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## Hydrogen sulfide

Snijder, Pauline

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# CHAPTER

## SODIUM THIOSULFATE ATTENUATES ANGIOTENSIN II-INDUCED HYPERTENSION, PROTEINURIA AND RENAL DAMAGE

PM Snijder\*  
AS Frenay\*  
AM Koning  
M Bachtler  
A Pasch  
AJ Kwakernaak  
E van den Berg  
EM Bos  
JL Hillebrands  
G Navis  
HGD Leuvenink  
H van Goor

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\*These authors contributed equally to this manuscript

# 5

## ABSTRACT

Hypertension and proteinuria are important mediators of renal damage. Despite therapeutic interventions, the number of patients with end stage renal disease steadily increases. Hydrogen sulfide ( $H_2S$ ) is an endogenously produced gasotransmitter with vasodilatory, anti-inflammatory and antioxidant properties. These beneficial characteristics make  $H_2S$  an attractive candidate for pharmacological use in hypertensive renal disease. We investigated the protective properties of  $H_2S$  in angiotensin II (ang II)-induced hypertensive renal disease in rats. Treatment with the  $H_2S$  donor NaHS and major  $H_2S$  metabolite sodium thiosulfate (STS) during three weeks of ang II infusion reduced hypertension, proteinuria, oxidative stress and renal functional and structural deterioration. In an ex vivo isolated perfused kidney setup NaHS, but not STS, reduced intrarenal pressure. The effect of NaHS could partially be explained by its activation of the ATP-sensitive potassium channels. In conclusion, treatment with  $H_2S$  attenuates ang II-associated functional and structural renal deterioration, suggesting that intervention in  $H_2S$  production pathways has potential therapeutic benefit and might be a valuable addition to the already existing antihypertensive and renoprotective therapies.

## INTRODUCTION

Chronic kidney disease (CKD) is a highly prevalent disorder associated with extensive morbidity and mortality worldwide. Hypertension and proteinuria are major contributors to the progression of CKD. Both are important actors in enhancing structural and functional renal deterioration through changes in intrarenal hemodynamics and inflammation, thereby promoting the release of chemokines and reactive oxygen species (ROS)<sup>1-4</sup>. This results in stimulation of extracellular matrix synthesis and enhancement of cellular apoptosis. Increased activity of the renin-angiotensin-aldosterone system (RAAS) resulting in augmented angiotensin II (ang II) signaling, is often the underlying cause of hypertension and proteinuria. Functional RAAS modulation has afforded great progress in renoprotection by reducing blood pressure, proteinuria and the rate of renal function loss. Although RAAS blockade stands out as the most effective renoprotective treatment, in many cases renal disease ultimately progresses to end-stage renal failure with the deplorable need for dialysis or transplantation<sup>5, 6</sup>. This prompts for additional modes of intervention by either optimization of RAAS blockade based therapies or targeting other pathophysiological pathways involved in the development of CKD.

Hydrogen sulfide ( $H_2S$ ) is acknowledged as the third gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO), and modulates many physiological functions<sup>7</sup>. It is endogenously produced from the amino acid L-cysteine by cystathionine  $\gamma$ -lyase (CSE) and cystathionine  $\beta$ -synthase (CBS)<sup>8, 9</sup> and from 3-mercaptopyruvate (3MP) by 3-mercaptopyruvate sulfurtransferase (3-MST)<sup>10</sup>. In the vasculature  $H_2S$  functions as an endothelial cell-derived relaxing factor via direct activation of ATP-sensitive potassium ( $K_{ATP}$ ) channels<sup>11</sup>. Accordingly, CSE-deficient mice and CBS heterozygous mice develop hypertension<sup>12, 13</sup>. CSE can act as an endogenous modulator of oxidative stress, as CSE-deficient mice have increased renal damage after ischemia-reperfusion<sup>14</sup>. Exogenous treatment with the soluble sulfide salt NaHS attenuates the hypertensive effects of NO synthase (NOS) inhibition<sup>15</sup> and has preventive and therapeutic effects on renovascular hypertension by inhibiting plasma renin activity<sup>16</sup>. In addition,  $H_2S$  stimulates cellular proliferation<sup>17</sup> and angiogenesis<sup>18</sup>, and reduces inflammation<sup>19, 20</sup>. Endogenous  $H_2S$  functions as a signaling molecule by regulating protein activity through S-sulfhydration, which is a form of posttranslational modification<sup>21, 22</sup>. Furthermore,  $H_2S$  can play a detoxifying role during oxidative stress by direct scavenging of ROS or increasing the formation of the antioxidant glutathione<sup>23, 24</sup>. Progression of renal disease in a CKD model is associated with depletion of  $H_2S$  and its producing enzymes<sup>25</sup>. Recently, urinary sulfur metabolites were found to associate with a favorable cardiovascular risk profile and even improved survival in renal transplant recipients<sup>26</sup>. Given the cytoprotective features of  $H_2S$ , its deficiency may contribute to progression of CKD and its systemic complications.

$H_2S$  can be delivered in vivo via gaseous administration or through the use of soluble sulfide salts like NaHS and  $Na_2S$ . In addition, several slow-release  $H_2S$  donors have been developed. Another possibility is the use of thiosulfate (TS), a major metabolite of  $H_2S$ . Increasing evidence grounds the idea that a dynamic conversion exists between the two

substances<sup>27-29</sup>. In humans, the short term therapeutic use of sodium TS (STS) has been proven safe<sup>30</sup> for the treatment of calciphylaxis<sup>31, 32</sup>. STS is also proposed to be an antioxidant<sup>32</sup> and useful in case of cyanide poisoning<sup>33</sup> or cisplatin toxicity<sup>34</sup>. Furthermore, vasodilating properties of TS itself have been described<sup>35</sup>

The vasodilating and cytoprotective features of H<sub>2</sub>S make it an attractive therapeutic candidate for reducing the damaging effects of hypertension and proteinuria. In the experimental setting, ang II infusion causes hypertension, proteinuria and renal damage<sup>36</sup>. We used this model to investigate the renoprotective properties of sulfide containing compounds.

## 5

## MATERIALS AND METHODS

### Animals

Male Sprague Dawley rats (240-280 gram, Harlan, Zeist, the Netherlands) were housed under standard conditions with a 12 hour light-dark cycle at the animal research facility with *ad libitum* access to food and water. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local ethics committee for animal experiments.

### Ang II infusion and NaHS or STS treatment

Osmotic minipumps (model 2004, Alzet, Cupertino, CA, USA) were placed subcutaneously under general anesthesia (2% Isoflurane/O<sub>2</sub>) for continuous administration of ang II (435 ng/kg/min, n = 7/group; Bachem, Weil am Rhein, Germany) or vehicle (0.9% NaCl, n = 6). Post-operatively, all rats received a subcutaneous injection of 50 µg/kg buprenorphin (Schering-Plough, Houten, the Netherlands) for analgesic purposes and were allowed to recover from surgery at 37°C in a ventilated incubator. At placement of the pumps ang II-infused rats were randomized to either 0.9% NaCl, NaHS (5.6 mg/kg/day; Sigma, Zwijndrecht, the Netherlands) or STS (1 g/kg/day; Sigma, Zwijndrecht, the Netherlands) treatment. 0.9% NaCl-infused rats received treatment with 0.9% NaCl. During the three weeks of infusion rats received intraperitoneal (ip) injections with one of the compounds twice a day. At baseline, blood was collected via orbital puncture. On a weekly basis body weight was measured and rats were placed in metabolic cages for collection of 24-hour urine. Chlorhexidin was added to the urine as an antiseptic agent to prevent bacterial growth. After three weeks blood pressure was measured under general anesthesia (2% Isoflurane/O<sub>2</sub>) via an intra-aortic probe (Cardiocap/5, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Subsequently, rats were sacrificed and blood was collected in heparin and EDTA containing tubes and centrifuged for 10 minutes at 1000 rcf. Plasma was collected and stored at -80°C. Kidneys were perfused with 0.9% NaCl. Coronal slices were fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemical analysis or immediately snap frozen in liquid nitrogen and stored at -80°C for molecular analysis.

## Plasma and urine biochemical analysis

Plasma and urine levels of creatinine, urea and electrolytes were determined by standard assays from Roche on the Roche Modular (Roche Diagnostics GmbH, Mannheim, Germany) according to routine procedures in our clinical chemical laboratory. Urinary protein levels were determined with the pyrogallol red molybdate method<sup>37</sup>. Urinary TS was determined by a specific HPLC method as described previously<sup>30, 38</sup>. In short, 25  $\mu$ L of urine was derivatized with 5  $\mu$ L of 46 mM monobromobimane, 25  $\mu$ L of acetonitrile, and 25  $\mu$ L of 160 mM HEPES/16 mM EDTA pH 8 buffer (Invitrogen, Carlsbad, CA, USA) for 30 minutes in the dark. Derivatization of thiol groups was stopped by 50  $\mu$ L of 65 mM methanosulfonic acid (Fluka, Buchs, Switzerland) and proteins were removed by recentrifugation.

## Qualitative real-time polymerase chain reaction

Rat renal tissue containing cortex and medulla was homogenized in lysis buffer and total RNA was extracted using the TRIZOL method (Invitrogen, Carlsbad, USA). RNA concentrations were measured by a nanodrop UV-detector (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized using Superscript II with random hexamer primers (Invitrogen, Carlsbad, USA). Gene expression (Applied Biosystems, Foster City, CA, USA) was determined by qualitative realtime-PCR (qRT-PCR) based on the Taqman methodology. HPRT was used as a housekeeping gene with the following primers (Integrated DNA Technologies) and probe (Eurogentec): Forward: 5'-GCC CTT GAC TAT AAT GAG CAC TTC A-3', Reverse: 5'-TCT TTT AGG CTT TGT ACT TGG CTT TT-3' and Probe: 6-FAM 5'-ATT TGA ATC ATG TTT GTG TCA TCA GCG AAA GTG-3' TAMRA. The other primers were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD ID's used were: Coll3a1 (Collagen 3) Rn01437683\_m1, Acta2 ( $\alpha$ SMA) Rn01759928\_g1, Havcr1 (KIM-1) Rn00597703\_m1, CTH (CSE) Rn00567128\_m1, CBS Rn00560948\_m1, Mpst (3-MST) Rn00593744\_m1, Renin Rn00561847\_m1, TGF- $\beta$ 1 Rn00572010\_m1 and Cybb (NOX2) Rn00576710\_m1. The qRT-PCR reaction mixture contained 20 ng cDNA template and 5  $\mu$ L PCR-mastermix. Nuclease free water was added to a total volume of 10  $\mu$ L. All assays were performed in triplicate. The thermal profile was 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the delta Ct. Results were expressed as  $2^{-\Delta Ct}$ .

## Immunohistochemistry

For immunostaining, deparaffinized sections were subjected to heat-induced antigen retrieval by overnight incubation with 0.1 M Tris/HCl buffer (pH 9.0) at 80°C (ED1,  $\alpha$ SMA, KIM-1, desmin) or by incubation with EDTA buffer (pH 8.0) heated by a microwave (Collagen 3). Endogenous peroxidase was blocked with 0.075% H<sub>2</sub>O<sub>2</sub> in phosphate buffered saline (PBS, pH 7.4) for 30 minutes. Primary antibodies for macrophages

(mouse anti-CD68 ED1, MCA341R AbD, 1:750, Serotec Ltd, Oxford, UK),  $\alpha$ SMA (mouse anti-SMA, clone 1A4 A2547, 1:10.000, Sigma, Zwijndrecht, the Netherlands), Collagen 3 (goat anti-type 3 Collagen, 1330-01, 1:75, Southern Biotech, Birmingham, Alabama, USA), Desmin (mouse anti-desmin NCL-DES-DER11, 1:500, Novocastra, Rijswijk, the Netherlands) or KIM-1 (rabbit anti-KIM-1 peptide 9, 1:400, gift V. Baily) were incubated for 60 minutes at room temperature. Binding was detected using sequential incubation with peroxidase-labeled secondary and tertiary antibodies (Dakopatts, Glostrup, Denmark) for 30 minutes. All antibodies were diluted with PBS supplemented with 1% BSA. At the secondary and tertiary antibody dilutions 1% normal rat serum was added. Peroxidase activity was developed using 3,3'-diaminobenzidine tetrachloride for 10 minutes containing 0.03%  $H_2O_2$ . Counterstaining was performed using Mayer's hematoxylin. Appropriate isotype and PBS controls were consistently negative.

### Analysis of histopathological changes

Kidney sections were scanned using an Aperio Scanscope GS (Aperio Technologies, Vista, CA, USA). The extent of fibrotic changes ( $\alpha$ -SMA, Collagen 3), glomerular damage (desmin) and proximal tubular ischemic damage (KIM-1) were determined using the Aperio positive pixel analysis v9.1 algorithm. For  $\alpha$ -SMA, Collagen 3 and KIM-1 the ratio between the relative cortical staining intensity and the total cortical surface area was used. For desmin the ratio between glomerular staining intensity and total cortical glomerular area was calculated. Interstitial macrophages were counted manually by random selection of thirty renal cortical high powered fields. Histopathological analysis was performed in a blinded fashion.

### Urinary malondialdehyde measurements

Malondialdehyde (MDA), a major breakdown product of lipid peroxides, is generated after oxidative stress. MDA is a thiobarbituric acid-reactive substance and can be fluorescently measured after binding to thiobarbituric acid. Twenty  $\mu$ L urine was incubated with 90  $\mu$ L of 3% SDS and 10  $\mu$ L of 0.5 M butylated hydroxytoluene followed by addition of 400  $\mu$ L 0.1 N HCl, 50  $\mu$ L 10% phosphotungstic acid and 200  $\mu$ L 0.7% 2-Thiobarbituric acid. The reaction mixture was incubated for 30 minutes at 95°C. After adding 800  $\mu$ L of 1-butanol, the samples were centrifuged at 960 g for 10 minutes. Two hundred  $\mu$ L of the 1-butanol phase was fluorescently measured using 530 nm excitation and 590 nm emission wavelengths.

### Isolated perfused kidney setup

To investigate the effect of NaHS and STS on intrarenal pressure we used an *ex vivo* isolated perfused kidney (IPK) setup. After induction of anaesthesia with 2% Isoflurane/ $O_2$ , both kidneys and renal vessels from five healthy rats were isolated via a midline incision and subsequently a cannula was placed in the renal artery. Depending on the

renal vascular anatomy either the right or left kidney was used. After placement in the IPK setup, the kidney was continuously perfused via the renal artery with warmed (37°C) and oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub> gas mixture) Krebs-Ringer Bicarbonate (KRB) solution complemented with albumin and creatinine at a pH of 7.5 ± 0.05 and a PO<sub>2</sub> ≈ 60 kPa, by using a roller pump (Ismatec mv-ca/04; Ismatec, Glattbrugg, Switzerland) delivering a constant flow of 8 mL/min throughout the experiment. The composition of the perfusion solution was as follows: 118.6 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 6.1 mM glucose, 7.1 mM creatinine and 50 g/L albumin. After connecting the kidney, the flow was gradually increased to 8 mL/min. Vascular responses were monitored by an electromechanical pressure transducer (Cobe; Arvada, CO) connected to a computer interface (LabView, National Instruments, Austin, TX). After an equilibration period, when renal vascular pressure had stabilized, 1-2 μM of phenylephrine (PE) (Sigma, Zwijndrecht, the Netherlands) was added to the perfusate to obtain a stable pre-contraction pressure of 200-250 mmHg. When the PE-induced vasoconstriction had reached a plateau, kidneys were subjected to subsequent doses of NaHS (1 μM, 10 μM, 100 μM, 1 mM and 5 mM (n=5)) or STS (1 μM (n=5), 10 μM, 100 μM (n=2), 1 mM, 5 mM (n=5)) for 1 minute. To investigate the role of K<sub>ATP</sub> channels in NaHS-induced vasodilation, kidneys (n=5) were continuously perfused with 1 mM of Glibenclamide (Sigma, Zwijndrecht, the Netherlands) and subjected to subsequent doses of NaHS. After every dose we conducted a washout period of 4 minutes or continued when the intrarenal pressure had returned to baseline.

### Statistical analysis

Data were analyzed and graphed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Statistical analyses were performed using t-tests, Mann-Whitney U tests, two-way ANOVA, one-way ANOVA or Kruskal-Wallis tests where appropriate. Bonferroni, Dunnett's or Dunn's postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov-Smirnov test. Statistical significance was accepted at p<0.05. All data are expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise.

## RESULTS

### Rat characteristics – body weight and electrolytes

At baseline, there were no significant differences in body weight between the groups (Table 1). After 3 weeks, vehicle treated ang II-infused rats had a significantly lower body weight compared to NaCl-infused controls (p<0.001). Treatment with NaHS partially prevented ang II-induced weight loss (NaHS: p<0.05) (Table 1). Plasma sodium was significantly lower in vehicle treated rats compared to controls (p<0.001). No differences between groups were observed in plasma potassium and calcium levels (Table 1). In STS treated rats, urinary sodium excretion was significantly higher



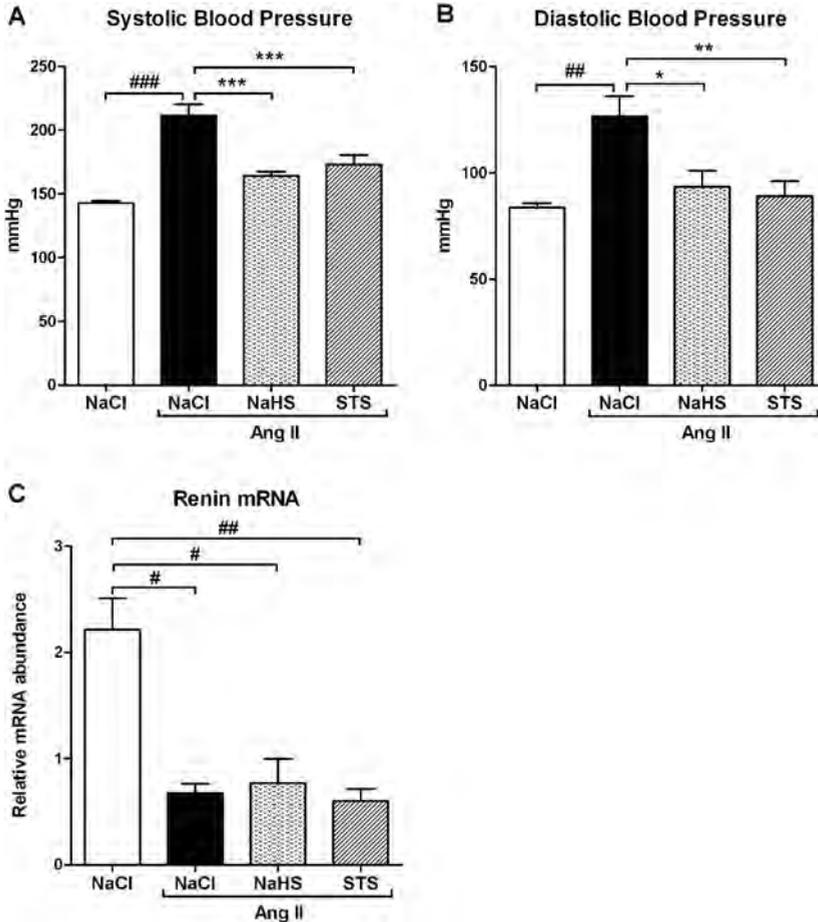


Figure 1 – Treatment with NaHS and STS attenuated ang II-induced hypertension and ang II infusion caused downregulation of renin mRNA expression. Infusion with ang II increased (A) systolic blood pressure by 48% and (B) diastolic blood pressure by 51%. Treatment with NaHS and STS significantly attenuated the ang II-induced hypertension. (C) Infusion with ang II caused downregulation of renin mRNA levels in all groups. (# $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. Ang II + NaCl)

(NaHS:  $14 \pm 2$  mmol/L,  $p < 0.01$ ; STS:  $14 \pm 2$  mmol/L,  $p < 0.01$ ) compared to vehicle treated rats (Figure 2A and B). From week 2 onwards, the 24-hour urinary protein excretion was significantly moderated by NaHS and STS treatment (NaHS:  $179 \pm 75$  mg/24 h,  $p < 0.001$ ; STS:  $197 \pm 58$  mg/24 h,  $p < 0.01$ ) (Figure 2C).

### STS increased the excretion of urinary TS

From week 1 onwards, the excretion of urinary TS ( $p < 0.001$ ) was significantly increased in ang II-infused rats treated with STS. No differences were observed in the other groups (Figure 3).

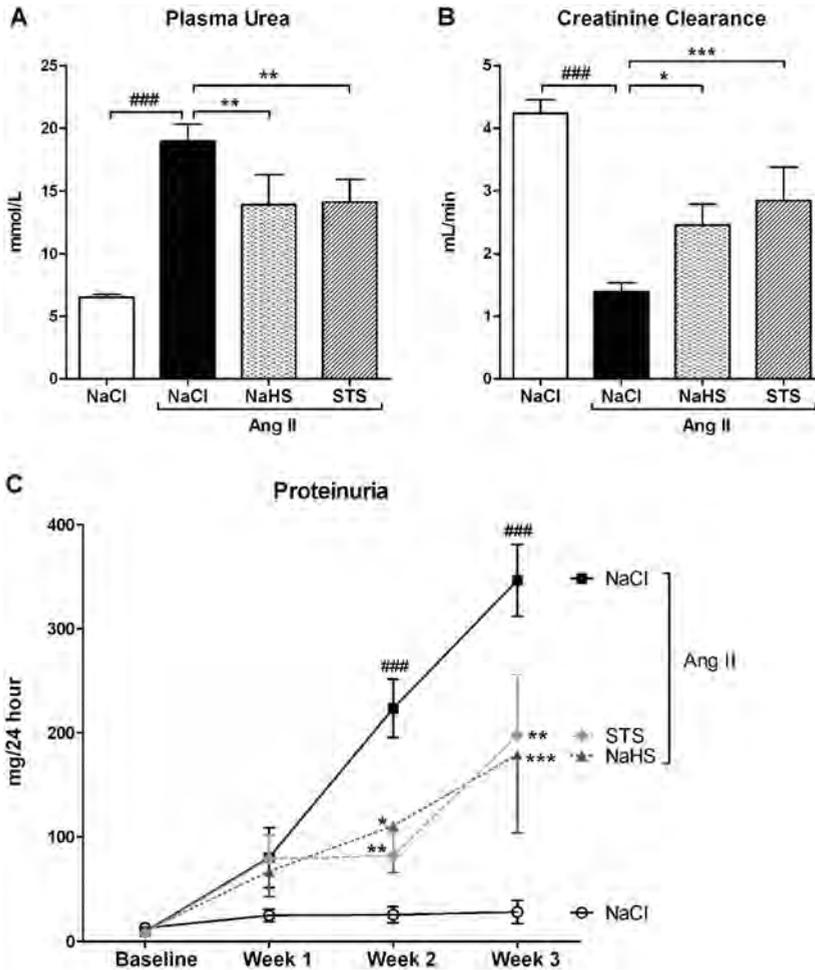


Figure 2 – Reduction of renal function loss and proteinuria in NaHS and STS treated rats. Three weeks of ang II infusion decreased renal function as evidenced by a 67% decrease in (A) creatinine clearance and a 2.9-fold increase in (B) plasma urea. Furthermore, (C) proteinuria was significantly increased from week 2 onwards. Treatment with NaHS and STS reduced renal function loss by preserving the creatinine clearance and plasma urea levels. In addition, the development of proteinuria was diminished in NaHS and STS treated rats from week 2 onwards. (### $p < 0.001$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. Ang II + NaCl)

### Effect of NaHS and STS treatment on tubular and glomerular damage

After 3 weeks, kidney injury molecule-1 (KIM-1) mRNA and protein expression were increased in ang II-infused rats treated with vehicle compared to controls ( $p < 0.001$ ). Both NaHS and STS treated rats showed less tubular damage as evidenced by a 2-fold reduction in proximal tubular damage at mRNA and protein level compared to vehicle treated animals ( $p < 0.05$ ) (Figure 4A, B and C). Also, the glomerular damage marker desmin was increased after three weeks of ang II infusion ( $p < 0.001$ ). Treatment

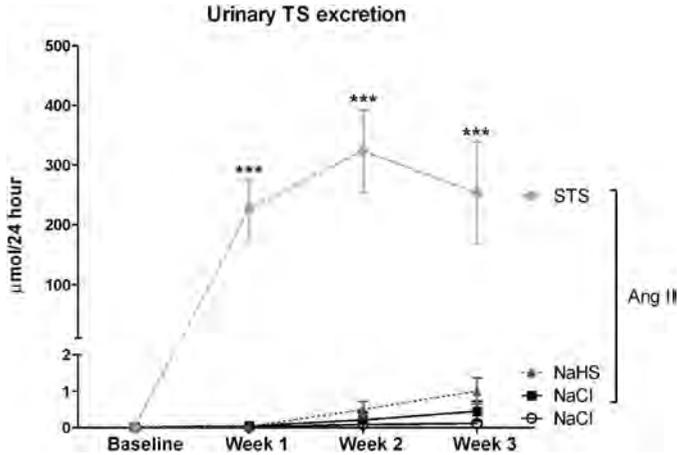


Figure 3 – Elevated urinary TS excretion in STS treated animals. In rats treated with STS, the excretion of urinary TS was increased from week 1 onwards. In all other groups no differences in urinary TS levels were observed. (\*\*\*) $p < 0.001$  vs. Ang II + NaCl)

with NaHS, but not STS, decreased the glomerular protein levels of desmin by 40% ( $p < 0.01$ ) (Figure 5A and B).

### Influx of macrophages is reduced by treatment with STS

Ang II infusion increased the number of ED1 positive cells in the renal interstitium more than 2-fold compared to NaCl infused controls ( $204 \pm 46$  vs.  $86 \pm 12$ ,  $p < 0.05$ ). STS and NaHS decreased the number of interstitial macrophages to near control levels (STS:  $73 \pm 21$ ,  $p < 0.01$ ; NaHS  $114 \pm 18$ ,  $p = 0.06$ ) (Figure 6A and B).

### Treatment with NaHS and STS reduced oxidative stress

Expression of NOX2 mRNA was increased 1.6-fold after ang II infusion ( $p < 0.05$ ) (Figure 7A). Furthermore, the urinary excretion of malondialdehyde (MDA) was increased more than 2-fold in ang II rats treated with vehicle ( $p < 0.001$ ) (Figure 7B). Simultaneous treatment with either NaHS or STS decreased NOX2 mRNA expression by 38% ( $p < 0.01$ ) and 39% ( $p < 0.01$ ), and urinary MDA levels by 35% ( $p < 0.05$ ) and 30% ( $p < 0.05$ ), respectively (Figure 7A and B).

### Protective effects of NaHS and STS treatment against renal interstitial changes

TGF- $\beta$ , a growth factor with proliferative and fibrotic effects on myofibroblasts, is significantly upregulated during ang II infusion ( $p < 0.05$ ). In rats treated with NaHS and STS, levels of TGF- $\beta$  mRNA return to near control values ( $p < 0.01$ ) (Figure 8A). Ang II infusion significantly increased mRNA ( $p < 0.05$ ) and protein ( $p < 0.001$ ) expression of

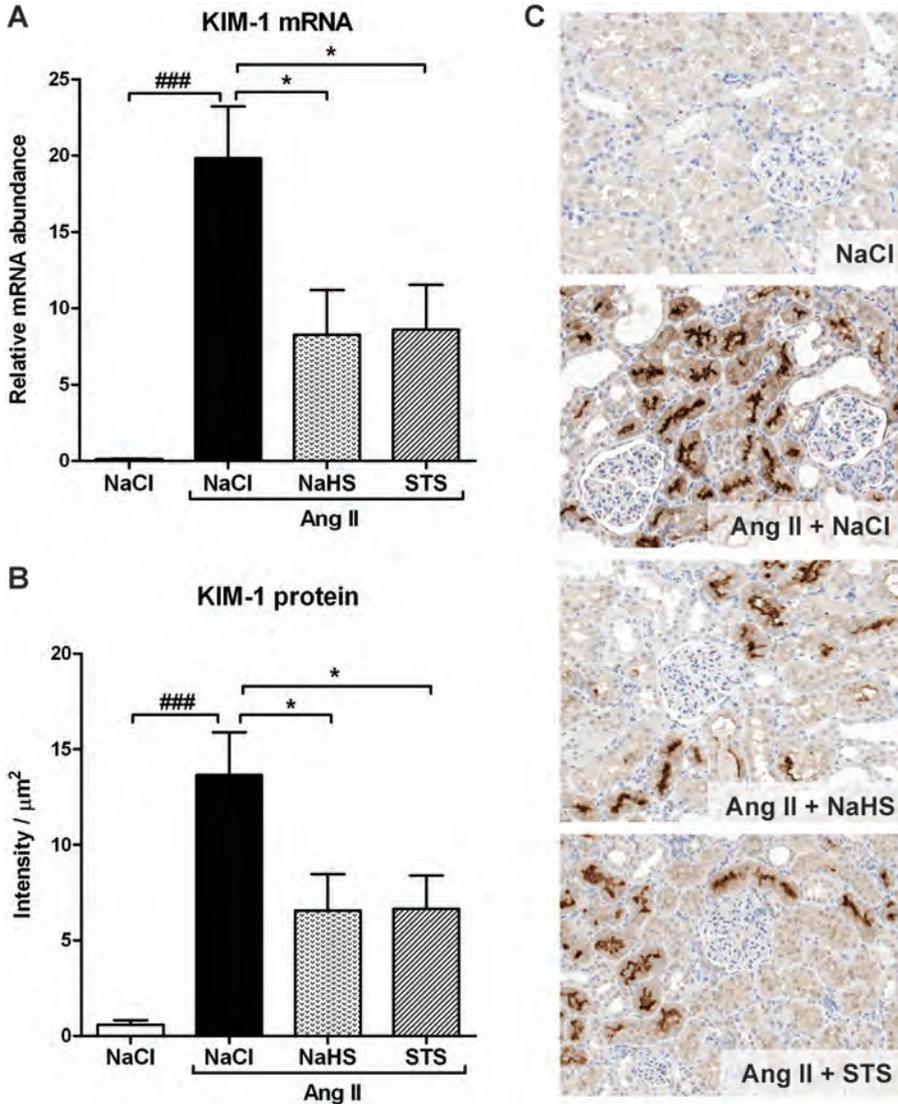


Figure 4 – Effect of NaHS and STS treatment on tubular damage. (A) KIM-1 mRNA and (B) KIM-1 protein levels were increased by ang II infusion. Rats treated with NaHS and STS had less tubular damage as evidenced by reduced KIM-1 protein and mRNA levels. (C) Representative photomicrographs of KIM-1 stained renal sections. (### $p < 0.001$  vs. control, \* $p < 0.05$  vs. Ang II + NaCl)

the pre-fibrotic marker  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), indicating ongoing interstitial myofibroblast transformation. STS, but not NaHS, decreased the mRNA and protein expression of  $\alpha$ SMA by 50% ( $p < 0.05$ ) (Figure 8B, C and D). The pre-fibrotic effects of ang II were accompanied by increased fibrotic damage, as evidenced by a significantly higher expression of Collagen 3 mRNA and protein ( $p < 0.05$ ). Treatment with NaHS

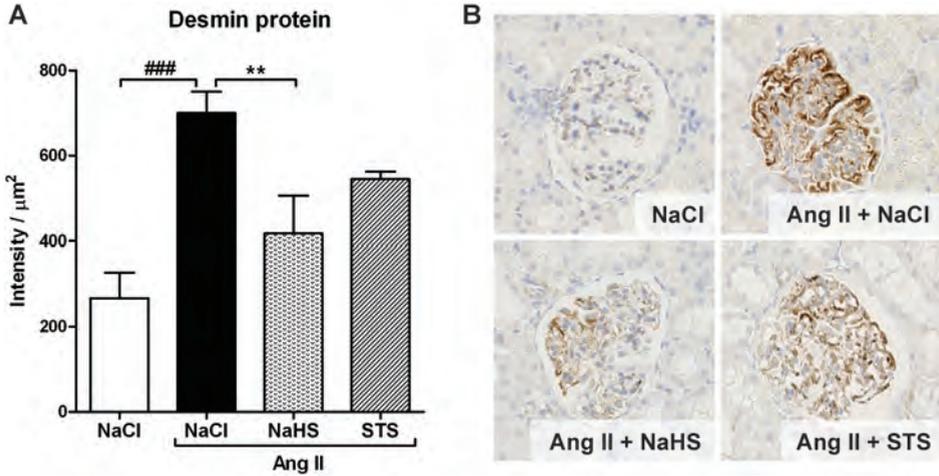


Figure 5 – Effect of NaHS and STS treatment on glomerular damage. (A) Desmin protein levels were increased in glomeruli of ang II-infused animals treated with vehicle. Treatment with NaHS reduced glomerular desmin levels by 40%. Treatment with STS only showed a trend towards decreased desmin levels. (B) Representative photomicrographs of desmin stained renal sections. (### $p < 0.001$  vs. control, \*\* $p < 0.01$  vs. Ang II + NaCl)

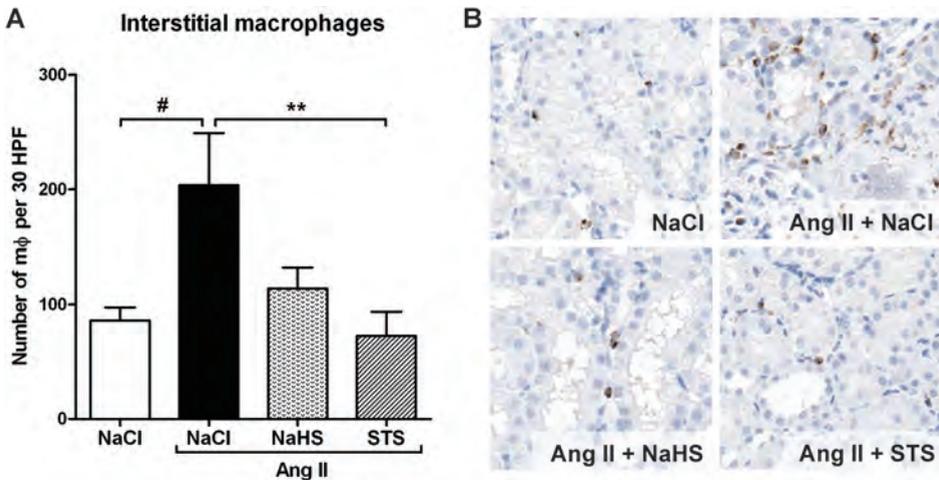


Figure 6 – Interstitial inflammation is reduced by treatment with STS. (A) The influx of macrophages was increased 2-fold in ang II-infused rats treated with vehicle. Treatment with STS, but not NaHS, reduced the number of interstitial macrophages 3-fold. (B) Representative photomicrographs of ED-1 stained renal sections. (# $p < 0.05$  vs. control, \*\* $p < 0.01$  vs. Ang II + NaCl)

and STS reduced the development of fibrosis, resulting in a reduction of Collagen 3 mRNA ( $p < 0.05$ ) and protein (NaHS:  $p < 0.05$ ; STS:  $p < 0.001$ ) to near control levels (Figure 8E, F and G).

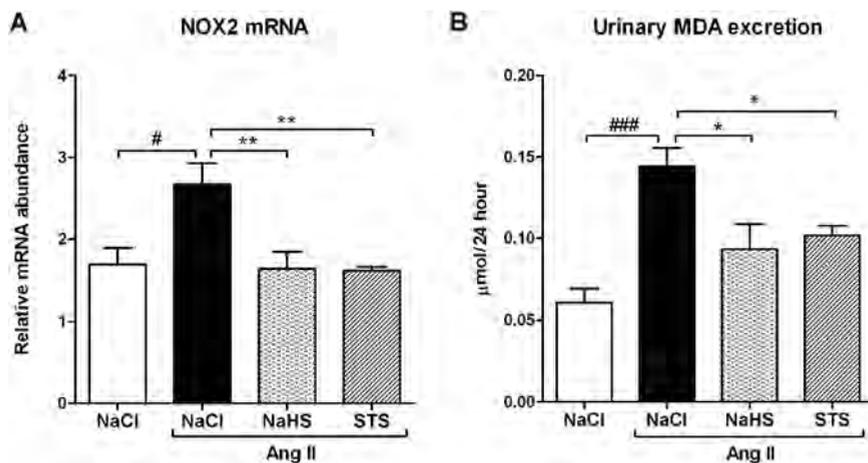


Figure 7 – Treatment with NaHS and STS reduced oxidative stress. After three weeks of ang II infusion, (A) NOX2 mRNA and (B) urinary MDA levels were increased 1.6-fold and 2-fold, respectively. Treatment with NaHS and STS reduced NOX2 mRNA expression by 38% and 39% and urinary MDA levels by 35% and 30%, respectively. (# $p < 0.05$ , ### $p < 0.001$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$  vs. Ang II + NaCl)

### Restoration of CSE, CBS and 3-MST mRNA expression with NaHS and STS treatment

Renal mRNA expression of CSE, CBS and 3-MST was significantly decreased after 3 weeks of ang II infusion ( $p < 0.01$ ). Expression levels were partially restored in ang II-infused rats that received NaHS and STS ( $p < 0.05$ ) (Figure 9A, B and C).

### Vasodilatory effects of NaHS in the isolated perfused kidney setup

Administration of 1 and 5 mM NaHS lowered intrarenal pressure by 44% ( $p < 0.001$ ) and 39% ( $p < 0.05$ ) compared to baseline pressure during phenylephrine (PE) constriction. Lower concentrations of NaHS had no effect on intrarenal pressure. To investigate the role of  $K_{ATP}$  channels in NaHS-induced vasodilation kidneys were perfused with Glibenclamide, which diminished the vasodilatory potency of 1 mM NaHS ( $p < 0.05$ ). STS showed no effect on intrarenal pressure (Figure 10).

## DISCUSSION

Exogenous treatment with NaHS and STS reduces hypertension, proteinuria, renal damage and renal function loss associated with ang II infusion. Furthermore, we are the first to show that STS, a clinically applicable compound, has promising renoprotective properties. These data suggest that intervention in sulfur pathways has protective potential in hypertension and hypertensive renal disease.

The effects of exogenous  $H_2S$  on blood pressure reduction are in line with previous literature showing its preventive and therapeutic properties in experimental

hypertension. Endogenous H<sub>2</sub>S shortage is suggested to be involved in the pathogenesis of hypertension<sup>11-13, 15, 39</sup>. One of the underlying mechanisms in blood pressure reduction by H<sub>2</sub>S is its direct effect on vascular smooth muscle cells by sulfhydration and subsequent activation of K<sub>ATP</sub>-channels<sup>11</sup>. The effects of H<sub>2</sub>S on proteinuria are described less clearly and not in a rectilinear fashion<sup>40, 41</sup>. The antiproteinuric effects of H<sub>2</sub>S in our model, as structurally evidenced by preservation of podocytes, can be partially explained by the reduction of systemic and intrarenal pressures. Mechanistic evidence for this comes from our ex vivo isolated perfused kidney (IPK) experiments in which we observed a reduction in intrarenal pressure by NaHS. Blockage of the K<sub>ATP</sub>-channels partly decreased the vasodilatory potency of NaHS, suggesting that other mechanisms are involved as well. One of these might be crosstalk between H<sub>2</sub>S and NO, since the vasorelaxant effects of H<sub>2</sub>S are partly NO-dependent. Inhibition of NO using N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) decreased the potency of H<sub>2</sub>S in aortic rings<sup>42</sup>. The in vivo effects of STS were unmistakable, but could not be related to direct changes in intrarenal pressure in our IPK setup. Since it is known that TS can be converted to H<sub>2</sub>S in vivo, we suggest that the IPK setup lacks the proper physiological conditions necessary for this conversion.

H<sub>2</sub>S directly affects the RAAS, as shown by its capacity to inhibit renin activity and ACE activity<sup>16</sup>. Since we infused ang II, the contribution of this mechanism is probably low or undetectable because of the existing negative feedback loop between ang II and renin. Indeed we found low mRNA levels of renin in all the ang II treated groups. Furthermore, H<sub>2</sub>S can decrease ang II-induced activation of mitogen-activated protein kinases and the binding affinity of the angiotensin-1 (AT-1) receptor in a dose-dependent manner<sup>43</sup>.

Although it is plausible that blood pressure regulation is the primary mechanism of action of H<sub>2</sub>S in our model, it is known that renal injury in ang II-induced hypertension can be independent of an elevated blood pressure. This suggests additional protective modes of action of H<sub>2</sub>S. Since we did not include a group receiving conventional antihypertensive drugs during ang II infusion, we can not determine whether the protective effects of H<sub>2</sub>S are solely mediated by blood pressure reduction.

Inflammation is one of the major consequences of renal exposure to hypertension and proteinuria<sup>4</sup>. STS completely prevented ang II-induced influx of interstitial macrophages, whereas NaHS only showed a trend. H<sub>2</sub>S is widely known for its anti-inflammatory properties and its role in modulation of leukocyte influx<sup>19, 44-48</sup>. Since proteinuria causes tubular damage and subsequent production of chemokines and attraction of macrophages<sup>49</sup>, the anti-inflammatory effects of H<sub>2</sub>S can also be explained by a reduction in proteinuria and a subsequent decline in tubular damage, as evidenced by decreased KIM-1 mRNA and protein levels.

Treatment with H<sub>2</sub>S influences renal fibrotic pathways as evidenced by a reduction in collagen 3 mRNA and protein, and a downregulation of TGFβ mRNA in both treatment groups. The attenuation of fibrosis by H<sub>2</sub>S treatment is in concordance with literature showing decreased fibrosis in various organs following H<sub>2</sub>S exposure<sup>46, 50-52</sup>.

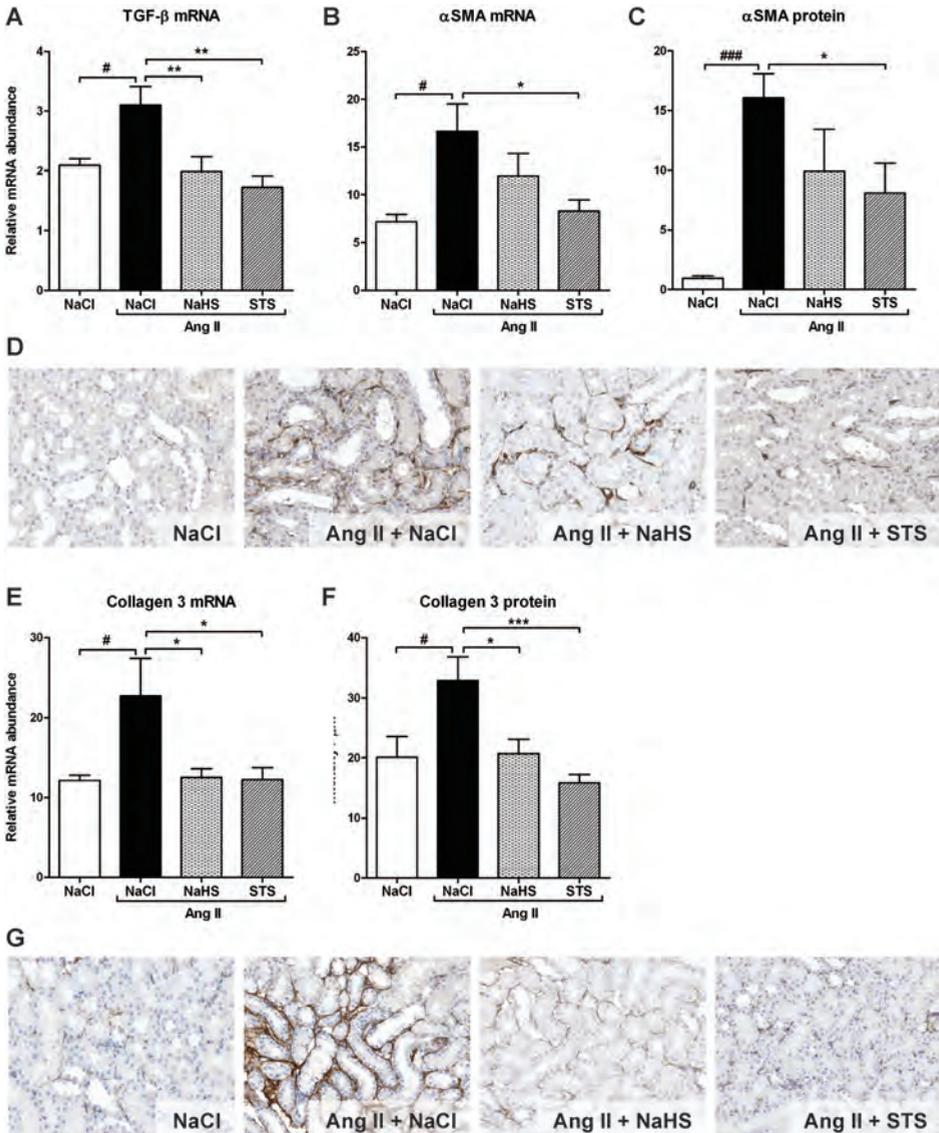


Figure 8 – Effect of NaHS and STS on renal fibrosis. (A) In rats infused with ang II and treated with vehicle, renal TGF- $\beta$  mRNA levels were upregulated. Treatment with NaHS and STS prevented the upregulation of TGF- $\beta$ . Ang II infusion caused upregulation of (B)  $\alpha$ SMA mRNA and (C)  $\alpha$ SMA protein expression. STS, but not NaHS, decreased the levels of  $\alpha$ SMA protein and mRNA. (D) Representative photomicrographs of  $\alpha$ SMA stained renal sections. (E) Collagen 3 mRNA and (F) Collagen 3 protein levels were increased in ang II-infused rats treated with vehicle. In NaHS and STS treated rats the formation of Collagen 3 was decreased. (G) Representative photomicrographs of Collagen 3 stained renal sections. (# $p$ <0.05, ### $p$ <0.001 vs. control, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 vs. Ang II + NaCl)

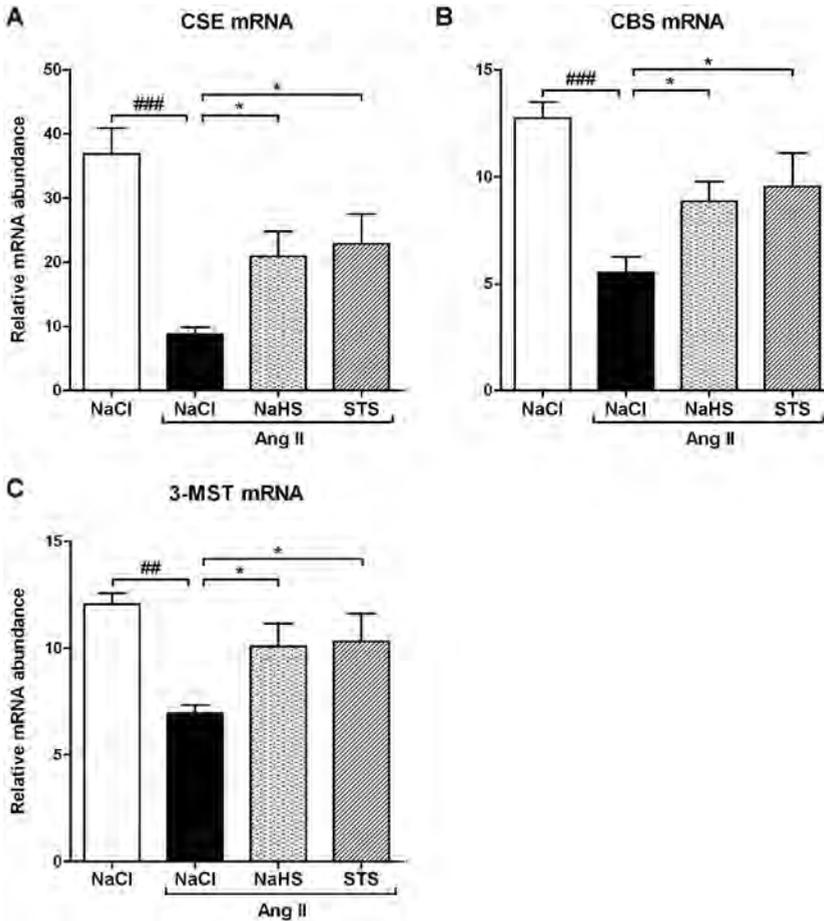


Figure 9 – Treatment with NaHS and STS restored the expression of CSE, CBS and 3-MST mRNA. After three weeks of ang II infusion, renal mRNA levels of (A) CSE, (B) CBS and (C) 3-MST are decreased. Treatment with NaHS and STS partially prevented this down regulation. (## $p < 0.01$ , ### $p < 0.001$  vs. control, \* $p < 0.05$  vs. Ang II + NaCl)

STS outperformed NaHS with regard to decreased  $\alpha$ SMA levels, which might be related to the lower level of interstitial macrophages in the former. Other studies showed an inhibiting effect of  $H_2S$  on  $\alpha$ SMA formation<sup>53, 54</sup>.

Another functional property of  $H_2S$  relates to the inhibition of ROS production. Ang II induces oxidative stress by activating NADPH oxidases via the AT-1 receptor<sup>55</sup>. We found diminished ROS production in  $H_2S$  treated animals as evidenced by lower urinary MDA levels. This might be related to the prevented upregulation of NOX2 in both  $H_2S$  treated groups, which is in line with literature showing the effect of  $H_2S$  on NADPH oxidases<sup>56</sup>.  $H_2S$  can also directly scavenge ROS, increase the intracellular glutathione levels, and reduce the amount of ROS produced through modulation of

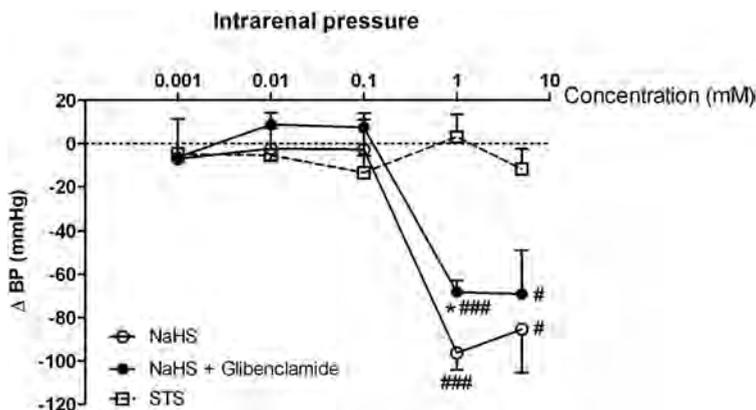


Figure 10 – NaHS lowered intrarenal pressure partly via activation of  $K_{ATP}$ -channels. In an IPK setup a stable pre-constriction pressure of 200-250 mmHg was induced by perfusion with 1-2  $\mu$ M PE. Administration of 1 and 5 mM NaHS lowered intrarenal pressure. Simultaneous perfusion with 1 mM Glibenclamide reduced the vasodilatory properties of 1 mM NaHS significantly. Concentrations of NaHS below 1 mM and all concentrations of STS had no effect on intrarenal pressure. (# $p < 0.05$ , ### $p < 0.001$  vs. baseline pressure, \* $p < 0.05$  vs. NaHS)

mitochondrial ROS production<sup>14, 23, 24</sup>. Loss of endogenous  $H_2S$ -production causes increased susceptibility to renal ischemia<sup>14</sup>. reduction of ROS plays an important role in the development of hypertension<sup>57</sup> and treatment with antioxidants reduces blood pressure in experimental models for hypertension<sup>58, 59</sup>.

Interestingly, we observed a decline in CSE, CBS and 3-MST mRNA levels in the ang II-infused animals treated with vehicle. Considering the vasorelaxing properties of  $H_2S$  we expected a compensatory increase in these enzymes. However, levels of  $H_2S$  and/or its producing enzymes were also decreased in other models of disease in which one might expect compensatory upregulation<sup>14, 25, 60-62</sup>. There is a concordance between progression of renal disease and the decline in  $H_2S$  producing capacity of renal tissue<sup>25</sup>, suggesting that depleted levels of endogenous  $H_2S$  production enhance renal damage. We have not measured  $H_2S$  since the reliability of the available techniques is controversial. Treatment with NaHS and STS restored the expression of CSE, CBS and 3-MST which is probably related to a reduction in proteinuria and subsequent salvage of tubular cells, which are major producers of these enzymes. These expression data suggest that there is a shortage in renal endogenous  $H_2S$  production in hypertensive renal disease, which implies that intervention in the  $H_2S$  producing pathway might be valuable to increase renal levels of sulfide.

One detailed study on the production of  $H_2S$  from thiosulfate showed that the amount and rate of  $H_2S$  production varies significantly between species and organs. Interestingly, the magnitude and rate of  $H_2S$  production was greatly amplified by the reducing agent DTT in the presence of tissue, with the most notable effects occurring in the liver.  $H_2S$  production was influenced by the amount of oxygen present, with

increased H<sub>2</sub>S production in hypoxic circumstances<sup>29</sup>. These interesting results give us a small clue on the relative H<sub>2</sub>S production from thiosulfate. However, absolute values must be interpreted with caution because of the lack of a reliable measurement method for H<sub>2</sub>S. The observation that STS as a major H<sub>2</sub>S metabolite<sup>27-29</sup> has similar protective effects as NaHS, provides us with exciting possibilities for the translation into clinical use. While short term treatment with STS is well tolerated, the long term side effects should be further explored. One of the drawbacks for long term administration to renal patients is the currently available route of delivery. To date, STS is only given intravenously to patients with calciphylaxis because oral forms of the compound with a validated intestinal uptake have not been developed. It is unknown whether the observed effects of STS are solely mediated by conversion to H<sub>2</sub>S or by unknown direct effects of STS itself. Animals treated with STS had significantly increased sulfate levels, indicating that conversion to H<sub>2</sub>S and sulfite took place before forming sulfate<sup>28</sup>. Effects of STS on blood pressure in humans have not been described extensively; however there are indications that STS exhibits vasodilating effects and antioxidant properties<sup>32, 35, 63</sup>, probably via the conversion to H<sub>2</sub>S<sup>27-29</sup> but also direct effects of STS have been suggested<sup>32</sup>.

Taken together, our data reveal novel protective modalities of H<sub>2</sub>S treatment in experimental renal disease. Therefore, intervention in H<sub>2</sub>S related pathways may have therapeutic potential in hypertension and hypertensive renal damage and deserves further exploration for clinical application.

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