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ORIGINAL ARTICLE

Hydrogen sulfide treatment reduces blood pressure and oxidative stress in angiotensin II-induced hypertensive mice

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Hydrogen sulfide (H_2S) is increasingly recognized as a gasotransmitter with protective effects in the cardiovascular system. The aim of the study was to examine the effects of chronic NaHS treatment on blood pressure, vascular function and oxidative stress in an *in vivo* model of hypertension and oxidative stress. Male C57Bl6/J mice were rendered hypertensive with 0.7 mg kg^{-1} per day angiotensin II (AngII) for 14 days administered *via* implanted mini-pumps. The mice were treated with NaHS (10 μmol kg^{-1} per day) to deliver H_2S or an inhibitor of cystathionine-γ-lyase, DL-propargylglycine (PPG 30 mg kg^{-1} per day) *via* intraperitoneal (i.p.) injection. Systolic blood pressure was measured and vascular function examined by myography. Vascular superoxide production was measured by lucigenin-enhanced chemiluminescence. AngII infusion significantly increased systolic blood pressure ($P<0.001$). This increase was significantly attenuated by treatment with NaHS ($P<0.001$). Both aortic endothelial function and NO bioavailability were significantly attenuated in the AngII group ($P<0.01$) but this attenuation was reversed by NaHS treatment. Similarly, aortic superoxide anion production was significantly enhanced by AngII ($P<0.01$), and this was reversed by NaHS treatment, and also exacerbated by PPG treatment ($P<0.001$). These data show that in a mouse model of hypertension and oxidative stress induced by AngII, exogenous H_2S treatment *in vivo* reduces blood pressure, endothelial dysfunction and vascular oxidative stress, while inhibiting endogenous H_2S production *in vivo* is deleterious. This furthers the evidence that H_2S is a vasoprotective molecule that may be a useful treatment target in cardiovascular disease.
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Keywords: NADPH oxidase; oxidative stress; superoxide; vasoprotection

INTRODUCTION

Hydrogen sulfide (H_2S) is a gasotransmitter[1] reported to have numerous physiological effects in diverse processes including metabolism, inflammation, the nervous system and the cardiovascular system. The cardiovascular effects of this molecule are currently of major interest and include vascular relaxation, cardioprotective and vasculoprotective effects.[2,3]

In the cardiovascular system, H_2S is produced primarily by the pyridoxyl'5'phosphate-dependent enzyme cystathionine-γ-lyase (CSE, EC 4.4.1.1), which is present in both endothelial cells[4] and vascular smooth muscle cells.[5,6] Inhibition of CSE, with the irreversible inhibitor DL-propargylglycine (PPG), leads to an elevation of vascular tone in isolated aorta[7] and an increase in blood pressure *in vivo* in rats.[8] Most importantly, mice deficient in CSE display early endothelial dysfunction and hypertension.[4] H_2S is additionally reported to be produced by 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) in concert with cysteine aminotransferase (EC 2.6.1.75) to metabolize cysteine.[9]

A key aspect of the biology of H_2S is its anti-oxidant effects. H_2S is a potent one-electron chemical reductant that is theoretically capable of scavenging free radicals by single electron or hydrogen atom transfer.[10] Thus, H_2S may participate in many reactions[11] and is reported to scavenge reactive oxygen and nitrogen species.[11–16] However, the kinetics, reactivity and mechanism of H_2S interactions with reactive oxygen species (ROS) are poorly understood under physiological conditions.[10] Further cytoprotective effects of NaHS have been attributed to its ability to decrease lipid peroxidation,[12] increase glutathione levels and boost endogenous anti-oxidant defences.[17]

Oxidative stress is an important feature in a number of cardiovascular disease states including hypertension, diabetes and atherosclerosis.[18,19] Blood vessels express NADPH oxidases; enzyme assemblies that contain catalytic 'NOX' subunits (or 'NOXs'), of which at least three isoforms, NOX1, NOX2 and NOX4, are responsible for the production of ROS in the vasculature. Both NADPH oxidase activity and expression are upregulated in

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cardiovascular disease, with NOX2 playing a key role in the vascular dysfunction associated with these pathologies.[20] Thus, strategies to limit oxidative stress in cardiovascular disease are sought and the antioxidant capacity of H_2S makes it an attractive candidate. It is well recognized that angiotensin II (AngII) can increase ROS generation in the vasculature, predominantly *via* activation of NOXs such as NOX2. Indeed, AngII-mediated NADPH oxidase activation and ROS production has been implicated in atherosclerosis and hypertension.[21] As such, the AngII-infusion model of hypertension exploits the ability of AngII to increase NADPH oxidase activity[22] and superoxide generation, and is useful as a model of both increased oxidative stress and hypertension.[23]

While to date there have been many *in vitro* studies examining the anti-oxidant effects of H_2S, *in vivo* studies focusing on vascular effects are lacking, so the physiological relevance of such findings is yet to be fully explored. The aim of this study was to investigate whether or not exogenous H_2S can ameliorate vascular oxidative stress *in vivo* and thereby confer vasoprotection, using the AngII-induced oxidative stress and hypertension model in mice. These studies are an important next step from the *in vitro* evidence of H_2S as an anti-oxidant[16] to proving its capacity as a vasoprotectant *in vivo*.

MATERIALS AND METHODS

Animals

All experimental procedures involving the use of animals were approved by the RMIT Animal Ethics Committee before the commencement of this project. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23 revised 1996). All animals were housed in the RMIT Animal Facility, RMIT University, Bundoora West Campus on a 12-h day/night cycle at room temperature of 20 ± 2 °C. Male C57BL/6J mice, 10 weeks of age were purchased from the Animal Resource Centre, Western Australia.

Angiotensin II-induced hypertension

Mice (C57Bl6/J, male 10 weeks) were anesthetized with 2% isoflurane in oxygen, and an osmotic mini-pump (Alzet micro-osmotic pump model 1004; Alzet DURECT Corporation, Cupertino, CA, USA) was implanted subcutaneously between the scapulae. Mice were infused for 2 weeks with angiotensin II (AngII) 0.7 mg kg^{-1} per day, prepared in AngII buffer (composition: 3 ml of 5 M NaCl (150 mM NaCl), 1 ml of 1 M acetic acid (10 mM) in 100 ml dH_2O). The animals were divided into four groups; (1) no treatment, (2) AngII infusion, (3) AngII infusion and PPG 3 mg kg^{-1} per day i.p., (4) AngII infusion and NaHS 10 μmol kg^{-1} per day, i.p.

Blood pressure measurement

The systolic blood pressure of the animals was measured 1 day before the insertion of the mini-pumps and 7 and 14 days after the procedure, using the non-invasive tail cuff apparatus (ADInstruments, Sydney, NSW, Australia). Systolic blood pressure was averaged from four to six consecutive measurements taken at intervals of 1–2 min. After measurement of systolic blood pressure on day 14, mice were culled in a humane manner *via* CO_2 asphyxiation (95% CO_2, 5%O_2), followed by cervical dislocation and decapitation. The aorta was dissected out and washed in ice-cold oxygenated Krebs' solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 5.5, EDTA 0.026, pH 7.4).

Myograph experiments

Abdominal aortic rings (~2 mm length) were mounted in 7 ml myograph chambers, where they were maintained in Krebs' solution at 37 °C, continuously supplied with carbogen (95% O_2, 5%CO_2). Changes in isometric force were recorded using Myograph Interface model 610M (ADInstruments) and the Myodac data acquisition system (Myodac 2.01, Myonic Software, Copenhagen, Denmark). The aortic rings were allowed to equilibrate for 20 min under zero force then a 5-mN resting tension was applied. Following a 15-min equilibration period at 5 mN, the aortic rings were maximally contracted using the thromboxane A_2 mimetic, U46619 1 μM ($U4_{max}$). The aortic rings were washed with Krebs' solution and the tension allowed to return to baseline. Concentration response curves to the endothelium-dependent dilator acetylcholine (ACh 1 nM–10 μM), the NO donor sodium nitroprusside (SNP 0.1 nM–10 μM), the K_{ATP} channel opener levcromakalim (LKM 1 nM-10 μM), NaHS (1 μM–3 mM) or the CSE substrate L-cysteine (10 μM–3 mM) were constructed in vessels pre-constricted with the thromboxane analogue U46619. Pre-contraction to U46619 was submaximal (~50% $U4_{max}$) and not significantly different between groups. At the end of each concentration response curve, 10 μM nifedipine was added to test vascular smooth muscle cell function. In the NO bioavailability experiments, the vessels were contracted to 20–30% of $U4_{max}$ using titrated concentration of U46619, and after the contraction stabilized (~10 min), L-NAME 100 μM was added. After the contraction to L-NAME stabilized for 30 min, the contraction value was taken and compared with $U4_{max}$.

Superoxide production from vascular tissue

NADPH oxidase-driven superoxide production in abdominal aorta was measured using lucigenin-enhanced chemiluminescence. Sections of abdominal aorta (3 mm long) were pre-incubated for 45 min at 37 °C in Krebs-HEPES buffer (composition (mM): NaCl 99.9, KCl 4.7, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.2, D-glucose 11.0, $NaHCO_3$ 25.0, $CaCl_2 \cdot 2H_2O$ 2.5, Na HEPES 20.0, pH 7.4) containing diethylthiocarbamic acid (1 mM) to inactivate superoxide dismutase, and NADPH (100 μM) as a substrate for NADPH oxidase. Diphenylene iodonium (1 μM) was used to inhibit NADPH oxidase in some wells. In all, 300 μl of Krebs-HEPES buffer containing lucigenin (5 μM) was placed in separate wells of a white 96-well Opti-plate which was loaded into a Polarstar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37 °C over a 30-min period. After background counting was completed, a single ring of aorta was added to each well, in semi-darkness, and photon emission was measured for 30 min. Superoxide production for each ring segment was calculated by subtracting the background chemiluminescence signal from the signal in the presence of the artery (10^3 counts per second) and then normalized to dry tissue weight (in mg).

CSE activity assay

The H_2S production rate in various tissues was measured as described previously[24] with modifications. In brief, tissues were collected from mice, weighed and homogenized in ice-cold potassium phosphate buffer, pH 7.4. The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.4, 10 mM L-cysteine, 2 mM pyridoxyl'5'phosphate and 10% (w/v) tissue homogenates. Cryovial test tubes (2 ml) were used to trap H_2S, each containing 0.5 ml 1% zinc acetate and a filter paper of 4 cm^2 to increase the air/fluid surface area. The reaction was performed in 50 ml falcon tubes, each falcon tube contained a trapping solution and a reaction mixture tube, and it was sealed by a double layer of parafilm. The reaction was initiated by transferring the tubes into a 37 °C shaking water bath. After incubation for 90 min, the tubes were put on ice for another 30 min to stop the reaction and to ensure complete trapping of H_2S. The contents of the trapping tube were transferred into test tubes each containing 3.5 ml water. Subsequently, 0.5 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate prepared in 7.2 M HCl was added followed by 0.4 ml of 30 mM $FeCl_3$ prepared in 1.2 M HCl. The absorbance of the resulting solution was measured at 670 nm after 20 min with a spectrophotometer. The H_2S produced from the reaction was calculated from a calibration curve of standard H_2S solution prepared by dissolving Na_2S in deoxygenated water under anoxic conditions. A single calibration curve was used to calculate H_2S concentration from each experiment. H_2S production was normalized to the wet tissue weight and expressed as μmol g^{-1} per minute.

Data analysis and statistics

Results are expressed as mean ± standard error of the mean (s.e.m.) with the number of experiments denoted by *n*. Concentration response curves to ACh were expressed as a percentage reversal of the U46619 pre-contraction. These data were computer fitted to a sigmoidal curve using non-linear regression

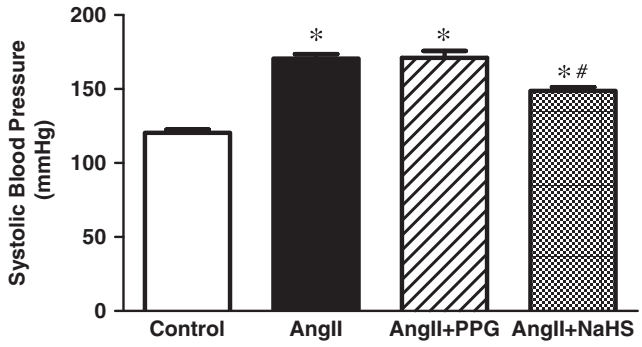

Figure 1 Effect of NaHS (10 μmol kg^{-1} per day) and the CSE inhibitor PPG (3 mg kg^{-1} per day) on the AngII (0.7 mg kg^{-1} per day)-induced increase in systolic blood pressure in C57Bl6/J mice. Systolic blood pressure was measured on day 14 after commencing the AngII infusion. *$P<0.001$ *vs.* Control, $^{\#}P<0.001$ *vs.* AngII, one-way ANOVA, *post hoc* Dunnett's test, $n=10$–12.

(Graphpad Prism 5, Graphpad Software Inc, La Jolla, CA, USA) to provide an estimate of the concentration of agonist causing a 50% relaxation (pEC_{50} value; in –log mol l^{-1}). Statistical analysis was performed using either unpaired *t*-tests or by one-way analysis of variance (ANOVA) with *post hoc* tests applied as appropriate and as stated in the text (GraphPad Prism, Version 5). $P<0.05$ was considered as statistically significant.

Drugs and reagents

All drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All drugs were dissolved in dH_2O, with the exception of LKM, which was dissolved in methanol.

RESULTS

Effect of AngII infusion on systolic blood pressure

There was no difference in the systolic blood pressure between the experimental groups at the commencement of the study (data not shown). AngII infusion (0.7 mg kg^{-1} per day) for 14 days caused a significant increase in systolic blood pressure (Control 120 ± 2 *vs.* AngII 171 ± 3 mm Hg, $P<0.001$). Concomitant treatment with NaHS (10 μmol kg^{-1} per day, i.p.) significantly attenuated this rise in systolic blood pressure ($P<0.001$); however, treatment with the CSE inhibitor PPG (3 mg kg^{-1} per day, i.p.) had no effect on the AngII-induced increase in systolic blood pressure (Figure 1).

Vascular responses in aorta from AngII-infused mice

Effect of AngII infusion on endothelial function. In aortic rings the vasorelaxant response to the endothelium-dependent vasodilator ACh was significantly reduced in the AngII and the AngII + PPG-treated groups compared with the control group ($P<0.001$). Combining AngII + NaHS reversed this endothelial dysfunction ($P<0.001$, Figure 2a, Table 1).

Effect of AngII infusion on endogenous nitric oxide bioavailability. The contraction response to L-NAME (100 μM) was significantly reduced in the AngII and the AngII + PPG-treated groups compared with that of the control group ($P<0.001$), indicative of decreased endogenous NO bioavailability. Combining AngII + NaHS restored the response to L-NAME to a level comparable to that of the control ($P<0.001$, Figure 2b, Table 1).

Effects of AngII infusion on vascular smooth muscle function. The sensitivity to the vasorelaxant response to the NO donor SNP was significantly reduced in the AngII and the AngII + PPG-treated

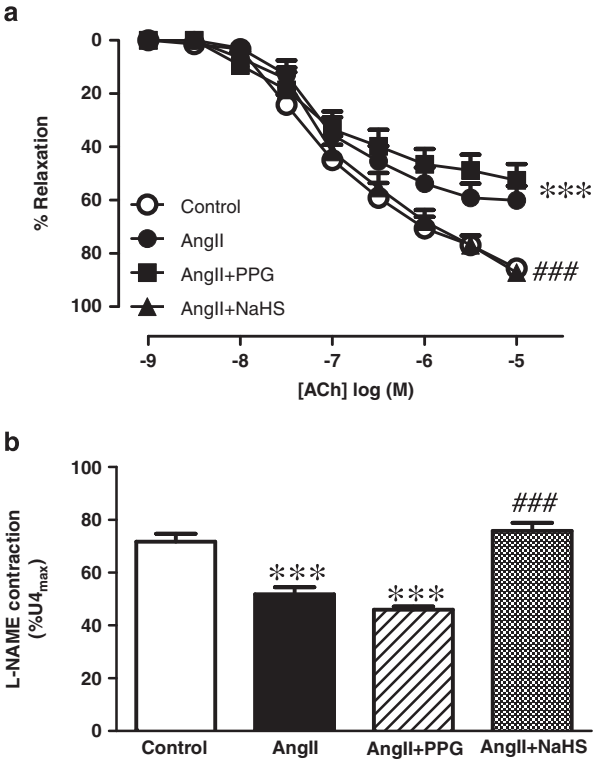

Figure 2 (**a**) Concentration response curves to the endothelium-dependent vasodilator acetylcholine (ACh), in aorta from mice chronically infused with AngII for 14 days and treated with either NaHS or the CSE inhibitor PPG. ○ Control, ● AngII, ▲ AngII + NaHS and ■ AngII + PPG. Response is expressed as % reversal of the pre-contraction to U46619. ***$P<0.001$ max relaxation *vs.* control, $^{\#\#\#}P<0.001$ max relaxation *vs.* AngII, one-way ANOVA, *post hoc* Dunnett's test, $n=8$–10. (**b**) Endogenous vascular nitric oxide bioavailability determined by the contraction response to L-NAME (100 μM) in aortic rings from mice chronically infused with AngII for 14 days and treated with either the CSE inhibitor PPG or NaHS. The contraction response to L-NAME is presented as percentage of the maximum contraction to U46619 1 μM. ***$P<0.001$, *vs.* control, $^{\#\#\#}P<0.001$ *vs.* AngII, one-way ANOVA, *post hoc* Dunnett's test, $n=4$.

groups compared with that of the control ($P<0.05$). However, the maximal relaxation response was unaffected. Combining AngII + NaHS restored the sensitivity to SNP back to the level of the control (Figure 3a). The vasorelaxant response to the K_{ATP} channel opener LKM was not different across the treatment groups (Figure 3b, Table 1).

Effect of AngII infusion treatment on NADPH-dependent vascular superoxide production. Chronic angiotensin infusion (14 days) in mice lead to a significant increase in NADPH-dependent superoxide generation in isolated aortae ($P<0.01$), an effect that was further augmented by combining AngII + PPG ($P<0.001$). Combining AngII + NaHS significantly reduced superoxide production in the aorta of these mice, back to the levels seen in the control group ($P<0.01$, Figure 4).

Effect of AngII infusion on vascular CSE activity. In aorta the vasorelaxation response to NaHS was unaffected by AngII infusion, or any of the treatments (PPG or NaHS) indicating no change in the ability of the vessels to respond to exogenous H_2S (Figure 5a). Maximum vasorelaxation induced by the CSE substrate, L-cysteine was significantly inhibited in the PPG-treated group ($P<0.01$) and slightly inhibited by AngII infusion alone, indicative of a reduction in

Table 1 Supplementary data for myograph experiments

Treatment	F_{max}	n	*% Pre-contraction*	*pEC_{50}*	*Max relaxation*
ACh response					
Control	19 ± 1	8	59 ± 3	7.01 ± 0.07	82 ± 3
AngII	18 ± 1	9	65 ± 2	7.07 ± 0.10	59 ± 3***
AngII + NaHS	15 ± 1	5	58 ± 3	6.84 ± 0.09	85 ± 4
AngII + PPG	15 ± 1	10	62 ± 3	7.24 ± 0.16	51 ± 4***
SNP response					
Control	17 ± 1	8	52 ± 6	7.86 ± 0.06	94 ± 2
AngII	17 ± 2	8	50 ± 5	7.35 ± 0.09$^{\phi}$	95 ± 4
AngII + NaHS	16 ± 2	7	54 ± 5	7.25 ± 0.03	93 ± 1
AngII + PPG	17 ± 2	9	55 ± 6	7.72 ± 0.08	94 ± 3
LKM response					
Control	19 ± 3	4	62 ± 3	7.686 ± 0.07	71 ± 2
AngII	20 ± 2	4	51 ± 3	7.541 ± 0.05	68 ± 2
AngII + NaHS	18 ± 2	4	60 ± 2	7.525 ± 0.05	69 ± 2
AngII + PPG	19 ± 3	4	62 ± 3	7.658 ± 0.11	66 ± 4
L-NAME response					
Control	19 ± 3	4	35 ± 1	—	—
AngII	24 ± 2	4	29 ± 2	—	—
AngII + NaHS	18 ± 2	4	33 ± 4	—	—
AngII + PPG	14 ± 2	4	38 ± 2	—	—

Abbreviations: ACh, acetylcholine; AngII, angiotensin II; LKM, levcromakalim; PPG, DL-propargylglycine; SNP, sodium nitroprusside.
Values are presented as mean ± s.e.m. F_{max} (Maximum contraction to U46619), Pre-contraction ($\%F_{max}$).
***$P<0.001$ *cf* control, one-way ANOVA.
$^{\phi}P<0.05$.

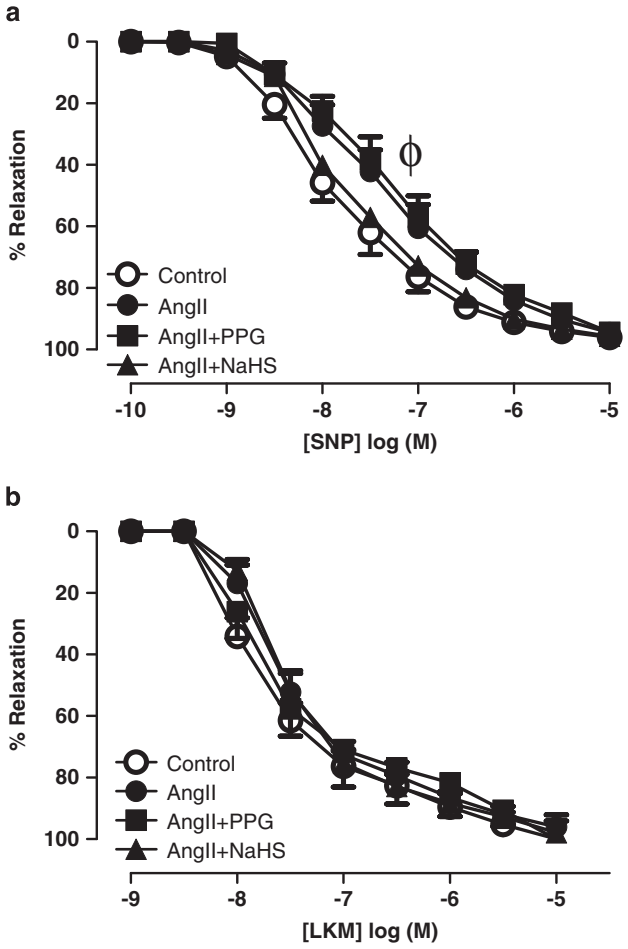

Figure 3 Concentration response curves to the (**a**) NO donor SNP and (**b**) K_{ATP} channel opener LKM in aorta from mice chronically infused with AngII for 14 days and treated with either the CSE inhibitor PPG or NaHS. ○ Control, ● AngII, ▲ AngII + NaHS and ■ AngII + PPG. Responses are expressed as % reversal of the pre-contraction to U46619. $\Phi = P<0.05$ EC_{50}, *vs.* control, ANOVA, *post hoc* Dunnett's test, $n = 5–9$.

endogenous H_2S generation. Treatment with NaHS reversed the inhibitory effect of Ang II infusion on L-cysteine mediated vasorelaxation (Figure 5b). In addition, H_2S production in aorta from AngII-infused mice was virtually abolished by PPG treatment ($P<0.001$) and significantly attenuated in the AngII-treated group compared with the control ($P<0.05$). Treatment with NaHS restored aortic H_2S production back to the levels of the control ($P<0.05$, Figure 5c).

DISCUSSION

This study presents data that highlight the potential importance of H_2S as a vasoprotectant molecule. Chronic AngII infusion in mice for 14 days caused hypertension, increased vascular superoxide production, endothelial dysfunction, reduction in endogenous NO bioavailability, and a decrease in NO signalling and endogenous H_2S production *via* CSE. All these effects were reversed by supplying exogenous H_2S. The increase in vascular superoxide production and a decrease in endogenous H_2S production were exacerbated by inhibition of the endogenous H_2S producing enzyme CSE. This study supports previous reports of the anti-oxidant,[16,25] cardioprotective,[26] and cytoprotective[27,28] effects of H_2S and extends the repertoire of H_2S to specifically include vasoprotectant actions *in vivo*.

AngII infusion caused a marked increase in systolic blood pressure that was reduced by NaHS. NaHS is a known vasorelaxant with effects in both conduit arteries[7,29] and resistance-like vessels.[30–32] The molecular mechanism of vasorelaxation induced by H_2S is controversial, with roles for K^+ channels, Ca^{2+} channels and Cl^- channels all being implicated. More recently, it has been reported that H_2S is a phosphodiesterase inhibitor and cGMP-dependent protein kinase (PKG-I) activator[33,34] and that vasorelaxation is reliant on underlying cGMP production. While the mechanism(s) *via* which H_2S donors elicit vasorelaxation remain to be fully elucidated it is well recognized that H_2S donors are effective vasodilators both *in vitro* and also *in vivo*. Indeed, intravenous administration of NaHS[8] or the H_2S donor compound GYY4137[35] have been shown to reduce blood pressure *in vivo*. It is most likely that the vasorelaxant actions of H_2S contribute to the NaHS-induced antihypertensive effect observed in the current study, but this is possibly not the only reason NaHS reduces blood pressure in the AngII-infusion model of hypertension. AngII activates AT_1 receptors (AT_1Rs) to cause vasoconstriction and signals by markedly increasing vascular NADPH oxidase activity.[36] The increased superoxide production will lead to a reduction in NO bioavailability, which in turn leads to increased vascular tone,[21,36] thus a reduction in superoxide levels by H_2S could also impede the development of increased vascular tone to AngII.

Another possible mechanism of the protective effects seen by NaHS in this study is an interaction with the AT_1R itself. It is well known that the AT_1R has two extracellular disulfide bonds that are important for AngII binding. When these disulfide bonds are reduced by thiol-reducing agents (for example, dithiothreitol[37] or *N*-acetylcysteine[38] in

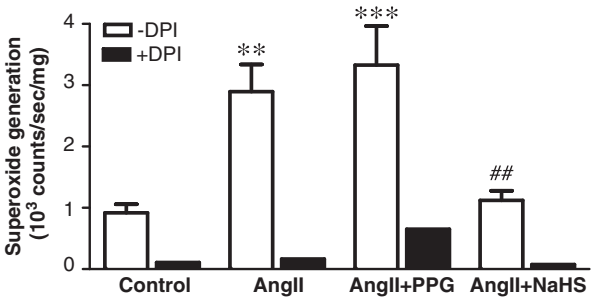

Figure 4 NADPH (100 μmol l^{-1})-stimulated superoxide levels in mouse aortic rings, after 14 days infusion with AngII (0.7 mg kg^{-1} per day) with additional treatment with either the CSE inhibitor PPG (3 mg kg^{-1} per day) or NaHS (10 μmol/kg^{-1} per day). Superoxide was measured by lucigenin (5 μmol l^{-1})-enhanced chemiluminescence in the absence (open bars) and presence (closed bars) of the NADPH oxidase inhibitor diphenylene iodonium (DPI) (1 μmol l^{-1}). Values are expressed as 10^3 counts per second per milligram of dry tissue. $^{**}P<0.01$, $^{***}P<0.001$, *vs.* control, $^{\#\#}P<0.01$ *vs.* AngII; one-way ANOVA, *post hoc* Dunnett's test, $n=9$–13.

cellular binding assays, AngII binding to the AT_1R is inhibited. Similarly, dithiothreitol reduces the vasocontraction response to AngII in *in vitro* vascular assays[39] thus it is established that reducing agents can decrease AngII binding to the AT_1R and block downstream signalling events. Interestingly, AT_2 receptor-mediated effects are enhanced by dithiothreitol.[40] H_2S is a known thiol reducing agent and there is evidence that it too can inhibit AngII-mediated signalling.[41] Thus, a possible mechanism in this study is that NaHS is causing an inhibition of AngII binding to AT_1R, ameliorating AngII-mediated signalling and inhibiting the progression of the pathology of the model and furthermore NaHS may be enhancing AT_2 receptor-mediated effects (vasodilatation and anti-proliferative effects) that counteract the effects of the AT_1R.[42]

NaHS treatment inhibited NADPH-dependent superoxide production in aorta *ex vivo* as assessed by lucigenin-enhanced chemiluminescence. It has previously been shown that H_2S can inhibit NADPH oxidase in cell-based studies, where it inhibits NOX1 expression and Rac1 activity in vascular smooth muscle cells[43] and gp91phox expression in endothelial cells.[44] Additionally, recent *in vitro* studies have shown an anti-oxidant and vasoprotective effect of NaHS.[16] This is the first report to show that chronic treatment with an H_2S donor can affect superoxide production *in vivo* in a hypertension model. Previous work has shown a similar effect in an atherosclerosis model[45] suggesting a role for H_2S as a vascular anti-oxidant. In addition, H_2S has been reported to act as a scavenger for a variety of ROS, including superoxide,[16,46,47] hydrogen peroxide,[43] peroxynitrite[12,13] and hypochloride.[15] Thus, H_2S may both inhibit vascular ROS production and act as a scavenger of ROS, increasing its anti-oxidant potential.

Endothelium-derived NO is destroyed by oxidative stress as NO and superoxide rapidly react to form peroxynitrite.[48,49] The detrimental outcomes of this reaction are twofold. First, the beneficial vascular actions of NO are impaired and second peroxynitrite is a highly reactive molecule which can cause protein nitration and lipid peroxidation.[50] Further, peroxynitrite causes single-strand DNA breakage and it oxidizes tetrahydrobiopterin, an important endogenous nitric oxide (eNOS) co-factor. Depletion of tetrahydrobiopterin is one trigger for the uncoupling of eNOS which then produces superoxide instead of NO[51] adding to the oxidant load. This increased oxidative stress is an important factor in the development of hypertension.[52] An important finding of this

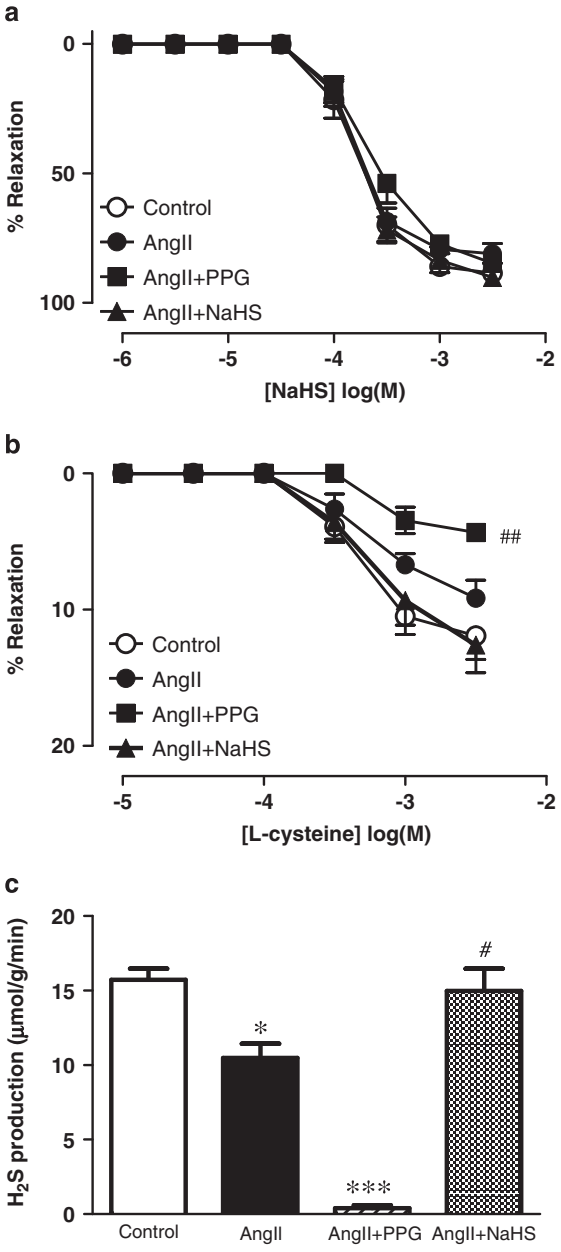

Figure 5 Concentration response curves in aorta from mice chronically infused with AngII for 14 days and treated with either the CSE inhibitor PPG or NaHS, $n=4$–6. ○ Control, ● AngII, ▲ AngII + NaHS and ■ AngII + PPG. (**a**) The vasorelaxation response to exogenous H_2S *via* NaHS, (**b**) the vasorelaxation response to the CSE substrate L-cysteine, $^{\#\#}P<0.01$ maximum relaxation *vs.* control, one-way ANOVA, $n=5$. (**c**) Aortic H_2S production assessed by the methylene blue assay $^{*}P<0.05$, $^{***}P<0.001$, *vs.* control, $^{\#}P<0.05$ *vs.* AngII, one-way ANOVA, *post hoc* Dunnett's test, $n=4$.

study is that NaHS treatment prevented endothelial dysfunction caused by AngII infusion. The endothelial dysfunction in this model is known to be due to increased superoxide production since it is reversed by scavengers of ROS and exacerbated by the deficiency of key endogenous anti-oxidants.[53,54] The endothelial impairment shown in this study is likely to be due to a reduction in NO bioavailability. NO bioavailability was assessed in this study by examining the contractile response to L-NAME, an inhibitor of eNOS. These experiments reflect the availability of endogenous NO

release which is controlling basal tone.[55] The contractile response to L-NAME was significantly impaired in the AngII-treated group whereas the response in the NaHS-treated group was comparable to that of the control. These data confirm that NO bioavailability is impaired under oxidative stress, and that NaHS treatment can reverse this impairment. Thus, the ability of H_2S to inhibit NADPH oxidase-derived superoxide would protect the vasculature from peroxynitrite-mediated damage. Additionally, H_2S donors have been shown to elevate glutathione levels,[17] increase the activity of glutathione peroxidase and glutathione reductase,[56] increase the activity and expression of superoxide dismutase,[57,58] and increase eNOS phosphorylation[59] and activity[60] and expression[61] implying H_2S has the capacity for a multi-faceted enhancement of anti-oxidant mechanisms and the protection of endogenous NO.

In addition to the impaired endogenous NO bioavailability in the AngII-infusion model, there was also impairment in the response to the NO donor SNP. The vasorelaxation response to the K_{ATP} channel opener LKM was the same in all groups, indicating that vascular smooth muscle cell vasorelaxant function *per se* was not affected; thus, the attenuation of SNP-induced relaxation indicates a problem in downstream NO signalling, rather than damage to the vascular smooth muscle. While maximal relaxation was preserved, the EC_{50} for SNP was significantly shifted to the right in the AngII-infused animals, compared with the control and the NaHS-treated group. This may suggest that the superoxide production of the aorta was sufficient to scavenge exogenously generated NO, or interfere with downstream NO signalling. Treatment with NaHS restored the sensitivity to the NO donor. There are a number of possible explanations for this effect. It may be that H_2S is promoting cGMP-mediated signalling by inhibiting phosphodiesterase.[33] Additionally, the activity and expression of the sGC can be directly impaired by oxidative stress due to oxidation of its ferrous heme which prevents NO-mediated activation of sGC.[62] As a scavenger of ROS and a chemical reductant, NaHS may also be acting to protect the ferric form of sGC and therefore preserving the receptor for NO in the vascular smooth muscle cell, providing a further explanation for the improvement of both endothelial function and sensitivity to SNP in the NaHS-treated group.

PPG is a widely used inhibitor of CSE, which acts by irreversibly binding to the pyridoxyl-5-phosphate binding site.[63] Despite quite poor cell permeability PPG does exhibit selectivity in inhibiting CSE[64] and is a well-used pharmacological tool in this field. On the basis of using PPG as an inhibitor of CSE, at the same concentrations, previous studies have shown that endogenous H_2S is involved in the regulation of basal blood vessel tone[7] and indeed blood pressure.[8] In this study, treatment with the CSE inhibitor PPG did not further increase blood pressure, or endothelial dysfunction, but did increase superoxide production and reduce vascular CSE activity. That inhibiting endogenous H_2S production causes little further exacerbation of the deleterious effects of AngII infusion is not unexpected. A simple explanation for this is that endogenous H_2S is not sufficient to ameliorate the hemodynamic and vascular changes induced by AngII. In addition, the H_2S production data suggest that AngII infusion leads to a decrease in endogenous H_2S anyway, so it follows that inhibiting CSE with PPG has little effect.

Interestingly, the NaHS treatment reversed the inhibitory effect of Ang II infusion on vascular H_2S production, as L-cysteine-induced vasorelaxation was maintained in the Ang II infusion with the NaHS treatment. This suggests that the activity of CSE is sensitive to increased oxidative stress. To our knowledge, it has not been reported previously that NaHS can protect L-cysteine bioavailability, but this is a possibility. The data we present indeed indicate that NaHS treatment *in vivo* can protect CSE activity in the vasculature from Ang II infusion; however, we did not measure CSE protein levels or conduct other CSE activity assays to further investigate this. It is also possible that other sources of H_2S are upregulated under conditions of CSE inhibition. It is reported that 3-mercaptopyruvate sulfurtransferase and cysteine aminotransferase can also produce H_2S in vascular cells,[9] although the regulation of this enzyme is not well understood, especially not *in vivo*. H_2S can also be produced by CBS, although this enzyme is generally associated with the neural production.[65] Further potential sources of H_2S are from bound or acid-labile sulfur storage sites in mitochondria[66] and other non-enzymatic sources, although the physiological relevance of these is yet to be determined. A more perplexing finding is that NaHS treatment restored CSE activity, as observed with both the vasorelaxation responses to L-cysteine and *via* aortic H_2S production. The reason for this is unclear, but suggests that H_2S may regulate CSE activity.

More work is still required though to examine the pharmacokinetics of NaHS. The $t_{1/2}$ and the fate of the administered NaHS are not well understood, and it is noted that the present results are obtained from a single daily i.p. dose of NaHS for a relatively short period. The dose of NaHS used is appropriate as it has returned H_2S production back to control levels, thus compensating for the decrease in endogenous H_2S that is observed with chronic AngII infusion. H_2S may react with methemoglobin to form sulfhemoglobin that acts as a sink for H_2S, which subsequently releases H_2S upon reduction. Another possibility for this effect is that H_2S may signal through protein sulfhydration;[67] thus, it is possible that NaHS treatment has a longer lasting effect. The field is plagued with a lack of tools, in particular selective and specific blockers of H_2S producing enzymes and reliable scavengers although consistent and stable donors of H_2S are now becoming available. Improvements in these will be most useful for advances in the study of H_2S biology.

CONCLUSIONS

These data show that in a mouse model of oxidative stress induced by AngII, exogenous H_2S treatment *in vivo* reduces blood pressure, vascular oxidative stress, endothelial damage and protects NO bioavailability. Inhibiting endogenous H_2S production *in vivo* exacerbated vascular superoxide formation. These data provide evidence that H_2S is a vasoprotective molecule that may be a useful treatment target in cardiovascular disease.

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