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Sodium thiosulfate attenuates angiotensin II-induced hypertension, proteinuria and renal damage [1]

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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.

1. 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) [1–4]. 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 [5,6]. 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 [7]. It is endogenously produced from the amino acid L-cysteine by cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) [8,9], and from 3-mercaptopyruvate (3MP) by 3-mercaptopyruvate sulfurtransferase (3-MST) [10]. In the vasculature H_2S functions as an endothelial cell-derived relaxing factor via direct activation of ATP-sensitive potassium (K_{ATP}) channels [11]. Accordingly, CSE-deficient mice and CBS heterozygous mice develop hypertension [12,13]. CSE can act as an endogenous modulator of oxidative stress, as CSE-deficient mice have increased renal damage after ischemia-reperfusion [14]. Exogenous treatment with the soluble sulfide salt NaHS attenuates the hypertensive effects of NO synthase (NOS) inhibition [15] and has preventive and therapeutic effects on renovascular hypertension by inhibiting plasma renin activity [16]. In addition, H_2S

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stimulates cellular proliferation [17] and angiogenesis [18], and reduces inflammation [19,20]. Endogenous H_2S functions as a signaling molecule by regulating protein activity through S-sulfhydration, which is a form of posttranslational modification [21,22]. Furthermore, H_2S can play a detoxifying role during oxidative stress by direct scavenging of ROS or increasing the formation of the antioxidant glutathione [23,24]. Progression of renal disease in a CKD model is associated with depletion of H_2S and its producing enzymes [25]. Recently, urinary sulfur metabolites were found to associate with a favorable cardiovascular risk profile and even improved survival in renal transplant recipients [26]. 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 [27–29]. In humans, the short term therapeutic use of sodium TS (STS) has been proven safe [30] for the treatment of calciphylaxis [31,32]. STS is also proposed to be an antioxidant [32] and useful in case of cyanide poisoning [33] or cisplatin toxicity [34]. Furthermore, vasodilating properties of TS itself have been described [35].

The vasodilating and cytoprotective features of H_2S 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 [36]. We used this model to investigate the renoprotective properties of sulfide containing compounds.

2. Materials and methods

2.1. 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.

2.2. 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_2) 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. During the three weeks of infusion, rats received intraperitoneal (ip) injections with one of the compounds twice a day. Control rats received 0.9% NaCl infusion via osmotic minipumps, as well as they were daily administered with 0.9% NaCl via ip injections. 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_2) 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.

2.3. 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 [37]. Urinary TS was determined by a specific HPLC method as described previously [30,38]. In short, 25 μL of urine was derivatized with 5 μL of 46 mM monobromobimane, 25 μL of acetonitrile, and 25 μL 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 μL of 65 mM methanosulfonic acid (Fluka, Buchs, Switzerland) and proteins were removed by recentrifugation.

2.4. 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, Wilminton, 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 IDs used were: Coll3a1 (Collagen 3) Rn01437683_m1, Acta2 (αSMA) Rn01759928_g1, Havcr1 (KIM-1) Rn00597703_m1, CTH (CSE) Rn00567128_m1, CBS Rn00560948_m1, Mpst (3-MST) Rn00593744_m1, Renin Rn00561847_m1, TGF-β1 Rn00572010_m1 and Cybb (NOX2) Rn00576710_m1. The qRT-PCR reaction mixture contained 20 ng cDNA template and 5 μl PCR-mastermix. Nuclease free water was added to a total volume of 10 μl. 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 $2^{-\Delta Ct}$.

2.5. 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, αSMA, 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_2O_2 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), αSMA (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