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Sodium thiosulfate improves renal function and oxygenation in L-NNA-induced hypertension in rats

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Sodium thiosulfate, a reversible oxidation product of hydrogen sulfide, has vasodilating and anti-oxidative properties, making it an attractive agent to alleviate damaging effects of hypertension. In experimental settings, inhibition of nitric oxide synthase causes hypertension, renal dysfunction and damage. We hypothesized that thiosulfate would attenuate renal injury and improve renal function, hemodynamics and the efficiency of oxygen utilization for sodium reabsorption in hypertensive renal disease. Additionally, thiosulfate co-administration would further improve these variables when compared to inhibiting the renin-angiotensin system alone. Nitric oxide synthase was inhibited in Sprague Dawley rats by administering N- ω -nitro-L-arginine (L-NNA) in the food for three weeks. After one week, rats were split into two groups; without and with thiosulfate in the drinking water. In a parallel study, rats given N- ω -nitro-L-arginine and the angiotensin converting enzyme inhibitor lisinopril at a relatively low dose in their food were divided into two groups; without and with thiosulfate in the drinking water. Treatment with thiosulfate alleviated hypertension (mean 190 vs. 229 mmHg), lowered plasma urea (mean 11.3 vs. 20.0 mmol/L) and improved the terminal glomerular filtration rate (mean 503 vs. 260 μ l/min/100 gbw), effective renal plasma flow (mean 919 vs. 514 μ l/min/100 gbw) and oxygen utilization for sodium reabsorption (mean 14.3 vs. 8.6 μ mol/ μ mol). Combining thiosulfate with lisinopril further lowered renal vascular resistance (mean 43 vs. 63 mmHg/ml/min/100 gbw) and prevented glomerulosclerosis. Thus, our results suggest that thiosulfate has therapeutic potential in hypertensive renal disease and might be of value when added to standard antihypertensive therapies.

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KEYWORDS: ACE inhibitor; chronic kidney disease; hydrogen sulfide; hypertension; sodium thiosulfate

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Translational Statement

Antihypertensive therapies, such as angiotensin-converting enzyme (ACE) inhibitors, generally reduce the progression of hypertensive renal disease. However, inhibition of ACE alone is not always effective, especially in the later stages of renal disease. Novel therapies exerting additive effects on top of inhibiting ACE are required. We here report that sodium thiosulfate (STS) in itself is renoprotective in rats with hypertensive renal disease. STS on top of the ACE inhibitor lisinopril revealed additive effects on the improvement of terminal hemodynamics and in reducing glomerular injury. STS, which is already clinically approved, might be beneficial in treating hypertensive renal disease in patients.

Chronic kidney disease (CKD) is a global health problem associated with high morbidity and mortality.¹ Hypertension is a well-known risk factor in the acceleration of the progression of CKD. Functional modulation of the renin-angiotensin system (RAS) has shown great progress in renoprotection by reducing blood pressure, proteinuria, and the rate of decline in renal function.² However, in many cases, CKD further develops to end-stage disease, thereby requiring the need for dialysis or transplantation. Therefore, new therapeutic approaches are required by either improving therapies inhibiting the RAS or targeting other pathophysiological pathways.

Hydrogen sulfide (H₂S) is the third member of the endogenous gasotransmitter family alongside nitric oxide (NO) and carbon monoxide.³ The enzymatic production of H₂S depends on 3 enzymes, namely cystathionine γ -lyase (CSE), cystathionine β -synthase, and, to a lesser extent, 3-mercapto-pyruvate sulfurtransferase.⁴ Under stressful conditions, cystathionine

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β -synthase and CSE translocate from the cytoplasm to mitochondria and promote the production of H_2S and adenosine triphosphate (ATP).⁵ H_2S is serially oxidized to persulfide, thiosulfate, sulfite, and sulfate and can be reformed again from sulfite and thiosulfate, but not sulfate.^{4,6} A key physiological and pharmacological action of H_2S is to mediate vasorelaxation, which may involve activation of ATP-sensitive potassium channels.^{7,8} Lower levels of H_2S -generating enzymes and plasma H_2S were found in patients and experimental animal models exhibiting hypertension, and similar differences are also observed in patients with CKD.^{9–11} In addition, H_2S has been shown to have antioxidative and anti-inflammatory effects and to affect renal sodium reabsorption.^{12–14}

In experimental settings, H_2S can be delivered via inhalation or by parenteral administration of soluble sulfide salts such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S). Another possibility is the use of sodium thiosulfate (STS, $Na_2S_2O_3$), an intermediate oxidation product of H_2S . Thiosulfate produces H_2S through a nonenzymatic and an enzymatic glutathione-dependent pathway. Glutathione disulfide, H_2S , and labeled sulfite are produced when mitochondria are incubated with oxygen, glutathione, and [^{35}S] thiosulfate.¹⁵ In humans, the use of STS has been approved for short-term treatment of calciphylaxis and has also been useful in the treatment of cyanide poisoning and cisplatin toxicity.¹⁶ Vasodilating and antioxidative properties of STS have also been described.¹⁷ These beneficial effects of STS make it an attractive therapeutic candidate for reducing the damaging effects of hypertension.

In this study, we investigated the renoprotective effects of STS in a rat model of chronic deficiency of NO. N- ω -nitro-L-arginine (L-NNA) was used to induce hypertension in conscious rats, as previously performed at our laboratory.^{18,19} Mean arterial pressure (MAP) increases within the first 6 hours after administration of L-NNA.¹⁹ Chronic inhibition of NO synthase (NOS), by either L-NNA or its methylester L-NAME (N- ω -nitro-L-arginine methylester), leads dose-dependently to severe hypertension, proteinuria, glomerulosclerosis, and lower glomerular filtration rate (GFR) and effective renal plasma flow (ERPF).^{20,21} Additionally, efficiency of oxygen consumption for tubular sodium reabsorption (TNa/QO₂), a measure for transport efficiency, is lower in this model.¹⁹ The inhibition of RAS has been repeatedly shown to be protective in this model.¹⁸ We hypothesized that the administration of STS improves renal function, hemodynamics, and TNa/QO₂ in L-NNA-induced hypertension. In addition, we investigated whether administering STS on top of the angiotensin-converting enzyme (ACE) inhibitor lisinopril would further improve renal function, structure, and TNa/QO₂ compared with lisinopril alone.

RESULTS

Treatment with STS leads to a higher urinary excretion of sodium, thiosulfate, and sulfate

Chronic deficiency of NO was induced by administration of L-NNA in the food (40 mg/kg per day) for 3 weeks. To

investigate whether oral administration of STS with the drinking water (2 g/kg per day) is taken up by the body, urine samples were collected weekly in conscious rats. Natriuresis was significantly greater in L-NNA+STS-treated rats than in rats treated with L-NNA alone (Figure 1a). The excretion of potassium in the urine was not significantly different between the groups, suggesting similar food intake (Figure 1b). Urine samples from week 2, collected under fasting conditions, were used for the measurement of thiosulfate, sulfate, and NO metabolites. The excretion of thiosulfate and particularly sulfate in urine was markedly higher in the L-NNA+STS-treated group at week 2 (Figure 1c and d). These data indicate that in L-NNA-treated rats, administration of STS with the drinking water leads to high systemic levels of these H_2S metabolites. As expected during administration of L-NNA, nitrite and nitrate excretion was low and not significantly different between the groups (Supplementary Figure S1A and B).

Administering STS for 2 weeks attenuates L-NNA-induced hypertension and renal damage

To assess the effects of STS on hypertension and renal function, treatment with STS in the drinking water was started after 1 week of L-NNA and maintained for 2 weeks (Figure 2a). No differences in body weight (BW) were detected between the L-NNA and the STS groups (Figure 2b). Inhibition of NOS rapidly induced progressive hypertension (229 ± 14 vs. 149 ± 8 mm Hg) and treatment with STS lowered systolic blood pressure (SBP) by 17% (190 ± 25 mm Hg) (Figure 2c). Additionally, administration of L-NNA for 3 weeks increased proteinuria (215 ± 98 vs. 22 ± 10 mg/d) and plasma urea (20.8 ± 10 vs. 7.2 ± 0.5 mmol/l) and lowered creatinine clearance (290 ± 77 vs. 675 ± 321 μ l/min per 100 g BW) compared with these levels at baseline (Figure 2d–f). After treatment with STS, compared with the L-NNA group, loss of renal function was partially prevented as evidenced by lower plasma urea levels (11.3 ± 2.7 mmol/l) and a higher creatinine clearance (529 ± 225 μ l/min per 100 g BW).

Administration of STS for 2 weeks improved terminal renal hemodynamics

After 3 weeks of L-NNA treatment, hemodynamic parameters were determined via gold-standard inulin and para-aminohippuric acid clearance during a terminal experiment under isoflurane anesthesia. Treatment with STS, compared with the L-NNA treatment, resulted in a higher GFR (503 ± 55 vs. 260 ± 45 μ l/min per 100 g BW) and ERPF (919 ± 157 vs. 514 ± 216 μ l/min per 100 g BW) (Figure 3a and b). Filtration fraction was similar between the groups (Figure 3c). A trend ($P = 0.054$) toward a lower hematocrit level was found (Figure 3d). Under isoflurane anesthesia, MAP was not significantly different (152 ± 23 vs. 125 ± 33 ; $P = 0.14$). After treatment with STS, compared with L-NNA treatment, a higher calculated renal blood flow (RBF) and lower renal vascular resistance (RVR) were found (Figure 3e and f). There was a correlation between calculated RBF and RBF directly measured with a Transonic flow probe (1PRB Probe;

Figure 1 | Sodium thiosulfate (STS) treatment with STS leads to a high urinary excretion of sodium, thiosulfate, and sulfate. (a) Natriuresis and (b) kaliuresis measured in urine samples collected for 24 hours in conscious N- ω -nitro-L-arginine (L-NNA) and L-NNA+STS rats. Urine samples collected for 24 hours under fasting conditions at week 2 were used to measure (c) thiosulfate and (d) sulfate excretion. Vertical dotted lines indicate start of treatment with STS. Mean $\pm$ SD. L-NNA ($n = 8$), L-NNA+STS ($n = 7$). * $P < 0.05$, *** $P < 0.001$. Asterisks refer to comparisons made between the 2 groups. BW, body weight.

Transonic Europe B.V., Elstloo, the Netherlands) ($r = 0.59$; $P = 0.02$; $n = 14$).

STS treatment leads to a greater TNa/QO₂ ratio

To investigate whether treatment with STS had an effect on TNa/QO₂, arterial blood and renal venous blood was drawn at the end of the experiment for blood gas analysis. TNa (32 ± 8 vs. $15 \pm 7 \mu\text{mol/min}$) was higher in the STS group than in the L-NNA group (Figure 4a), while renal QO₂ was not altered (Figure 4b). Thus, TNa/QO₂ (14 ± 3 vs. $9 \pm 3 \mu\text{mol}/\mu\text{mol}$) was higher after 2 weeks of treatment with STS (Figure 4c).

Effect of STS treatment on glomerular damage

Periodic acid–Schiff staining results indicated no significant differences in glomerulosclerosis and mesangial matrix expansion between the groups after 2 weeks' administration of STS (Table 1). Likewise, immunohistochemistry and semi-quantitative analysis of α -smooth muscle actin (α -SMA) showed no significant differences in intensity of α -SMA staining. The effect of treatment with STS on the influx of inflammatory cells was also determined. ED1 staining for macrophages showed no significant differences in the number of glomerular and interstitial macrophages between the groups. Representative morphological pictures are shown in Figure 5.

Combining STS and lisinopril does not further attenuate hypertension-induced renal injury

The effects of STS on top of lisinopril for 2 weeks were investigated to see whether this would further improve blood pressure and renal function and damage in L-NNA hypertensive rats compared with those treated with lisinopril alone (Figure 6a). BW was maintained during the whole study period (Figure 6b).

As in the L-NNA+STS group, natriuresis was significantly higher in the L-NNA+lisinopril+STS group compared with the L-NNA+lisinopril-alone group (Supplementary Figure S2A). No differences were detected in urinary excretion of potassium (Supplementary Figure S2B) but thiosulfate and sulfate excretion was significantly higher (Supplementary Figure S2C and D).

As expected from previous studies with L-NNA and L-NAME,^{22–24} the ACE inhibitor lisinopril alone had a marked antihypertensive effect, although at this dose, SBP did not fall to baseline levels (Figure 6c). However, proteinuria was completely reversible (Figure 6d). No differences were detected in SBP at week 3 between lisinopril alone and the combination of lisinopril+STS. Proteinuria, plasma urea, and creatinine clearance were not significantly different between groups (Figure 6d–f).

Figure 2 | Sodium thiosulfate (STS) administration attenuates N- ω -nitro-L-arginine (L-NNA)-induced hypertension and renal damage. (a) Study design: rats received L-NNA mixed in the food for 3 weeks, and treatment with STS was initiated after 1 week of inhibition of nitric oxide synthase for 2 weeks. Longitudinal measurements were performed weekly, whereas blood sampling was done at baseline and week 3 only. (b) Body weight (BW), (c) systolic blood pressure, and (d) proteinuria are shown over the whole experimental period in L-NNA- and L-NNA+STS-treated rats. Baseline and week 3 urine and plasma samples were used to measure (e) plasma urea and calculate (f) creatinine clearance. Vertical dotted lines indicate start of treatment with STS. Mean $\pm$ SD. L-NNA only ($n = 8$), L-NNA+STS ($n = 6-7$). * $P < 0.05$, *** $P < 0.001$. Asterisks refer to comparisons between the L-NNA and L-NNA+STS groups. PAH, para-aminohippuric acid.

Combining STS and lisinopril further lowers RVR

The ACE inhibitor lisinopril alone caused a considerable rise in GFR (565 ± 200 vs. $260 \pm 100 \mu\text{l}/\text{min per } 100 \text{ g BW}$; $P = 0.02$), ERPF (1386 ± 251 vs. $514 \pm 216 \mu\text{l}/\text{min per } 100 \text{ g BW}$; $P < 0.001$), and RVR (63 ± 16 vs. $199 \pm 106 \text{ mm Hg}/\text{ml}/\text{min per } 100 \text{ g BW}$; $P = 0.02$). Combining lisinopril and STS did not further lead to a higher GFR and ERPF, although a trend ($P = 0.07$) was observed for ERPF (Figure 7a and b). No difference was detected in filtration fraction and hematocrit between the groups (Figure 7c and d). Under isoflurane anesthesia, MAP was not significantly different (128 ± 6 vs. $131 \pm 34 \text{ mm Hg}$; $P = 0.85$). A trend ($P = 0.08$) toward a higher RBF compared with lisinopril alone was observed (Figure 7e). STS on top of lisinopril, compared with lisinopril

alone, further lowered RVR (43 ± 9 vs. $63 \pm 16 \text{ mm Hg}/\text{ml}/\text{min per } 100 \text{ g BW}$) (Figure 7f).

Combining STS and lisinopril does not further improve TNa/QO₂

No differences were detected in TNa and QO₂ between the groups (Figure 8a and b). There was a trend toward a lower TNa/QO₂ ($P = 0.07$) compared with lisinopril alone (Figure 8c).

Combining STS and lisinopril improves glomerular injury

Lisinopril alone had no significant effect on glomerulosclerosis (2.7 ± 5.4 vs. 1.7 ± 1.6 ; $P = 0.61$), but when compared with L-NNA, a trend toward a lower mesangial

Figure 3 | Sodium thiosulfate (STS) treatment improves terminal renal hemodynamics. (a) Glomerular filtration rate, (b) effective renal plasma flow, and (c) filtration fraction were determined using gold-standard clearance methodology during a terminal experiment under isoflurane anesthesia. In addition, (d) hematocrit levels were measured and (e) renal blood flow and (f) renal vascular resistance were calculated. Mean $\pm$ SD. N- ω -nitro-L-arginine (L-NNA) ($n = 5$), L-NNA+STS ($n = 6$). * $P < 0.05$, ** $P < 0.01$. BW, body weight.

matrix expansion was observed ($4.8 \pm 7.0\%$ vs. $22.7 \pm 19.0\%$; $P = 0.07$). At the structural level, compared with lisinopril alone, STS plus lisinopril completely prevented glomerulosclerosis (Table 2). No differences in the intensity of α -SMA staining or number of glomerular and interstitial macrophages were detected between the groups. Representative morphological pictures are shown in Figure 9.

DISCUSSION

Our findings demonstrate that (i) STS orally administered via the drinking water is taken up by the body and metabolized as evidenced by the high urinary excretion of sodium, thiosulfate, and sulfate; (ii) treatment with STS attenuates L-NNA-induced hypertension, renal function, and hemodynamics; (iii) treatment with STS leads to a greater TNa/QO_2 ; and (iv)

Figure 4 | Efficiency of oxygen utilization for sodium reabsorption (TNa/QO_2) is higher in sodium thiosulfate (STS)-treated rats. Blood gas analysis of arterial and renal venous blood during a terminal experiment was performed to calculate (a) tubular sodium absorption (TNa), (b) renal oxygen consumption (QO_2), and (c) TNa/QO_2 in N- ω -nitro-L-arginine (L-NNA) and L-NNA+STS rats. Mean $\pm$ SD. L-NNA ($n = 5$), L-NNA+STS ($n = 6$). ** $P < 0.01$. BW, body weight.

Table 1 | Renal injury scores after 3 weeks of inhibition of NOS and 2 weeks of STS

Variable	L-NNA	L-NNA + STS
n	8	7
Total glomerulosclerosis score	1.7 ± 1.6	1.7 ± 2.0
Mesangial matrix expansion (%)	22.7 ± 19.1	18.1 ± 10.2
α-SMA (intensity/μm ²)	55.5 ± 26.0	59.4 ± 24.1
Glomerular MΦ (MΦ/glomerulus)	1.35 ± 0.66	1.14 ± 0.34
Interstitial MΦ (MΦ per 20 fields)	249 ± 168.5	198 ± 80.4

α-SMA, α-smooth muscle actin; L-NNA, N-ω-nitro-L-arginine; MΦ, macrophage; NOS, nitric oxide synthase; STS, sodium thiosulfate.

Data are expressed as mean ± SD.

STS on top of lisinopril, compared with lisinopril alone, further improves RVR and inhibits renal injury. These data suggest that intervention with a H₂S donor such as STS has therapeutic potential in hypertensive renal disease.

The novel observation that oral administration of the H₂S donor STS exerts renoprotective effects in the L-NNA model offers opportunities for translation into clinical practice. In the majority of preclinical studies, STS has been given via i.p. injections at concentrations ranging from 0.4 to 1.0 g/kg BW.^{2,12,25} In patients with CKD with calciphylaxis, i.v. infusion is the preferred route of administration with doses ranging from 5 to 25 g.¹⁶ Oral administration of STS as

maintenance therapy for calcific uremic arteriolopathy was well tolerated with no reported side effects.²⁶ Oral STS has also been used to reduce calcium phosphate nephrolithiasis in hypercalciuric rats.²⁷ Treatment with STS in our study showed a markedly higher sulfate excretion, consistent with extensive conversion to H₂S and sulfite prior to oxidative biotransformation to sulfate.²⁸

That exogenously administered H₂S donor alleviates hypertension extends previous findings acquired in different models of hypertension. Low levels of CSE, 3-mercapto-pyruvate sulfurtransferase, and endogenous H₂S have been shown to be involved in the development of hypertension both in patients and in animal models.^{10,11,29,30} Treatment with NaHS for 6 weeks reduced SBP by 19% in L-NAME-induced hypertensive rats.³¹ In addition to the reduction in the generation of NO, both expression of CSE and levels of plasma H₂S were less after administration of L-NAME. Chronic administration of L-NNA also down-regulates the expression of CSE, suggesting that members of the family of gasotransmitters interact with one another.³² NaHS attenuated the inhibition of the generation of H₂S, but generation of NO stayed the same between L-NAME- and NaHS-treated rats.³¹ The exogenous administration of NaHS lowered the blood pressure in renovascular hypertensive rats, spontaneously hypertensive rats, salt-sensitive Dahl Salt rats and angiotensin II-infused rats.^{2,30,33–35} A key physiological action of H₂S is to mediate vasorelaxation, which involves an enhancement of endogenous NO signaling, activation of vascular smooth muscle K_{ATP} channels or the induction of Keap1/Nrf2 signaling.^{7,8,36} In our L-NNA model, the former is less likely considering that it relies on the systemic inhibition of endogenous NOS. STS may react with cysteine through various reactions involving transsulfuration enzymes within the smooth muscle cells to produce H₂S.³⁷ H₂S mediates vasorelaxation via cysteine-S-sulphydration of K_{ATP} channels and subsequent hyperpolarization of the membrane potential.⁷ Additionally, H₂S nonselectively inhibits phosphodiesterases, thereby preventing the inactivation of cyclic adenosine monophosphate and cyclic guanosine monophosphate.³⁸ STS may also recouple endothelial NOS via restoration of tetrahydrobiopterin to its fully reduced state, thereby restoring bioavailable NO.³⁹ However, as our model is based on the inhibition of endogenous NOS, we believe that this mechanism plays a lesser role in our present study.

That STS was able to improve renal function and attenuate renal damage in a model of chronic deficiency of NO is a novel finding. A lower proteinuria, plasma urea, and improved creatinine clearance after treatment with STS have been reported in angiotensin II-infused rats.² NaHS improved creatinine clearance in 5/6 nephrectomized rats and spontaneously hypertensive rats.^{35,40} Higher GFR, RBF, and filtration fraction were found after acute intrarenal arterial infusion of NaHS in normotensive Sprague Dawley rats, but no differences in MAP were detected.⁴¹ H₂S has been shown to improve hypoperfusion in diabetic mice.⁴² Using gold-standard clearance methodology, we now showed for the

Figure 5 | Representative morphological pictures from paraffin-embedded kidney sections stained for (a) periodic acid-Schiff (PAS), (b) α-smooth muscle actin (α-SMA), and (c) ED1 for macrophages (MΦ) are shown for the N-ω-nitro-L-arginine (L-NNA)- and sodium thiosulfate (STS)-treated groups. Arrows indicate the presence of ED1-positive macrophages. Bars = 200 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 6 | Combining sodium thiosulfate (STS) and lisinopril (Lis) does not further attenuate hypertension-induced renal injury. (a) Study design: effects of STS on top of Lis for 2 weeks on blood pressure and renal function and injury were investigated in N- ω -nitro-L-arginine (L-NNA) hypertensive rats. Longitudinal measurements of (b) body weight (BW), (c) systolic blood pressure, and (d) proteinuria are shown over the whole experimental period in rats treated with only Lis or with Lis+STS. In (c), the horizontal dashed line indicates the baseline level of the systolic blood pressure. (e) Plasma urea and (f) creatinine clearance at baseline and at the end of the treatment period is reported. Vertical dotted lines indicate start of Lis or Lis+STS. Mean $\pm$ SD. L-NNA+Lis ($n = 5$), L-NNA+Lis+STS ($n = 5-6$). PAH, para-aminohippuric acid.

first time that GFR, ERPF, and RBF were improved after chronic treatment with STS in L-NNA hypertensive rats. An even further lowered RVR was observed when STS was combined with lisinopril. In addition, there was markedly less glomerular damage in the combination treatment, suggesting that orally given STS might well be of value when added to standard antihypertensive therapies.

The antihypertensive and renoprotective effects of STS in our study were achieved in the face of a large increase in sodium load. Although, high sodium intake is generally considered detrimental in the setting of hypertensive renal disease,^{43,44} this probably only pertains to sodium chloride.⁴⁵ Beneficial effects of sodium bicarbonate,⁴⁶⁻⁴⁸ sodium citrate,^{49,50} sodium nitrate,⁵¹ and sodium nitrite^{52,53} in this setting are well documented. Our findings show that STS can be included in this group of antihypertensive and

renoprotective sodium salts. The administration of high doses of STS in patients with calcific uremic arteriolopathy has been found to be beneficial with no reported hypertension or other side effects related to STS.^{26,54}

Administration of STS attenuated the lower TNa/QO₂ recently reported in this model.¹⁹ In the present study, QO₂ was unaltered, whereas TNa was significantly higher after treatment with STS, resulting in a higher TNa/QO₂. The higher TNa results from the improvement in GFR observed after treatment with STS. Much of the oxygen consumed by the kidney is utilized for the production of ATP to support sodium transport, and the remainder is utilized for other non-transport-related activities, typically referred to as “basal metabolism.” However, in the interpretation of the TNa/QO₂ ratio as a measure of transport efficiency, a fixed basal metabolism is assumed, which is unaffected by changes in sodium transport.⁵⁵ In reality, basal

Figure 7 | Sodium thiosulfate (STS) on top of lisinopril (Lis) further lowers renal vascular resistance. Terminal renal hemodynamics were measured after 3 weeks of N- ω -nitro-L-arginine (L-NNA) and 2 weeks of treatment. (a) Glomerular filtration rate, (b) effective renal plasma flow, and (c) filtration fraction were determined using gold-standard clearance methodology in rats anesthetized with isoflurane. (d) Hematocrit levels were measured and (e) renal blood flow and (f) renal vascular resistance were calculated for rats treated with only Lis or Lis+STS. Mean $\pm$ SD. L-NNA+Lis ($n = 5$), L-NNA+Lis+STS ($n = 5$). * $P < 0.05$. BW, body weight.

metabolism is subject to considerable variation and alterations in GFR and sodium transport can independently change basal O_2 consumption. Therefore, the TNa/QO_2 ratio should be interpreted cautiously as it can change independently of the efficiency of sodium transport.

Emerging evidence exist that H_2S functions as an O_2 sensor in the kidney, more specifically in the medulla.⁵⁶

Although H_2S generation is independent of O_2 , its metabolism relies highly on the presence of O_2 . Under normoxic conditions, H_2S is metabolized into sulfate. Hypoxia will lead to accumulation of H_2S , which helps to restore O_2 levels by enhancing RBF and suppressing tubular transport in the kidney.⁵⁷ Chronic inhibition of NOS for 2 weeks has been shown to induce hypoxia in the renal medulla.¹⁹ H_2S itself

Figure 8 | Combining sodium thiosulfate (STS) and lisinopril (Lis) tends to decrease the efficiency of oxygen utilization for sodium reabsorption (TNa/QO_2). Blood gas analysis of arterial and renal venous blood was used to calculate (a) tubular sodium reabsorption (TNa), (b) renal oxygen consumption (QO_2), and (c) TNa/QO_2 in the N- ω -nitro-L-arginine (L-NNA)+Lis and L-NNA+Lis+STS groups. Mean $\pm$ SD. L-NNA+Lis ($n = 4$), L-NNA+Lis+STS ($n = 5$). BW, body weight.

Table 2 | Renal injury scores after 3 weeks of inhibition of NOS and 2 weeks Lis or Lis + STS

Variable	Lis	Lis + STS
n	5	5
Total glomerulosclerosis score	2.7 ± 5.4	0 ± 0 ^a
Mesangial matrix expansion (%)	4.8 ± 6.9	1.7 ± 1.46
α-SMA (intensity/μm ²)	45.4 ± 20.5	46.9 ± 21.5
Glomerular MΦ (MΦ/glomerulus)	1.36 ± 0.87	1.08 ± 0.47
Interstitial MΦ (MΦ per 20 fields)	216.2 ± 134.1	139.7 ± 91.6

α-SMA, α-smooth muscle actin; Lis, lisinopril; MΦ, macrophage; NOS, nitric oxide synthase; STS, sodium thiosulfate.

^aP < 0.05.

Data are expressed as mean ± SD.

might serve as an electron donor for the production of ATP in mitochondria in hypoxic conditions.⁵ H₂S inhibits the activity of the tubular Na⁺-K⁺-2Cl⁻-cotransporter and the Na⁺-K⁺-ATPase pump.⁴¹ Combined with the higher GFR and higher sodium load after oral administration of STS, this led to a higher natriuresis. Thus, both hemodynamic and bioenergetic effects of H₂S may have been involved in our model.

In an additional set of experiments, we investigated whether STS on top of the ACE inhibitor lisinopril was superior over lisinopril alone to improve the hypertensive renal pathophysiology induced by L-NNA. There is a paucity of

information about the effects of H₂S in combination with inhibition of RAS. In rats with experimental diabetic nephropathy, the combination of NaHS and losartan, compared with NaHS or losartan alone, showed a larger reduction in MAP and recovered histopathological changes.⁵⁸ In the unilateral ureteral obstruction rat model, less renal fibrosis and infiltration of inflammatory cells were observed after treatment with low doses of NaHS. These effects were similar to those observed after treatment with enalapril after unilateral ureteral obstruction.⁵⁹ Treatment with allicin, the main organosulfur compound in garlic, in 5/6 nephrectomized rats improved blood pressure, proteinuria, and glomerular abnormalities to the same extent as losartan treatment did.⁶⁰ It has been known that H₂S affects the RAS directly, by inhibiting the activity of renin.⁶¹

In our present study, only the combination of lisinopril plus STS led to significantly lower glomerulosclerosis. Treatment with STS lowered SBP, but did not improve proteinuria. Lisinopril alone further lowered SBP and abolished proteinuria. Although ACE inhibition can effectively treat renal damage in the model of NOS inhibition,^{62,63} the low dose of lisinopril used in this study, deliberately chosen to allow observation of additional effects of STS, was apparently too low to improve glomerulosclerosis. This may be due to the residual high blood pressure observed under lisinopril alone. These data suggest that H₂S exerts additional renoprotective effects independent of its blood pressure- and proteinuria-lowering effects. H₂S has been described to have antioxidant properties via activation of the Keap1/Nrf2 signaling pathway, ameliorating oxidative stress.⁶⁴ Additionally, H₂S might play a role in preserving mitochondrial integrity.⁶⁵ In combination with further lowering of blood pressure when STS is used on top of lisinopril, these additional mechanisms might be involved in the improvement of glomerular damage in our model.

To conclude, we demonstrated that orally administered STS improves renal function and hemodynamics in L-NNA-induced hypertensive rats. STS treatment on top of lisinopril showed a further improvement in RVR and glomerular damage, suggesting that multiple pathways might be involved. Approaches targeting the H₂S pathway might have therapeutic potential in hypertensive renal disease. Further investigation on treatment with STS in hypertensive renal disease is warranted to gain more insight into its mechanisms of action.

METHODS

All procedures were approved by the Animal Ethics Committee of the University of Utrecht (CCD: AVD1150020171484) and were in accordance with the Dutch Codes of Practice for the Care and Use of Animals for Scientific Purposes.

Study design

Male Sprague Dawley rats (300–350 g; Envigo, Venray, the Netherlands) were cohoused in a climate-controlled facility with a 12-hour light/dark cycle. Rats had access to water and standard rat chow (CRM-E; Special Diet Services, Witham, Essex, UK) *ad libitum*. After a baseline period, NOS was inhibited by administering L-NNA

Figure 9 | Representative morphological pictures from paraffin-embedded kidney sections stained with (a) periodic acid-Schiff (PAS), (b) α-smooth muscle actin (α-SMA), and (c) ED1 for macrophages (MΦ) are shown for the N-ω-nitro-L-arginine (L-NNA) + lisinopril (Lis) and the L-NNA + Lis + sodium thiosulfate (STS) groups. Arrows indicate the presence of ED1-positive macrophages. Bars = 200 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

(40 mg/kg per day; Sigma-Aldrich, Zwijndrecht, the Netherlands) with the food for 3 weeks.²¹ After 1 week of inhibition of NOS, animals were divided into 2 groups: (i) L-NNA only (L-NNA; $n = 8$) and (ii) L-NNA with STS (2 g/kg per day; Sigma-Aldrich) administered in the drinking water for 2 weeks (L-NNA+STS; $n = 7$). The dose of STS was determined in a pilot study in healthy Sprague Dawley rats in which the dose of both 1 and 3 g/kg per day led to high thiosulfate and free thiol excretion (Supplementary Figure S3A and B). In a parallel study, rats were divided in 2 groups: (i) L-NNA with lisinopril (1 mg/kg per day; Sigma-Aldrich) mixed in the food ($n = 5$) and (ii) L-NNA with both lisinopril in the food and STS in the drinking water ($n = 6$). Of note, this dose of lisinopril is very low in comparison to most other studies with lisinopril in models of CKD.^{66–69} Starting from baseline, SBP measurements via tail-cuff plethysmography and 24-hour urine collection was performed weekly. Blood was sampled from the tail vein at baseline and after 3 weeks of administration of L-NNA. Body weight was monitored every 3 days. At the end, renal hemodynamic evaluation, blood gas analysis, and morphological analysis were performed in all groups.

Urine collection and analysis

Rats were individually housed in metabolic cages with access to food and water for 24-hour urine collection. For the measurements in week 2 (Figure 1c and d), rats received only drinking water supplemented with 2% glucose. The excretion of thiosulfate, sulfate, and NO metabolites were determined in these samples. Proteinuria was measured by BIO Rad protein assay (Bio-Rad Laboratories B.V., Venendaal, the Netherlands). Concentrations of sodium and potassium were determined by flame photometry (model 420; Sherwood Scientific, Cambridge, UK). Creatinine was measured enzymatically (Creatinine PAP FS; DiaSys Diagnostic Systems, Holzheim, Germany). Thiosulfate and sulfate concentrations were measured by ion chromatography-mass spectrometry, as described.⁷⁰ Nitrite and nitrate concentrations were analyzed without dilution using a dedicated high-pressure liquid chromatography system;⁷¹ because the presence of L-NNA in the urine produced an additional peak that eluted right before nitrate in this system, concentrations of the latter were cross-validated by ion chromatography-mass spectrometry.

Plasma collection and analysis

A blood sample of 0.5 ml was drawn from the tail vein into ethylenediaminetetraacetic acid anticoagulant-coated tubes in rats anesthetized with isoflurane (4% induction and 2.5% maintenance). Plasma was extracted by centrifugation at 3500g for 15 minutes at 4 °C and used for determination of urea (Urea CT FS; DiaSys Diagnostic Systems) and creatinine.

Assessment of renal hemodynamics and blood gas analysis

Renal function was assessed in rats after 3 weeks of L-NNA administration as described.^{72,73} Briefly, rats were anesthetized with isoflurane (4% induction and 2% maintenance) and artificially ventilated (UNO, Zevenaar, the Netherlands). The femoral artery was cannulated for the collection of plasma. Urine was collected via the bladder and left ureter. The jugular vein was cannulated for the infusion of inulin and para-aminohippuric acid, to calculate GFR and ERPF, respectively. RBF was calculated as ERPF/1-hematocrit. Blood gas variables from the left renal vein and femoral artery were analyzed on a blood gas analyzer (i-STAT; Abbott, Hoofddorp, the Netherlands). QO_2 ($\mu\text{mol}/\text{min}$) was calculated from the

arteriovenous difference in oxygen content ($\text{oxygen content} = [\text{Hb}] \cdot O_2 \text{ saturation} \cdot 1.34 + pO_2 \cdot 0.003$) multiplied by RBF².

Renal histology

Kidneys were fixed for at least 24 hours in 10% formalin and embedded in paraffin. Kidney sections of 3 μm were stained with periodic acid-Schiff and scored for focal glomerulosclerosis. Focal glomerulosclerosis score was obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and summing these scores.⁷⁴

For immunostaining, deparaffinized sections were subjected to heat-induced antigen retrieval overnight with 0.1 mol/l tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 9.0) at 80 °C. Endogenous peroxidase was blocked with 0.03% H_2O_2 for 30 minutes. Sections were incubated with primary antibody for α -SMA (mouse anti-SMA, clone 1A4 A2547, 1:10,000; Sigma-Aldrich) or macrophages (mouse anti-ED1, MCA341R; Serotec, Oxford, UK) for an hour, followed by sequential incubation with peroxidase-labeled secondary and tertiary antibodies for 30 minutes. One percent of normal rat serum was added to the dilutions. Peroxidase activity was stopped using 3,3'-diaminobenzidine and counterstained with Mayer's hematoxylin. Kidney sections were scanned with the Aperio Scanscope GS (Aperio Technologies, Vista, CA). The degree of fibrotic changes was determined with Aperio positive pixel analysis V9.1 algorithm. The ratio between relative cortical staining intensity and total cortical surface area was calculated. Glomerular and interstitial macrophages were counted manually by random selection of 20 high-powered fields. Analysis was performed by an experienced technician in a blinded manner.

Statistical analysis

Data were analyzed using GraphPad Prism 7.0 software (GraphPad, San Diego, CA). Data are presented as mean $\pm$ SD. For longitudinal measurements, a 2-way analysis of variance for repeated measures was performed, followed by Bonferroni *post hoc* test. Comparison between baseline and after 3 weeks of L-NNA administration was done with a paired *t* test and comparisons between groups were done using unpaired *t* tests.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. (A) Nitrite and (B) nitrate excretion measured in urine collected under fasting conditions in conscious rats. Mean $\pm$ SD. L-NNA ($n = 8$), L-NNA+STS ($n = 7$).

Figure S2. Treatment with STS led to higher excretion of sodium and sulfate. Urine samples were collected for 24 hours to measure (A) natriuresis and (B) kaliuresis in the L-NNA+Lis+STS group and the L-NNA+Lis group. Additionally, rats were placed under fasting conditions in the metabolic cages in week 2 to measure (C) thiosulfate and (D) sulfate excretion. Mean $\pm$ SD. L-NNA+Lis ($n = 5$), L-NNA+Lis+STS ($n = 6$). * $P < 0.05$, *** $P < 0.001$.

Figure S3. In a pilot study in 4 healthy Sprague Dawley rats, STS was orally administered via the drinking water at 3 incremental doses to investigate which dose would be optimal. **(A)** Thiosulfate and **(B)** free thiol excretion was measured in urine samples collected for 24 hours from conscious rats. Mean $\pm$ SD.

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