of diabetes.

MDA: malondialdehyde, H2O2: hydrogen peroxide, CAT: catalase, SOD: superoxide dismutase. NaHS: sodium hydrogen sulfide, L-NAME:Nitro L-arginine methyl ester. Data represent the mean ± SEM of 6 observations; # significant difference from the control group at p<0.05, * significant difference from diabetic group at p<0.05, ○: significant difference from diabetes + NaHS group at p<0.05.

### 3.8. Histopathological changes:

Normal histological structure of the glomeruli and tubules at the cortex are shown in photomicrograph 6A of kidney of control animals. Focal inflammatory cells infiltration was detected in between the congested glomeruli and the degenerated as well as necrosed tubules at the cortex shown in photomicrograph 6B of kidneys of diabetic rats. Photomicrograph 6C of rat kidney from group receiving NaHS treatment showed degeneration in the lining epithelium of some cortical tubules. Photomicrograph 6D kidney of rat receiving both L-NAME+ NaHS, showed degenerative change associated with vacuolization of the lining endothelium of the glomeruli, eosinophilic casts were detected in the tubular lumen at the corticomedullary portion an intracellular vacuolization was detected in the endothelial cells of the intima.

**Figure 6:** Representative photomicrographs showing kidney tissue stained by H&E and showing the following

Control: A) Normal histological structure of the glomeruli and tubules at the cortex of kidney

Diabetic group: B) Focal inflammatory cells infiltration in between the congested glomeruli and the degenerated as well as necrosed tubules at the cortex of kidney.

Diabetes + NaHS: C) degeneration in the lining epithelium of some cortical tubules of kidney.

Diabetes + NaHS + L-NAME: D) degenerative change associated with vacuolization of the lining endothelium of the glomeruli of kidney.

### 4. Discussion

In this study, diabetes model was induced by feeding the rats with HFD for 12 weeks followed by a single injection of STZ. Four weeks later, an obvious increase in blood urea nitrogen and serum creatinine was observed which can be considered asmarkers of nephropathy. Cystathionine gamma layase (CSE) mRNA expression and activity were decreased in kidney tissue of diabetic rats emphasize the role of the endogenously produced H2S via the CSE biosynthetic pathway in normal kidney physiology. Our results showed that tissue level of malondialdehyde (MDA) was elevated with subsequent decrease in superoxide dismutase (SOD) and catalase (CAT) activity. Renal nuclear factor kappa B (NF-kB) expression, the activity of caspase-3 enzyme (an apoptotic enzyme), transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) concentrations were all elevated in HFD/STZ diabetic rats.

Treatment with an H2S donor (NaHS) attenuated the increase in serum level of urea and creatinine induced by diabetes and reduced diabetes induced oxidative stress and inflammatory processes. Administration of L-NAME with NaHS treatment blocked some of the protective effect of NaHS on diabetes induced nephropathy.

Hyperglycemia and hyperinsulinemia may be considered aswell-known factors contributing to diabetic nephropathy and can lead to end-stage renal failure [19]. Treatment with NaHS attenuated the hyperglycemia and the increase in HOMA IR score induced by diabetes. Inhibition of NO synthesis by administration of L-NAME with NaHS had no significant effect on the above mentioned effects of NaHS.

In our study, diabetic rats showed a decrease in CSE activity and mRNA expression in kidney tissue. This data is supported by the finding of Zhou and his colleagues [20] who showed that
the induction of diabetes resulted in decreased H₂S production and decreased protein expression of CSE in renal tissue. The observed high serum urea and creatinine in our diabetic model can be directly attributed to the reduced CSE activity, as supplementation with NaHS reversed these effects. Previous studies reported that CSE expression was reduced in a model of kidney fibrosis induced by unilateral ureteral obstruction in mice [21, 22]. Decreased H₂S level has been recently reported in chronic kidney disease patients [23] and also in vivo and in vitro animal models of chronic and acute kidney diseases [8]. The protective role of H₂S on diabetic nephropathy can be explained by the work of Beltowski who reported that H₂S can restore oxygen balance and result in increased blood flow and glomerular filtration rate of kidneys [9]. L-NAME in combination with NaHS prevented the protective effect of NaHS on creatinine serum level which can be attributed to the deleterious effect of L-NAME on kidney functions. The observed nephropathy in our study can also be attributed to the oxidative stress status in diabetics. Increased reactive oxygen species with subsequent reduction of antioxidant capacity is considered as main causes for development of diabetic nephropathy [24]. In this study, we measured MDA (as a marker of lipid peroxidation) and H₂O₂ production in renal tissue. Defensive antioxidant enzymes SOD and CAT were also estimated in renal tissue. In our diabetic model, renal tissue analysis revealed a high level of MDA and H₂O₂ production with low levels of SOD and CAT enzymes. Exogenous H₂S attenuated the increase of MDA and H₂O₂ induced by diabetes. SOD and CAT levels were markedly elevated in diabetic rats treated with NaHS for four weeks. The protective effect of H₂S donor can be explained by its ability to reduce ROS accumulation by increasing the activity of ROS-scavenging enzymes. Similar findings were also shown in a study published in 2014 by Jiang and his colleagues who showed that NaHS treatment attenuated MDA level and increased SOD activity in rats with unilateral ureteric obstruction as a model to induce renal damage [22]. This effect of NaHS on oxidative stress in a non-diabetic model supports the role of hydrogen sulfide as an antioxidant agent regardless of its effects on glycemic status. Inhibition of nitric oxide production by L-NAME treatment abolished the protective effects of NaHS on free radicals production and antioxidants capacity which indicate the role of NO in antioxidant activity of H₂S. The role of NO in mediating H₂S antioxidant activity can be explained by the work of Filiers, et al who studied the effect of H₂S on NADPH oxidase 4 (NOX4) which modulates insulin stimulated generation of H₂O₂ [25]. They concluded that inhibition of NO synthesis by L-NAME attenuates the inhibitory effect of H₂S on NOX4. Oxidative stress can also lead to increased activation of inflammatory pathways which is known as a hallmark of type 2 diabetes induced nephropathy [19]. NF-κB stimulates caspase-3 activity and lead to apoptotic cell death. It also stimulates the release of cytokines and growth factors, including transforming growth factor-β (TGF-β1) and tumor necrosis factor-α (TNF-α). These factors play an important role in the pathogenesis of diabetic complications including nephropathy [26]. For example, TGF-β1 increases the expression of mRNA of glucose transporter 1 (GLUT-1) in renal mesangial cells, resulting in increased glucose uptake by kidney cells and accelerated metabolic abnormalities [27]. In this study, HFD/STZ model caused increased NF-κB expression in kidney tissues with subsequent increase in the level of TNF-α, TGF-β and caspase-3 activity. Our data demonstrated that an H₂S donor (NaHS) diminished the stimulating effects of diabetes on NF-κB pathways. Ford and his colleagues established that one possible mechanism of H₂S to protect kidney is by attenuating apoptosis via decreasing caspase-3 activity [28] where they show that treating the animals with exogenous H₂S resulted in lowering of caspase-3 activity. Our data show that co-administration of L-NAME with NaHS abolished the protective effects of NaHS on inflammatory status despite preserving the H₂S improvement of BGL in diabetic rats. This can be explained by a previous study showing that NO inhibits the NF-κB activation [29]. Thus, L-NAME administration as NOS synthase inhibitor resulted in sustained activation of NF-κB. This is supported by pervious work showing the stimulatory effect of H₂S on eNOS activity [30, 31] which led to reduced activation of NF-κB pathway [32]. Thus, inhibition of NOS by L-NAME can be considered as a downstream blocking effect on H₂S induced reduction in inflammation. These results indicate that the protective anti-inflammatory effects of H₂S are dependent on NO availability. Exogenous source of H₂S can protect kidney from diabetes induced renal failure. The protective effects of H₂S can be attributed to its effects on BGL, insulin resistance and its anti-oxidant and anti-inflammatory effects. This improvement was showed in the reduction of urea and creatinine serum levels and improvement of the histopathological changes. Inhibition of NO blocked the protective effect of our hydrogen sulfide donor leading to increasing oxidative stress and inflammatory responses.

5. Conclusion

H₂S has a protective role in diabetes induced renal impairment. This potential effect may be due to the decrease in hyperglycemia, insulin resistance, oxidative stress and inflammation status. Nitric oxide presence is found to be important in some of H₂S actions such as its effect on expression of CSE enzyme and its role as antioxidant and anti-inflammatory agent through reduction of MDA and H₂O₂ production and decreased levels of NF-κB, TGF-β and TNF-α in spite of absence of the effect of L-NAME on NaHS glycemic status.

References


