Exogenous administration of thiosulfate, a donor of hydrogen sulfide, attenuates Angiotensin II-induced hypertensive heart disease in rats

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Summary

Background and purpose: Hypertension is an important mediator of cardiac damage and remodelling. Hydrogen sulfide (H_2S) is an endogenously produced gasotransmitter with cardioprotective properties. However, clinical use is not within reach. We therefore investigated the protective effects of sodium thiosulfate (STS), a clinically applicable H_2S donor substance, in Angiotensin II (Ang II)-induced hypertensive cardiac disease in rats.

Experimental approach: Male Sprague Dawley rats were infused with Ang II (435 ng/kg/min) or saline (control) for three weeks via subcutaneously placed osmotic minipumps. During these three weeks rats received intraperitoneal injections (n=7/group) with either STS ($Na_2S_2O_3$, 1 g/kg/day), NaSH (5.6 mg/kg/day) or vehicle (0.9% NaCl).

Key results: Compared to controls, Ang II infusion caused an increase in systolic and diastolic blood pressure with associated cardiac damage as evidenced by cardiac hypertrophy, an increase in ANP mRNA, cardiac fibrosis, and increased oxidative stress. Treatment with NaSH and STS prevented the development of hypertension and the increase in ANP mRNA levels. Furthermore, the degree of cardiac hypertrophy, the extent of histological fibrosis in combination with the expression of pro-fibrotic genes, and the levels of oxidative stress were all significantly decreased.

Conclusions and Implications: Ang II-induced hypertensive cardiac disease can be attenuated by treatment with STS and NaSH. Although blood pressure regulation is the most plausible mechanism of cardiac protection, the anti-fibrotic and anti-oxidant properties of released sulfide may also be present. Our data show that H_2S might be a valuable addition to the already existing antihypertensive and cardioprotective therapies.

Keywords: thiosulfate, hydrogen sulfide, hypertension, angiotensin II, fibrosis, cardiac damage

Abbreviations

3-MST, 3-mercaptopyruvate sulfurtransferase; Ang II, angiotensin II; ANP, atrial natriuric peptide; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CV, cardiovascular; Cybb/NOX2, cytochrome b (558) subunit beta/NADPH oxidase 2; DBP, diastolic blood pressure; H_2S, hydrogen sulfide; K_{ATP} channels, ATP-sensitive potassium channels; NO, nitric oxide; NOx, nitrite and nitrate; MDA, malondialdehyde; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; (S)TS, (sodium) thiosulfate; SBP, systolic blood pressure; WGA, wheat germ agglutinin

Introduction

Hypertensive heart disease is a major global health problem and a considerable cause of cardiovascular (CV) morbidity and mortality. Hypertension is a key contributory factor in CV disease, especially cerebrovascular disease and heart failure (Drazner, 2011). The heart's response to increased stress such as hypertension is captured by the term "cardiac remodelling", which encompasses myocyte hypertrophy, formation of cardiac fibrosis and vascular rarefaction. Remodelling is associated with activation of various stress responses including the induction of neurohormonal and inflammatory pathways. Progressive remodelling may perpetuate into left ventricular dysfunction and heart failure (Jia *et al.*, 2012, Mann, 2002). Angiotensin II (Ang II) is a well-established mediator in the pathogenesis of hypertensive heart disease. In addition to its hypertensive effect, Ang II induces cardiac remodelling by directly exerting pro-hypertrophic effects through enhancing inflammatory responses and activation of several pro-fibrotic factors via a number of signalling pathways (Wynn, 2008, Shahbaz *et al.*, 2010). Blockade of the renin-angiotensin-aldosterone system (RAAS) is able to regress cardiac remodelling and has evolved into the cornerstone of CV disease management. Despite the use of RAAS intervention and other antihypertensive treatments, many patients with hypertensive heart disease still develop heart failure (Koitabashi and Kass, 2011). Therefore, additional modes of intervention are warranted.

Hydrogen sulfide (H_2S) is recognized as a biologically important gaseous signalling molecule with a myriad of physiological functions (Wang, 2002). H_2S is endogenously produced from the degradation of L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ–lyase (CSE) (Singh *et al.*, 2009, Abe and Kimura, 1996) and from D-cysteine by 3-mercaptopyruvate sulfurtransferase (3-MST) (Shibuya *et al.*, 2013). H_2S is serially oxidized to persulfide, thiosulfate (TS, S_2O_3), sulfite and sulfate (Hildebrandt and Grieshaber, 2008, Kabil and Banerjee, 2010). Similar to nitric oxide (NO), H_2S functions as an endothelial cell-derived relaxing factor

via direct activation of ATP-sensitive potassium (K_{ATP}) channels (Mustafa *et al.*, 2011). Accordingly, deprivation of endogenously produced H_2S contributes to the development of hypertension (Yang *et al.*, 2008, Sen *et al.*, 2010). Furthermore, H_2S has beneficial effects on oxidative stress (Bos *et al.*, 2013), inflammation and fibrosis (Snijder *et al.*, 2013). In various cardiovascular disease states a reduction in H_2S plasma and tissue levels was observed, suggesting a possible role for H_2S in its pathogenesis (Kovacic *et al.*, 2012, Kondo *et al.*, 2013, Jiang *et al.*, 2005, Zhao *et al.*, 2008). Exogenous treatment with H_2S is cardioprotective in various experimental models of cardiac injury. The pathways implicated in the cardioprotective actions of H_2S are multiple. While most of the studies focus on acute myocardial protection, there are some elegant studies investigating the protective effects of H_2S in experimental models for chronic heart failure (Szabo *et al.*, 2011). The effects of H_2S on Ang II-induced hypertensive heart disease have not been investigated.

For the administration of H_2S in experimental models, soluble sulfide salts such as NaSH and Na_2S are often used. Other ways of delivery include H_2S gas or slow-release H_2S donors. A novel approach is the use of sodium thiosulfate (STS, $Na_2S_2O_3$), an endogenous actor in the enzymatic pathways of H_2S production in mammalian cells. Increasing evidence grounds the idea that a dynamic conversion exists between STS and H_2S (Ubuka *et al.*, 2001, Olson *et al.*, 2013). In humans, the short term therapeutic use of STS has been proven safe for the treatment of calciphylaxis (Singh *et al.*, 2011), providing us with the possibility of using H_2S related therapies in a clinical setting.

The vasodilating and cytoprotective features of H_2S make it an attractive candidate for therapeutic reduction of the cardiovascular alterations associated with hypertension. We

investigated the protective properties of sulfide releasing compounds in Ang II-induced hypertensive heart disease in rats.

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 committee for animal experiments (DEC 6412B). With regard to the execution of our animal experiment we followed the ARRIVE guidelines.

Ang II infusion and NaSH or STS treatment

Osmotic minipumps (model 2004, Alzet, Cupertino, CA, USA) were placed subcutaneously under general anaesthesia (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 STS (1 g/kg/day; Sigma, Zwijndrecht, the Netherlands), NaSH (5.6 mg/kg/day; Sigma, Zwijndrecht, the Netherlands) or 0.9% NaCl treatment. Rats receiving 0.9% NaCl via osmotic minipumps received treatment with 0.9% NaCl. During the three weeks of infusion rats received intraperitoneal 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.

After three weeks blood pressure was measured under general anaesthesia (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. Hearts were perfused with 0.9% NaCl and weighed after sacrifice. The hearts were dissected and mid-papillary slices were fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemical analysis, upper parts were immediately snap frozen in liquid nitrogen and stored at -80°C for molecular analysis.

Cardiomyocyte size

To investigate cardiac hypertrophy, we visualized the membrane of the cardiomyocytes and measured its size. After deparaffinization, mid-papillary cardiac sections were incubated with wheat germ agglutinin (WGA)-FITC (1:100, L4895-2 mg, Sigma, Zwijndrecht, the Netherlands) for 30 minutes in room temperature. Nuclei were stained with 4,6-diamino-2-phenylindole (6-H-1200, Vector Laboratories). Images were captured with a fluorescence microscope. Per slide the size (short axis) of 75-100 WGA-stained cardiomyocytes was measured with NIH ImageJ software.

Cardiac fibrosis

To investigate cardiac fibrosis Masson's Trichrome staining was used on paraffin embedded mid-papillary cardiac sections. Subsequently, cardiac sections were scanned using an Aperio Scanscope GS (Aperio Technologies, Vista, CA, USA). The extent of collagen deposition was determined using the Aperio positive pixel analysis v9.1 algorithm. The ratio between the fibrotic surface area and the total cardiac surface area was calculated. Histopathological analysis was performed in a blinded fashion.

Qualitative Real-time Polymerase Chain Reaction

Rat cardiac tissue was homogenized in lysis buffer and total RNA was extracted using the TRIZOL method (Invitrogen, Carlsbad, USA). 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. GAPDH was used as a housekeeping gene. The primers of atrial natriuric peptide (ANP) and Cybb (NOX2) were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD ID's used were: Cybb (NOX2) Rn00576710_m1 and Nppa (ANP) Rn00561661_m1. The primers used for Fibronectin and galectin-3 were: FN1 (Fibronectin) Forward – GTACCACTGGCCACACCTAC Reverse – TGTCAGCCTGCACGTCCAAC; Lgals3 (galectin-3) Forward – CCCGCTTCAATGAGAACAAC Reverse – ACCGCAACCTTGAAGTGGTC (both from Sigma, Zwijndrecht, the Netherlands). For Cybb (NOX2) and ANP, the qRT-PCR reaction mixture contained 20 ng cDNA template and 5µl PCR-mastermix. Nuclease free water was added to a total volume of 10 µl. For fibronectin and galectin-3, qRT-PCR was performed in a volume of 20 µl containing 10 ng cDNA and 15 µl PCR mastermix (SYBR GREEN Applied Biosystems; 5 ml P/N 4309155). All assays were performed in triplicate. The thermal profile was 2 minutes at 50°C, 15 minutes at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min 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 $2^{-\Delta Ct}$.

Plasma nitrite and nitrate (NOx)

Measurement of combined nitrite and nitrate (NOx) was used as a marker for NO production. We colorimetrically measured plasma NOx applying the Griess reaction after reduction of nitrate to nitrite.

Urinary Malondialdehyde measurements

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

Plasma and urinary calcium measurements

Plasma and urinary calcium levels were determined by standard assays (Roche Diagnostics GmbH, Mannheim, Germany) in our clinical chemical laboratory.

Statistical analysis

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

Results

STS and NaSH treatment attenuates Ang II-induced hypertension

Infusion with Ang II significantly increased systolic (211 ± 9 mmHg *vs*. 143 ± 2 mmHg $p< 0.001$) and diastolic (127 ± 10 mmHg *vs.* 84 ± 2 mmHg, $p<0.01$) blood pressure compared to controls. Simultaneous treatment with either STS or NaSH decreased systolic blood pressure (SBP) by 18% (173 ± 7 mmHg, $p<0.001$) and 22% (164 ± 3 mmHg, $p<0.001$), and diastolic blood pressure (DBP) by 30% (89 ± 7 mmHg, $p<0.01$) and 26% (93 ± 8 mmHg, $p<0.05$), respectively (Figure 1, Table 1).

Effects of STS and NaSH on cardiac hypertrophy

After 3 weeks, Ang II-infused rats treated with vehicle had a lower body weight compared to NaCl-infused controls ($p<0.001$). Treatment with NaSH, but not with STS, resulted in a higher bodyweight compared to treatment with vehicle (NaSH: $p<0.01$; (Figure 2A). The heart-to-body weight ratio of vehicle treated Ang II-infused rats was significantly increased compared to 0.9% NaCl-infused controls ($p<0.001$) (Figure 2B), suggesting that these animals developed hypertrophy subsequent to Ang II infusion. To confirm this, we investigated the cardiomyocyte size by analyzing WGA stained cardiac sections. Indeed, we observed cardiomyocyte hypertrophy in the vehicle treated Ang II-infused rats as evidenced by an increased cardiomyocyte size ($p<0.001$) (Figure 2C and D). Treatment with STS and NaSH prevented the development of cardiac hypertrophy as evidenced by a preserved heart-to-body weight ratio (STS: $p<0.05$, NaSH: $p<0.01$) and cardiomyocyte size ($p<0.001$) (Figure 2A, B, C and D, Table 1).

Treatment with STS and NaSH reduces ANP mRNA levels

ANP mRNA expression, a hallmark of the re-activation of the fetal gene program, was significantly increased in hearts of Ang II-infused rats treated with vehicle compared to 0.9% NaCl-infused controls ($p<0.01$). In hearts of STS and NaSH treated rats the relative ANP expression was significantly reduced compared with vehicle treated rats ($p<0.05$) (Figure 3, Table 1).

Rise in plasma NOx after infusion with Ang II

After three weeks of infusion with Ang II, plasma NOx levels were increased by 71% compared to 0.9% NaCl-infused control animals (23.2 ± 1.7 μmol/L *vs.* 13.6 ± 0.3 μmol/L, $p<0.001$). In the STS and NaSH treated groups, plasma NOx levels were lower compared to NOx levels in Ang II-infused rats treated with vehicle (STS: 19.0 ± 0. 9 μmol/L, NaSH: 16.3 ± 1.2 μmol/L) (Figure 4, Table 1).

Protective effects of STS and NaSH against cardiac fibrosis

Fibrosis, as measured by collagen deposition in Masson stained sections 3 weeks after Ang II infusion, was markedly increased in animals treated with vehicle compared to 0.9% NaCl-infused controls ($p<0.05$). Treatment with STS or NaSH reduced collagen deposition by 52% for both compounds (STS: $p<0.01$, NaSH: $p<0.05$) (Figure 5A and B, Table 1). Cardiac mRNA levels of the pro-fibrotic genes fibronectin and galectin-3 were assayed. Fibronectin was massively increased in Ang II-infused animals treated with vehicle ($p<0.001$), while only a moderate increase was detected in animals of the STS and NaSH treated groups (Figure 6A, Table 1). Galectin-3 mRNA expression was increased 3.2-fold in hearts of vehicle treated Ang II-infused rats compared to control ($p<0.001$). In hearts of STS and NaSH treated mice the relative galectin-3 expression was reduced by 29% ($p<0.05$) and 44% ($p<0.01$), respectively (Figure 6B, Table 1).

Treatment with STS and NaSH reduces oxidative stress

Cybb (NOX2) is a member of the NADPH oxidase family that is responsible for the formation of reactive oxygen species (ROS). Cybb (NOX2) mRNA expression is increased 2-fold after Ang II infusion ($p<0.01$) (Figure 6A). Also, the urinary excretion of MDA, which is considered a biomarker of oxidative stress, is increased more than 2-fold in Ang II rats treated with vehicle ($p<0.001$) (Figure 6B). Simultaneous treatment with either STS or NaSH resulted in a decrease in Cybb (NOX2) mRNA expression by 30% ($p<0.05$) and 40% ($p<0.01$), and urinary MDA levels by 30% ($p<0.05$) and 35% ($p<0.05$), respectively (Figure 7A and B, Table 1).

Unchanged plasma and urinary calcium levels

No significant differences between groups were observed in plasma calcium levels. Urinary excretion of calcium was increased in Ang II-infused rats treated with vehicle compared to 0.9% NaCl-infused controls ($p<0.05$). In both STS and NaSH treated groups, urinary calcium levels were not different from vehicle treated Ang II-infused animals (Figure 8A and B, Table 1).

Discussion

In the present study we demonstrated that exogenous administration of STS and NaSH attenuates the development of hypertension and associated hypertensive heart disease. Blood pressure reduction is the probable mechanism of action, but secondary effects such as reducing oxidative stress and fibrosis might also play a role. Furthermore, we are the first to show in a hypertensive rat model that the clinically applicable compound STS has promising cardioprotective properties.

For the administration of H_2S in experimental models soluble sulfide salts are often used, while occasionally gaseous H_2S is administrated. Recently, slow release H_2S donors such as GYY4137 are becoming more popular. TS is an old player in the field, but recently rediscovered as H_2S producing substance. TS is an intermediate of sulfur metabolism and a metabolite of H_2S that can also lead to the production of H_2S (Ubuka *et al.*, 2001, Olson *et al.*, 2013). In this study, we used STS because this is a non-toxic substance that is associated with the production of H_2S. A recent study showed that inhalation of H_2S markedly increased TS levels during endotoxemia in mice. In addition, administration of STS dose-dependently improved survival after LPS challenge in mice (Tokuda *et al*., 2012). The dosage of STS used in this study is based on previous experimental studies (Asplin *et al.*, 2009, Tokuda *et al.*, 2012, Shirozu *et al.*, 2013) and STS usage in humans (Cicone *et al.*, 2004, Farese *et al.*, 2011). The dosage range of STS in experimental studies is 0.5-2 g/kg given daily till 3 times a week; in humans even 12.5 g and 25 g of STS have been infused without adverse effects. In humans, the effect of intravenous STS infusion on plasma TS concentrations has been studied (Farese *et al.*, 2011). In our rat study we did not measure plasma TS concentrations, to our knowledge there are no studies available that have done so in rats. It has to be taken into account that in our rat model STS was given intraperitonealy and not intravenously, the kinetics of this route is not known. The clinical use of H_2S is not within reach, but STS has been used in clinical practice for decades. To date, short

term treatment with intravenous STS is safely used in patients for the treatment of calciphylaxis (Singh *et al.*, 2011). However, one of the disadvantages for long-term administration might be the route of delivery, since orally administrated STS is quickly degraded in the stomach and a validated oral form of the compound has not been developed yet. Furthermore, the long term effects of STS administration should be further explored.

A substantial body of literature demonstrates the cardioprotective effects of H_2S. Many of these studies focused on the beneficial effects of H_2S in acute myocardial infarction induced by coronary artery ligation (Szabo *et al.*, 2011). There is only one study describing beneficial effects of STS in a hypotensive high cardiac output model (Sen *et al.*, 2008). In humans, effects of STS on blood pressure have not been described extensively; however there are indications that STS exhibits vasodilating effects (Thomas and McGinnis, 2002, Hayden *et al.*, 2005, Hayden and Goldsmith, 2010). We observed that STS has similar protective effects as NaSH thereby providing us with exciting possibilities for the translation into clinical use.

The reduction in blood pressure observed in this study is in line with previous literature showing the vasorelaxing capacities of H_2S. Homozygous CSE knockout and heterozygous CBS-deficient mice both develop hypertension (Yang *et al.*, 2008, Sen *et al.*, 2010), suggesting a role for endogenous H_2S production in the pathogenesis of hypertension. Furthermore, exogenous administration of H_2S causes vasodilation in experimental models for hypertension (Mustafa *et al.*, 2011, Roy *et al.*, 2012, Zhong *et al.*, 2003). A proposed mechanism is the activation of K_{ATP} channels through sulfhydration (Mustafa *et al.*, 2011), but it is also suggested that the increased bioavailability of NO might play a role in the vasodilative effects of H_2S (Zhao and Wang, 2002). However, we observed that the antihypertensive effect of H_2S is not accompanied by an increase in plasma NOx as compared to Ang II-infused animals suggesting that the vasodilating effects of H_2S are not related to an increased bioavailability of NO in our model. The increased NOx

plasma levels in the Ang II-infused animals treated with vehicle might be compensatory and due to the high blood pressure. The ability of H_2S to reduce renin and the activity of angiotensin converting enzyme also contributes to its blood pressure reducing effects (Lu *et al.*, 2010). However, the contribution of this mechanism in this particular model is probably low or undetectable due to continuous Ang II infusion and its negative feedback on the production of renin.

In our model the prevention of hypertension by STS and NaSH is likely the ground for the attenuation of cardiac hypertrophy. In the STS and NaSH treated groups we observed a concordant smaller cardiomyocyte size and a lower heart-to-body weight ratio compared to untreated animals. The lower expression of ANP mRNA in these groups further supports the unloading of the heart. In overload-induced models for heart failure, treatment with H_2S attenuated the development of hypertrophy. Conversely, CSE knockout mice with little endogenous H_2S production showed an increase in cardiac hypertrophy compared to wild type mice (Kondo *et al.*, 2013).

Although we postulate that blood pressure regulation is the primary mechanism of action in our model, it is known that Ang II causes damage independent of an elevated blood pressure (Mervaala *et al.*, 2000). This enables additional protective modes of action of H_2S. We observed that treatment with H_2S influences cardiac fibrotic pathways as evidenced by a reduction in fibronectin mRNA and Masson positive surface area. It is not certain if H_2S has any direct effects in fibrosis. Recent studies have demonstrated a potential link between the Ang II-associated fibrotic response and NADPH oxidases in cardiac fibroblasts (Lassegue and Griendling, 2010). Therefore the anti-fibrotic effects of H_2S might be related to the reduction in Cybb (NOX2) we observed in this study. However, we also observed a reduction in galectin-3 mRNA, a protein with established fibrotic properties (de Boer *et al.*, 2009). Recently it was shown that genetic and

pharmacological inhibition of galectin-3 prevents cardiac remodelling by interfering with myocardial fibrogenesis (Yu *et al.*, 2013). Ang II-induced cardiac fibrosis was significantly decreased in galectin-3 knockout mice (Yu *et al.*, 2013).

Ang II induces oxidative stress by activating NADPH oxidases via the angiotensin-1 receptor (Agarwal *et al.*, 2004). H_2S is a known anti-oxidant and can directly scavenge ROS, increase intracellular glutathione levels and reduce the amount of ROS produced through modulation of mitochondrial ROS production (Bos *et al.*, 2013, Kimura *et al.*, 2010, Kimura and Kimura, 2004). In concordance with previous literature, we observed that treatment with NaSH reduced oxidative stress as evidenced by low Cybb (NOX2) mRNA and urinary MDA levels. These anti-oxidant effects may also have contributed to the protective effects of STS and NaSH in this study. The production of reactive oxygen species due to the abundant presence of Ang II causes endothelial dysfunction and can therefore also contribute to the development of hypertension (Rathaus and Bernheim, 2002). Accordingly, treatment with anti-oxidants is able to reduce blood pressure in experimental models for hypertension (Mullan *et al.*, 2002, Racasan *et al.*, 2004).

From our data we are unable to discriminate whether the protective effects of STS can be completely attributed to the conversion of H_2S or if there are direct effects of STS. One of the proposed direct effects of STS is calcium chelation based on the fact that there are studies showing that calcium chelation inhibits some effects of Ang II (Brinson *et al.*, 1998, Du *et al.*, 1999). However, in contrast to what has been published repeatedly, other studies show that STS is not a strong calcium chelator (Pasch *et al.*, 2008, O'Neill *et al.*, 2012). Our data indicate that calcium chelation did not play a major role in the protective effects of STS in this model. However, the calcium excretion measurements (figure 8B) are possibly underpowered, so we must be careful drawing firm conclusions from these data.

In this model the beneficial effects are likely related to a reduction in blood pressure, but also the attenuation of oxidative stress and fibrosis may contribute to the protection against cardiac remodelling. We have extended these findings by demonstrating that STS has marked protective properties, which brings us closer to translation into clinical use. Whether the protective effects of STS are solely mediated by the conversion to H_2S or by additional direct effects of STS, is currently not known. H_2S is a promising agent in the battle against hypertensive heart disease and might be a valuable addition to the already existing cardioprotective therapies.

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Author contributions

PMS, ASF, RAdB, AP, JH, HGDL and HvG designed research; PMS, ASF, HGDL and HvG performed research; RAdB, AP and JH contributed new reagents/analytic tools; PMS, ASF, RAdB, AP, JH, HGDL and HvG analyzed data; and PMS, ASF and HvG wrote the paper.

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Table 1. Overview of all parameters

	NaCl (%)	Ang II + NaCl (%)	Ang II + NaSH (%)	Ang II + STS (%)
SBP	100 (1)	148 (6) ###	115 (2) ***	121 (5) ***
DBP	100 (2)	146 (11) ##	108 (9) *	103 (10) **
BW week 3	100 (7)	70 (2) ###	88 (5) *	85 (5)
Heart-to-BW ratio	100 (2)	160 (3) ###	125 (9) **	134 (7) *
Cardiomyocyte size	100 (1)	139 (4) ###	104 (2) ***	101 (3) ***
ANP mRNA	100(10)	928 (255) ##	332 (103) *	274 (63)*
NOx plasma	100 (2)	171 (13) ###	119 (25) **	140 (6) *
Fibrosis	100(20)	222 (43) #	108 (11) *	107 (9) **
Fibronectin mRNA	100(12)	429 (33) ###	229 (57) **	230 (59) *
Galectin-3 mRNA	100(16)	320 (27) ###	178 (29) **	228 (22) *
NOX2 mRNA	100 (7)	201 (26) ##	121 (17) **	140 (11) *
MDA excretion	100(14)	237 (18) ###	154 (25) *	167 (10) *
Plasma calcium	100 (4)	104 (6)	103 (5)	109 (3)
Calcium excretion	100(44)	567 (44) #	342 (110)	698 (150)

Percentage of change relative to control (NaCl). ###p<0.001, ##p<0,01, #p<0.05 vs. NaCl; ***p<0.001, **p<0.01, *p<0.05 vs. Ang II + NaCl. (SEM)

Figure legends

Figure 1. Treatment with STS and NaSH ~~prevents the development of~~ reduces Ang II-induced hypertension ~~by Ang II~~

Three weeks of Ang II infusion caused hypertension in the vehicle treated animals (n=7). Systolic blood pressure was increased by 48% and diastolic blood pressure by 51%. Treatment with STS (n=7) and NaSH (n=6) prevented the development of hypertension by Ang II. (SBP: ###p<0.001 vs. control, ***p<0.001 vs. Ang II + NaCl; DBP: $$p<0.01 vs. control, &&p<0.01 vs. Ang II + NaCl, &p<0.05 vs. Ang II + NaCl).

Figure 2. Attenuated cardiac hypertrophy by treatment with STS and NaSH

(A) Ang II-infused vehicle treated animals (n=7) had a lower bodyweight than controls (n=6). Simultaneous treatment with NaSH (n=6), but not with STS (n=7), resulted in a higher bodyweight compared with Ang II-infused vehicle treated animals. (B) After three weeks of Ang II infusion the heart-to-body weight ratio was increased in vehicle treated rats (n=7) compared to 0.9% NaCl-infused controls (n=6). Treatment with STS (n=7) and NaSH (n=6) attenuated the increase in heart weight. (C) To confirm this finding we measured the average cardiomyocyte size in WGA stained cardiac sections. STS (n=7) as well as NaSH (n=6) prevented the Ang II-induced increase in cardiomyocyte size. (D) Representative photomicrographs of WGA stained cardiac sections. (###p<0.001 vs. control, ***p<0.001, **p<0.01, *p<0.05 vs. Ang II + NaCl).

Figure 3. Reduced cardiac mRNA levels of ANP in STS and NaSH treated rats

Cardiac mRNA levels of ANP were 9-fold increased after three weeks of Ang II infusion (n=7) compared to 0.9% NaCl-infused controls (n=6). Treatment with STS (n=7) and NaSH (n=6)

reduced ANP mRNA expression by 70% and 64%, respectively. (##p<0.01 vs. control, *p<0.05 vs. Ang II + NaCl). ANP mRNA levels were normalized to GAPDH.

Figure 4. Ang II infusion causes a rise in plasma NOx levels

Plasma NOx levels were increased in Ang II-infused animals treated with vehicle (n=7). In STS (n=7) and NaSH (n=6) treated animals we observed no significant increase in plasma NOx levels. (###p<0.001 vs. control, **p<0.01, *p<0.05 vs. Ang II + NaCl).

Figure 5. STS and NaSH treatment prevented the development of cardiac fibrosis

(A) The relative fibrotic area was 2.2-fold increased in hearts of Ang II-infused animals treated with vehicle (n=7) compared to 0.9% NaCl-infused control animals (n=6). Treatment with STS (n=7) and NaSH (n=6) attenuated the deposition of collagens. (B) Representative photomicrographs of Masson stained cardiac sections. (#p<0.05 vs. control, **p<0.01, *p<0.05 vs. Ang II + NaCl).

Figure 6. Treatment with STS and NaSH prevents the upregulation of fibronectin and galectin-3

(A) Fibronectin and (B) galectin-3 mRNA levels were 4.3-fold and 3.2-fold increased in Ang II-infused animals treated with vehicle (n=7), respectively. In STS (n=7) and NaSH (n=6) treated animals, no increase in fibronectin and galectin-3 mRNA levels were observed. (###p<0.001 vs. control, **p<0.01, *p<0.05 vs. Ang II + NaCl). Fibronectin and galectin mRNA levels were normalized to GAPDH.

Figure 7. Treatment with NaSH and STS reduces oxidative stress

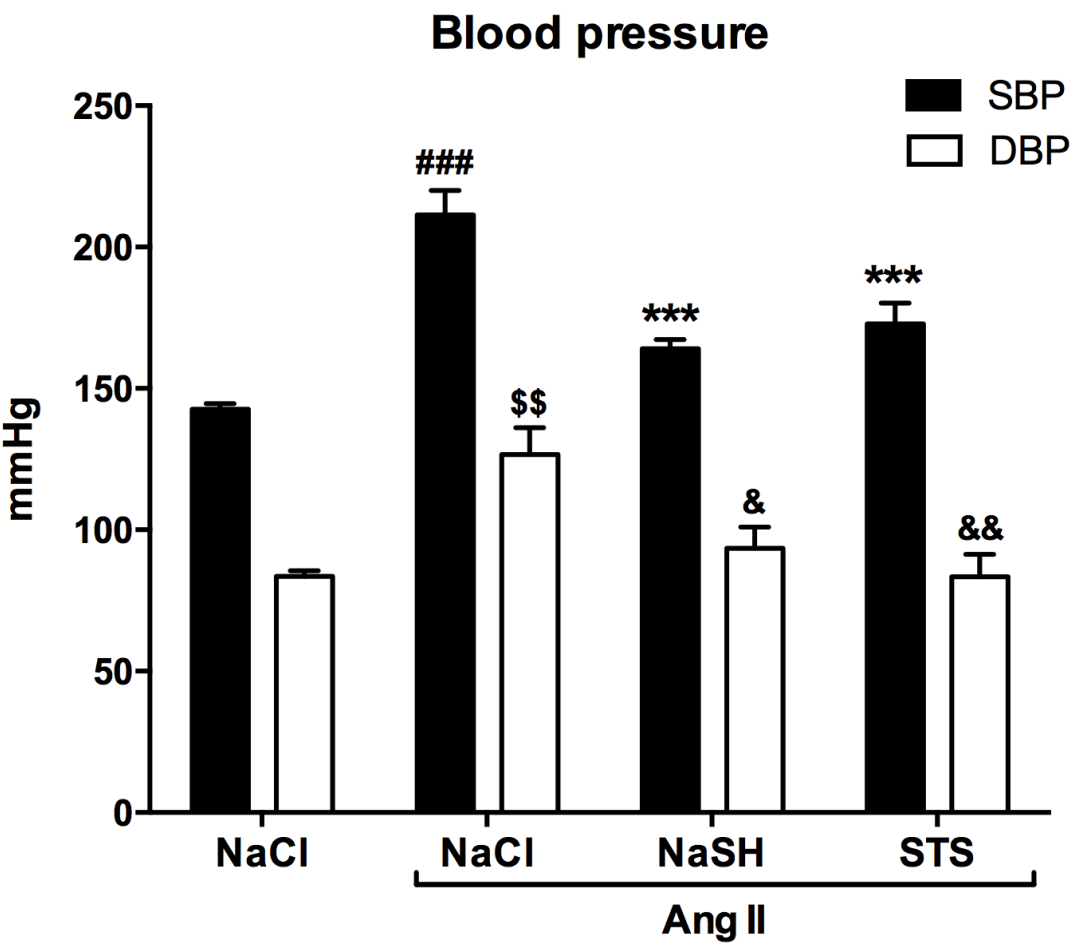
(A) Cardiac levels of Cybb (NOX2) mRNA and (B) urinary MDA levels were increased in vehicle treated animals after three weeks of Ang II infusion (n=7). Simultaneous treatment with either STS (n=7) or NaSH (n=6) prevented the development of oxidative stress, as evidenced by near-control Cybb (NOX2) mRNA and urinary MDA levels. (###p<0.001, ##p<0.01 vs. control, **p<0.01, *p<0.05 vs. Ang II + NaCl). NOX2 mRNA levels were normalized to GAPDH.

Figure 8. Unchanged urinary clacium levels upon STS treatment

(A) Plasma calcium levels were unaltered upon infusion with Ang II (n=7) and simultaneous treatement with NaSH (n=6) and STS (n=7). (B) After three weeks of Ang II infusion (n=7) the calcium excretion was increased compared to controls (n=6). Urinary calcium levels from STS (n=7) and NaSH (n=6) treated animals were not different from Ang II-infused animals treated with vehicle. (#p<0.05 vs. control).

Statement of conflicts of interest

A.P. has support from an unrestricted research grant provided from Köhler Chemie. The other authors declare no conflicts of interest.



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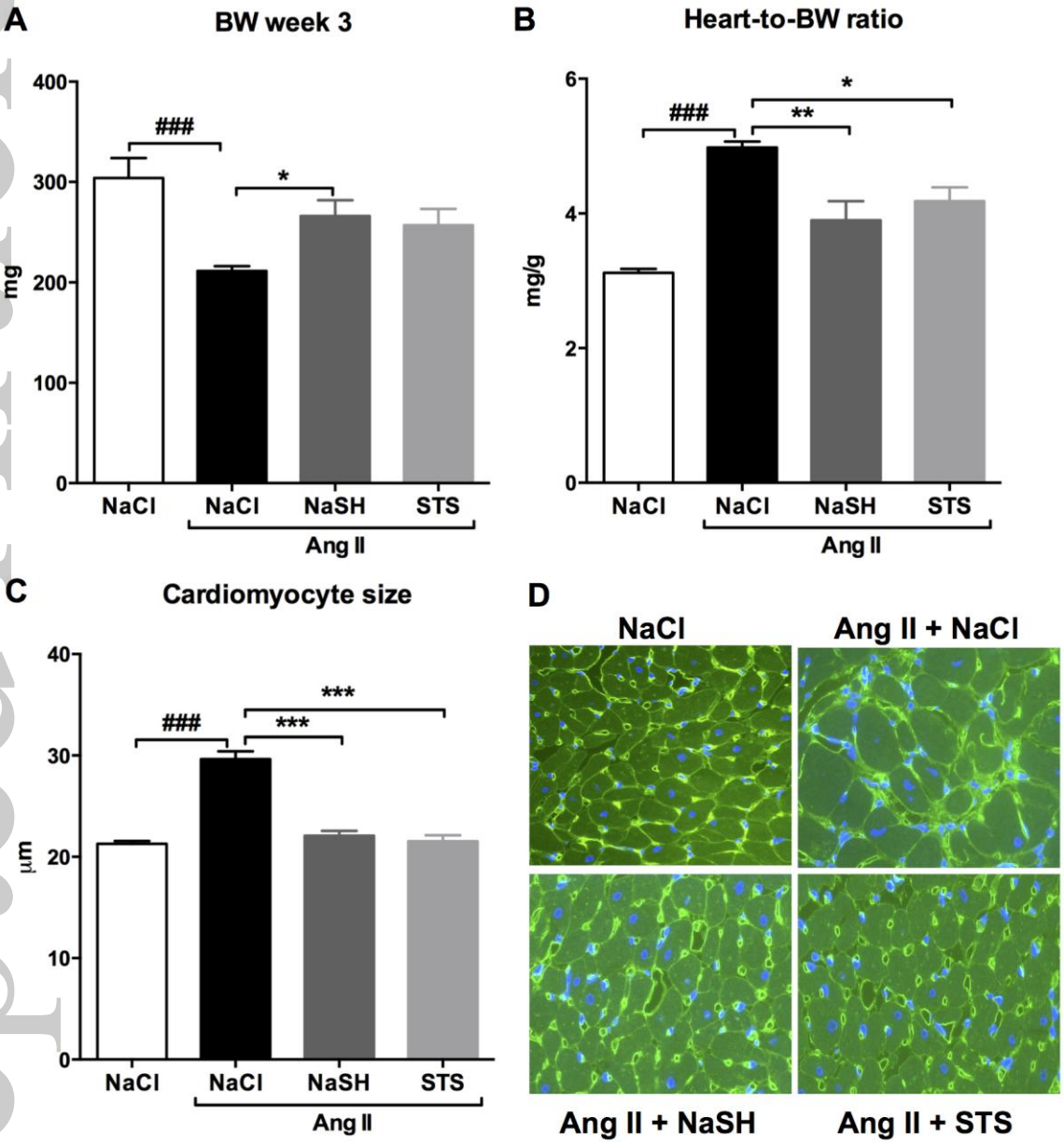

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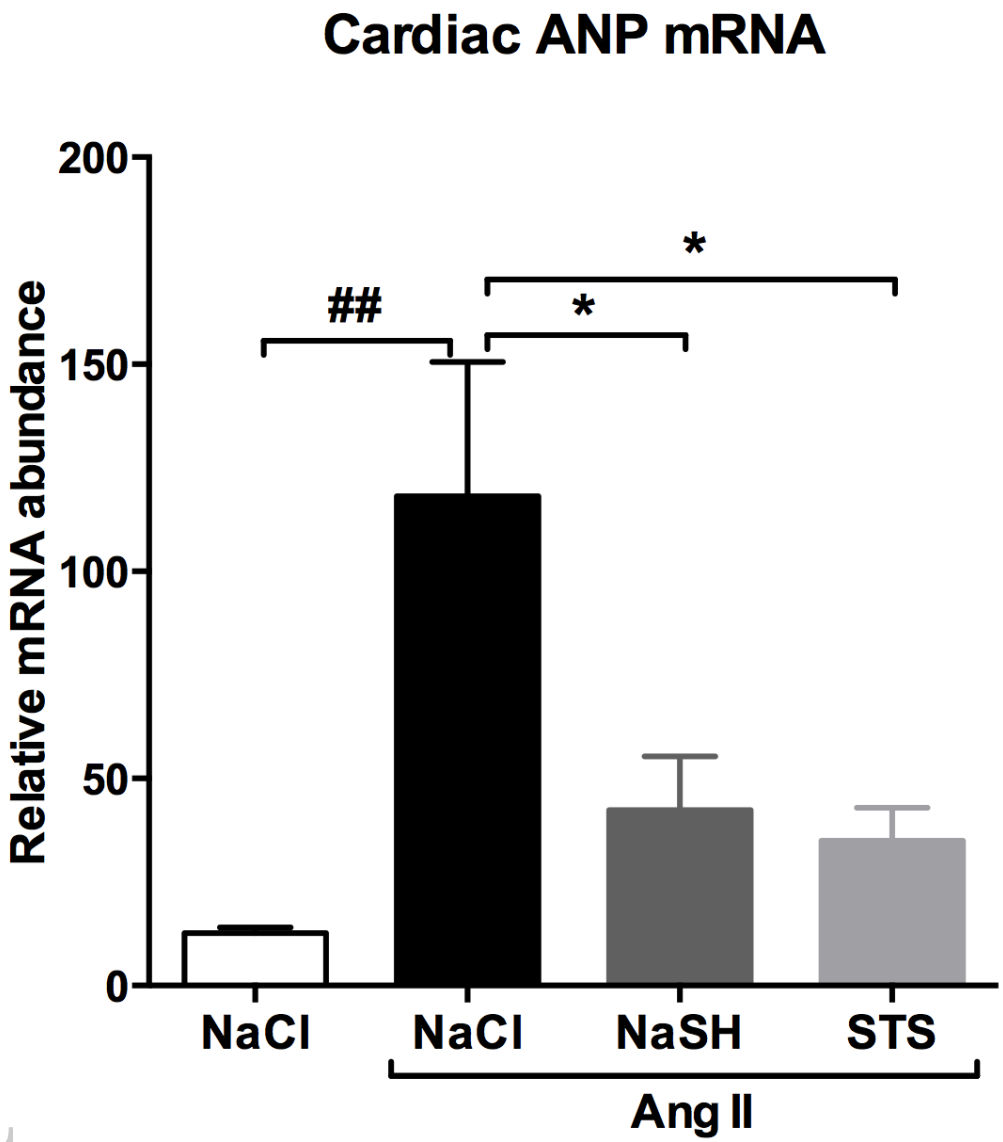

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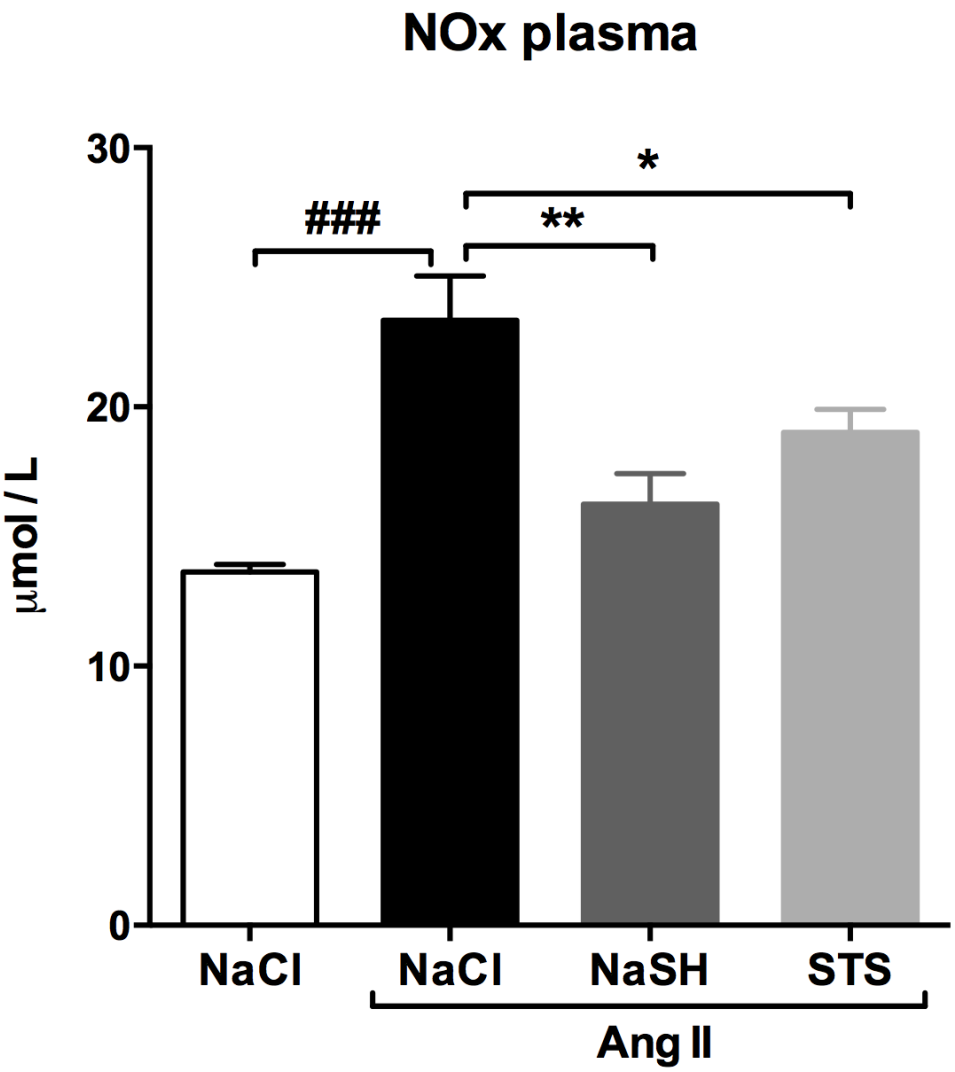

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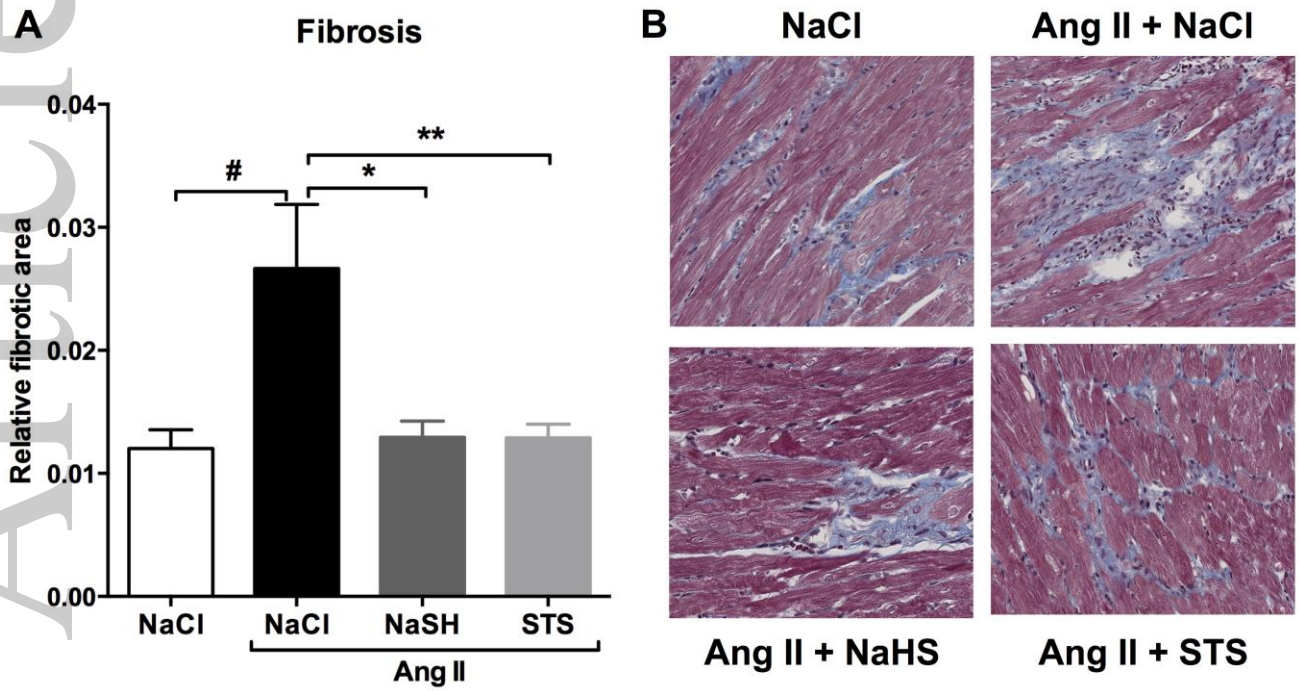

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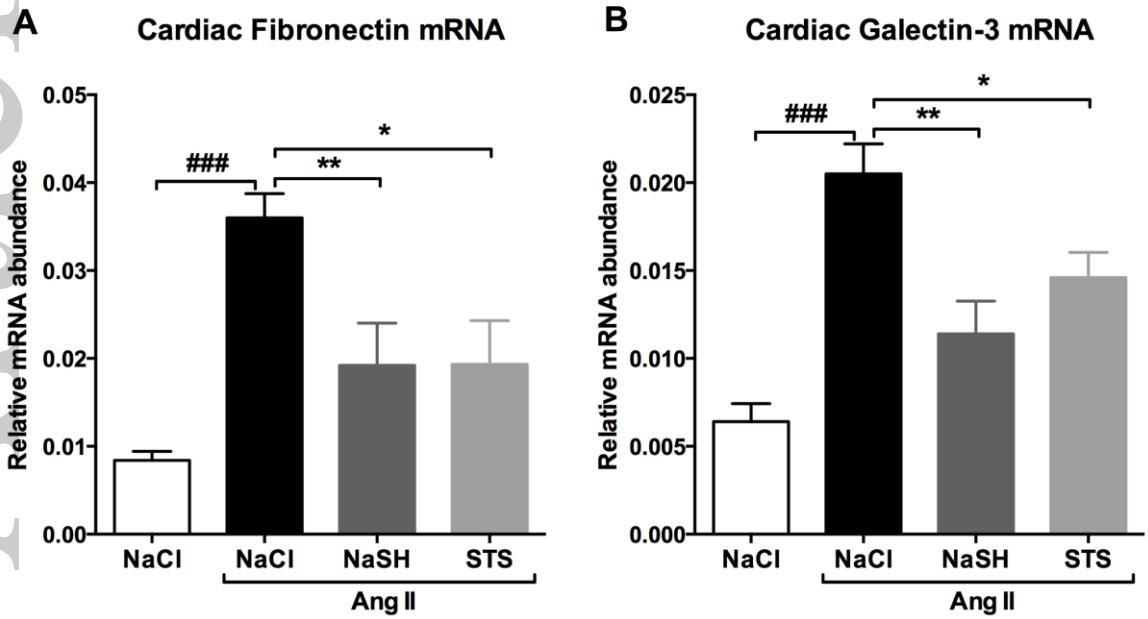

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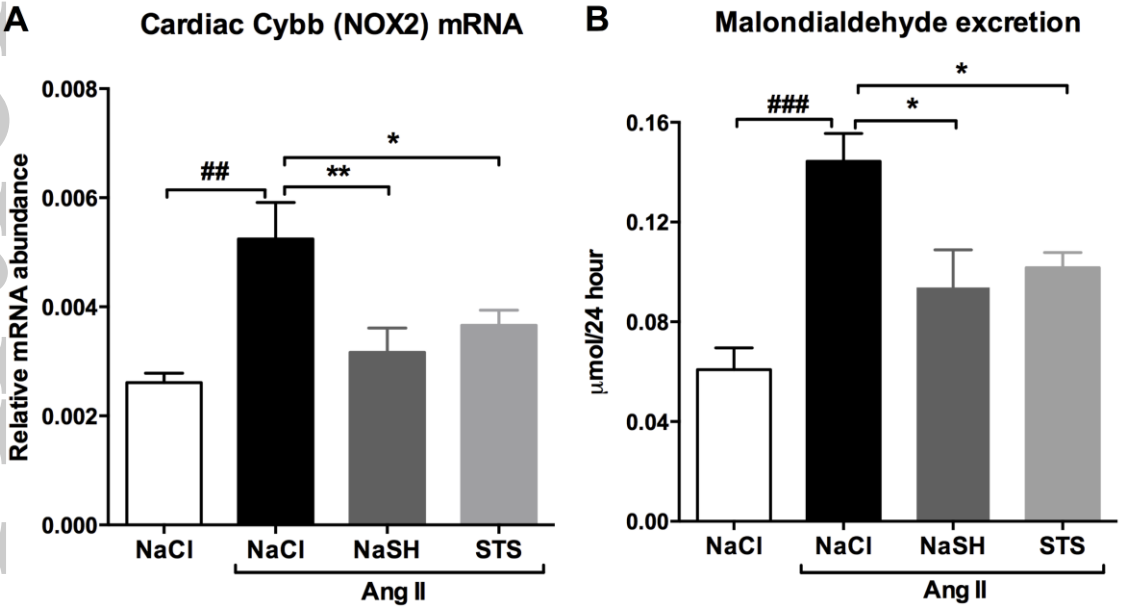

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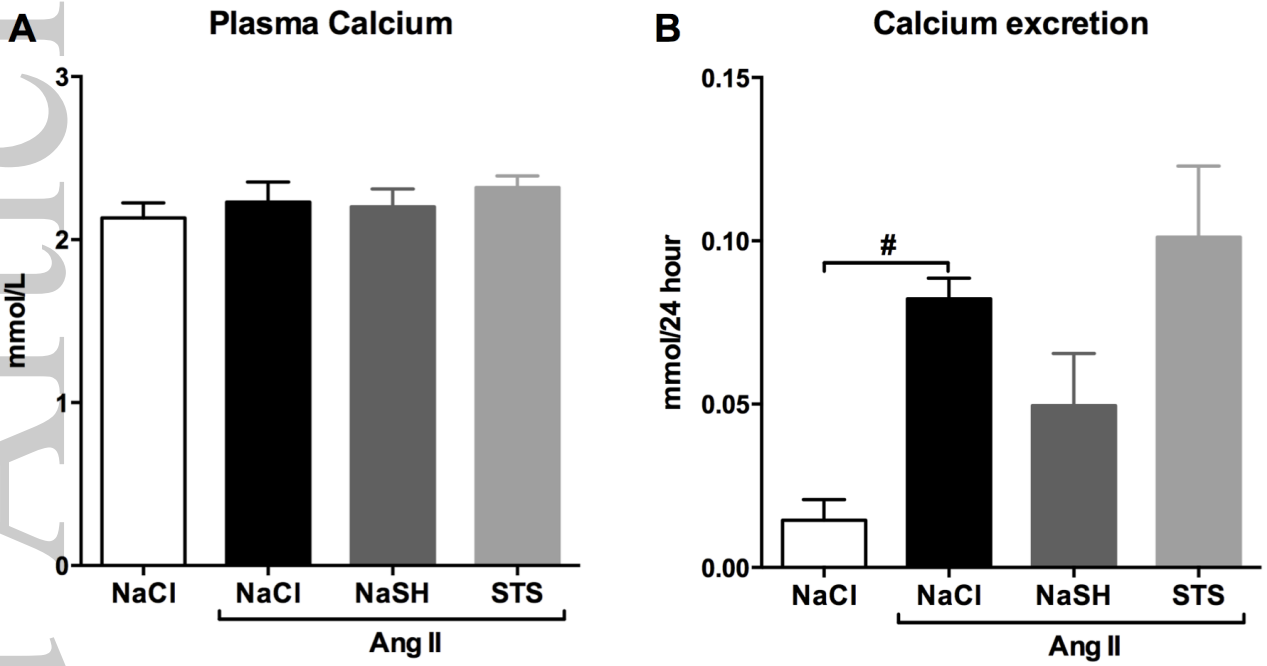

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