

The Effects of Chronic Hydrogen Sulfide Treatment on Hemodynamics and
Vasomotor Function in Adult Spontaneously Hypertensive Rats

by

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Abstract

The endothelial layer of blood vessels is able to produce a number of vasoactive substances, and these substances can work to either relax or contract the underlying vascular smooth muscle. A hallmark of hypertension is the development of endothelial dysfunction, a shift in the balance of these substances to a state of increased contraction. Hydrogen sulfide (H₂S) has recently garnered much interest as a gaseous signaling molecule with the discoveries that it can relax isolated blood vessels and lower blood pressure in young spontaneously hypertensive rats (SHR). Here we investigate whether chronic H₂S treatment (56 μmol/kg of the H₂S donor sodium hydrosulfide (NaHS), once daily for 5 weeks) can lower the blood pressure of adult aged SHR when compared to normotensive control Wistar Kyoto rats (WKY), and whether there are changes in the endothelium-dependent relaxation and contraction pathways. Invasive hemodynamic measurements including systolic, diastolic, and mean blood pressure, as well as heart rate were measured. Isolated vessel myography was performed on the common carotid artery to determine whether there were changes in the endothelium-dependent and independent relaxation and contraction pathways. This was achieved using a number of dose response curves. Changes in endothelium dependent dilation to ACh, VSM sensitivity to NO and H₂S, and NO bioavailability were tested with dose response curves using ACh, SNP (an NO donor), H₂S and indomethacin, respectively. TP receptor sensitivity, as well as COX-mediated constriction in quiescent vessels was also examined by using the TP receptor agonist U46619 and L-NAME (eNOS inhibitor), respectively. Biochemical analyses included Western blotting to assess protein levels of CSE (H₂S generating enzyme) and eNOS (NO generating enzyme) as well as determining prostacyclin production.

Determination of H₂S concentration in the blood via a sulfide electrode was also performed to confirm that the H₂S treatment was effective. There were no main effects of H₂S treatment in any of the hemodynamic measurements taken. ACh dose response revealed a blunting in the recontraction at 10⁻⁵ and 10^{-4.5} log M concentrations (p<0.05) in SHR treated with H₂S. No effects were observed, however, in any other myography protocol. Western blot analysis revealed no difference in the protein expression of CSE or eNOS with H₂S treatment, and there were no differences in prostacyclin production with H₂S treatment. In conclusion, these data suggest that H₂S may not be an effective treatment for hypertension in adult SHR, in contrast to previous work finding a similar dosing regimen to be effective at lowering blood pressure in young SHR. Further work must be completed to ascertain the mechanism for the alteration in the ACh dose response curve and to determine at what time point the H₂S treatment becomes ineffective.

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LIST OF ABBREVIATIONS

AA	<i>Arachidonic acid</i>
AC	<i>Adenylate cyclase</i>
ACh	<i>Acetylcholine</i>
ATP	<i>Adenosine triphosphate</i>
AUC	<i>Area under the curve</i>
Ca ²⁺	<i>Calcium</i>
[Ca ²⁺] _i	<i>Intracellular calcium</i>
CaMKII	<i>Calcium/calmodulin-dependent protein kinase</i>
cAMP	<i>Cyclic adenosine monophosphate</i>
CBS	<i>Cystathionine-β-synthase</i>
CCA	<i>Common carotid artery</i>
cGMP	<i>Cyclic guanosine monophosphate</i>
COX-1	<i>Cyclooxygenase 1</i>
COX-2	<i>Cyclooxygenase 2</i>
CSE	<i>Cystathionine-γ-lyase</i>
EDCF	<i>Endothelium-dependent contracting factors</i>
EDHF	<i>Endothelium-dependent hyperpolarizing factors</i>
EDRF	<i>Endothelium-dependent relaxing factors</i>
eNOS	<i>Endothelial nitric oxide synthase</i>
GTP	<i>Guanosine-5'-triphosphate</i>
H ₂ S	<i>Hydrogen sulfide</i>
INDO	<i>Indomethacin</i>
iNOS	<i>Inducible nitric oxide synthase</i>

IP	<i>Prostacyclin receptor</i>
K _{ATP} channel	<i>ATP-sensitive potassium channel</i>
KCL	<i>Potassium chloride</i>
L-NAME	<i>L-NG-Nitroarginine Methyl Ester</i>
LV/BW	<i>Left ventricle to body weight ratio</i>
MC	<i>Maximum contraction</i>
MR	<i>Maximum relaxation</i>
mRNA	<i>Messenger ribonucleic acid</i>
NADPH oxidase	<i>Nicotinamide adenine dinucleotide phosphate-oxidase</i>
NaHS	<i>Sodium hydrosulfide</i>
nNOS	<i>Neuronal nitric oxide synthase</i>
NO	<i>Nitric oxide</i>
OCT	<i>Optimal cutting temperature compound</i>
PDE	<i>Phosphodiesterase</i>
PE	<i>Phenylephrine</i>
PGI ₂	<i>Prostacyclin</i>
PLA ₂	<i>Phospholipase A2</i>
PKA	<i>Protein kinase A</i>
PKG	<i>Protein Kinase G</i>
ROS	<i>Reactive oxygen species</i>
SAOB	<i>Sulfide antioxidant buffer</i>
SERCA	<i>Sarcoendoplasmic reticulum calcium-ATPase</i>
sGC	<i>Soluble guanylyl cyclase</i>

SHR	<i>Spontaneously hypertensive rat</i>
SNP	<i>Sodium nitroprusside</i>
SOD	<i>Superoxide dismutase</i>
TP	<i>Thromboxane-prostanoid receptor</i>
VSM	<i>Vascular smooth muscle</i>
WKY	<i>Wistar Kyoto rat</i>

Introduction:

The endothelium is a single layer of cells that lines the luminal surface of blood vessels. Prior to 1980, this layer was thought to be a barrier between the circulating blood and the vascular smooth muscle (VSM) and nothing more. Then in 1980, the seminal work by Furchgott and Zawadzki led to the realization that the endothelium was able to synthesize and release a factor that caused relaxation of the underlying smooth muscle ¹. This factor was termed an endothelium-dependent relaxing factor (EDRF), and was later identified as nitric oxide (NO) ². The realization that the endothelium was able to produce EDRFs led to a paradigm shift in vascular research, to a focus on what role the endothelium played in the maintenance of vascular tone.

It is now well established that along with EDRFs, the endothelium is also able to synthesize and release a number of endothelium-dependent contracting factors (EDCF) and endothelium-dependent hyperpolarizing factors (EDHF) that also act on the VSM to cause contraction and relaxation, respectively. It is the balance and interplay between these three groups of factors that plays a large part in the endothelial control of vascular tone. Two of the most important EDRFs are the previously mentioned NO, and prostacyclin (PGI₂). In a normotensive state, these two molecules work to relax the overlying smooth muscle in response to certain mechanical or pharmacological stimuli. In diseased states however, such as hypertension, a balance shift occurs which leads to underactive EDRF pathways and overactive EDCF pathways ³. Endothelial dysfunction that results from this shift is recognized as a hallmark of vascular disease, and a major predictor of future cardiovascular complications.

Along with NO, more gasotransmitters have been identified as being important signaling molecules, including carbon monoxide and hydrogen sulfide (H₂S). H₂S has garnered much attention in the last ten years, with more and more evidence being presented suggesting that apart from being a highly toxic gas with an offensive odour, at lower concentrations it is actually a signaling molecule important to the maintenance of vascular tone ⁴.

In the following sections, the role of NO and PGI₂ in the regulation of vascular tone will be outlined in detail, including how that role changes in endothelial dysfunction. Further detail will also be given on EDHF relaxation and the role of reactive oxygen species (ROS). Finally, H₂S biology will be examined, with emphasis on its role as a vasodilator and its potential importance in the progression and possible treatment of hypertension.

Nitric Oxide Signaling:

Nitric oxide is a gaseous signaling molecule that is able to diffuse from the endothelial cell to the VSM and cause relaxation. NO is produced by any of the three nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). As the name suggests, most NO produced in the endothelium comes from eNOS, which can be activated by a Ca²⁺-dependent pathway. In response to external stimuli (cell-surface receptor activation, shear stress), intracellular Ca²⁺ concentrations ([Ca²⁺]_i) increase in the endothelium which in turn leads to Ca²⁺ binding to calmodulin to form a Ca²⁺-calmodulin complex. This complex then binds to eNOS, leading to its activation and the production of NO. Once activated, eNOS utilizes the

amino acid L-arginine and oxygen as substrates to synthesize NO, which then diffuses to the VSM. The main physiological target of NO is soluble guanylyl cyclase (sGC), which converts GTP to cGMP, leading to activation of protein kinase G (PKG) and a subsequent decrease in $[Ca^{2+}]_i$. The lower levels of Ca^{2+} in the VSM lead to decreased formation of calcium-calmodulin complex, leading to decreased cross-bridge formation and smooth muscle relaxation ⁵ (Figure 1).

In diseases associated with endothelial dysfunction, such as hypertension, major differences in NO signaling are observed that lead to deficits in smooth muscle relaxation. NO bioavailability is markedly reduced with endothelial dysfunction, in part due to increased ROS ³. The following discussion on ROS pertains only to its involvement in NO interaction and scavenging. A detailed section outlining the more diverse role of ROS in the endothelium and ROS-H₂S interactions will be discussed later. Increases in ROS, such as superoxide anion, react with the unstable NO molecule to create peroxynitrite. Peroxynitrite in itself is a dangerous and destructive molecule and can cause damage to surrounding protein structures. More importantly however, the formation of peroxynitrite decreases the amount of NO that is able to relax the VSM, effectively reducing NO bioavailability. Experimental models that introduce exogenous superoxide or inhibit superoxide dismutase (SOD) show decreased NO bioavailability. In contrast, treating vessels with SOD mimetics or inhibiting the pro-oxidant enzyme NADPH oxidase show increases in NO bioavailability ⁶⁻⁸.

Prostacyclin:

Prostacyclin (PGI₂) is important both in EDRF signaling as well as in the progression to endothelial dysfunction. Upon flow stimulus or cell surface receptor stimulation, resultant increases in endothelial [Ca²⁺]_i cause Ca²⁺-dependent phospholipase A₂ (PLA₂) activation. This leads to the release of arachidonic acid (AA) from the cell membrane, which can be metabolized by a number of enzyme systems, most notably by cyclooxygenase-1 and 2 (COX-1, 2), into endoperoxides⁹. The endoperoxides are then synthesized into a number of bioactive prostaglandins through their respective synthases¹⁰. Of these prostaglandins, PGI₂ is one of the most abundantly produced, and it is released by the endothelium to diffuse to the VSM. PGI₂ interacts with the prostacyclin receptor (IP) on the surface of the VSM. This receptor in turn undergoes a conformational change and stimulates adenylate cyclase (AC) through a G-protein coupled response. AC then converts ATP to cAMP, which causes activation of protein kinase A (PKA) leading to a decrease in [Ca²⁺]_i and relaxation (Figure 2).

While a normotensive vessel demonstrates relaxation with stimulation of the PGI₂ pathway, a very different scenario is observed during endothelial dysfunction. In 1986, Luscher et al.⁹ noted that in the aorta of spontaneously hypertensive rats (SHR), higher concentrations of acetylcholine (ACh) cause a re-constriction of the vessel while a blunting of the relaxation response was observed at low concentrations of ACh, an effect not seen in the Wistar-Kyoto (WKY) controls. A corroboratory result was also observed in quiescent rings from WKY and SHR animals, where rings without endothelium from both strains and with endothelium from the WKY showed no development in tension in response to the addition of ACh, but the SHR rings with endothelium began developing

tension at higher ACh concentrations. Upon further investigation with a number of inhibitors, these investigators concluded that the metabolite causing contraction was a product of AA metabolism through the COX pathway. Further research has shown that the metabolite responsible for the EDCF response in hypertension is PGI₂¹¹ (Figure 2).

The transition to endothelial dysfunction in hypertension involves many changes to the COX mediated PGI₂ production pathway. The first major change involves the expression levels of COX enzymes in the endothelium. As mentioned previously, the COX enzyme exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells with a 20-fold higher expression in endothelial cells than in VSM³. COX-2 has a much lower expression level in both tissues, but this can be increased at sites of inflammation. SHR animals show a much higher expression of COX-1 than WKY controls¹², as well as increased expression of certain prostanoid synthases, in particular prostacyclin synthase¹³. This suggests that higher prostanoid production as well as increased prostanoid conversion to PGI₂ could play a major role in the greater EDCF response seen in hypertension¹².

Each of the prostanoids released by COX preferentially stimulates a specific VSM surface receptor. In the case of PGI₂ this receptor is the IP receptor, which leads to smooth muscle relaxation. Thromboxane A₂ is another prostanoid released by COX that stimulates the thromboxane-prostanoid (TP) receptor, which causes smooth muscle contraction by opening receptor-mediated and voltage gated Ca²⁺ channels, leading to increased [Ca²⁺]_i¹⁴. In SHR animals, PGI₂ loses its ability to cause relaxation. Due to a dysfunction in the IP receptor, the overflow of PGI₂ begins to cause contraction by stimulating the TP receptor^{15,16}. Interestingly, the protein and mRNA expression of TP

receptors is not different between SHR and WKY animals ¹⁷, yet the SHR vessels do seem to become hyper-responsive to certain prostanoids such as PGH₂ ¹⁸. Downstream signaling cascades that are activated by TP receptor stimulation might also be altered in hypertension. This could be attributed to Rho-ROCK activation in the VSM, which could lead to enhanced EDCF actions in the VSM ¹⁹. Increased COX-1 expression and PGI₂ production, IP receptor dysfunction, TP receptor hypersensitivity and enhancements to downstream signaling cascades all play a role in overactive EDCF activation.

Changes in both the bioavailability of NO and the actions of PGI₂ are important steps in the progression of endothelial dysfunction. More and more literature is being produced that supports the idea that the changes in both of these pathways are related to an increased level of oxidative stress, as apparent from an increased production of ROS and a decreased ability to scavenge excess (Figure 3).

Reactive Oxygen Species:

In a normotensive state, physiological levels of ROS can aid in normal cell homeostasis, with several antioxidant mechanisms in place to ensure excess ROS is quenched to ensure proper cell function. During endothelial dysfunction, however, pathological level of ROS begin to accumulate, leading not only to impairments in proper cell signaling pathways and cell function, but also to impairments in the ability for ROS scavenging by antioxidant mechanisms ²⁰.

Unchecked ROS production can be detrimental to relaxation signaling pathways. ROS is produced at many different locations in both the endothelium and the VSM, mainly: NADPH oxidase, xanthine oxidase, COX-1 and 2 as well as uncoupled eNOS ²¹.

The most common ROS are superoxide, hydrogen peroxide and peroxynitrite^{3,5}. A significant amount of superoxide in the vasculature is produced in the endothelium, as considerable decreases in superoxide production can be seen in SHR and WKY vessels that have had the endothelium removed²². NADPH oxidase protein expression, as well as activity, is increased in hypertension leading to increased production of superoxide²³. Superoxide can inhibit sGC, decreasing the effect that any still available NO has on VSM relaxation²⁴⁻²⁶ as well as inactivating Ca²⁺-activated potassium channels²⁷. As mentioned previously, superoxide can combine with NO to form the potent oxidant peroxynitrite, decreasing NO bioavailability. Peroxynitrite can act to inhibit guanylyl cyclase, reducing the effectiveness of NO signaling as well as inhibiting superoxide dismutase, and this leads to further increases in superoxide^{26,28}. Peroxynitrite can also work to uncouple eNOS by oxidizing complexes within the enzyme and by reducing co-factor availability. Production of NO is then compromised and superoxide is produced instead (Figure 3).

Hydrogen Sulfide:

Hydrogen sulfide (H₂S), best known for its offensive “rotten egg” odor, has been known to be a very toxic gas for almost 300 years²⁹. As a broad-spectrum poison, all body systems are affected with overexposure, with the central nervous system and the pulmonary system being most affected. H₂S toxicity involves inhibition of the mitochondrial cytochrome oxidase and the major lethal consequence is loss of central respiratory drive, by way of respiratory center brainstem lesions³⁰. Experiments in the late 1800s and early 1900s have shown that many bacteria have the ability to synthesize and produce H₂S, but in the late 1980s *endogenous* levels of H₂S were found in the rat

brain³¹. In 1996, Abe and Kimura³² published a seminal paper that described H₂S production in rat hippocampus by an enzyme-regulated pathway with the ability to influence long-term potentiation in the brain. Since then, H₂S has garnered much interest as an important gasotransmitter with sites of action all over the body. In 2002, Zhao et al. presented work that H₂S had the ability to relax blood vessels in a dose dependent manner⁴. Then in 2008, Yang et al. demonstrated that genetic knockout mice missing the enzyme to synthesize H₂S demonstrated advanced hypertension. Some known cardiovascular actions of H₂S will be examined in more detail, as this thesis examines cardiovascular responses to exogenous H₂S.

H₂S is produced endogenously by two pyroxidal-5'-phosphate-dependent enzymes. Cystathionine-β-synthase (CBS) is found primarily in brain tissue, with little expression in the vasculature^{4,32}. The opposite holds true for cystathionine-γ-lyase (CSE), as it is the main H₂S synthase in the vasculature, with little evidence that it is found in the central nervous system³². CSE and CBS both use the substrate L-cysteine to synthesize H₂S. CSE activation and production of H₂S seems to be triggered in a manner very similar to that of NO. Upon endothelial stimulation by methacholine (1 μM for ten minutes, a muscarinic cholinergic receptor stimulant), H₂S levels in cultured endothelial cells tripled compared to being incubated with vehicle only. Production of H₂S also seems to be calcium-calmodulin dependent, as the addition of the calcium ionophore A23187 significantly increased H₂S production, whereas the presence of the calmodulin antagonist W7 abolished the increased H₂S production³³.

H₂S has been proposed as the newest member of the gasotransmitter family, which includes NO and carbon monoxide^{34,35}. H₂S, however, seems to work more as an

EDHF than an EDRF. Being a small gaseous molecule, H₂S can readily pass through membrane barriers to access specific sites of action. H₂S has been shown to directly act upon K_{ATP} channels on the VSM due to the fact that it can sulfhydrate K_{ATP} channel subunits, and its relaxation effect is mimicked by pinacidil (a K_{ATP} channel opener) and abolished by glibenclamide (a K_{ATP} channel blocker)³⁶. Opening of these K_{ATP} channels causes smooth muscle hyperpolarization with an end result of VSM relaxation (Figure 4). H₂S has been shown to relax a number of different artery types, from isolated rat aorta³⁷ to mesenteric arteries³⁸. Contributing to the idea that H₂S could be an important vasodilator, CSE knockout mice have been shown to demonstrate pronounced hypertension³³, and animals with L-NAME-induced hypertension³⁹, high blood flow-induced pulmonary hypertension⁴⁰, and hypoxic pulmonary hypertension⁴¹ all have decreased CSE mRNA expression and H₂S production. H₂S could also play a part in the treatment of hypertension. Interestingly, young hypertensive animals (4 weeks old) that are treated chronically with sodium hydrosulfide (NaHS, a H₂S donor) injections show a decrease in blood pressure, as well as increases in CSE mRNA expression and H₂S production^{42,43}. This indicates that H₂S could be very important in the maintenance of blood pressure and the progression of hypertension.

H₂S and NO seem to have a very complicated relationship. There are many studies that show conflicting, and oftentimes opposite, effects that one molecule has on the other. This is most notably seen with their functional interactions. In rat aorta, the actions of SNP were shown to be decreased in the presence of 60μM NaHS³⁷. It was hypothesized that this reduction in efficacy could be due to a newly formed S-nitrosothiol, which in the short term would decrease NO bioavailability and lead to a

decrease in SNP effect, but could also act as a NO buffer for which could later release NO for future effect. In stark contrast to this, Hosoki et al. found that the addition of 30 μ M NaHS had additive effects to SNP dilation⁴⁴. It is difficult to determine which effect represents true physiological actions, as the studies used different animal strains (Sprague Dawley and WKY, respectively), precontraction drugs (phenylephrine and norepinephrine, respectively) and tissue preparations (aortic rings and helical strips, respectively). In the end, it is likely that there are functional interactions of H₂S and NO that are complex, resulting in a combined action not simply being a summation of their individual effects.

NO seems to have a positive effect on H₂S production. Incubation of cultured vascular smooth muscle cells with SNAP results in an increase in the transcriptional levels of CSE, possibly allowing for increased H₂S production^{36,45}. Incubation of homogenized vascular tissue with SNAP also increases the accumulation of H₂S³⁷. In animals treated with the NOS inhibitor L-NAME, CSE activity and expression were significantly reduced, leading to reductions in H₂S production³⁹. There is no evidence as of yet to show that NO can directly interact with CSE and alter its activity.

While the effects of NO on H₂S production seem to be mostly positive, the effects that H₂S has on NO production do not seem to be straightforward. Treatment with NaHS has been shown to decrease NO formation, activity of eNOS and to decrease L-arginine transport⁴⁶. Treatment with H₂S can also work to directly inhibit all three of the NOS isoforms^{47,48}. Interestingly, recent evidence shows that H₂S might actually increase NO production and increase eNOS activity. Meng et al. showed that in the rat corpus cavernosum, treatment with NaHS increases the expression of both eNOS mRNA and

eNOS protein content. This increase in protein also led to an increased production of NO⁴⁹. Evidence has also been presented that H₂S can stimulate an increase in [Ca²⁺]_i by opening intracellular Ca²⁺ channels⁵⁰. The opening of these channels and subsequent increase in [Ca²⁺]_i then causes an increase in calcium/calmodulin complex formation, leading to direct activation of eNOS. This also leads to the activation of calcium/calmodulin-dependent protein kinase (CaMKII) which can activate eNOS on Ser1177, leading to increased NO production⁵⁰. This rise in [Ca²⁺]_i has been shown to be from mainly intracellular stores, and not due to decreased extrusion of Ca²⁺ by inhibition of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA)⁵¹.

There also seems to be evidence supporting H₂S as an antioxidant. H₂S is a powerful reducing agent and has been shown to interact with many different ROS, as well as antioxidant systems. Exogenous administration of H₂S has been shown to directly scavenge superoxide as well as hydrogen peroxide in rat models of myocardial injury^{52,53}. H₂S inhibits superoxide formation in cultured vascular smooth muscle cells that have been treated with the TP receptor agonist U46619⁵⁴. Exogenous H₂S can also inhibit and decrease expression of NADPH oxidase, leading to decreased superoxide formation^{54,55}. Peroxynitrite is another molecule that can react with H₂S. H₂S has been shown to significantly reduce the amount of peroxynitrite-mediated tyrosine nitration to a similar extent as reduced glutathione, an antioxidant known to be a powerful scavenger of peroxynitrite⁵⁶. In relation to antioxidant systems, H₂S can induce glutathione synthesis and reduce oxidized glutathione^{57,58}. Experimental depletion of glutathione in glioma cells can also increase the expression of CSE protein⁵⁹. These data suggest that H₂S may

play an important role as an antioxidant, and that the blood pressure lowering effects it has may be due to scavenging of ROS and increasing NO bioavailability.

Purpose:

The following study aims to elucidate the effects of chronic sodium hydrosulfide on endothelium-dependent vasomotor function of the CCA. The CCA is an ideal vessel for these experiments due to its superficial location, lack of side branches and important role in controlling brain blood flow. Vasomotor functions will be investigated, for the first time, in both the adult SHR and WKY animal models. Changes in associated biochemical parameters and in hemodynamic measures will also be examined.

Specifically, the following will be examined:

1. Whether there is a blood pressure lowering effect of chronic H₂S in adult SHR animals with established hypertension.
2. Effects of chronic H₂S on endothelium-dependent relaxation. This will include possible changes in endothelium dependent dilation to ACh, VSM sensitivity to NO and H₂S, and NO bioavailability.
3. Effects of chronic H₂S on endothelium-dependent contractions. This will include examining TP receptor sensitivity as well as COX-mediated constriction in quiescent vessels.
4. Effects of chronic H₂S on the expression of a number of important proteins including eNOS and CSE. We will also be examining the possible differences in the production of PGI₂.

Hypotheses:

1. Chronic H₂S treatment will improve endothelium-dependent and possibly endothelium-independent relaxations in the CCA of SHR animals but not in WKY. This will be observed as decreased re-constriction to higher concentrations of ACh, and increased relaxation in vessels incubated with indomethacin.
2. Chronic H₂S treatment will decrease the endothelium-dependent contractions in SHR animals. This will be observed as a decrease in the ACh stimulated tension seen in quiescent SHR vessels incubated with L-NAME, and less tension development with U46619 stimulation.
3. Chronic H₂S treatment will decrease blood pressure in adult SHR animals, but have no effect on WKY animals. This will be observed as a decrease in systolic, diastolic and mean arterial blood pressure.
4. Chronic H₂S treatment will result in decreased PGI₂ production in SHR but not in WKY animals.

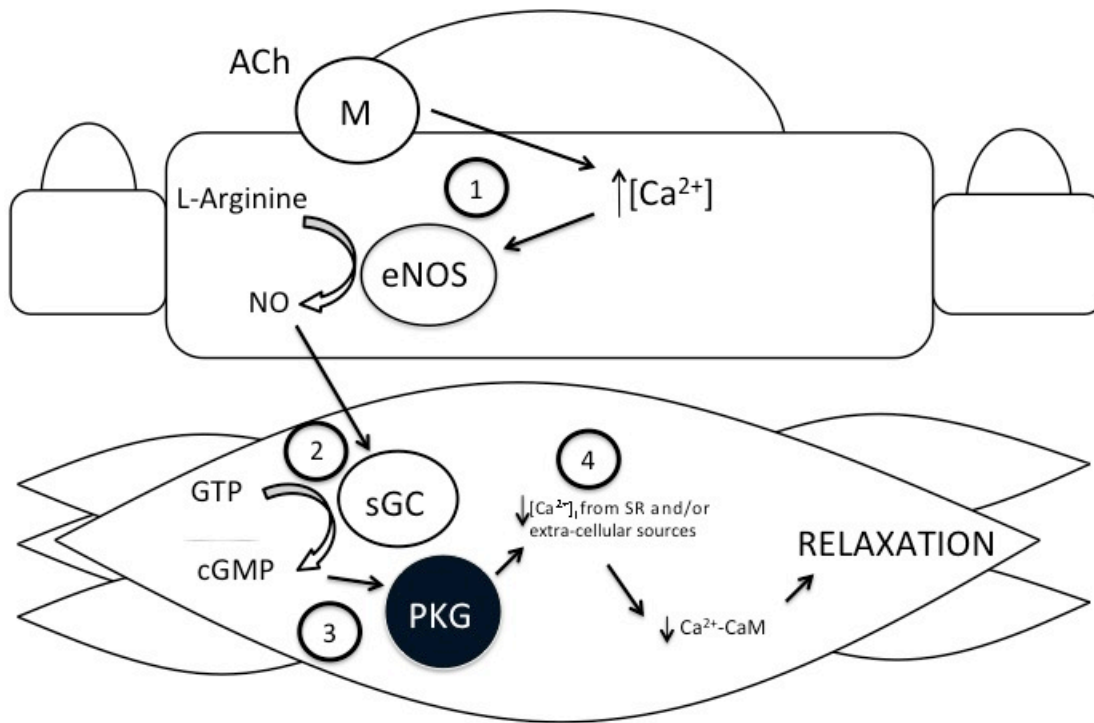


Figure 1: Activation of the muscarinic receptor by ACh causes an increase in intracellular calcium concentrations, leading to the activation of eNOS and the conversion of L-Arginine to NO (1). NO then diffuses to the VSM where it activates soluble guanylyl cyclase (sGC), converting GTP to cGMP (2). cGMP then activates PKG (3), leading to relaxation of the VSM via decreased formation of Ca^{2+} -CaM and less cross-bridge formation (4).

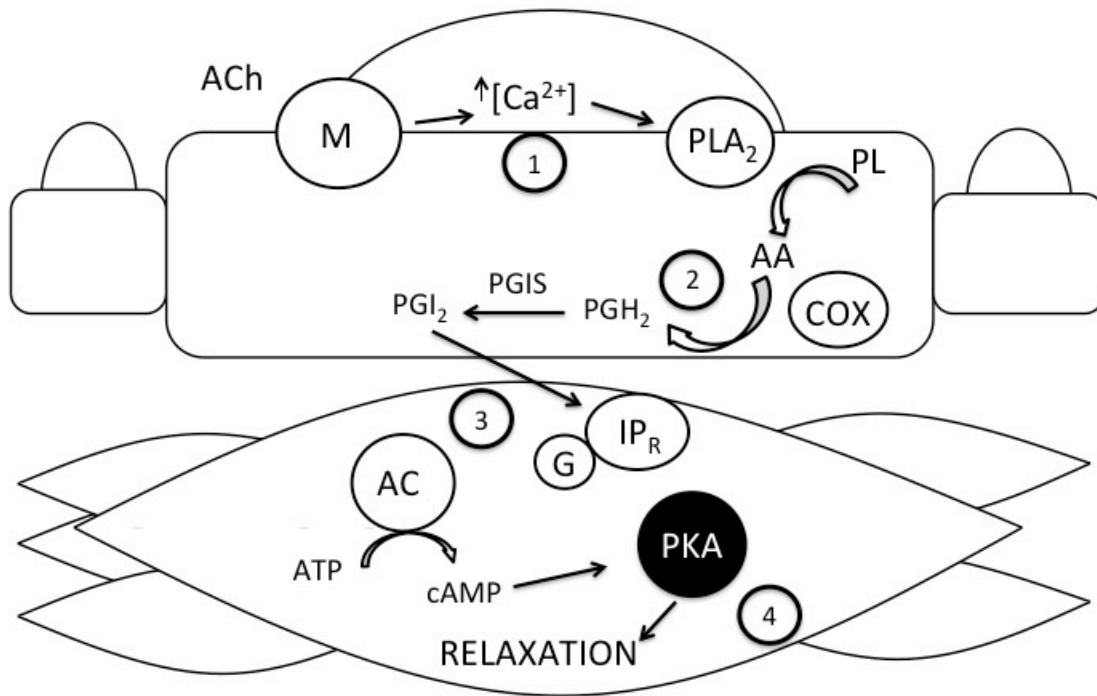


Figure 2: Activation of the muscarinic receptor by ACh causes an increase in intracellular calcium concentrations, activating PLA₂ which releases arachidonic acid from the cell membrane (1). Arachidonic acid is then converted to PGH₂ by COX-1, with PGH₂ being converted to PGI₂ by PGIS (2). PGI₂ then activates the IP receptor on the VSM, which activates AC via a G-protein coupled response (3). AC then converts ATP to cAMP which activates PKA, leading to VSM relaxation (4).

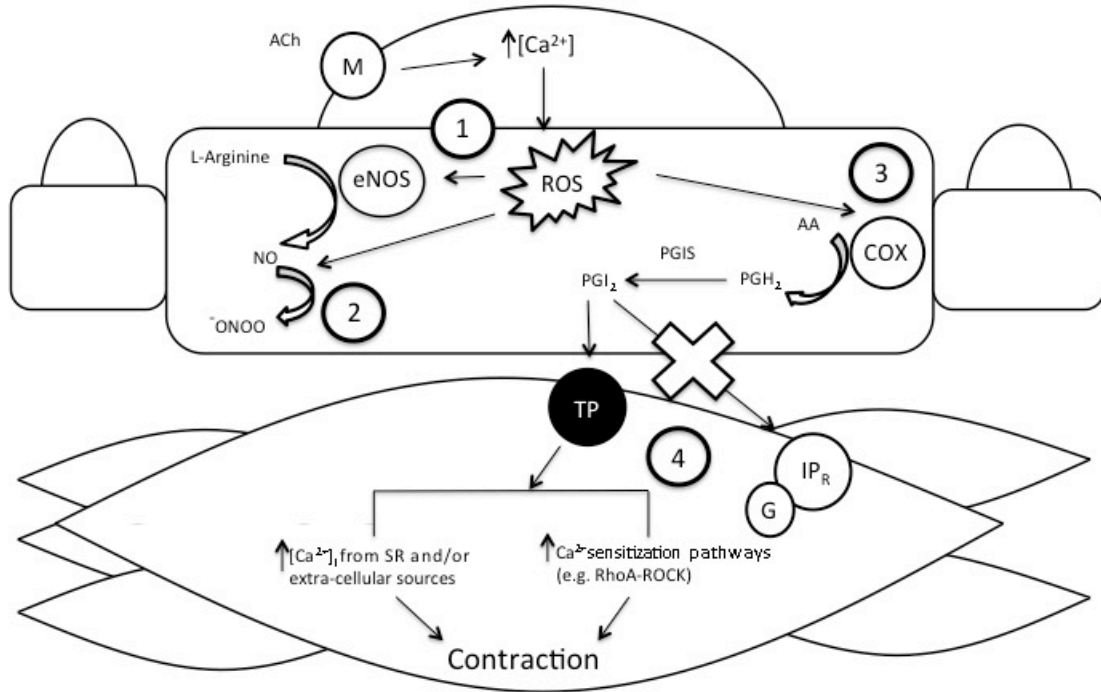


Figure 3: The hallmark of hypertension is the progression of endothelial dysfunction. Increases in ROS production and decreases in ROS scavenging create a pro-oxidative environment in the endothelial cell. This ROS can react with eNOS, uncoupling it and causing it to produce less NO and instead produce superoxide (1). Superoxide can also react with NO to create the potent oxidant peroxynitrite, leading to decreased NO bioavailability and causing more oxidative damage (2). Increased ROS increases the expression of COX-1, leading to increased production of PGI_2 (3). PGI_2 loses its affinity for the IP receptor and instead stimulates the TP receptor, causing contraction instead of relaxation via increased $[Ca^{2+}]_i$ and increased Ca^{2+} sensitization(4).

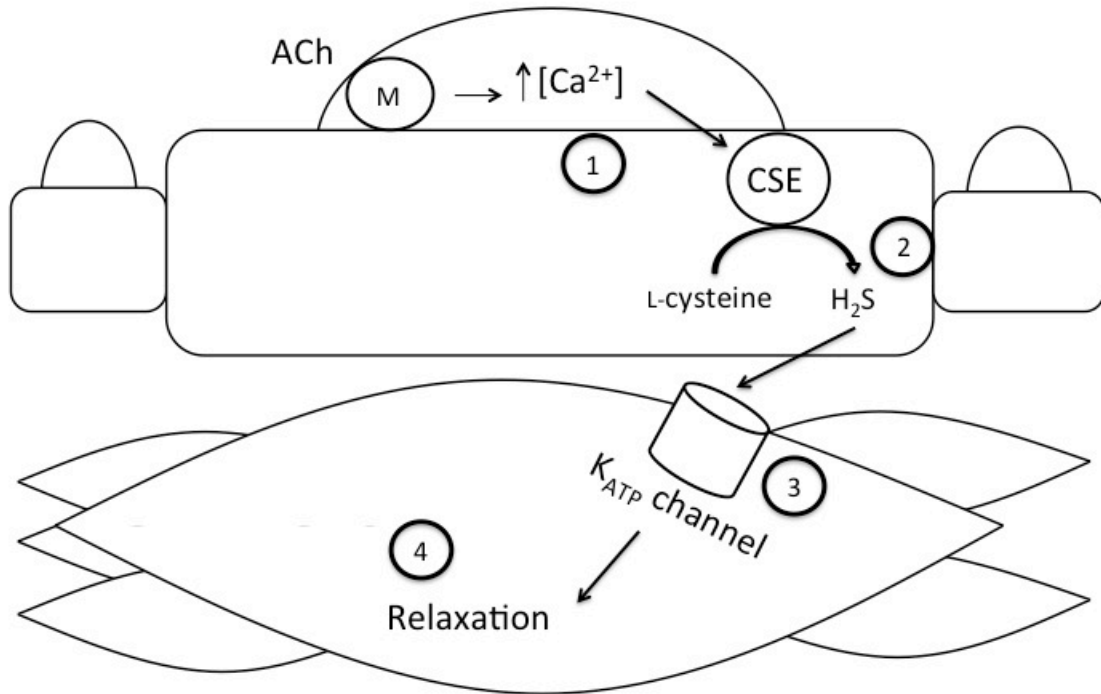


Figure 4: Activation of the muscarinic receptor by ACh causes an increase in intracellular calcium concentration which activates CSE in a calcium-calmodulin dependent manner (1). CSE then converts L-cysteine to H_2S (2), which crosses over to the VSM and opens K_{ATP} channels (3), leading to relaxation of the VSM (4).

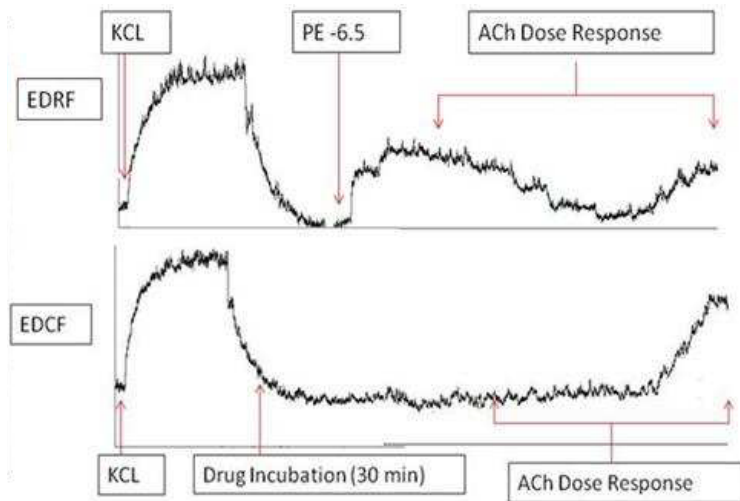


Figure 5: EDRF protocol (top tracing): After vessel viability has been tested with exposure to 60 mM KCL, vessels are washed with buffer and precontracted with 10^{-6} log M PE. ACh is then administered at increasing concentrations to assess endothelium-dependent dilation.

EDCF protocol (bottom tracing): After vessel viability has been tested with exposure to 60 mM KCL, vessels are washed with buffer and incubated with the drug of choice for 30 minutes. ACh is then administered at increasing concentrations to assess endothelium-derived contractions.

Methods:

Animals:

Male Spontaneously Hypertensive rats (SHR, n=40) and male Wistar Kyoto rats (WKY, n=37) were obtained from the University of Waterloo breeding colony. Animals were group-housed in a temperature and humidity controlled environment and acclimated to a 12-hour reverse light cycle. Animals had free access to standard chow (Harlan) and tap water *ad libitum*. All procedures involving rats were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Sodium Hydrosulfide Treatment:

Recent studies have shown that 56 μ mol/kg of sodium hydrosulfide (NaHS) over 5 weeks results in a significant decrease in blood pressure in young (4 weeks old) SHR animals⁴². Other studies show that an increased or decreased dose over a longer period of time causes no additional blood pressure decreases in the animals⁴³. Therefore the above-mentioned dose is appropriate for the current experiments. At 14-17 weeks of age, animals were weighed and the 5-week, daily subcutaneous injection protocol began. A stock of solution of 1mol/L NaHS was made fresh daily in sterile saline solution. 560 μ L of this stock was added to 9.44 ml of sterile saline to make a 56 mmol/L injection solution. Injecting 0.42cc of this solution into a 420g animal yielded a final dose of 56 μ mol/kg. Dose was adjusted as accurately as possible based on weight. SHR and WKY animals were randomly split into control and H₂S treatment groups, with the control groups receiving weight-adjusted subcutaneous injections of saline solution and the H₂S

treatment group receiving the NaHS injection solution. Hemodynamic measures and vasomotor assessments were performed approximately 15 hours after their final dose.

Hemodynamic Measures:

A subset of animals (SHR; n=13, WKY; n=15) was used for direct hemodynamic measures via the left common carotid artery. Animals were injected with sodium pentobarbital (50-65 mg/kg i.p.; Bimeda-MYC, Cambridge, ON) and the level of anesthetization was monitored through the withdrawal reflex from a toe pinch. To ensure proper levels of anesthetization, additional sodium pentobarbital was injected until withdrawal reflex was absent. Animals were placed supine on a heating pad at 38°C (Gaymar TP-500, Orchard Park, New York and Temp-Pad; Seabrook Medical Systems). Following a small incision into the neck, blunt dissection was used to isolate the left common carotid artery. The artery was cleaned with saline and a small incision was made at the cephalic end. The calibrated Mikro-Tip pressure catheter (Model SPR-320, 2F Mikro-Tip Pressure Transducer Catheter, Millar Instruments, INC., Houston, TX) was then inserted into the artery and secured with silk suture (4-0 silk, Look, Reading, PA). A stable systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were measured for 5-7 minutes. Following hemodynamic measures, animals were sacrificed via exsanguination.

Vasomotor Assessment:

A different subset of animals (SHR; n=27, WKY; n=22) was used for vasomotor assessment experiments. Following anesthetization with sodium pentobarbital, animals

were sacrificed by exsanguination. Both common carotid arteries were excised and placed in 4° C Krebs-Henseleit buffer (concentration (mmol/L): 131.5 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, 0.023 EDTA, 13.5 NaHCO₃, and 11.2 glucose). The arteries were cut into 2 mm rings with a surgical blade under a dissecting microscope (Zeiss; VWR, Mississauga, ON) and then mounted on a wire myograph system (vascular myography unit, Radnoti Glass Technology Inc., Monrovia, CA). The arterial rings were suspended between a fixed titanium wire attached a glass foot and a titanium triangle attached to a force transducer (Model MLT0201/D, ADInstruments). Mounted arterial segments were then immersed in tissue baths warmed to 38° C and filled with 5 mL oxygenated (95%O₂/5%CO₂) Krebs buffer.

Previous work in our lab ¹⁹ has established the optimal basal length of the CCA to be at 2.85g of tension. To achieve this we increase the tension to 2.85g for ten minutes followed by a wash with new buffer. This is then repeated two more times until the vessel holds steadily at 2.85g. Tension viability was then tested by the addition of 60mM KCL to elicit a contraction for 30 minutes followed by three 5-minutes washouts with buffer in preparation for the vasomotor experiments.

Myography Experimental Protocols:

1) ACh, SNP and H₂S Dilation Dose-Response Curves

Phenylephrine (PE) (10⁻⁶ mol/L) was used to pre-contract vessel segments until a stable plateau was reached. To assess vasorelaxation function of the CCA, an ACh dose-response curve (10⁻⁹-10⁻⁴ mol/L and half-concentrations) was constructed to determine endothelium-dependent relaxation, and an SNP dose-response curve (10⁻¹⁰-10⁻⁴ mol/L

and half-concentrations) was constructed to determine endothelium-independent relaxation. Indomethacin (INDO, 10^{-5} mol/L), a COX-1 and 2 inhibitor, was used for one preparation to eliminate the endothelium-dependent contraction component and allowing for the assessment of NO-bioavailability as established in our previous experiments. Vessels were incubated with INDO for 30 minutes prior to PE pre-contraction¹⁹. A H₂S (10^{-5} - 10^{-3} and half-concentrations) dose-response curve was also constructed to determine if there were differences in vascular smooth muscle sensitivity to H₂S across treatment groups.

II) ACh and U46619 Constriction Dose-Response Curves

Quiescent vessel segments (non pre-constricted) were used for the examination of EDCF responses. The eNOS inhibitor L-NAME was added and allowed to incubate for 30 minutes. At the end of incubation an ACh dose-response curve was constructed to assess COX-mediated constriction. A dose-response curve was also constructed using the TP receptor agonist U46619 (10^{-9} - 10^{-6} and half-concentrations) to assess possible changes in VSM TP receptor sensitivity.

Biochemical Analysis:

6 keto PGF_{1α} Competitive Enzyme Immunoassay – Prostacyclin Production:

Following the peak contraction (highest concentration) in the ACh dose-response curve incubated with L-NAME, buffer from the tissue bath was collected and immediately frozen in liquid nitrogen and stored at -80° C. The concentration of 6-keto-PGF_{1α} (stable metabolite of PGI₂) in the buffer was assessed using a competitive EIA kit (Cayman Chemical), as an index of the PGI₂ production by the vessel segments¹⁹.

Western Blotting:

Two vessel segments not used in the vasomotor assessment experiments were frozen in liquid nitrogen and stored at -80°C . Vessel segments were hand homogenized with a glass homogenizer (Kontes-Glass, Vineland, NJ) in 150 μL of ice-cold extraction buffer (10mM NaH_2PO_4 , 1% SDS, 6M urea at pH7.4). Homogenates were then incubated at 60°C for 3 hours with intermittent vortexing, centrifuged (12 min, 12000rpm) and supernatant removed and stored at -80°C . Protein concentrations were then assessed using the BCA protein assay method. Samples were combined with the BCA working reagent (50 parts bicinchoninic acid + 1 part Copper II sulfate) and compared to a bovine albumin protein standard curve using spectrometry (OD 527nm, SpectraMax plus 384, Molecular Devices, Sunnyvale, CA). Samples were prepared for Western blotting by diluting to $1\mu\text{g}/\mu\text{l}$.

Samples underwent electrophoresis (30 $\mu\text{g}/\text{lane}$ at 120V for 60-80mins), after 5 minutes of denaturing at 95°C , in sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) ranging from 7.5% to 12% depending on the molecular weight of the protein of interest. Proteins were then transferred onto PVDF membranes (25V for 40 min). Immunodetection began by blocking membranes with 5% bovine serum albumin (BSA) for 1 hour followed by incubation with protein specific antibodies (eNOS 1:750, CSE 1:500). Following primary antibody incubation, membranes were incubated with secondary antibody made up in either horseradish peroxidase-conjugated anti-mouse or anti-rabbit. Membranes were then washed with enhanced horseradish peroxidase/luminol chemiluminescence reagents (Amersham, Little Chalfont, UK) and detected using a syngene system (Syngene, Cambridge, UK)¹⁹.

Statistical Analysis:

For vasomotor assessment experiments, tension values in the contraction curves were expressed as a percentage of KCL pre-contraction. Relaxation curves were expressed as a percentage of relaxation from the PE pre-contraction. Dose-response curves were analyzed using point-by-point analysis, where strain and treatment differences were compared using a student-t test between individual points for a given concentration. Strain and treatment differences for maximal response to the highest drug concentration and area under the curve were analyzed using 2 way ANOVA. Western blots were analyzed using student-t test ($\alpha=0.05$). Hemodynamic measures and PGI₂ production assay were analyzed using 2 way ANOVA ($\alpha=0.05$),

Results:

Animal Characteristics and Hemodynamics:

There was no significant difference in final body weight between SHR and WKY animals, as well as no difference seen between treatment groups (Table 1). As previously reported, SHR animals displayed significantly increased heart rate, mean arterial pressure, systolic blood pressure and diastolic blood pressure when compared to the WKY controls ($P < 0.0001$). There were no main effects of H₂S treatment on any of the above parameters. SHR CON and SHR H₂S treated animals had a significantly higher LV/BW ratio (index of hypertension induced cardiac hypertrophy) when compared to WKY CON animals. H₂S treatment, however, had no effect on LV/BW ratio.

Vasomotor Function:

Viability Measures:

KCL Contraction:

There was found to be a very small but significant change in the force produced from a 30 min 60mM KCL exposure when comparing all SHR vs. all WKY (Table 2). There were no differences when comparing H₂S treatment groups to their respective control groups (Table 2).

Phenylephrine Pre-Contraction

Neither strain nor H₂S treatment had a main effect on tension development to 10⁻⁶ log M phenylephrine (Table 2).

Vasorelaxation:

The results for each vasomotor function test are presented in two subsections: SHR and WKY control groups will be examined to present strain differences; followed by within strain comparisons to examine differences between control groups and chronic hydrogen sulfide treatment.

Endothelium-Dependent Vasorelaxations-ACh

Comparison of SHR and WKY control

Point-by-point analysis revealed that WKY displayed much greater relaxation to concentrations of 10^{-6} to 10^{-4} log M than SHR (Figure 6). WKY demonstrated greater relaxation to a maximal dose (MR) of ACh ($p < 0.0001$)(Table 3) as well as a greater area under the curve (AUC)($p < 0.05$)(Table 3).

Comparison within strain

Point-by-point analysis revealed significant differences in the SHR H₂S treatment group compared to SHR controls at the 10^{-5} and $10^{-4.5}$ log M concentrations ($p < 0.05$)(Figure 7). There were, however, no statistically significant H₂S treatment differences with respect to maximal relaxation or AUC (Table 3).

Endothelium-Independent Vasorelaxations-SNP

Comparison of SHR and WKY control

Point-by point analysis revealed that WKY displayed a slightly greater relaxation to SNP at the 10^{-10} log M concentration than SHR (WKY: $-0.7 \pm 1\%$, SHR: $3 \pm 1\%$,

p=0.044)(Figure 8). There were no differences (p=0.2, p=0.6 respectively) between SHR and WKY with respect to AUC and maximal relaxation (Table 3).

Comparison within strain

No H₂S treatment differences were observed in SNP relaxation curves with point-by-point analysis in either SHR or WKY treatment groups (Figure 8). There were also no H₂S treatment differences in maximal relaxation or AUC observed (Table 3).

Hydrogen Sulfide Mediated Vasorelaxations

Comparison of SHR and WKY control

No differences were observed with the point-by-point analysis (Figure 9). A strain difference was observed, with WKY having a greater AUC than SHR (p<0.05)(Table 3) but MR displaying no statistically significant differences (p=0.08).

Comparison within strain

No chronic H₂S treatment differences were observed in the acute H₂S relaxation curves with point-by-point analysis in either SHR or WKY groups (Figure 10). There were also no differences in maximal relaxation or AUC from the experiments (Table 3).

Endothelium-Dependent Vasorelaxations-ACh and INDO

Comparison of SHR and WKY control

No differences were observed with point-by-point analysis (Figure 11). AUC and MR were not significantly different between SHR and WKY controls (Table 3).

Comparison within strain

No H₂S treatment differences were observed in ACh relaxation curves incubated with indomethacin with point-by-point analysis in either SHR or WKY groups (Figure 12). There were also no differences in maximal relaxation or AUC (Table 3).

Vasoconstrictions:

Endothelium-Dependent Vasoconstrictions-ACh and L-NAME

Comparison of SHR and WKY control

Point-by-point analysis revealed that SHR displayed a greater development in tension at concentrations 10⁻⁶ log M to 10⁻⁴ log M than WKY (Figure 13). SHR demonstrated a greater AUC than WKY (p<0.0001)(Table 4) as well as higher tension development at a maximal dose of ACh (p<0.0001)(Table 4).

Comparison within strain

No H₂S treatment differences were observed in ACh tension development experiments incubated with L-NAME with point-by-point analysis in either SHR or WKY groups (Figure 14). There were also no differences in maximal contraction or AUC between either H₂S treatment group and its respective control (Table 4).

Thromboxane-Prostanoid Receptor Sensitivity-U46619

Comparison of SHR and WKY control

No statistically significant differences were observed in either point-by point, AUC or maximal contraction analysis between strains (Figure 15)(Table 4).

Comparison within strain

There were no statistically significant differences in tension development with U46619 with point-by-point analysis (Figure 15). Maximal contractions and AUC analysis were not significantly different (Table 4).

Biochemical Assessments:

6 keto PGF_{1α} Competitive Enzyme Immunoassay – Prostacyclin Production:

As expected, there was a significant increase in prostacyclin production by the SHR CON group compared to the WKY CON group (WKY: 139±23 pg/ml, SHR: 341±30 pg/ml, p<0.0001). H₂S treatment did not affect prostacyclin production in either WKY (132±20 pg/ml) or SHR (353±66 pg/ml) (Figure 16).

Western Blotting

Due to tissue limitations there was not sufficient WKY H₂S treatment data to be included in the analysis. Therefore, multiple student's t-tests were performed between SHR CON vs. WKY CON as well as SHR CON vs. SHR H₂S groups.

eNOS and CSE Protein Expression:

Similar to previous reports¹¹, SHR CON displayed an increase in eNOS protein content when compared to the WKY CON (SHR: 2.005±0.27, WKY:1±0.18 both arbitrary normalized units, p=0.02)(Figure 17). There was no statistically significant difference between the SHR CON and the SHR H₂S groups (SHR CON: 2.005±0.27,

SHR H₂S: 2.610±0.63, both arbitrary normalized units, p=0.31)(Figure 17). There were no strain or H₂S treatment differences in CSE expression (Figure 18).

Table 1: Animal Characteristics and Hemodynamic Parameters

	WKY		SHR	
	Control	H ₂ S	Control	H ₂ S
Weight (g)	375.1±4	377.1±5	368.5±5	373.2±7
Left ventricle to body weight ratio α	2.036±0.015	2.068±0.029	2.618±0.062	2.564±0.044
Heart rate (bpm) α	349±11	344±19	442±6	415±11
CCA Blood Pressure (mmHg)				
Systolic α	121.1±6.2	113.3±8.1	212.8±6.2	218.3±5.3
Diastolic α	94.8±2.5	88.1±6.4	163.5±4.2	159.9±4.7
Mean α	108.3±3.6	101.1±7.6	185.2±4.9	184.8±4.9

Animal characteristics and hemodynamic parameters. α represents differences between strains. Values are mean \pm s.e.m.: significance level $p < 0.05$.

Table 2: Vasomotor Function Viability Measures

Drug Condition	WKY		SHR	
	Control	H ₂ S	Control	H ₂ S
KCl pre-constriction (g) α	1.18±0.04	1.17±0.05	1.23±0.02	1.28±0.04
PE pre-constriction (g)	1.01±0.06	1.00±0.09	1.05±0.05	1.07±0.05

Vasomotor function viability measures: Maximum contraction to 60mM KCL and 10^{-6} log M PE. α represents differences between strains. Values are mean \pm s.e.m.: significance level $p < 0.05$.

Table 3: Vasomotor Assessment Parameters (Vasorelaxation)

Drug Condition		WKY		SHR	
		Control	H ₂ S	Control	H ₂ S
Endothelium dependent relaxations (ACh)	MR (%) α	100.6±3	99.4±2	78.7±6	81.8±3
	AUC α	356±14	362±19	314±16	326±13
Endothelium independent relaxations (SNP)	MR (%)	107.2±2	105.9±2	104.7±2	106.9±1
	AUC	430±18	429±14	413±9	414±7
Hydrogen sulfide relaxations	MR (%)	97.6±4	96.0±2	91.1±4	91.8±2
	AUC α	69±7	64±1	57±4	59±2
Endothelium dependent relaxations (INDO, ACh)	MR (%)	106.0±7	101.5±2	105.2±3	105.5±2
	AUC	386±34	385±12	351±15	350±12

Vasomotor assessment parameters: Response to a maximal dose (MR) expressed as a percent relaxation from PE pre-contraction and area under the curve (AUC) of vasorelaxation assessments. α represents differences between strains. Values are mean \pm s.e.m.: significance level $p < 0.05$.

Table 4: Vasomotor Assessment Parameters (Vasocontraction)

Drug Condition		WKY		SHR	
		Control	H ₂ S	Control	H ₂ S
Endothelium dependent contractions (L-NAME, ACh)	MC (%) α	33.4±9	27.6±8	63.6±6	62.8±4
	AUC α	63±11	48±13	110±12	110±6
TP receptor sensitivity (U46619)	MC (%)	142.4±4	140.0±5	132.7±5	137.1±4
	AUC	253±7	264±7	249±9	268±8

Vasomotor assessment parameters: Response to a maximal dose (MC) expressed as a percent of KCL contraction and area under the curve (AUC) of vasocontraction assessments. α represents differences between strains. Values are mean \pm s.e.m.: significance level $p < 0.05$.

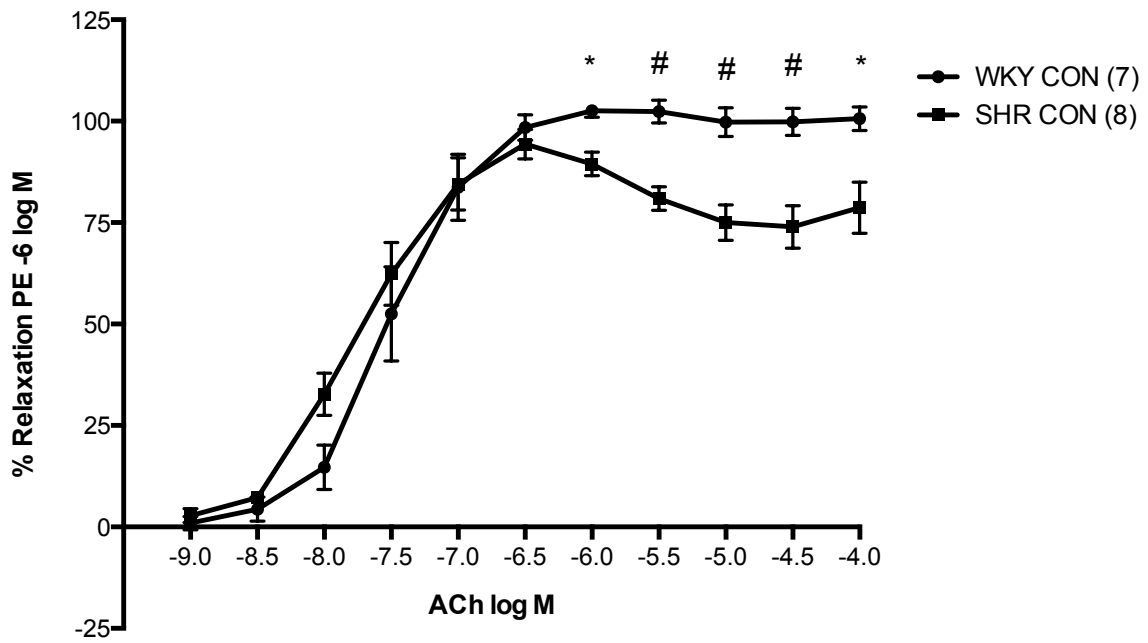


Figure 6: Endothelium-dependent relaxation to ACh expressed as a percent relaxation from PE pre-contraction. WKY CON vs. SHR CON. Values are mean \pm s.e.m., n per group in parenthesis: Significance levels *= p <0.05, #= p <0.001.

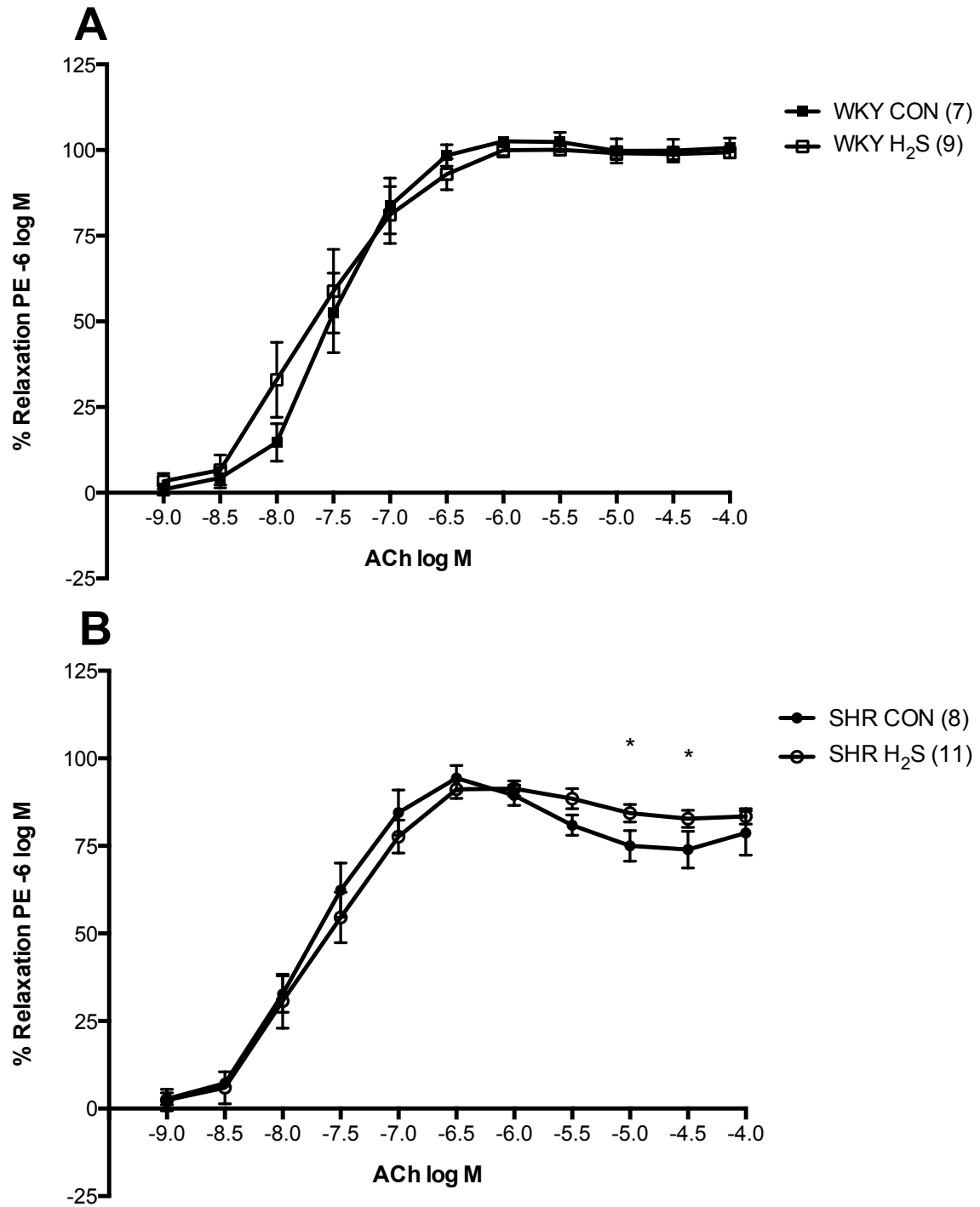


Figure 7: Endothelium-dependent relaxation to ACh expressed as a percent relaxation from PE pre-contraction. WKY (A) and SHR (B). Values are mean \pm s.e.m., n per group in parenthesis: Significance levels $\ast = p < 0.05$.

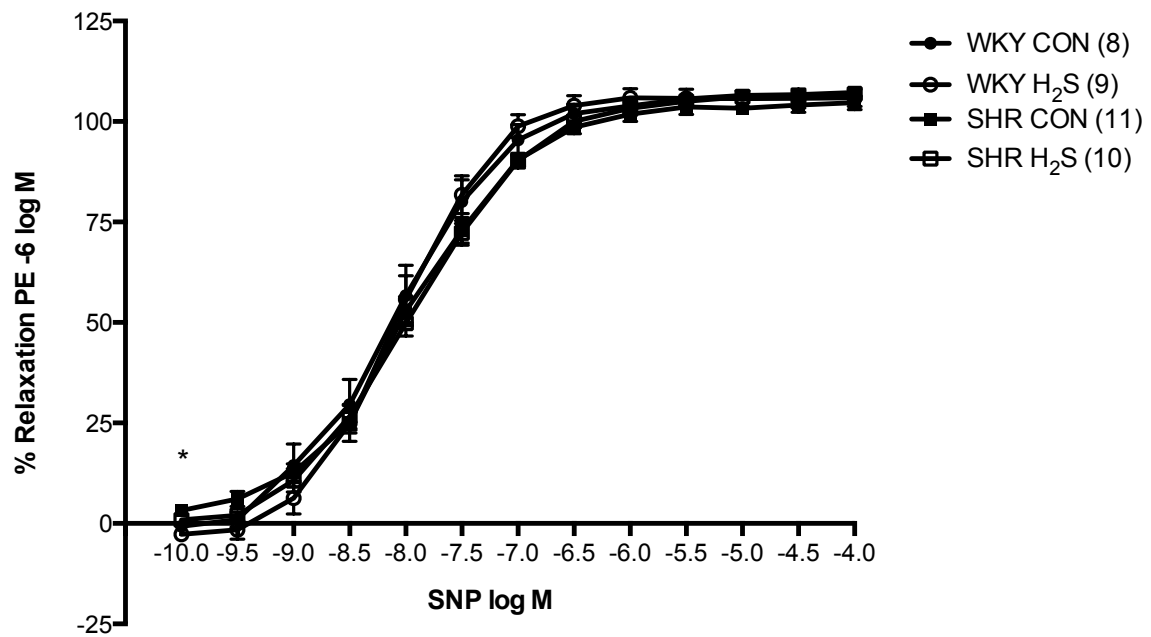


Figure 8: Endothelium-independent relaxation to SNP expressed as a percent relaxation from PE pre-contraction. All groups. Values are mean \pm s.e.m., n per groups in parenthesis: Significance levels $*=p < 0.05$ between SHR CON and WKY CON.

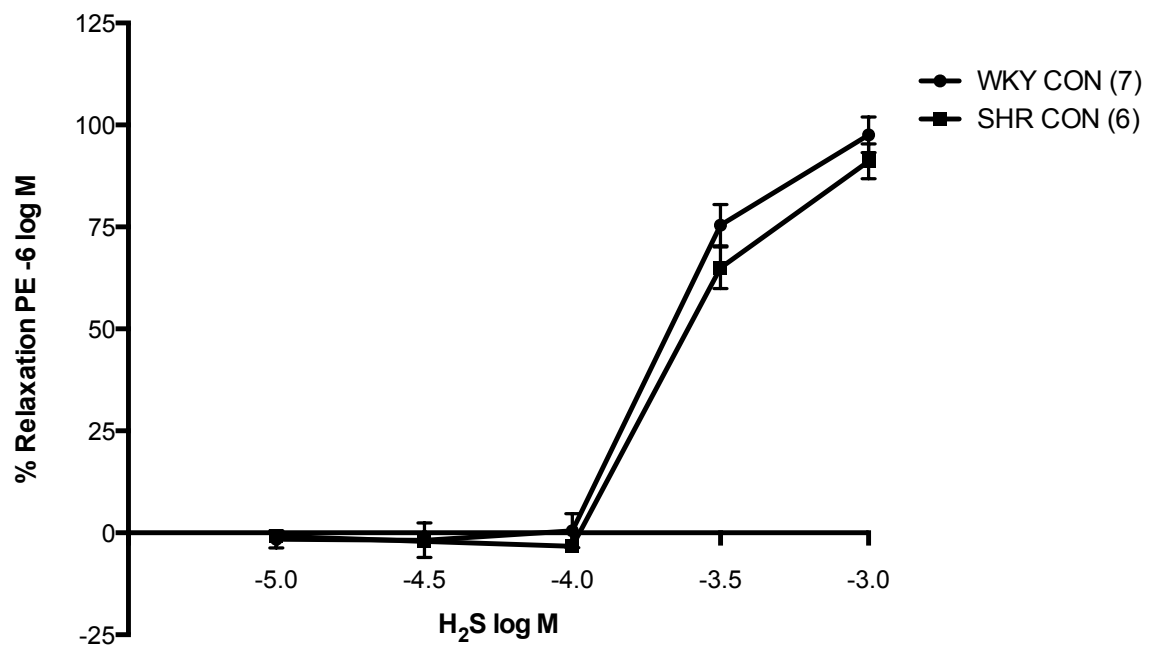


Figure 9: Relaxation to H₂S expressed as a percent relaxation from PE pre-contraction. WKY CON vs. SHR CON. Values are mean \pm s.e.m., n per groups in parenthesis.

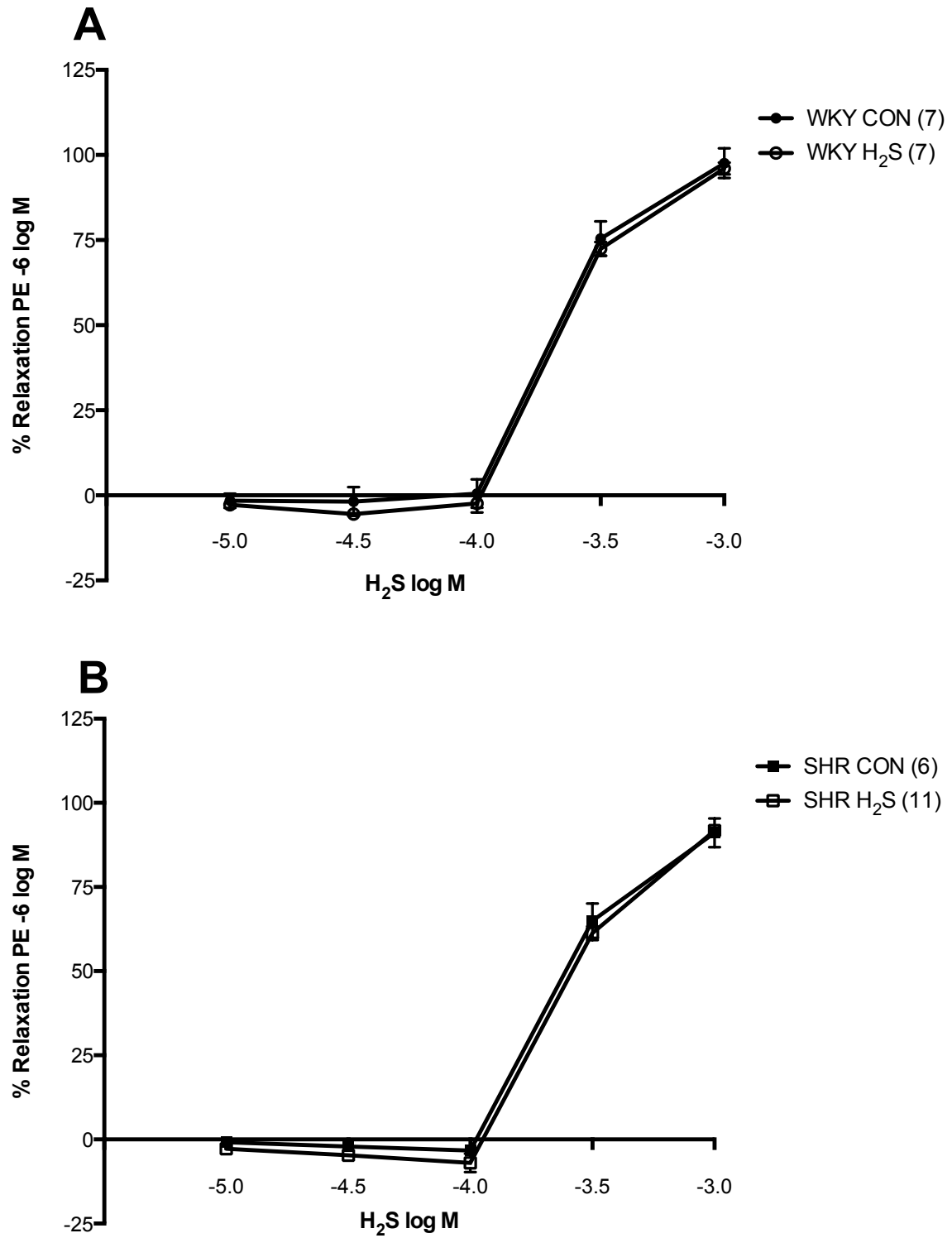


Figure 10: Relaxation to H₂S expressed as a percent relaxation from PE pre-contraction. WKY (A) and SHR (B). Values are mean \pm s.e.m., n per groups in parenthesis.

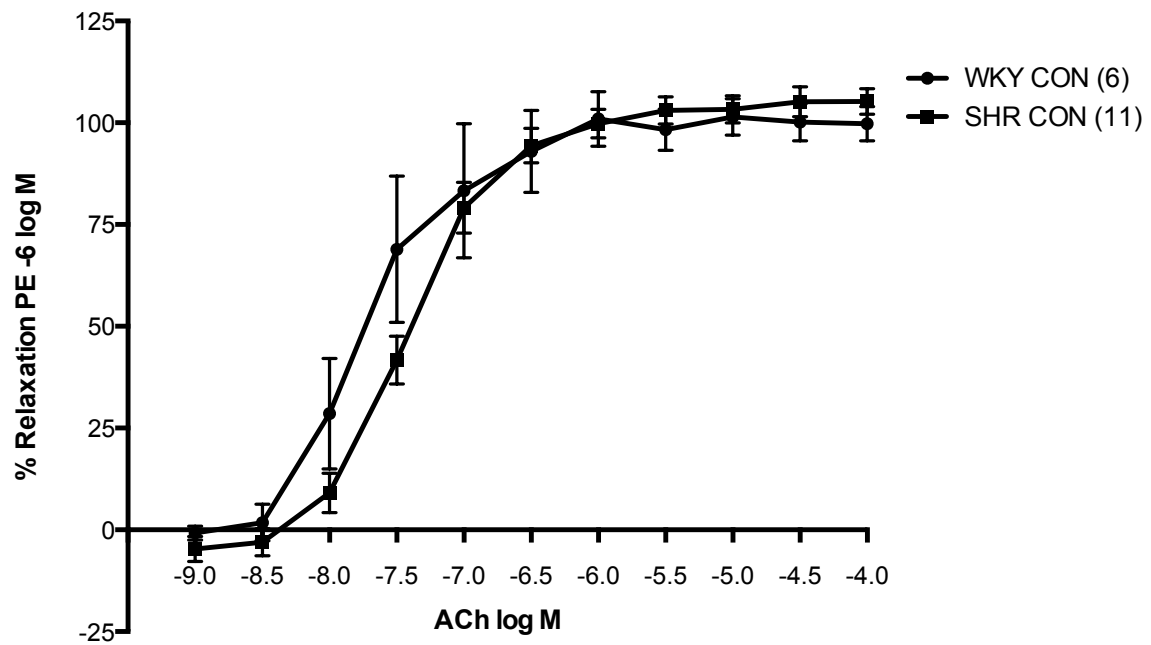


Figure 11: Endothelium-dependent relaxation to ACh following incubation with indomethacin, expressed as a percent relaxation from PE pre-contraction. WKY CON vs. SHR CON. Values are mean \pm s.e.m., n per groups in parenthesis.

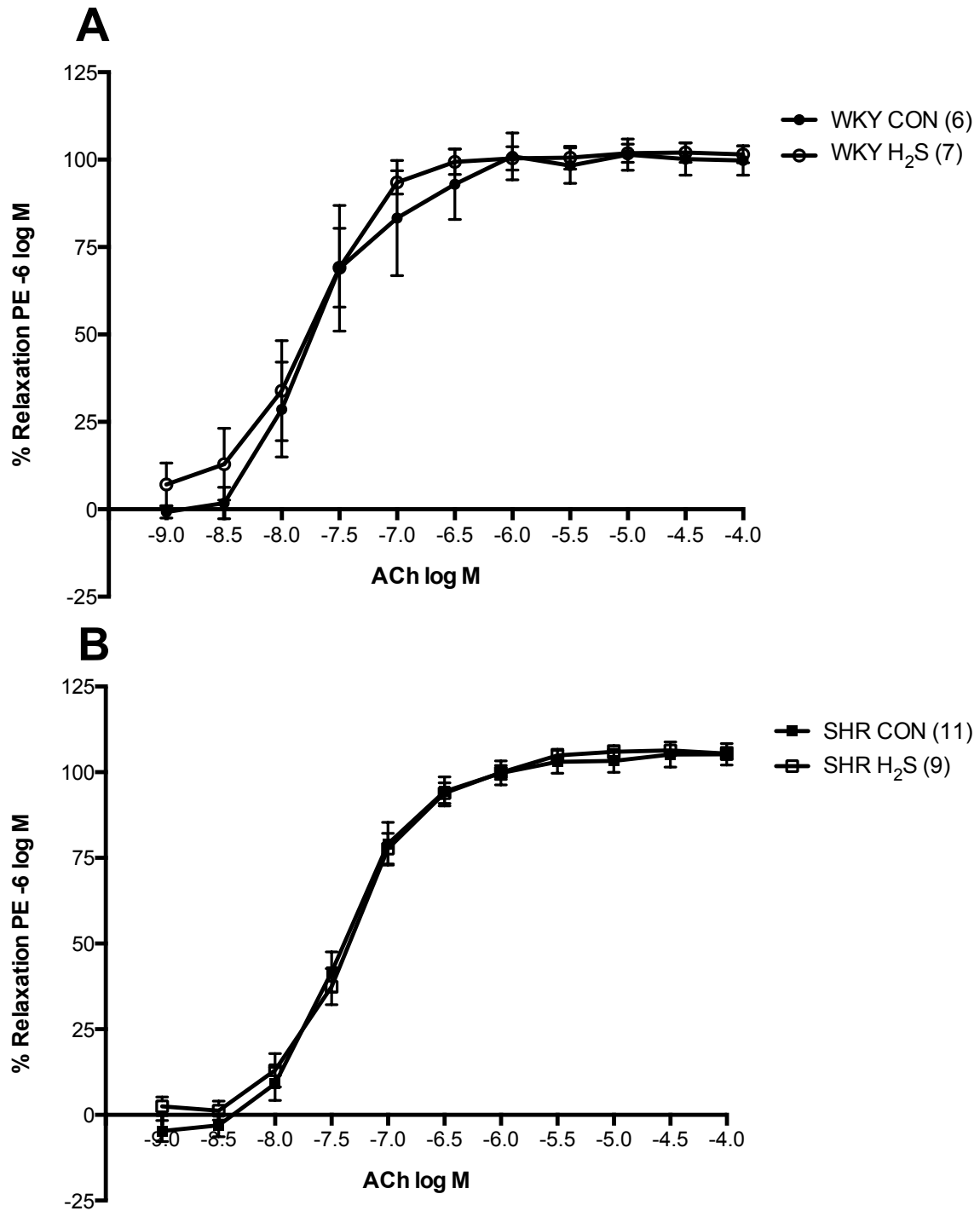


Figure 12: Endothelium-dependent relaxation to ACh following incubation with indomethacin, expressed as a percent relaxation from PE pre-contraction. WKY (A) and SHR (B). Values are mean \pm s.e.m., n per group in parenthesis.

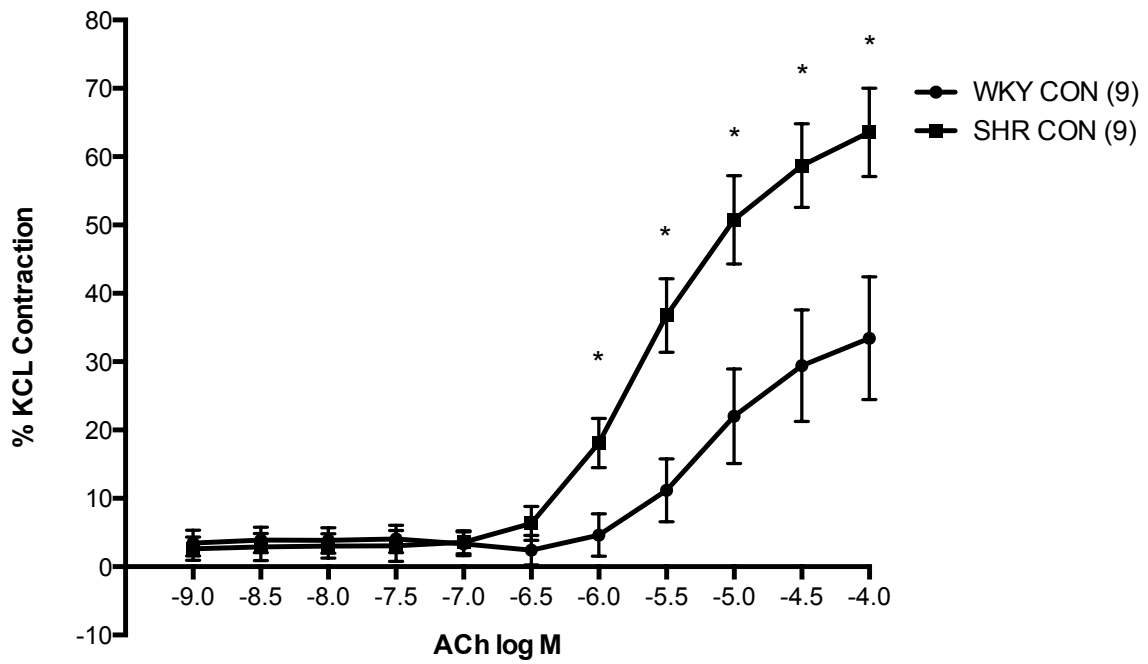


Figure 13: Endothelium-dependent contractions of quiescent rings to ACh following incubation with L-NAME, expressed as a percent of KCL contraction. WKY CON vs. SHR CON. Values are mean \pm s.e.m., n per groups in parenthesis: Significance levels $*=p<0.05$.

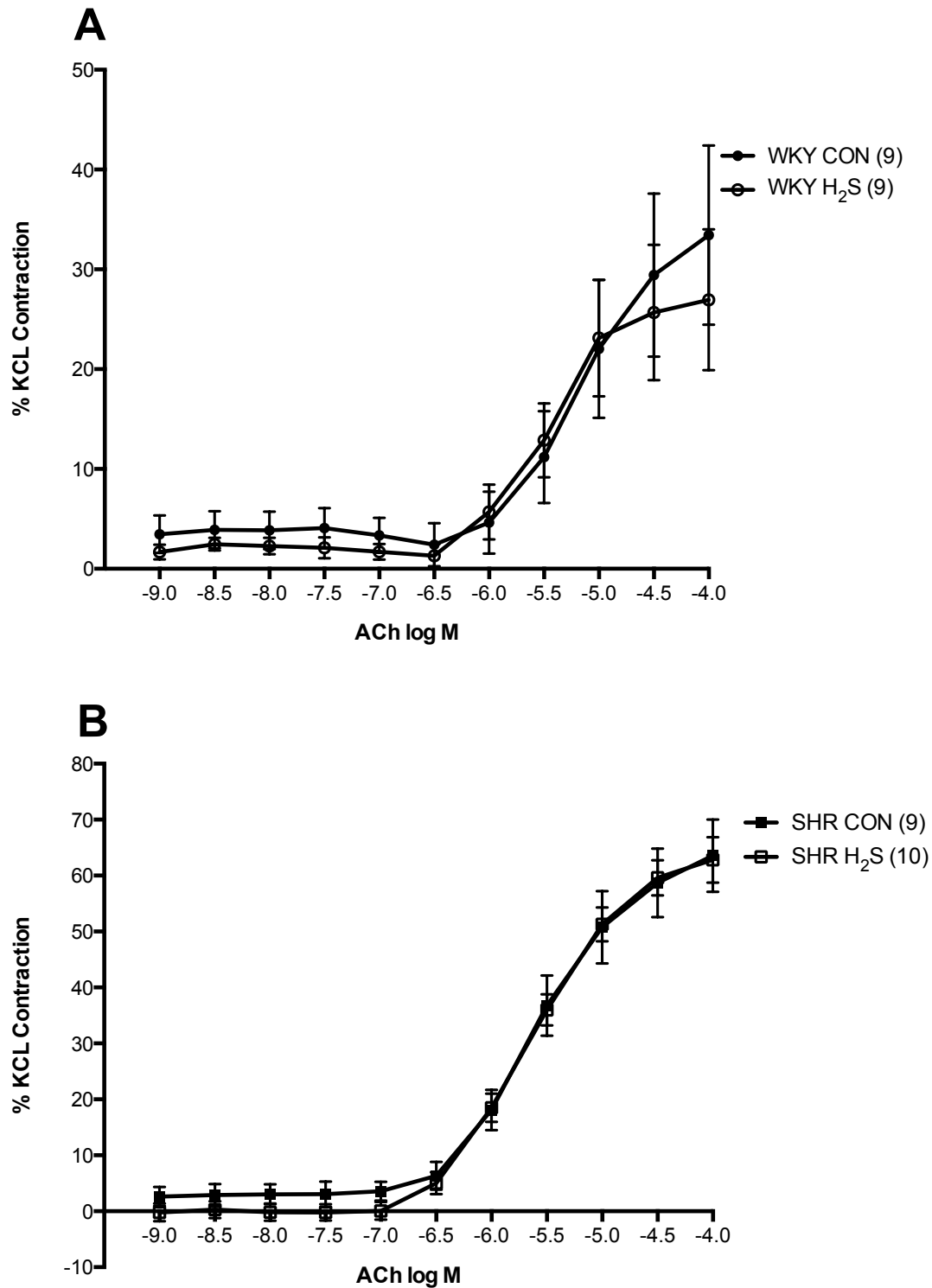


Figure 14: Endothelium-dependent contractions of quiescent rings to ACh following incubation with L-NAME, expressed as a percent of KCL contraction. WKY (A) and SHR (B). Values are mean \pm s.e.m., n per groups in parenthesis.

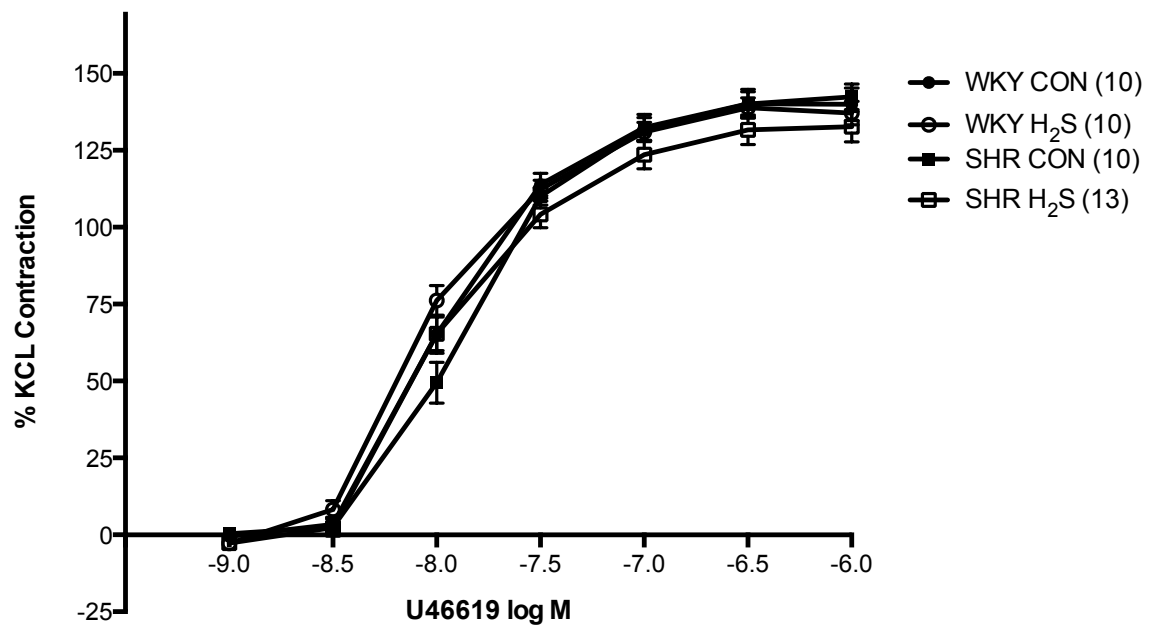


Figure 15: Endothelium-independent contractions to TP receptor agonist U46619 in quiescent rings, expressed as a percent of KCL contraction. All groups. Values are mean \pm s.e.m., n per groups in parenthesis.

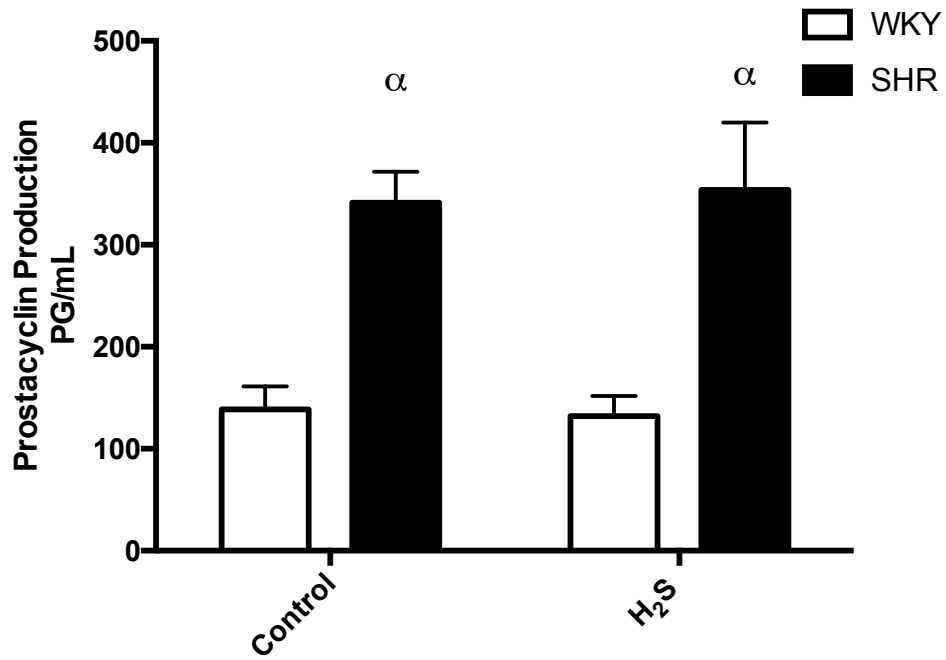


Figure 16: Prostacyclin production from CCA segments stimulated with ACh 10^{-4} log M. $n=9$ per group. α represents differences compared to WKY CON. Values are mean \pm s.e.m.: significance level $p<0.05$.

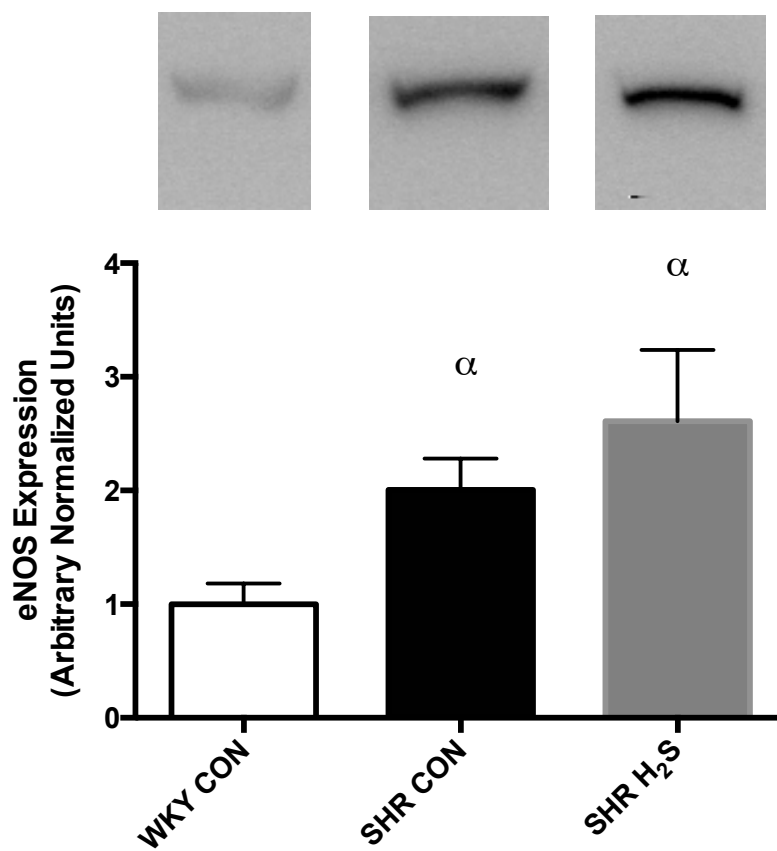


Figure 17: Protein expression values for eNOS. Values normalized to WKY CON group. $n=9$ for WKY CON, SHR H₂S, $n=13$ for SHR CON. α represents differences compared to WKY CON. Non-continuous lanes illustrating quality of blots are included for control and treatment groups. Values are mean \pm s.e.m.: significance level $p<0.05$.

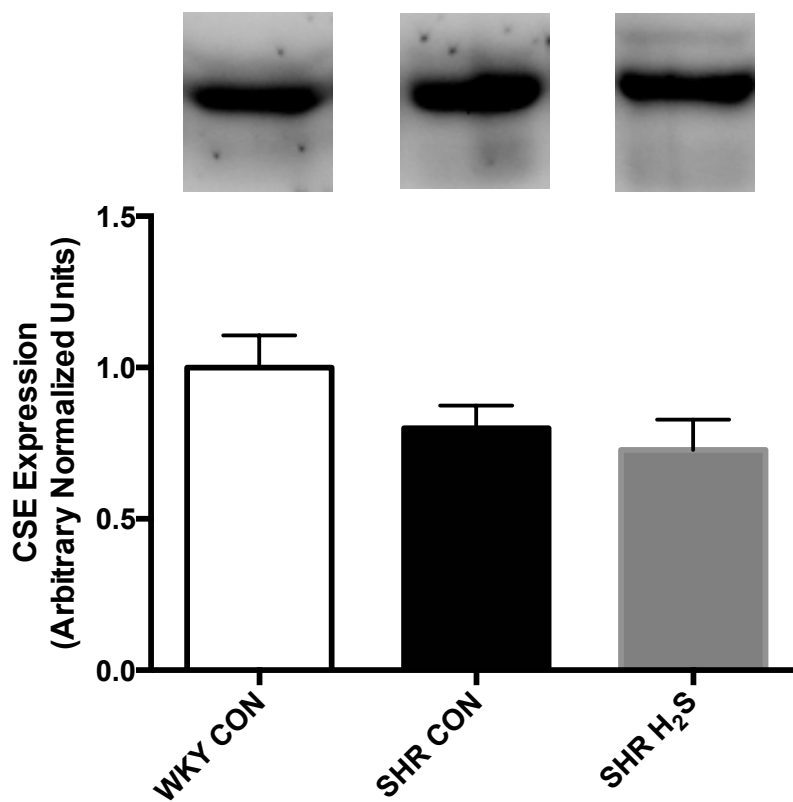


Figure 18: Protein expression values for CSE. Values normalized to WKY CON group. n=8-10 per group. Non-continuous lanes illustrating quality of blots are included for control and treatment groups. Values are mean \pm s.e.m.

Discussion:

The purpose of this study was to attempt to elucidate the effects that chronic sodium hydrosulfide injections have on endothelium-dependent vasomotor functions of the CCA, as well as examining changes in associated biochemical parameters and in hemodynamic measures in both the adult SHR and WKY animal models. The main findings of this study are:

1. Chronic hydrogen sulfide treatment did not lower blood pressure in adult spontaneously hypertensive rats.
2. Chronic hydrogen sulfide treatment improved relaxation to ACh in SHR treated with H₂S.
3. Chronic hydrogen sulfide treatment did not decrease endothelium-dependent contractions in the carotid artery.
4. Chronic hydrogen sulfide treatment did not decrease PGI₂ produced by the carotid artery.
5. Chronic hydrogen sulfide treatment did not alter protein expression levels of eNOS or CSE in SHR.

Animal Characteristics:

While previous studies conducted by our laboratory have consistently shown that SHR have a greater final body weight than the WKY animals^{12,19}, this study was unable to replicate those results. The discrepancy may be due to the fact that many of the WKY

animals used in the myography studies were older than planned, and so may have added weight due to their age. This is also the first study from our lab to use SHR animals bred from our breeding colony, so these animals may differ slightly genetically from the ones purchased from Harlan with respect to the body weight differences observed in the past. Vasomotor responses and blood pressure, however, were consistent with previous measures obtained from our lab, confirming that they are a viable model to be used in this study and in the future. H₂S treatment did not cause changes in body weight, which is consistent with previous reports⁴³.

Hemodynamics:

SHR displayed a mean arterial pressure of 185.2±4.9 mmHg compared to 108.3±3.6 mmHg in the WKY (Table 1). This confirms that the SHR were indeed hypertensive, as these mean arterial pressure values are in line with values found in the literature^{12,60}. Previous studies utilizing chronic H₂S treatment and SHR have shown that following H₂S treatment, the blood pressure in the animals is significantly decreased^{42,43}. Contrary to these results, our study demonstrated that after 5 weeks of H₂S treatment, no change in blood pressure was observed in the SHR. Some differences may arise from the different blood pressure measure protocols used. Both Yan et al.⁴² and Shi et al.⁴³ performed blood pressure measurements on conscious animals, while the current study performed blood pressure measurements on anesthetized animals. One possible explanation for these conflicting results is the age difference in the animals used in the previous studies and ours. We used animals 14 weeks of age, where the other investigations used animals starting at 4 weeks of age. In newborn and 2-week old SHR,

vascular resistance is reduced when compared to age-matched WKY ⁶¹. Progressing to 4-6 weeks of age, SHR display still unchanged vascular resistance, albeit with a higher cardiac output ⁶². SHR aged 3-4 weeks display significantly higher blood pressure than age matched WKY controls, but while WKY animals reach their adult blood pressure at around 10 weeks of age, SHR blood pressure continues to steeply increase until approximately 20 weeks of age ⁶³. This leads to the possibility that beginning the chronic treatment with H₂S at 14 weeks of age might be past the stage at which it can effectively decrease blood pressure. Thus, at 4 weeks of age, H₂S might be able to slow the progression of vascular remodeling and the progressive increase in blood pressure, while at 14 weeks, H₂S may be unable to reverse the structural changes and increase in blood pressure that have already taken hold. Further experimentation needs to be completed to systematically examine when the H₂S treatment becomes unable to lower blood pressure, as well as the mechanisms for this loss of effect. As it stands, the data indicate that chronic H₂S treatment does not decrease blood pressure in “adult” animals with established hypertension.

The other difference between previous studies and our own was the site of bolus injection. While studies in the literature use an intraperitoneal injection, we used a subcutaneous injection for animal care reasons. Over the course of a 5-week injection protocol, the likelihood of organ puncture or developing a serious infection are decreased with subcutaneous injections compared to intraperitoneal injections. A long-term subcutaneous injection regimen is also more comfortable for the animal ⁶⁴. This change in location however may have changed how the treatment affected the vasculature. With an intraperitoneal injection, the bolus injection would have bathed the mesenteric artery bed

with NaHS, possibly allowing for an acute relaxation of the entire mesenteric arterial bed. It is unclear from the literature exactly how long this dilation would have lasted, but it may have been long enough to play a role in slowing the progression of vascular remodeling in the young rats. With the subcutaneous injection, we can assume that there would not be this acute dilation of the entire mesenteric arterial bed⁶⁴. Instead, there would be a large bolus collected under the skin while the NaHS is distributed through the bloodstream. Our confirmatory experiments have shown (Appendix A) that within ten minutes of a subcutaneous injection there is an increase in the H₂S content in the blood, and Bucci et al.⁶⁵ recently showed that a subcutaneous injection of 1 μmol/kg of NaHS caused a decrease in blood pressure of 20 mmHg in mice that lasted 10-15 minutes, demonstrating that a subcutaneous injection does indeed introduce H₂S into the bloodstream and cause a reduction in blood pressure.

There is also evidence that this H₂S increase might not be beneficial to an adult SHR. A recent study showed that in cultured endothelial cells, exposure to different concentrations of NaHS caused an increase in cytosolic calcium originating from intracellular pools⁶⁶. Expanding on this, Kida et al. recently demonstrated that incubation of bovine aortic endothelial cells with NaHS resulted in an increase in cytosolic calcium, which led to activation of eNOS, which in turn increased the concentration of NO⁵⁰. While all these processes may be beneficial and aid in relaxation in young SHR, it may exacerbate endothelial dysfunction in adult animals. SHR have established endothelial dysfunction, with an increase in intracellular calcium thought to be one of the major contributors³. Should this increase in calcium occur in an endothelial cell with endothelial dysfunction, the increased calcium could also lead to COX-1 activation,

further increasing the amount of ROS and concentration of PGI₂. Increasing NO production and superoxide production simultaneously could lead to greater concentrations of peroxynitrite, furthering damage and reducing NO-bioavailability. Further work investigating the role of H₂S stimulated increases in intracellular calcium concentrations where endothelial dysfunction is present must be performed in order to determine whether it exacerbates endothelial dysfunction.

Evidence has also been presented showing that H₂S can stimulate PLA₂ mobilization and subsequent release of arachadonic acid⁶⁷. In this study the authors demonstrated that low concentrations of H₂S constricted the perfused mesenteric bed, and that this constriction was due to increased arachadonic acid but was COX- and NOS-independent. Again, in a young animal this increase in arachadonic acid may be beneficial, but with endothelial dysfunction, the increase in arachadonic acid could elicit negative effects through the COX- mediated production of PGI₂.

Vasomotor responses:

To the best of our knowledge this is the first study to assess the EDRF and EDCF signaling mechanisms in a hypertensive model following chronic H₂S treatment. SHR animals display impaired relaxation to ACh. With PE pre-contraction, vessels from the SHR show normal relaxation characteristics to low and moderate concentrations of ACh, but at higher concentrations they show a marked recontraction. This recontraction, a hallmark of endothelial dysfunction, has been shown to be due to alterations in NO and PGI₂ signaling pathways³. The current study confirmed that the SHR had endothelial dysfunction, apparent in the development of tension after 10⁻⁶ M ACh (Figure 6).

In agreement with our hypothesis, H₂S treatment in the SHR slightly improved relaxation to ACh with point-by-point analysis of certain concentrations. These differences reached significance at 10⁻⁵ and 10^{-4.5} log M (p<0.05) and close to significance at 10^{-5.5} and (p=0.07) (Figure 7). However, the effects were modest compared to what was hypothesized based on previous related studies. ACh stimulation triggers many cellular signaling cascades in the endothelium, so molecular inhibitors and receptor agonists were used to investigate any changes that might have occurred to the NO, PGI₂ and H₂S signaling pathways to account for the increased relaxation observed in H₂S treated animals.

NO Signaling Pathway:

Through the use of SNP and INDO, an NO donor and COX-1 inhibitor respectively, we are able to assess VSM sensitivity to NO, and NO bioavailability in the vessel, respectively. Here we demonstrate that with the comparison of SHR and WKY control animals, both the SNP and INDO dose-response curves exhibit no differences between strains (Figure 8 and 11, respectively). This result is supported in the literature where no strain differences are seen in either curve^{68,69}. In the current study we found that there were no changes to the SNP or INDO dose-response curve with chronic H₂S treatment (Figure 8 and 12, respectively). To the best of our knowledge, this is the first study to produce SNP and INDO dose-response curves in SHR that have been treated with H₂S, so we cannot confirm these results in the literature. Earlier studies have reported that the vasodilatory response elicited by H₂S does not involve the cGMP/PKG pathway^{4,70} and that at physiological concentrations, H₂S does not stimulate sGC³². Emerging evidence now suggests, however, that H₂S may stimulate PKG directly⁶⁵, as

well as inhibiting phosphodiesterase (PDE) activity⁷¹. Inhibition of PDE leads to increases in cGMP, and direct activation of PKG could be another possible mechanism for H₂S induced vasodilation. Altering both PDE and PKG could lead to those enzymes become more sensitive to upstream signals, and may contribute to the increased relaxation seen with ACh stimulation. Further work must be completed to assess the possible changes to these pathways with chronic H₂S treatment.

Though we attempted to make semi-quantitative assessments of ROS in carotid slices using dihydroethidium staining, this attempt was unsuccessful because of confounding factors affecting interpretation of the data (Appendix A). Therefore, we can only speculate as to the oxidative state of the endothelial cell. If there was no decrease in ROS levels due to the age of the animals, then it seems only logical that no increase in NO bioavailability would be revealed from incubation with INDO, and therefore no change in the INDO dose-response curve with H₂S treatment (Figure 12).

Previous work from our lab has demonstrated that SHR have increased expression of eNOS when compared to WKY controls¹², and we were able to confirm this in the current study (Figure 17). Interestingly, this higher expression did not yield an increase in NO bioavailability (Figure 11). This has been shown in the literature to be due to eNOS uncoupling, where ROS decreases the levels of tetrahydrobiopterin, important for stabilization of the eNOS dimer, which leads to increased production of ROS and reduces NO production⁷². Herein we demonstrate that in SHR treated with H₂S, there is a small but not significant increase in the expression levels of eNOS when compared to SHR control (Figure 17). While there is conflicting literature with respect to whether H₂S increases or decreases the expression of eNOS, there is evidence to suggest that eNOS

mRNA and protein expression can be enhanced when exposed to H₂S, supporting our findings ⁴⁹.

H₂S Signaling Pathway:

Using H₂S to induce acute relaxation revealed that the SHR controls had a slightly attenuated response compared to the WKY control animal (Table 2A). Though no specific point-by-point differences were observed (Figure 9), AUC was significantly higher in WKY controls, and maximal relaxation to the highest concentration of H₂S almost reached significance (p=0.08) (Table 2A). These differences suggest that there might be alteration in the VSM that negatively affect the SHR vessels in their ability to relax to exogenous H₂S. There is evidence that the SHR have differences in the expression levels of KIR6.1 and SUR2B, two important subunits of the K_{ATP} channel ⁷³. Considering that K_{ATP} channel activation is the mechanism by which H₂S induces relaxation, this lower expression of the K_{ATP} subunits could account for the slight decreased in the relaxation effect seen in SHR vessels. It remains to be seen whether these strain differences are present with 4-week-old SHR. H₂S treatment did not improve the relaxation response to exogenously applied H₂S (Figure 10). This demonstrates that chronic H₂S treatment did not alter the sensitivity of the VSM to H₂S. This study is the first to our knowledge that investigated NaHS relaxation in SHR treated with H₂S, so no literature is available to support the finding that H₂S treatment did not improve VSM sensitivity to H₂S. Further investigation is needed to determine whether chronic H₂S treatment has any effect on the expression of K_{ATP} channel subunits.

CSE protein expression was found to be no different between WKY and SHR controls, as well as no different with H₂S treatment (Figure 18). Studies using SHR and

chronic H₂S treatment have shown that with hypertension, there is a decrease in CSE mRNA expression when compared to WKY controls, and that mRNA expression returns to WKY control values after H₂S treatment⁴². It is difficult, however, to relate our findings to evidence available in the literature due to our examination of protein expression, while others examine mRNA expression. It is oftentimes incorrect to assume that an increase in gene expression will lead to an equal magnitude increase in protein expression, as there are many steps between transcription and translation⁷⁴. Thus, increases in CSE mRNA with H₂S treatment as reported in the literature may not necessarily lead to increases in CSE protein content, as suggested by our current results.

PGI₂ Signaling Pathway:

Incubation with the eNOS inhibitor L-NAME was used to assess COX-mediated constriction. In agreement with the literature¹², SHR controls displayed a marked recontraction in quiescent rings when compared to WKY control animals (Figure 13). This confirms that the SHR animals suffered from endothelial dysfunction, and that a large part of the SHR vessel recontraction to ACh was due to overactive PGI₂ signaling. H₂S treatment did not reduce endothelium-dependent contractions in SHR (Figure 14). Although there is no evidence for the cAMP/PKA pathway contributing to the vasorelaxant effect of H₂S^{4,70}, there is evidence that H₂S may interact with the pathway in some ways. Lim et al. have presented evidence that NaHS (5-100μM) can reverse forskolin-stimulated increases in cAMP in cell lines from aortic smooth muscle cells⁷⁵. Whether this decrease in accumulation was due to AC inhibition or due to PDE stimulation was not tested. With adult SHR, this ability for H₂S to possibly decrease

cAMP may contribute to the decreased efficacy of the H₂S treatment, by further removing cAMP signaling from an already dysfunctional endothelium. It will be important, in the future, to determine the mechanisms in which H₂S interacts with the cAMP pathway, and whether this interaction changes with age. It will also be important to investigate whether these changes work in a negative manner with endothelial dysfunction.

During endothelial dysfunction, PGI₂ preferentially stimulates the TP receptor causing contraction of the VSM³. The sensitivity to the TP receptor agonist U46619 was not different between SHR and WKY controls, nor was sensitivity decreased with H₂S treatment (Figure 15). This leads us to believe that H₂S treatment does not alter the sensitivity of the TP receptor on the VSM. It has been demonstrated in the literature^{18,76} that there are no differences in tension development with the use of the selective TP receptor agonist U46619, and we were able to replicate these results in the present study.

As expected, PGI₂ production was significantly increased in the SHR compared to the WKY control (Figure 16). This increase in PGI₂ production in the SHR has been well documented and is thought to be one of the main contributors to the EDCF response observed in SHR³. H₂S treatment did not decrease the amount of PGI₂ produced by the SHR (Figure 16). This finding, along with the finding that H₂S treatment did not reduce the development in tension with SHR vessels incubated with L-NAME, further suggests that chronic H₂S treatment, in contrast to hypothesis #3, does not affect the EDCF pathway, and that any slight alterations in ACh mediated dilation are due to changes in the NO signaling pathway.

Limitations:

Tissue limitations made performing biochemical assessments difficult. Arteries that were used for hemodynamic measure were unable to be used for biochemical analysis due to the manipulation they underwent during the isolation and measurement procedures leaving only the opposite carotid artery for biochemical assessment. Vessels used for myography were unable to be used due to their incubation with different drugs and exposure to different pharmacological agonists. We planned on performing Western blots for COX-1, but due to tissue limitations we were unable to perform those experiments. There were original plans to perform a H₂S production assay where tissue is homogenized and analyzed to assess the H₂S production levels from CSE, but the shortage in tissue did not allow for this experiment to take place.

This study was limited by the way in which hemodynamic measures were taken. While a pressure catheter surgically inserted into the CCA is a very direct measure of blood pressure, the animal must be anesthetized to take the measurements. Thus, there is the risk that differing anesthetization levels of the animals may have an effect on blood pressure measurements, but we attempted to counteract this by a large number of animals for blood pressure measurement.

We had originally planned on assessing oxidative stress of vessel segments by performing dihydroethidium staining on cross sectional slices of tissue rings taken from the carotid artery remaining after the hemodynamic measures were taken. This staining proved difficult to perform and quantify, as some sections from the same artery differed substantially in the amount of fluorescence that appeared. More detail on the ROS experiments and the difficulties encountered can be found in appendix A.

Conclusion:

This study demonstrates that chronic H₂S treatment at 56 μmol/kg did not decrease blood pressure of the adult SHR, while only slightly improving ACh mediated dilation at certain ACh concentrations. While H₂S treatment did not improve vascular smooth muscle sensitivity to NO or NO bioavailability, the increased relaxation may have been due to other mechanisms that were not explored in the current study. H₂S treatment seems to have no effect on endothelium-dependent contracting factors, as PGI₂ mediated contractions, TP receptor sensitivity and PGI₂ production were unaffected. All previous studies that use a chronic bolus injection of NaHS have shown a decrease in blood pressure, though interestingly have chosen a very young age for the animals to begin treatment. The present study demonstrated that chronic H₂S treatment in the adult SHR did not lower blood pressure, and did not alter the EDCF signaling pathway. While ACh stimulated relaxation was slightly altered, the mechanisms behind it remain unclear.

Future Directions:

Further experimentation needs to be completed to systematically examine when the H₂S treatment becomes unable to lower blood pressure, as well as the mechanisms for this loss of effect. It would also be important to expand the vasomotor assessment investigations to gather a more thorough idea of what changes are happening with H₂S treatment. This can include a thorough investigation of how H₂S interacts with the cAMP pathway in a hypertensive model, as well as probing possible alterations to PDE and PKG activity. It will also be important to determine whether H₂S treatment alters PLA₂ expression and arachadonic acid levels in hypertensive animals. Further investigation is

needed to determine whether chronic H₂S treatment has any effect on the expression of K_{ATP} channel subunits as well as investigating the role of H₂S stimulated increases in intracellular calcium concentrations where endothelial dysfunction is present in order to determine whether it exacerbates endothelial dysfunction.

Appendix A:

Supplemental Experiments:

Superoxide Production:

Methods:

A segment of the right common carotid artery taken after hemodynamic measures was used to assess superoxide production using dihydroethidium fluorescence. Briefly, segments were frozen in OCT compound and transverse sections (10 μ m) were generated using a cryostat. Sections were then incubated with 10 μ mol/l dihydroethidium (Molecular Probes) for 30 minutes on a 37° C shaker in a light sealed box. Rings were visualized with an Axio Observer Z1 structured-illumination fluorescent microscope equipped with standard Red/Green/Blue filters, an AxioCam HRm camera, and AxioVision software (Carl Zeiss). Fluorescent images were analyzed using ImageJ software⁴³.

Difficulties:

The majority of difficulties came during the analysis of the acquired images. It was difficult to obtain consistent staining, even on arterial segments that were on the same slide. It is unclear whether the differences seen can be attributed to arterial segments not being equally coated with the dye, or whether it was exposure time while under the microscope. We had first decided to attempt to quantify fluorescence for multiple individual stained nuclei in a given file, however this proved to be technically difficult, time consuming, and at times biased. Others had reported quantifying the entire picture, and taking multiple pictures to be the average for a single animal, yet with an arterial slice there is area in the file that was not part of the artery, skewing the fluorescence

analysis. We decided to use a system where a box of set dimensions was placed over a section of artery and the fluorescence from that section was quantified. This was then repeated for the whole arterial segment, with a final average being the average for that arterial ring. Multiple rings were analyzed for a single animal, with the average fluorescence of the rings being the final average fluorescence for that animal. Again, this was difficult to replicate from file to file, as placing the boxes on the segment still left area in the artery that was not analyzed.

Silver/Sulfide Electrode Experiments:

Methods:

A small subset (n=8) of female Sprague-Dawley rats were used to confirm that the H₂S injection being administered subcutaneously was able to increase H₂S levels in the blood. Animals were injected with sodium pentobarbital (50-65 mg/kg i.p.; Bimeda-MYC, Cambridge, ON). The level of anesthetization was monitored through the withdrawal reflex from a toe pinch. To ensure proper levels of anesthetization, additional sodium pentobarbital was injected until withdrawal reflex was absent. Animals were then given a 56µmol/kg subcutaneous injection, and blood was collected after 10 minutes. Blood collection was taken from the right ventricle using an 18 gauge needle and approximately 5-6 ml of blood was obtained from each animal. The blood was then separated into three 2 ml eppendorf tubes, left to clot for 10 minutes, rimmed, centrifuged for 3 minutes at 8000 rpm, and plasma was collected. Following collection of blood, animals were sacrificed by exsanguination. 500 µl of plasma was then mixed with 250 µl deionized water and 250 µl sulfide anti-oxidant buffer (SAOB)(0.35g ascorbic acid, 0.67g disodium

EDTA in 10 ml of 2M NaOH). The sulfide electrode (Fisher Scientific, Pittsburgh, PA) was washed with distilled water, blotted dry and immersed in the sample. The electrode potential was recorded using a Fisher Accumet AB15 pH meter (Fisher Scientific, Pittsburgh, PA) when the reading stabilized. A standard curve was created using increasing concentrations of sodium sulfide to calculate the concentration of H₂S in the samples, as well as to ensure that readings taken on subsequent days could be compared⁷⁷. The average of each standard concentration was used and fit with a semilog line with the equation $Y = -33.32 \cdot \log(X) - 663.1$. Millivolt readings from the samples were then converted to concentrations and corrected using a dilution factor of two.

Results:

Standard Curve:

A sodium sulfide standard curve was prepared daily to allow for comparison of results. The standard was made up to 25, 50, 100 and 200 μM concentrations. The plot of electrode potential values to H₂S concentration is displayed in Figure 19.

Electrode Values:

Our results show that after an H₂S subcutaneous injection, the H₂S concentration in the plasma was significantly different when compared to animals that had been given an equal volume of saline (CON: 2.31±0.55 μM, H₂S: 13.24±4.40 μM, p<0.05)(Figure 20). This demonstrates that the H₂S injected subcutaneously is delivered to the blood after 10 minutes.

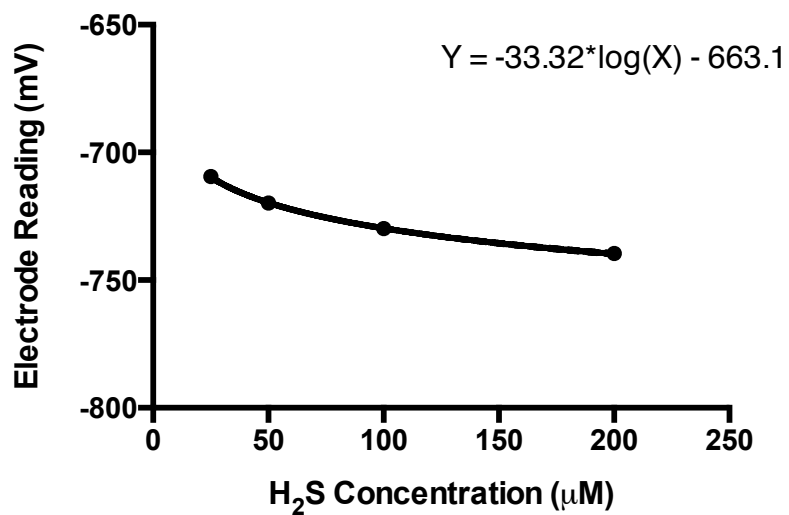


Figure 19: Standard Concentration Curve

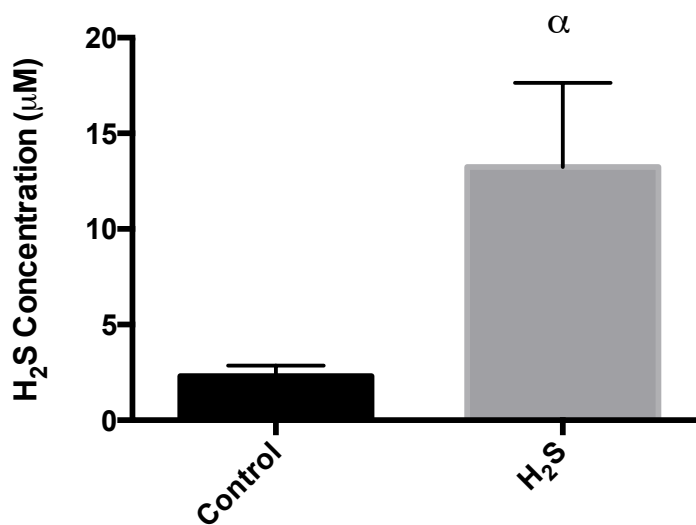


Figure 20: H₂S Concentration in µM. $n=4$ for each group. α represents differences with H₂S injection. Values are mean \pm s.e.m.: significance level $p < 0.05$.

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