INVESTIGATION INTO THE MECHANISMS OF HYDROGEN SULFIDE SIGNALING IN THE CARDIOVASCULAR SYSTEM AND THE EFFECTS OF AGE AND CALORIC RESTRICTION

By

BENJAMIN LEE PREDMORE

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To my Grandparents, Roy and Dorothy Predmore and my Parents, Roy and Donna Predmore

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The gasotransmitter hydrogen sulfide (H_2S) modulates vascular tone in vertebrates. Hydrogen sulfide and hypoxia elicit similar contractile responses in vertebrate smooth muscle, while both H_2S and nitric oxide (NO) elicit synergistic vasodilatory responses. Aging has negative impacts on the cardiovascular system, which can be attenuated by caloric restriction (CR). The mechanisms behind H_2S and hypoxia signaling and the synergy with NO are unknown, as well as whether aging and CR affect hydrogen sulfide signaling. I investigated the mechanisms through which aorta rings respond to hydrogen sulfide and hypoxia, how hydrogen sulfide regulates endothelial NO production, and how aging and CR affect the H_2S signaling system. I used bovine arterial endothelial cells and Fisher 344 x Brown Norway rats, 6-38 months of age, maintained on an *ad libitum* (AL) or CR diet. To investigate aging and CR, I measured protein and mRNA expression of the hydrogen sulfide producing enzymes cystathioneine γ–lyase (CSE) and cystathionine β–synthase (CBS) and the rate of hydrogen sulfide production from aorta and liver tissues, in addition to functional assessment using aorta rings. In the first study, I found that hypoxia and hydrogen sulfide elicit a triphasic, contraction-relaxation-contraction response. However, the mechanisms are not the same between hypoxia

and hydrogen sulfide. In the second study, I found that hydrogen sulfide stimulated a two-fold increase in NO production from endothelial nitric oxide synthase (eNOS). Phosphorylation of eNOS at Ser 1177 was also significantly increased, and inhibition of Akt attenuated this. In the third study, I found that the first phase contraction increased in sensitivity to hydrogen sulfide with age, while CR increased the magnitude of all phases. AL aorta CSE and CBS protein expression increased with age, but remained unchanged with CR. Liver CSE and CBS protein expression remained constant with age. Aorta CSE and CBS mRNA expression was higher with CR. Hydrogen sulfide production was also higher with CR in aorta and liver. I conclude that in rat aorta the triphasic responses to hypoxia and H_2S are mediated by different mechanisms, hydrogen sulfide up-regulates eNOS/NO production through an Akt-dependent mechanism, and the increased sensitivity to hydrogen sulfide and increased protein expression of CSE and CBS with age in aorta point to a drop in hydrogen sulfide bioavailability, while CR maintains CSE/CBS function. These studies reveal novel, age-sensitive mechanisms of hydrogen sulfide action to regulate vascular tone. They also illustrate the benefit of CR on the hydrogen sulfide signaling system. This is quite timely, given the emerging roles of hydrogen sulfide in cardiovascular pathologies.

CHAPTER 1
HYDROGEN SULFIDE: PAST, PRESENT AND FUTURE

Introduction

Hydrogen sulfide, a name often used to refer to sum of the chemical species H_2S, HS^- and S_2^-, has had a long and interesting development in the scientific literature. The "face" of hydrogen sulfide has changed within the last 40 years from an environmental and industrial toxin, to an energy source for chemosynthetic communities, and most recently as a gaseous physiological modulator, or gasotransmitter (Wang 2002), and a potential therapeutic agent in medicine. To familiarize the reader with the many aspects of hydrogen sulfide, this chapter will briefly examine the chemistry and toxicology of hydrogen sulfide as well as the chemosynthetic communities that it fuels, and then focus on its recent induction into the gasotransmitter family and its transition into biomedical research and therapeutic applications.

Hydrogen Sulfide is an Environmental and Industrial Toxin

There are many toxic and potentially lethal actions of hydrogen sulfide, the most significant of which is the reversible inhibition of the electron transport chain via cytochrome c oxidase (Nicholls 1975; Nicholls et al. 1982; Khan et al. 1990). This reversible inhibition can occur at 1-40 μM sulfide in isolated mitochondria (Nicholls et al. 1982; Bagarinao 1992), and is mechanistically very similar to cyanide poisoning (Nicholls et al. 1982; Shepherd et al. 2008). Other mechanisms of sulfide toxicity include opening of the mitochondrial permeability transition pore (Eghbal et al. 2004; Julian et al. 2005a); increasing production of reactive oxygen species (including superoxide) (Chen et al. 1972; Tapley et al. 1999; Eghbal et al. 2004; Julian et al. 2005a); out-competing oxygen for hemoglobin binding, causing formation of sulfhemoglobin (Bagarinao et al. 1992; Kraus et al. 1996; Völkel et al. 2000); and inhibition of about 20 other enzymes (Bagarinao 1992).

Various degrees of exposure to hydrogen sulfide can occur, and consequently the observed or experienced side effects vary. The effects of exposure to hydrogen sulfide can range from smelling a strong foul odor (> 3-5 ppm, described as the smell of rotten eggs), to olfactory nerve paralysis (> 150 ppm), to a headache (> 500 ppm), to dizziness and eventually loss of consciousness and death (500-1000 ppm or greater) (Beauchamp et al. 1984).

Hydrogen sulfide is produced in large quantities in some environments, and because of this many animals are intermittently or chronically exposed to hydrogen sulfide. Environmental hydrogen sulfide is a product of anaerobic, sulfate-reducing, bacterial metabolism (Bagarinao 1992; Attene-Ramos et al. 2007). Environments characterized by hydrogen sulfide include the anoxic layer of marine sediments (i.e. beaches, coastal lagoons, mangrove swamps, and salt marshes), stagnant basins and anoxic fjords, and the digestive tract of animals (Bagarinao 1992; Attene-Ramos et al. 2007). Hydrothermal vents and hydrocarbon seeps are also characterized by hydrogen sulfide, but the source of hydrogen sulfide at these sites is a mixture of geological and biological processes. These environments will be further characterized below.

Anthropogenic activities can also be large sources of hydrogen sulfide. Over 70 industries involve or produce concentrations of hydrogen sulfide that are often in toxic to lethal doses (> 50 ppm) (Beauchamp et al. 1984). These range from paper mills, tanneries, large-scale aquaculture, rayon production, petroleum and natural gas operations, sewage plants, and many other industries that involve livestock (i.e. dairy and pig farms), where hydrogen sulfide is produced from organic waste (Bagarinao 1992; Yalamanchili et al. 2008). Not surprisingly there are numerous published case-studies of workers in these industries who have experienced highly toxic yet sub-lethal doses (Tvedt et al. 1991; Fenga et al. 2002; Gangopadhyay et al. 2007), or lethal doses of hydrogen sulfide (Tatsuno et al. 1986; Yalamanchili et al. 2008). Hydrogen

sulfide poisoning still remains an everyday risk for those working in the petroleum, sewer, maritime, and mining industries (Yalamanchili et al. 2008).

Animals that are adapted to environments with hydrogen sulfide have avoidance behaviors in addition to antioxidant defenses and detoxification mechanisms to protect themselves from acute and/or chronic exposure to hydrogen sulfide (Bagarinao 1992). Most of these animals have a very similar set of detoxification mechanisms, including specialized hemoglobin that can bind both oxygen and hydrogen sulfide (Martineu et al. 1997; Zal et al. 1997; Zal et al. 1998; Hourdez et al. 2000), conversion of sulfide to thiosulfate (Levitt et al. 1999; Doeller et al. 2001), and storage of sulfide as both taurine and thiotaurine (Joyner et al. 2003). However, in some invertebrates, hydrogen sulfide can also be used as the terminal electron acceptor in aerobic respiration (Doeller et al. 2001; Kraus et al. 2004).

Hydrogen Sulfide is an Energy Source for Chemosynthetic Communities

The discovery of hydrothermal vent communities in 1977 greatly increased the rate of scientific publications regarding hydrogen sulfide (Lonsdale 1977). Not long after the discovery of hydrothermal vent communities, hydrocarbon seep communities were discovered (Hecker 1985). At these sites geothermal and volcanic activities (McMullin et al. 2000), or the pressure of rising salt-domes (Claypool et al. 1983), contribute to releasing hydrogen sulfide into the water column. This hydrogen sulfide fuels a unique chemosynthetic ecosystem.

Many invertebrates thrive in hydrothermal vents, including several species of tubeworms, such as *Riftia pachyptila* and *Tevnia jerichonana*, at the vents and *Lamellibrachia luymesi* and *Seepiophila jonesi*, at the seeps. These animals lack a mouth, gut and anus and instead live in a chitonous tube that contains a sack-like structure termed the trophosome. This sack contains bacterial symbionts that use energy from sulfide oxidation to fix carbon into organic molecules, some of which are provided to the host tubeworm for its nutrition (Hand et al. 1983). These

tubeworms have specialized hemoglobin to transport oxygen and hydrogen sulfide from the respiratory surface to the bacteria without the animal becoming poisoned itself (Martineu et al. 1997; Zal et al. 1997; Zal et al. 1998; Hourdez et al. 2000). In addition to specialized hemoglobin, hydrocarbon seep tubeworms also have posterior extensions, termed "roots", to obtain hydrogen sulfide from below the sediment-water interface (Julian et al. 1999).

Hydrogen Sulfide is a Gasotransmitter and Physiological Modulator

Since hydrogen sulfide has had a long history as a toxin, it was very surprising to find that hydrogen sulfide can be endogenously produced in a variety of animal tissues and that it has both neuromodulatory (Abe et al. 1996) and cardiovascular regulatory effects in mammals (Hosoki et al. 1997). Since its discovery of its neuromodulatory abilities (Abe et al. 1996), hydrogen sulfide has been added to the family of gasotransmitters (Wang 2002), which also includes nitric oxide (NO) and carbon monoxide (CO).

The discovery of gasotransmitters began with the work on the actions of NO by Murad, Furchgott and Ignarro from 1977-1986 (Furchgott 1999), who in 1988 shared the Nobel Prize in Physiology or Medicine for their work. The discovery of the gasotransmitter function of CO by Verma and colleagues followed shortly thereafter in 1993 (Verma et al. 1993). Finally, investigations of hydrogen sulfide as a physiological modulator began in 1996-1997 with the work of Abe and Kimura (Abe et al. 1996) and Hosoki, Matsuki and Kimura (Hosoki et al. 1997).

All three gasotransmitters, CO, NO and H_2S, share several similarities. They are endogenously produced, small gas molecules that are capable of physiological action (Wang 2002). They can easily diffuse across cell membranes to exert their function (Wang 2002). They do not require a mechanism of degradation or reuptake because they are all very reactive, and they use heme as a common sink (Wang 2002).

Enzymatic Production of Hydrogen Sulfide

All of the gasotransmitters are endogenously produced by enzymatic reactions. Nitric oxide is produce from L-arginine by nitric oxide synthase (NOS). Carbon monoxide is produced from heme by heme oxygenize (HO). In mammalian tissues, hydrogen sulfide is primarily produced from L-cysteine by two PLP (pyridoxal-5'-phosphate)-dependent, cysteine metabolic enzymes: cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS) (Julian et al. 2002; Zhao et al. 2003). However, several other enzymatic pathways exist for the production of hydrogen sulfide, including via cysteine amino transferase or cysteine lyase (Julian et al. 2002). CSE is expressed in endothelial cells and vascular smooth muscle cells (Hosoki et al. 1997; Wang 2002; Wang 2003; Yang et al. 2008), and is the predominant enzyme for hydrogen sulfide production in the cardiovascular system. In the CSE reaction (Figure 1-1), L-cysteine must first dimerize to form cystine, which is then transformed into pyruvate, thiocystine and NH_3 by CSE. CSE can then catalyze the reaction of thiocystine with other thiol compounds to from H_2S and CysSR (Julian et al. 2002). Alternatively, thiocystine may form H_2S and cystine non-enzymatically (Cavallini et al. 1962). CBS is the predominant enzyme for hydrogen sulfide production in the nervous system (Abe et al. 1996). In the CBS reaction (Figure 1-1), L-cysteine is hydrolyzed to yield equimolar amounts of H_2S and L-serine (Cavallini et al. 1962).

Julian and colleagues showed that hydrogen sulfide is produced in invertebrate tissues from CBS and CSE (Julian et al. 2002), revealing that gasotransmitters have a deeper phylogenic history than just vertebrates. Hydrogen sulfide has been shown to act as a signaling molecule in a variety of invertebrates (Julian et al. 1998; Julian et al. 2002; Gainey et al. 2005; Julian et al. 2005b), as has nitric oxide (Gainey et al. 2003; Palumbo 2005).

Regulation of Hydrogen Sulfide Production

Hydrogen sulfide production by CBS and CSE can be physiologically regulated, although

the mechanisms are poorly understood. Sex hormones appear to regulate brain hydrogen sulfide production by CBS, with males having higher hydrogen sulfide concentration in the brain than females (Eto et al. 2002b). This can be reversed either by castration of males or by testosterone injection in females (Eto et al. 2002b). CBS activity *in vitro* can also be regulated by S-adenosyl-methionine (SAM), an allosteric activator of CBS (Eto et al. 2002b). CSE also appears to be calcium-calmodulin dependent, and can be stimulated through muscarinic receptor activation of intracellular calcium (Yang et al. 2008).

Hydrogen sulfide production may also be regulated by the other gasotransmitters. NO and CO may bind to and inactivate CBS, with CO being the more potent inactivator than NO (Taoka et al. 2001; Puranik et al. 2006). In contrast, NO seems to stimulate hydrogen sulfide production in the cardiovascular system (Lowicka et al. 2007): NO donors stimulate CSE-dependent hydrogen sulfide production in aorta tissue homogenates in a cGMP-mediated manner (Zhao et al. 2003), and incubation of vascular smooth muscle cells with NO donors increases CSE protein and mRNA expression (Zhao et al. 2001). Sodium nitroprusside (SNP), an NO donor, increases the activity of brain CBS *in vitro*, but this effect results from chemical modification of the cysteine groups of CBS, rather than a direct action of NO itself (Eto et al. 2002a).

Physiological Actions of Hydrogen Sulfide

Abe and Kimura were the first to show a physiological role for hydrogen sulfide (Abe et al. 1996). They demonstrated that hydrogen sulfide was not only produced in the brain by CBS, but that it increased N-methyl-D-aspartic acid (NMDA) receptor-mediated responses and facilitated hippocampal long-term potentiation (Abe et al. 1996). Since then many actions have been reported. These include the ability to upregulate γ-aminobutyric acid B ($GABA_B$) receptors in the brain (Han et al. 2005), regulate cerebrovascular circulation (Leffler et al. 2006) and play a

role in cerebral ischemic damage after a stroke (Qu et al. 2006), mediate learning and memory formation (Partlo et al. 2001), stimulate L-type calcium channels in neurons (Garcia-Bereguiain et al. 2008), negatively regulate the hypothalamo-pituitary-adrenal axis (Dello Russo et al. 2000), and protect neurons from oxidative stress (Kimura et al. 2004; Kimura et al. 2006).

A multitude of functions for hydrogen sulfide have been reported in the cardiovascular system, particularly in the vascular smooth muscle. One of the most important sites of action of hydrogen sulfide is the ATP-sensitive K^+ channel (K_{ATP}) in smooth muscle cells, which causes hyperpolarization and relaxation (Zhao et al. 2001). However, hydrogen sulfide has also been shown to cause contraction, relaxation or multiphasic responses in aorta (Hosoki et al. 1997; Zhao et al. 2001; Dombkowski et al. 2005), mesenteric artery (Cheng et al. 2004; Tang et al. 2005), cerebral artery (Leffler et al. 2006), gastric artery (Kubo et al. 2007c), mammary artery (Webb et al. 2008), and pulmonary arteries (Dombkowski et al. 2005; Wang et al. 2008). These responses, however, all depend on the concentration of hydrogen sulfide and O_2, the specific vessel examined, and the animal model used (Dombkowski et al. 2004; Dombkowski et al. 2005). Hydrogen sulfide has also been associated with the pathology of a number of cardiovascular diseases, including hypertension and COPD (chronic obstructive pulmonary disease), in addition to hydrogen sulfides involvement in septic shock (Chunyu et al. 2003; Du et al. 2003; Hui et al. 2003; Yan et al. 2004; Chen et al. 2005). Additionally, hydrogen sulfide has recently been implicated in vasodilation of the corpus cavernosum (Srilatha et al. 2007; d'Emmanuele di Villa Bianca et al. 2009; Shukla et al. 2009) and the dysregulation of hydrogen sulfide has been linked to erectile dysfunction (Srilatha et al. 2006).

Hydrogen sulfide also affects the heart itself. Hydrogen sulfide preconditioning of rat cardiomyocytes induces a cardioprotection against ischemia and reperfusion injury (Hu et al.

2007). The proposed pathways in this protection include eNOS, K_{ATP} channels, protein kinase C, extracellular signal regulated kinase (ERK 1/2), and phosphatidylinositol 3-kinase/proteine kinase B (Akt) (Hu et al. 2007; Yong et al. 2008a). Other cardiovascular targets of hydrogen sulfide are β-adrenergic receptors (Yong et al. 2008b), carotid sinus baroreceptor (Xiao et al. 2007), NADPH oxidase-1, and Rac(1) (Ras-related C3 botulinum toxin substrate 1) (Muzaffar et al. 2008) and cyclooxygenase, potentially altering arachadonic acid metabolite levels as well (Koenitzer et al. 2007).

Recent evidence also suggests that hydrogen sulfide acts as an oxygen sensor in vertebrates and may be involved in vascular responses to hypoxia (Dombkowski et al. 2006; Olson et al. 2006; Olson 2008). The rationale is that the low oxygen levels during hypoxia allow accumulation of hydrogen sulfide in vascular tissue, which then modulates vascular tone.

A role for hydrogen sulfide in the gastrointestinal system has also been emerging. As in other tissues, hydrogen sulfide is produced in gastric and intestinal tissues from CBS and CSE (Fiorucci et al. 2006), and has had several demonstrated functions. Hydrogen sulfide has been reported to protect gastric mucosal epithelium from oxidative stress (Yonezawa et al. 2007) and enhance ulcer healing in rats (Wallace et al. 2007). Hydrogen sulfide also affects gut motility and secretion (Kubo et al. 2007c), relaxation of ileum (Hosoki et al. 1997), and can also inhibit motor patterns in human, rat and mouse colon (Gallego et al. 2008). In the liver, hydrogen sulfide regulates perfusion and biliary bicarbonate secretion (Fiorucci et al. 2005b; Fujii et al. 2005).

In addition to actions of hydrogen sulfide in the nervous, cardiovascular, and gastrointestinal systems, there is evidence that hydrogen sulfide is involved in insulin secretion, also working through K_{ATP} (Yang et al. 2005), as well as leukocyte adhesion and trafficking, via K_{ATP} (Zhang et al. 2007), and leukocyte-mediated inflammation (Zanardo et al. 2006).

Hydrogen sulfide has a protective role or antioxidant capacity in many systems. Hydrogen sulfide will readily scavenge hydrogen peroxide, and increase intracellular levels of reduced glutathione (GSH) (Kimura 2002; Pryor et al. 2006) and the hydrogen sulfide signaling system (including CSE and CBS) has both anti-oxidant (Kimura et al. 2004; Whiteman et al. 2004a; Kimura et al. 2006; Yan et al. 2006; Jha et al. 2008) and anti-inflammatory (Fiorucci et al. 2005a; Zanardo et al. 2006; Wallace 2007b) actions. Not surprisingly, there is large interest in its potential role as an NSAID and therapeutic agent for a variety of disorders, including hypertension (Fiorucci et al. 2007; Lowicka et al. 2007; Szabo 2007; Wallace 2007a; Wallace 2007b; Li et al. 2009). However, hydrogen sulfide is also reported to increase lipopolysaccharide-induced inflammation (Li et al. 2005).

One of the more dramatic actions of hydrogen sulfide is greatly reducing metabolism in mice, resulting in a suspended animation-like state (Blackstone et al. 2005). While the possibility of inducing a suspended animation has tremendous potential, this has not yet been duplicated in animals larger than the mouse (Haouzi et al. 2008). Nonetheless, this discovery has led the creation of the Ikaria Corporation, a gas-drug company investigating both therapeutic potential of NO and H_2S. Ikaria has produced a more stable form of Na_2S called IK-1001, which is purportedly suitable for injection, and INOmax® which is nitric oxide for inhalation. IK-1001 has been used in several research applications and finished Phase I clinical trials in 2008 (Elrod et al. 2007; Szabo 2007; Jha et al. 2008; Kiss et al. 2008). The metabolic effects of hydrogen sulfide are also seen in the nematode *Caenorhabditis elegans*, in which hydrogen sulfide increases thermotolerance and lifespan in (Miller et al. 2007). While still in research and development, it is clear that hydrogen sulfide has a very high therapeutic potential, much like NO and NO-donors.

Interactions between the Gasotransmitters

Hydrogen sulfide and the other gasotransmitters may interact with each other, but the interactions have not been firmly established. The early work on the interactions of hydrogen sulfide and NO indicated that the relationship was synergistic. In 1997 Hosoki et al showed that hydrogen sulfide increased the effects of the NO donor SNP by up to 13-fold (Hosoki et al. 1997). Later, Julian et al. showed that SNP potentiates hydrogen sulfide-induced contractions in the body wall of the echiuran worm *Urechis caupo* (Julian et al. 2005b)..

Not surprisingly, there have also been reports of negative interactions between the gasotransmitters. At a direct chemical level hydrogen sulfide can react with NO to form a nitrosothiol (Whiteman et al. 2006), and hydrogen sulfide can increase CO production from HO-1, which can then inhibit NO production from iNOS (Oh et al. 2006). In studies of hypertension and pulmonary vascular structural remodeling (PVSR), exogenous hydrogen sulfide inhibits the NO/NOS pathway and upregulates the CO/HO-1 pathway (Qingyou et al. 2004; Li et al. 2006). Moreover, during hypertension the expression and activity of CSE decreases with a concomitant decrease in plasma hydrogen sulfide concentration (Qi et al. 2004; Xiaohui et al. 2005), while plasma NO levels and eNOS expression levels increase (Zhong et al. 2003). Accordingly, application of hydrogen sulfide (as NaHS) rescues rats from hypertension (Du et al. 2003; Qingyou et al. 2004; Yan et al. 2004) and application of hydrogen sulfide lessens aorta structural remodeling, decreases NO levels, and increases CO levels (Qingyou et al. 2004; Yan et al. 2004; Li et al. 2006). Similarly, increasing expression of HO-1 prevents development of hypertension and inhibits PVSR (Zhao et al. 2001).

However, data have been published that contradict many of the interactions reported between the gasotransmitters and their respective enzymes. These discrepancies are likely the result of using different experimental systems, as well as differences in methodologies and

techniques while working with hydrogen sulfide, which could alter or skew the results (see Technical Considerations below). For example, while several studies have demonstrated the positive effect of hydrogen sulfide on NO signaling and NO production (Chapter 3, Hosoki et al. 1997; Yong et al. 2008a), several other studies show negative effects of hydrogen sulfide on NO production (Geng et al. 2007; Kubo et al. 2007b; Kubo et al. 2007c). This discrepancy is likely because the latter investigators waited for hours to look for an effect of hydrogen sulfide, when the positive effect on NO production can be observed in minutes (Chapter 3). The absence of a sustained effect is likely because H_2S is so volatile and can rapidly oxidize in solution (see below).

It is also unclear whether the action of hydrogen sulfide on K_{ATP} is universal. Rui Wang and colleagues show that hydrogen sulfide causes a direct activation of K_{ATP} and that glibenclamide (an K_{ATP} inhibitor) inhibits the action of hydrogen sulfide (Zhao et al. 2001; Wang 2002; Zhao et al. 2002; Tang et al. 2005; Yang et al. 2005). However, the precise mechanism for this activation still remains unknown, and others, including myself (Chapter 2), have not seen the same effect of glibenclamide. In these cases glibenclamide either does not work at all (Kiss et al. 2008), or only partially inhibits the relaxation to hydrogen sulfide (Chapter 2, Kubo et al. 2007a; Kubo et al. 2007b; Kubo et al. 2007c; Webb et al. 2008).

An additional receptor of hydrogen sulfide, the Cl^-/HCO_3^- exchanger, has recently been revealed (Kiss et al. 2008). This receptor, when inhibited using the anion exchanger inhibitor 4,4'-Diisothiocyanatostilbene-2,2'-disulfonate (DIDS), completely blocks the hydrogen sulfide relaxation (Kiss et al. 2008). From this it is evident that hydrogen sulfide is likely working through both K_{ATP} and the Cl^-/HCO_3^- exchanger. However, these receptors only apply to the

relaxation observed to hydrogen sulfide. The mechanism(s) behind the contractile, or multiphasic responses to hydrogen sulfide have yet to be identified.

Hydrogen Sulfide Chemistry and Technical Considerations

Hydrogen sulfide exists as a gas (H_2S, dihydrogen sulfide) and is a weak acid in solution, dissociating into HS^- and S_2^-. There are three commonly used methods to create hydrogen sulfide solutions in the laboratory. One way is to bubble distilled water or a physiological buffer directly with hydrogen sulfide gas. However, this method is not as accurate as using a known amount of a chemical donor. Two chemical donors, sodium (Na_2S) and sodium hydrosulfide (NaHS), are widely used instead of hydrogen sulfide gas. Na_2S crystals are clear, and once the oxidized portions are rinsed from the crystals with distilled water, they can be measured out as any other chemical. NaHS is in the form of yellow flakes, and the oxidized portions cannot be easily rinsed before weighing since the flakes immediately dissolve upon contact with water. Na_2S is argued to be a better hydrogen sulfide donor than NaHS for making solutions because of its higher purity. It is thought that oxidation products formed from the impurities in NaHS solutions may interfere with physiological experiments (Koenitzer et al. 2007). However, Na_2S is highly basic and may cause confounding effects by altering pH of buffers if used in high concentrations. Despite this, both donors have been commonly used in published studies. Working in a fume hood is recommended when dealing with hydrogen sulfide because higher levels of hydrogen sulfide can prevent its olfactory detection and these symptoms can rapidly progress during a high, acute exposure. Therefore, it is important at first notice of hydrogen sulfide gas to move to a well-ventilated area to avoid increased exposure and more severe symptoms.

In solution, hydrogen sulfide exists as three species: H_2S, HS^- and S_2^- (see Equation 1).

$$H_2S \Leftrightarrow HS^- + H^+ \Leftrightarrow S_2^- + H^+ \quad (1)$$

Because the pKa for the first dissociation is 7.02-7.04 (Chen 1972; Beauchamp et al. 1984) and the estimated pKa of the second dissociation is 12-15 (Chen 1972; Beauchamp et al. 1984; Bagarinao 1992), at a physiological pH of 7.4 hydrogen sulfide exists as approximately 1/3 H_2S and 2/3 HS^- with very little S_2^- (Beauchamp et al. 1984). This approximate relationship holds true in fresh- and salt-water environments, although variation in pH will shift the H_2S/HS^- equilibrium. Therefore when working with and discussing the effects of hydrogen sulfide, it is important to take into account not only H_2S gas, but the HS^- anion. Throughout this dissertation the term "hydrogen sulfide" will refer to the sum of the species H_2S, HS^- and S_2^-, unless otherwise specified.

Hydrogen sulfide gas is very volatile (Cline 1969; Julian et al. 1998; Dorman et al. 2002) and will readily come out of solution, causing a net loss of hydrogen sulfide. This loss is exacerbated by the fact that hydrogen sulfide will readily oxidize in the presence of divalent metals and oxygen (Tapley et al. 1999), a condition that is prevalent in most physiological buffers, blood, sea water, and extracellular fluid. Therefore, an additional complication that faces the experimenter when working with hydrogen sulfide is its ephemeral nature. This not only makes the detection of hydrogen sulfide in low quantities a challenge, but rigorous deoxygenation measures must be taken to make accurate stock solutions of hydrogen sulfide for experimentation. All solutions should also be made immediately prior to experimentation to assure the hydrogen sulfide concentration has not changed significantly due to oxidation and volatilization.

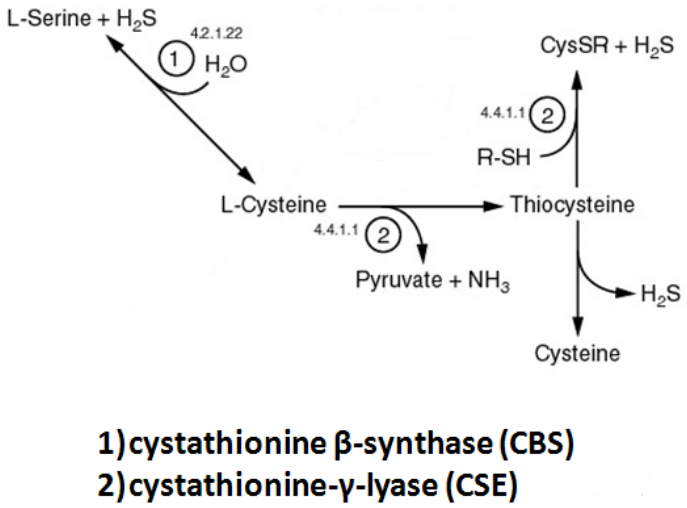

Figure 1-1. Enzymatic production of hydrogen sulfide by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Figure adapted from Julian et al. 2002. In the CBS reaction (1), L-cysteine is hydrolyzed to yield equimolar amounts of H_2S and L-serine. In the CSE reaction (2), L-cysteine must first dimerize to form cystine, which is then transformed into pyruvate, thiocystine and NH_3 by CSE. CSE can then catalyze the reaction of thiocystine with other thiol compounds to from H_2S and CysSR. Alternatively, thiocystine may form H_2S and cystine non-enzymatically.

CHAPTER 2
NITRIC OXIDE, ADENOSINE TRIPHOPHATE (ATP)-SENSITIVE POTASSIUM CHANNELS, AND ARACHIDONIC ACID METABOLITES MODULATE THE TRIPHASIC RESPONSE TO HYPOXIA AND HYDROGEN SULFIDE IN RAT AORTA

Abstract

Hypoxia and hydrogen sulfide elicit similar contractile responses in every vertebrate smooth muscle thus far tested, but the mechanism of each is poorly understood. In aorta preparations, the responses to hypoxia and hydrogen sulfide can be mediated by blockade of nitric oxide (NO), ATP-sensitive potassium channels (K_{ATP}), and arachidonic acid metabolites, but no study has determined whether the effects of blockade are similar in the same tissue preparation. We tested this with aortic rings from Fisher 344 and Fisher 344 x Brown Norway rats using standard vascular myography techniques. We found that both hypoxia and hydrogen sulfide elicit a triphasic, contraction-relaxation-contraction response. The NO synthase inhibitor L-NAME and the K_{ATP} inhibitor glibenclamide significantly reduced hypoxia-induced contraction phases, while the hypoxia-induced relaxation phase was reduced only by glibenclamide. An arachidonic acid metabolism inhibitor cocktail (AAM inhibitor cocktail) of esculetin (to inhibit lipoxygenase), clotrimazole (to inhibit Cytochrome P-450) and indomethacin (to inhibit cyclooxygenase) did not have an effect on either hypoxia-induced contraction or relaxation. In contrast, the hydrogen sulfide response was affected only by the AAM inhibitor cocktail, which reduced the second contraction phase. We conclude that in rat aortic smooth muscle the triphasic responses to hypoxia and hydrogen sulfide are mediated by different signaling mechanisms.

Introduction

Hydrogen sulfide (H_2S) is the newest member of the gasotransmitter family, joining nitric oxide (NO) and carbon monoxide (CO) (Wang 2002; Wang 2003). Hydrogen sulfide is produced

from L-cysteine by cystathionine γ-lyase in vascular smooth muscle and endothelial cells (Wang 2002; Wang 2003; Yang et al. 2008) and it elicits a variety of effects in the vasculature when applied exogenously (Dombkowski et al. 2005). Furthermore, hydrogen sulfide may be intrinsic to or interact with hypoxia signaling, since accumulation of endogenous hydrogen sulfide would be favored by hypoxia and exogenous hydrogen sulfide mimics the effects of hypoxia in smooth muscle (Olson et al. 2006). Consistent with this, the vascular response to hypoxia is decreased by inhibitors of hydrogen sulfide production and enhanced by addition of L-cysteine (Olson et al. 2006; Olson et al. 2008).

The mechanisms underlying hypoxia-induced vascular responses, and whether they are identical to hydrogen sulfide-induced responses, are poorly understood. Here we investigate the mechanisms of the vascular responses to hypoxia and hydrogen sulfide in rat thoracic aorta by blocking three potential downstream effectors: NO, which causes cyclic GMP-mediated relaxation; ATP-sensitive potassium channels (K_{ATP}), which cause relaxation by hyperpolarizing smooth muscle cells (Wang 2002; Wang 2003); and arachidonic acid metabolites (AAM), which can be vasoconstrictors or vasodilators (Kompanowska-Jezierska et al. 2008). We hypothesized that both hypoxia and hydrogen sulfide would cause a similar multiphasic response, potentially mediated by NO, K_{ATP}, and AAM.

While the contractile responses to hypoxia and hydrogen sulfide may both result from a reduction of bioavailable NO, the mechanisms may differ. Since NO synthase (NOS) is O_2 dependent, hypoxia may reduce NO bioavailability (Besse et al. 2002) thereby increasing vessel tension. Moreover, hypoxia may allow accumulation of endogenous hydrogen sulfide by decreasing its spontaneous oxidation (Olson et al. 2006). Hydrogen sulfide may itself reduce NO bioavailability by chemically reacting with NO to produce a nitrosothiol (Whiteman et al. 2006)

and reduce NO production by inhibiting NOS (Geng et al. 2007). Therefore, blockade of NO production by inhibiting NOS should eliminate or reduce hypoxia- and hydrogen sulfide-induced contractions if they are the result of inhibition of NOS and/or reduced NO bioavailability by hypoxia and hydrogen sulfide. Furthermore, K_{ATP} channels hyperpolarize smooth muscle cells and are activated by hydrogen sulfide (Wang 2002; Tang et al. 2005), so their blockade should reduce relaxation. Finally, hydrogen sulfide may directly interact with the hemes of cyclooxygenase and cytochrome P-450 (Koenitzer et al. 2007), potentially altering AAM production and consequently vascular tone. Therefore blockade of AAM may affect hypoxia- and hydrogen sulfide-induced responses.

Materials and Methods

Adult, male Fisher 344 x Brown Norway hybrid (N = 33) of ages 6-27 months of age were housed individually under standard conditions and provided with food and water. Older animals (19-27 mo) were used in the hypoxia and hydrogen sulfide inhibitor experiments, while younger animals were used to investigate the role of the endothelium in the hydrogen sulfide response (6-10 mo). There was no significant variability in the responses within these two age groups. To reduce potential anesthesia artifacts on responses to hydrogen sulfide (Dombkowski et al. 2005), euthanasia was performed by guillotine according to IACUC-approved methods. After dissection, the thoracic aorta was carefully cleaned of fat and sectioned into 5 mm long rings. Four rings were then randomly selected, with each receiving one of four treatments: control; 1 mmol/L L-NAME to block NOS; 1 μmol/L glibenclamide (a sulfonylurea derivative) to block K_{ATP}; or an AAM inhibitor cocktail consisting of 10 μmol/L each of esculetin, clotrimazole, and indomethacin to block AAM (Dombkowski et al. 2004). Vessel rings were attached with stainless steel wire to a force transducer and mounted in a tissue bath system (Radnoti Glass Technology, Monrovia, CA) containing 37°C Krebs bicarbonate buffer, pH 7.4, aerated with

95% air/ 5% CO_2. A resting tension of 1.5 g was applied and maintained as vessels were allowed to equilibrate for at least 1 h. This tension was found to give the best response based on a tension –response curve was generated for the Fisher 344 x Brown Norway hybrid rat aorta.

At the start of each experiment, vessels were maximally contracted with 80 mmol/L KCl, and subsequently washed twice with Krebs buffer. This was repeated after 30 min, at which point the vessels had returned to resting tension of 1.5 g. This procedure ensures optimum *in vitro* vessel activity (Dombkowski et al. 2005). The second KCl contraction was also used to normalize the responses to the other agonists. To check for an intact endothelium, acetylcholine (Ach, 10 μmol/L) was then added to cause relaxation. Vessels that failed to contract to KCl or relax to ACh were discarded. After two additional washes, the vessels were incubated with the respective inhibitors for 30 min and the tension on all rings was continuously adjusted to 1.5 g. To determine the response to hypoxia, vessels were precontracted with 1 μmol/L phenylephrine (PE) and the buffer aeration was switched from 95% air / 5% CO_2 to 95% N_2 / 5% CO_2 after the PE contraction had stabilized (approximately 5 min). To determine the response to hydrogen sulfide, vessels were precontracted with PE, as above, but aeration with air was continued and 300 μmol/L total hydrogen sulfide (referring to the sum of the chemical species: H_2S, HS^- and S^{2-}) was added to each bath as either sodium hydrosulfide (NaHS, N = 18) or sodium sulfide (Na_2S, N = 6). After the response completed and vessel tension had stabilized, baths were washed twice and vessels returned to resting tension (30 min). Some experiments were performed with the endothelium removed. To achieve this, the rings were gently rubbed on the inside with a stainless steel wire (Chung et al. 2007). Rings were then checked to make sure endothelium was removed by addition of 10 μmol/L ACh. In some experiments the components of the AAM inhibitor cocktail were added separately to investigate the individual contributions

of COX (indomethacin, N = 6), LOX (esculetin, N = 7), and Cyt P-450 (clotrimazole, N = 7). Of the 33 animals tested, the final number that responded to both KCl and ACh, and therefore that were used in subsequent statistical analysis were as follows: control treatment, N = 17 for hypoxia and N = 20 for hydrogen sulfide; L-NAME treatment, N = 16 for hypoxia and N = 14 for hydrogen sulfide; glibenclamide treatment, N = 18 for hypoxia and N = 15 for hydrogen sulfide; AAM inhibitor cocktail treatment, N = 14 for hypoxia and N = 11 for hydrogen sulfide; removal of endothelium, N = 12 for hydrogen sulfide (hypoxia not tested).

The magnitude of the hypoxia- and hydrogen sulfide-induced triphasic responses for each vessel were quantified in the following manner: the initial contraction was measured from the PE pre-contracted tension to the peak of the first contraction, the relaxation was measured from the peak of the first contraction to the base of the relaxation, and the second contraction was measured from the base of the relaxation to the peak of the second contraction. The magnitude of contractions and relaxations were subsequently normalized to the magnitude of the second KCl contraction for that vessel (i.e., KCl contraction force = 100%). Significant differences from control values were determined by one-way ANOVA with a Tukey post-hoc test. A two-tailed Student's t-test was used to compare the hypoxia-induced and hydrogen sulfide-induced control phases. For each combination of treatment and inhibitor, at least 13 rings were used for analysis. The statistical software used was JMP 7.0.1 (SAS Institute, Cary, NC USA), with $p < 0.05$ accepted as significant.

Total hydrogen sulfide concentrations were measured by a methylene blue assay (Gilboa-Garber 1971) and were approximately 75% of calculated values (with the difference presumably being due to volatilization and oxidation of sulfide). In physiological saline at pH 7.4, hydrogen sulfide dissociation is approximately 1/3 H_2S and 2/3 HS^- (Beauchamp et al. 1984). Therefore the

true hydrogen sulfide gas (H_2S) concentrations were typically *ca*. 75 μmol/L. Chemicals were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).

Results

Isolated rat aorta rings exposed to hypoxia alone showed a triphasic contraction-relaxation-contraction response that was complete in *ca*. 90 min (Figure 2-1a). Isolated rat aorta rings exposed to hydrogen sulfide alone showed a similar triphasic contraction-relaxation-contraction response (Figure 2-1b), although it was complete in *ca*. 30 min. The duration of all three phases of the hypoxia-induced triphasic response was consistently, and significantly longer in duration than the phases of the hydrogen sulfide -induced triphasic response (Figure 2-2; two-tail t-test, $p < 0.0001$ for all). The triphasic response was the same when elicited by NaHS or Na_2S as the hydrogen sulfide donor. When the hypoxia-induced response was compared to the hydrogen sulfide-induced response, the initial contraction and relaxation were significantly larger in magnitude during hypoxia (Figure 2-3; two-tail t-test, $p = 0.003$ and Figure 2-4; two-tail t-test; $p < 0.001$).

To test whether similar mechanisms are responsible for modulating the triphasic responses to hypoxia and hydrogen sulfide, we inhibited NO production with L-NAME, K_{ATP} channel opening with glibenclamide, and blocked AAM with an inhibitor cocktail of indomethacin, esculetin, and clotrimazole, which inhibit cyclooxygenase, cytochrome P-450, and lipoxygenase, respectively. When applied prior to hypoxia exposure, L-NAME significantly reduced the initial contraction phase, but this phase was not significantly affected by glibenclamide or the AAM inhibitor cocktail (Figure 2-3a, ANOVA, $p = 0.0397$). The relaxation phase in response to hypoxia was not significantly affected by any of the inhibitors compared to control, but glibenclamide significantly reduced the relaxation phase compared to L-NAME (Figure 2-4a, ANOVA, $p = 0.0340$). The second contraction phase in response to hypoxia was

significantly reduced by L-NAME and glibenclamide, but not by the AAM inhibitor cocktail (Figure 2-5a, ANOVA, $p = 0.0215$). In contrast, when applied prior to hydrogen sulfide exposure, none of the inhibitors significantly affected the initial contraction phase (Figure 2-3b, ANOVA, $p = 0.272$) or the relaxation phase (Figure 2-4b, ANOVA, $p = 0.254$). The second contraction phase in response to hydrogen sulfide was not significantly affected by L-NAME or glibenclamide, but was significantly reduced by the AAM inhibitor cocktail (Figure 2-5b, ANOVA, $p = 0.0176$).

To further investigate the role of endothelium-derived products on the hydrogen sulfide-induced response, we removed the endothelium from the aorta rings before experimentation. There were significant differences in all three phases of the hydrogen sulfide-induced triphasic response when comparing rings with no endothelium to control rings with an intact endothelium. Compared to control rings, removal of the endothelium significantly reduced the magnitude of the initial contraction phase (Figure 2-6, two-tail t-test, $p = 0.0027$), significantly increased the magnitude of the relaxation phase (Figure 2-6, two-tail t-test, $p = 0.0009$), and significantly reduced the magnitude of the second contraction phase (Figure 2-6, two-tail t-test, $p < 0.0001$). To determine the effect of L-NAME without manipulating the tension before addition of hydrogen sulfide as was done in the majority of the experiments, L-NAME was administered after precontraction with PE and the ring was allowed to further constrict until stable. This differed from the previous application of L-NAME which was 30 minutes before PE preconstruction, and where the tension was adjusted back down to 1.5 g manually after L-NAME was added. The initial contraction phase was significantly reduced while the second contraction phase remained ($N = 4$, data not shown). Finally, when the AAM inhibitor cocktail components

were added separately, indomethacin, esculetin, and clotrimazole did not have a significant effect on the second phase contraction (data not shown, N = 6-7, ANOVA, $p = 0.157$).

Discussion

This report demonstrates that hypoxia and hydrogen sulfide each elicit a characteristic, triphasic contraction-relaxation-contraction response in rat aorta. While others investigators have reported mono- or biphasic responses to hydrogen sulfide (Hosoki et al. 1997; Dombkowski et al. 2005; Koenitzer et al. 2007), this is the first report of a triphasic response to hydrogen sulfide in aorta. However, a triphasic response has been reported in rat pulmonary artery (Dombkowski et al. 2005) and in response to acute hypoxia in rat aorta (Besse et al. 2002). While aorta has been used in this study, and is commonly used to elicit the vascular actions of hydrogen sulfide (Hosoki et al. 1997; Zhao et al. 2001; Dombkowski et al. 2005; Olson et al. 2006; Koenitzer et al. 2007; Yang et al. 2008), it should be noted that this is a conduit vessel, not a resistance vessel. Changing the tone of the aorta will effectively alter blood pressure and flow to the entire vascular system not specific vascular beds, and would therefore not be an effective means of regulating blood flow, as it would in a resistance vessel. In preliminary experiments with pulmonary artery and mesenteric artery, I observed the same triphasic response as in aorta rings (Predmore, unpublished data). Therefore, the aorta is used here as an experimental proxy for the resistance vessels.

The concentration of hydrogen sulfide that accumulates in the aorta rings during hypoxia is unknown, and for that matter so is the hydrogen sulfide concentration during hypoxia in vivo. Therefore the incubation bath hydrogen sulfide concentrations reported are approximations of what may actually accumulate during hypoxia (300 μmol/L total hydrogen sulfide or ca. 75 μmol/L H_2S). This is within the range of previously reported plasma hydrogen sulfide concentrations, which range from 10 to 300 μmol/L (Whitfield et al. 2008), although note that it

has recently been argued that free hydrogen sulfide is actually significantly lower (<100 nmol/L) (Whitfield et al. 2008).

While the triphasic responses to hypoxia and hydrogen sulfide are broadly similar, they differ temporally, in magnitude, and in their interaction with NO, K_{ATP}, and AAM. The hypoxia-induced initial contraction and relaxation phases were significantly larger than the hydrogen sulfide-induced counterparts, whereas the second contraction phase was similar. Interestingly, the triphasic response to hydrogen sulfide is dose dependent: at concentrations of 100 and 600 μmol/L total hydrogen sulfide, the magnitude of the triphasic responses were similar, but at 900 μmol/L total hydrogen sulfide the magnitude of the phases increased slightly (data not shown). Although all phases of the hypoxia-induced triphasic response could be reduced in magnitude by one or more of the inhibitors, only the second contraction phase of the hydrogen sulfide-induced triphasic response was significantly affected by inhibitors, suggesting that the triphasic responses to hypoxia and hydrogen sulfide are differentially mediated.

Initial Contraction Phase

Our data suggest that the initial contraction phase in response to hypoxia is partially mediated by NO bioavailability but not by K_{ATP} or AAM, and that the initial contraction phase in response to hydrogen sulfide is not primarily mediated by NO bioavailability, K_{ATP} or AAM. However, there must be some endothelium-derived factor mediating this response to hydrogen sulfide, since removal of the endothelium severely reduced the initial contraction phase to hydrogen sulfide.

In the initial experiments with L-NAME the baseline tension was maintained constant throughout the incubation with L-NAME. Therefore any increase in tension after L-NAME addition was reduced back to baseline before the addition of PE, hypoxia, or hydrogen sulfide. The rationale behind this was to prevent the rings from maximally constricting after the PE was

added. If they were maximally constricted before addition of hypoxia or hydrogen sulfide, it would be impossible to tell if it was an effect of NOS blockade, or simply the mechanical limits of the rings to respond. In the experiments following when L-NAME was added after PE pre-contraction, care was taken to ensure that the maximal tension, as determined from the KCl contraction, was not reached before addition of hydrogen sulfide.

While the blockade of NO synthesis by L-NAME did not significantly alter the initial contraction phase to hydrogen sulfide in the initial experiments, there was a non-significant decrease in the hydrogen sulfide-induced initial contraction with L-NAME, similar to what was observed with hypoxia (Fig. 2-3a vs. 2-3b). However, when L-NAME was added after the PE precontraction, and the tension was not adjusted after L-NAME was added (which was the case in the previous experiments), there was a significant reduction of the initial contraction phase to hydrogen sulfide. Recent evidence also shows that hydrogen sulfide may cause a reduction of cyclic AMP (cAMP) (Lim et al. 2008). This may occur in addition to the reduction of free NO during hydrogen sulfide-induced contractions in vascular smooth muscle to mediate the observed contractions.

Relaxation Phase

Our data suggest that the relaxation phase in response to hypoxia is partially mediated by K_{ATP}, but not by NO or AAM, and that the relaxation phase in response to hydrogen sulfide is not mediated by NO, K_{ATP} or AAM. Glibenclamide significantly reduced the relaxation phase during hypoxia when compared to the L-NAME treatment (Fig. 2-4a), but it did not affect the response to hydrogen sulfide (Fig. 2-4b). This was surprising given that hydrogen sulfide activates K_{ATP} (Wang 2002; Tang et al. 2005). However, the glibenclamide concentration we used (1 μmol/L) may have been higher than that required for specific blockade of K_{ATP}, and may

have also blocked Cl^- channels (Sheppard et al. 1997). A blockade of Cl^- efflux could prevent depolarization of smooth muscle cells (Kitamura et al. 2001), thereby reducing the effects of K_{ATP} blockade. However, 10-100 μM glibenclamide is required for noticeable Cl^- inhibition (Sheppard et al. 1997; Robert et al. 2005). Recent evidence suggests that hydrogen sulfide-induced vasorelaxation may be due to a reduction in ATP concentration by metabolic inhibition followed by acidosis and activation of Cl^-/HCO_3^- exchangers (Kiss et al. 2008), and therefore may not primarily rely on K_{ATP} channels. It remains to be tested whether this occurs during the triphasic response for both hypoxia and hydrogen sulfide, since only a monophasic relaxation was observed in the aforementioned study. It is also interesting to note that the magnitude of the relaxation phase during hydrogen sulfide-induced responses increased when the endothelium was removed. This may be the result of removing the source of AAM that are vasoconstrictive (i.e. thromboxanes, and some prostaglandins) and that may interfere with the relaxation pathway(s) initiated by hydrogen sulfide (see below).

Second Contraction Phase

Our data suggest that the second contraction phase in response to hypoxia is partially mediated by NO bioavailability and potentially K_{ATP}, but not mediated by AAM, and that the second contraction phase in response to hydrogen sulfide is mediated by AAM but not by NO bioavailability or K_{ATP}. This could indicate that hypoxia continues to inhibit the NO/NOS system, whereas hydrogen sulfide induces production of one or more AAM. Both of these possibilities would lead to contraction. This is similar to the response of the initial contraction, in which the hypoxia-induced contraction may result from a decrease in NO bioavailability, whereas the hydrogen sulfide-induced contraction may result from an alternative pathway such as vasoconstrictive AAM or by decreasing cAMP (Lim et al. 2008). This is supported by the observation that removal of the endothelium significantly reduced the second contraction phase,

indicating that the response is derived from endothelium. While the involvement of specific enzymes and their products (i.e. cyclooxygenase, lipoxygenase or Cytochrome P-450) could not be determined by adding the components of the AAM inhibitor cocktail alone, there may be an additive or combined effect of multiple AAM causing the second phase contraction to hydrogen sulfide. Our data provide no support for a role for of K_{ATP} channels in hydrogen sulfide-induced contractions, which is also supported by Lim and colleagues (Lim et al. 2008), but our data do support a role for K_{ATP} channels in hypoxia-induced contractions. However, if glibenclamide non-specifically blocked chloride channels, and therefore Cl^- efflux, in addition to K_{ATP} (Sheppard et al. 1997; Kitamura et al. 2001), this could have artificially reduced the magnitude of the hypoxia-induced second contractions.

Conclusion

Our data show that hypoxia and hydrogen sulfide produce similar responses in rat aorta but that these responses result from different mechanisms. The data suggest that NO and K_{ATP} contribute to the triphasic response to hypoxia, while AAM play a role in the hydrogen sulfide response. However, no component of the triphasic response to any blockade was completely eliminated, indicating that more signaling pathways are involved, potentially including, but certainly not limited to, cAMP and metabolic inhibition. Additionally, the effect of the inhibitors on higher or lower hydrogen sulfide concentrations remains to be determined, since we tested only one concentration of hydrogen sulfide. Therefore, further investigations are warranted to illuminate how these two signaling events are mediated and interconnected, and whether these trends persist over a range of hydrogen sulfide concentrations.

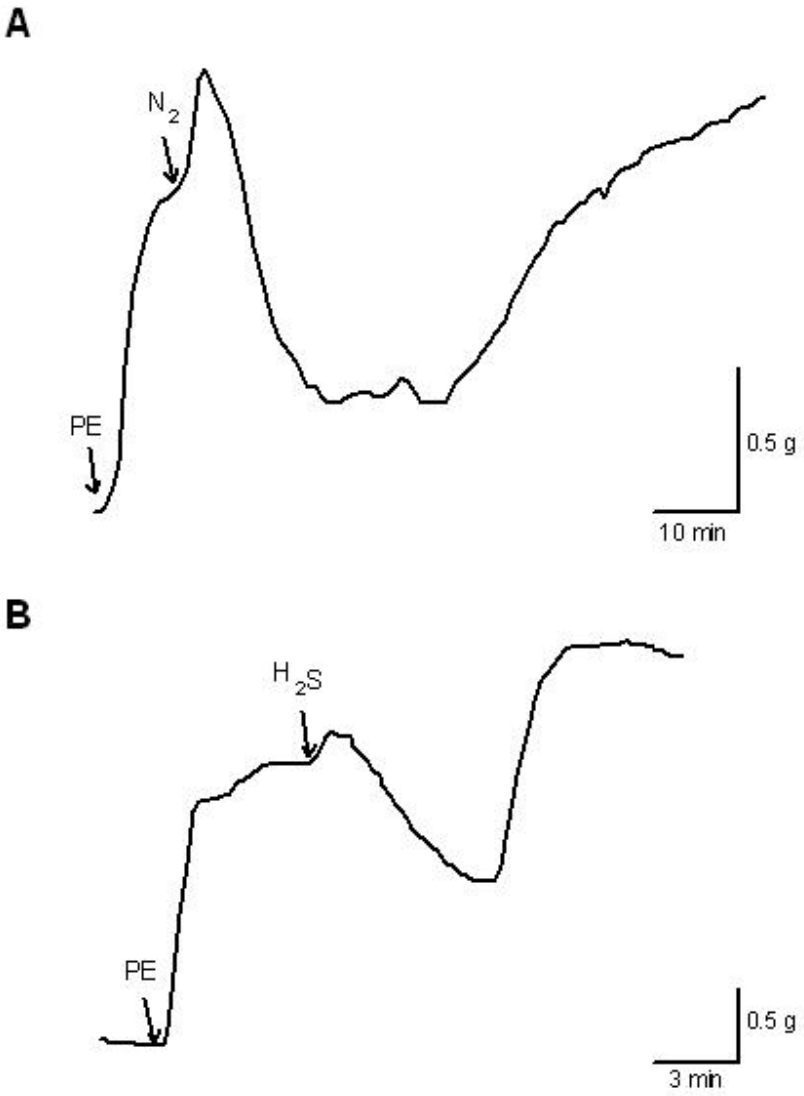

Figure 2-1. Representative tracings of the effect of (**a**) hypoxia (95% N_2/5% CO_2) or (**b**) hydrogen sulfide (H_2S; 300 µmol/L NaHS or Na_2S) on phenylephrine (PE) (1 µmol/L) pre-contracted rat aorta rings. Both stimuli produced a triphasic contraction-relaxation-contraction. Horizontal scale bar indicates time (min) and vertical scale bar indicates aorta ring tension (g). To reduce noise, data were smoothed in Sigma Plot using a negative exponential 1st degree polynomial function with a 0.02 (hypoxia) or 0.05 (hydrogen sulfide) sampling proportion and nearest neighbors bandwidth method.

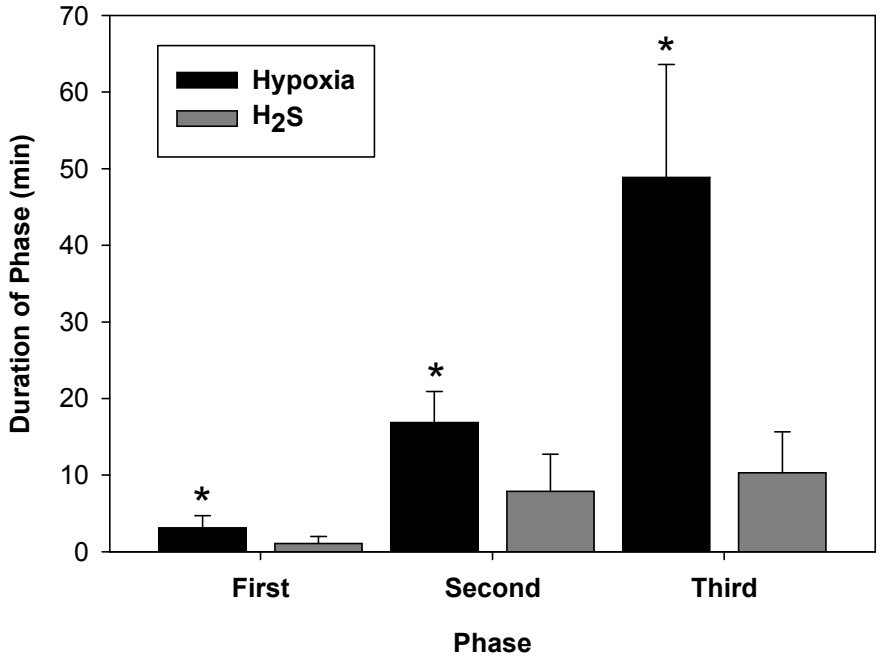

Figure 2-2. Duration of the triphasic response of phenylephrine (1 μmol/L) pre-contracted rat aorta rings to hypoxia (95% N_2/5% CO_2, N = 40) and hydrogen sulfide (H_2S; 300 μmol/L NaHS or Na_2S, N = 40). Treatment is on the X-axis, and response is on the Y-axis as % of the KCl contraction. All panels show mean ± SEM. Significant differences (asterisks) were determined using a two-tailed Student's t-test. The duration of the hypoxia-induced triphasic response is significantly longer than the hydrogen sulfide-induced triphasic response at every phase.

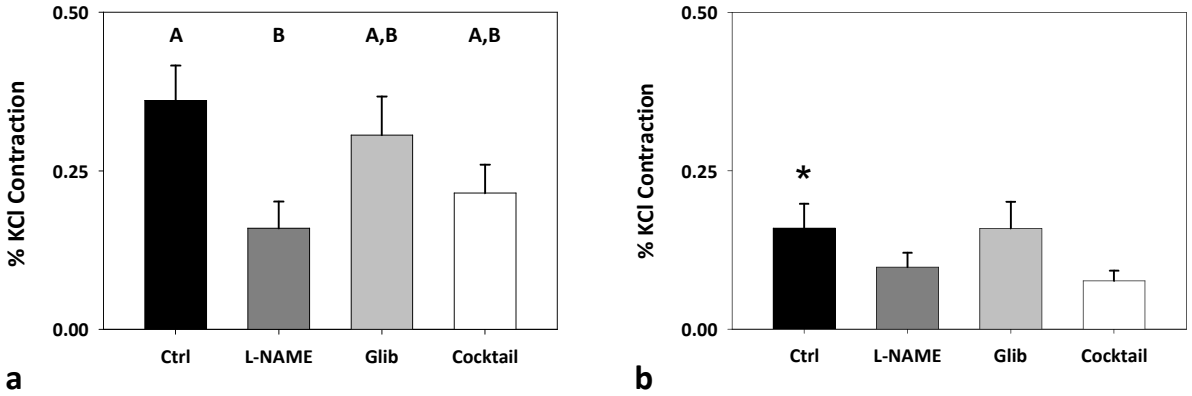

Figure 2-3. Magnitude of the initial contraction phase of the triphasic response of phenylephrine (1 µmol/L) pre-contracted rat aorta rings to hypoxia (95% N_2/5% CO_2) and hydrogen sulfide (H_2S; 300 µmol/L NaHS or Na_2S). Each ring received one of the following treatments: no inhibitors (Control; N = 20 hypoxia, N = 23 hydrogen sulfide), L-NAME (L-NAME; N = 18 hypoxia, N = 16 hydrogen sulfide), glibenclamide (Glibenclamide; N = 20 hypoxia, N = 17 hydrogen sulfide), or an AAM inhibitor cocktail of indomethacin, esculetin and clotrimazole (AAM inhibitor cocktail, N = 16 hypoxia, N = 13 hydrogen sulfide). Treatment is on the X-axis, and response is on the Y-axis as % of the KCl contraction. All panels show mean ± SEM, with hypoxia data in panel **a** and hydrogen sulfide data in panel **b**. Significant differences (different letters) were determined using one-way ANOVA with a Tukey post-hoc, in addition to a two-tailed Student's t-test (asterisks). Application of L-NAME 30 min prior to hypoxia significantly reduced the initial contraction phase (**a**). However, application of inhibitors during the hydrogen sulfide response did not significantly affect the initial contraction phase (**b**). The control hypoxia initial contraction was significantly greater in magnitude than the control initial contraction to hydrogen sulfide (**a** vs. **b**, asterisk).

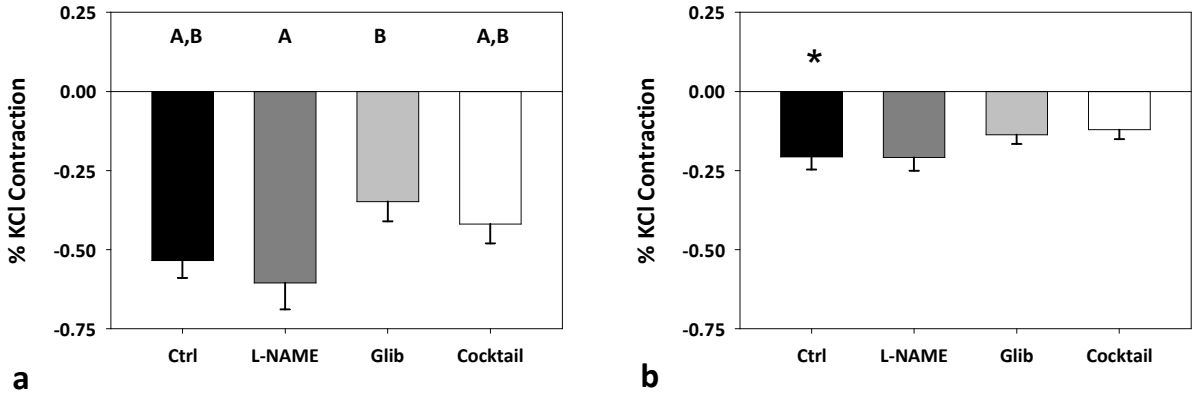

Figure 2-4. Magnitude of the relaxation phase of the triphasic response of phenylephrine (1 µmol/L) pre-contracted rat aorta rings to hypoxia (95% N_2/5% CO_2) and hydrogen sulfide (H_2S; 300 µmol/L NaHS or Na_2S). Each ring received one of the following treatments: no inhibitors (Control; N = 20 hypoxia, N = 23 hydrogen sulfide), L-NAME (L-NAME; N = 18 hypoxia, N = 16 hydrogen sulfide), glibenclamide (Glibenclamide; N = 20 hypoxia, N = 17 hydrogen sulfide), or an AAM inhibitor cocktail of indomethacin, esculetin and clotrimazole (AAM inhibitor cocktail, N = 16 hypoxia, N = 13 hydrogen sulfide). Treatment is on the X-axis, and response is on the Y-axis as % of the KCl contraction. All panels show mean ± SEM, with hypoxia data in panel **a** and hydrogen sulfide data in panel **b**. Significant differences (different letters) were determined using one-way ANOVA with a Tukey post-hoc, in addition to a two-tailed Student's t-test (asterisks). Application of glibenclamide 30 min prior to hypoxia significantly reduced the relaxation phase compared to L-NAME (**a**). However, application of inhibitors during the hydrogen sulfide response did not significantly affect the relaxation phase (**b**. The control hypoxia relaxation was significantly greater in magnitude than the control relaxation to hydrogen sulfide (**a** vs. **b**).

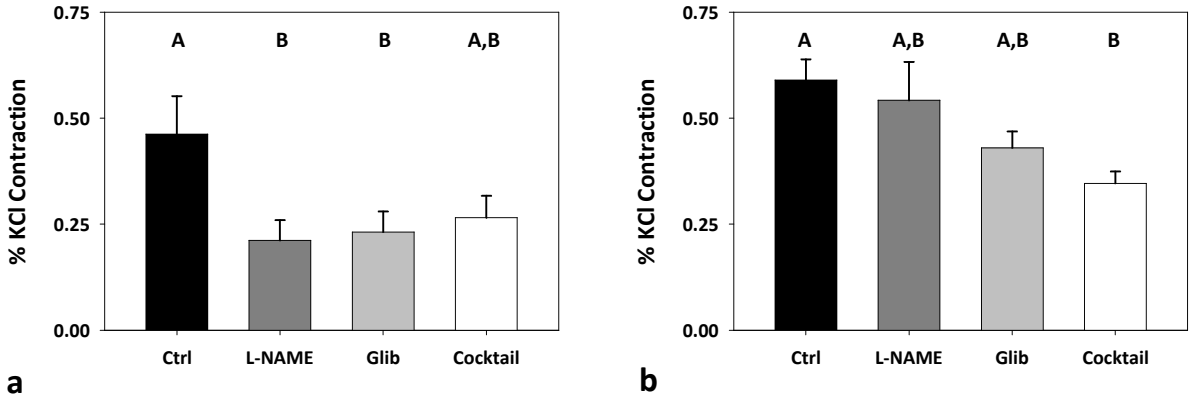

Figure 2-5. Magnitude of the second contraction phase of the triphasic response of phenylephrine (1 μmol/L) pre-contracted rat aorta rings to hypoxia (95% N_2/5% CO_2) and hydrogen sulfide (H_2S; 300 μmol/L NaHS or Na_2S). Each ring received one of the following treatments: no inhibitors (Control; N = 20 hypoxia, N = 23 hydrogen sulfide), L-NAME (L-NAME; N = 18 hypoxia, N = 16 hydrogen sulfide), glibenclamide (Glibenclamide; N = 20 hypoxia, N = 17 hydrogen sulfide), or an AAM inhibitor cocktail of indomethacin, esculetin and clotrimazole (AAM inhibitor cocktail, N = 16 hypoxia, N = 13 hydrogen sulfide). Treatment is on the X-axis, and response is on the Y-axis as % of the KCl contraction. All panels show mean ± SEM, with hypoxia data in panel **a** and hydrogen sulfide data in panel **b**. Significant differences (different letters) were determined using one-way ANOVA with a Tukey post-hoc. Application of L-NAME and glibenclamide 30 min prior to hypoxia significantly reduced the second phase contraction (**a**). Application of the AAM inhibitor cocktail 30 min prior to hydrogen sulfide significantly reduced the second contraction phase (**b**).

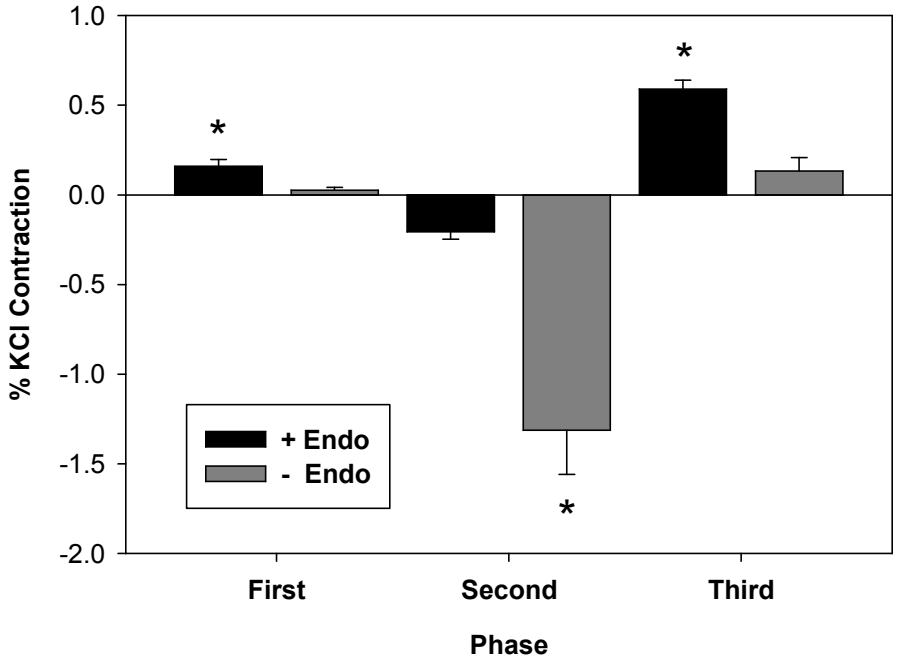

Figure 2-6. Magnitude of the triphasic response of phenylephrine (1 μmol/L) pre-contracted rat aorta rings to hydrogen sulfide (H_2S; 300 μmol/L NaHS or Na_2S) with (N = 19) and without (N = 12) an intact endothelium. Endothelium was removed by rubbing the inside of the ring with stainless steel wire. Treatment is on the X-axis, and response is on the Y-axis as % of the KCl contraction. All panels show mean ± SEM. Significant differences (asterisks) were determined using a two-tailed Student's t-test. Removal of the endothelium results in a significant reduction of the initial and second contraction phases to hydrogen sulfide, while it significantly increases the relaxation phase to hydrogen sulfide.

CHAPTER 3
HYDROGEN SULFIDE INCREASES NITRIC OXIDE PRODUCTION FROM ENDOTHELIAL CELLS BY A PROTEIN KINASE B (AKT)-DEPENDENT MECHANISM

Abstract

Hydrogen sulfide (H_2S) and nitric oxide (NO) are both gasotransmitters that can elicit synergistic vasodilatory responses in the in the cardiovascular system, but the mechanisms behind this synergy are unclear. In the current study we investigated the molecular mechanisms through which hydrogen sulfide regulates endothelial NO production. Initial studies were performed to establish the temporal and dose-dependent effects of hydrogen sulfide on NO generation using EPR spin trapping techniques. H_2S stimulated a two-fold increase in NO production from endothelial nitric oxide synthase (eNOS), which was maximal 30 min after exposure to 25-150 μmol/L hydrogen sulfide. Following 30 min hydrogen sulfide exposure, eNOS phosphorylation at Ser 1177 was significantly increased compared to control, consistent with eNOS activation. Pharmacological inhibition of Akt, the kinase responsible for Ser 1177 phosphorylation, attenuated the stimulatory effect of hydrogen sulfide on NO production. Taken together, these data demonstrate that hydrogen sulfide up-regulates NO production from eNOS through an Akt-dependent mechanism. These results implicate hydrogen sulfide in the regulation of NO in endothelial cells, and suggest that deficiencies in hydrogen sulfide signaling can directly impact processes regulated by NO.

Introduction

Hydrogen sulfide (H_2S) and nitric oxide (NO) are both gasotransmitters that function in the cardiovascular system (Hosoki et al. 1997; Wang 2003). Recent reports indicate that the NO and hydrogen sulfide signaling pathways interact on a variety of levels (Geng et al. 2007; Kubo et al. 2007b; Kubo et al. 2007d; Yong et al. 2008a). Hydrogen sulfide and NO interact synergistically *in vitro*, with hydrogen sulfide enhancing NO-mediated relaxation up to 13 fold in

isolated rat aorta (Hosoki et al. 1997), and treatment of Langendorff-perfused Sprague-Dawley rat hearts with hydrogen sulfide immediately following ischemia confers cardioprotection through NOS activation (Yong et al. 2008a). In contrast, other *in vitro* studies indicate that incubation with hydrogen sulfide decreases NO production in aortic rings (Geng et al. 2007; Kubo et al. 2007b), cell culture (Geng et al. 2007), and recombinant eNOS (Kubo et al. 2007d). However, in these studies hydrogen sulfide incubation occurred 1-6 h before measurement of NO production. Consequently, since hydrogen sulfide is volatile and oxidizes rapidly in the presence of oxygen and free divalent metals (Tapley et al. 1999), the key effects of hydrogen sulfide in those studies may have occurred before NO measurement began. In the present study we investigated the ability of hydrogen sulfide to acutely modulate NO production in the endothelium, with a specific focus on the mechanism of action.

Materials and Methods

Chemicals

Endothelial cell growth supplement was purchased from Upstate (Temecula, CA USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest quality available, unless otherwise noted.

Bovine Arterial Endothelial Cell Culture

Bovine Arterial Endothelial Cells (BAECs) were cultured in DMEM (1.0 g/L glucose) supplemented with 10% FBS, 1% penicillin/streptomycin and endothelial cell growth supplement (5 mg/L). Culture flasks were maintained in a 37° C incubator at 5.0% CO_2. Adherent endothelial cells were grown in 6-well plates for EPR studies and in 100 mm dishes for western blotting studies.

Hydrogen Sulfide Exposure

Sodium sulfide (Na_2S), a hydrogen sulfide donor, was prepared as a saturated stock solution in distilled water and maintained at 4° C. At this temperature, the concentration of a saturated solution of Na_2S is 1.72 mol/L. From this stock, hydrogen sulfide dilutions were made in Krebs buffer, of which 1.0 mL was added per well of a six-well plate, and 3.0 mL was added per 100 mm Petri dish. The concentration of total hydrogen sulfide (H_2S, HS^- and S^{2-}) in the dilutions was determined using a methylene blue assay, with a standard curve generated with deoxygenated, distilled water and Na_2S as the reference (Gilboa-Garber 1971). After 30 min in a 37° C incubator, a 150 μmol/L hydrogen sulfide solution prepared in Krebs buffer showed no detectable hydrogen sulfide, indicating virtually complete loss of hydrogen sulfide, presumably by oxidation and volatilization (data not shown).

Akt Blockade

The Akt inhibitor Triciribine was used to prevent the phosphorylation of eNOS (Dieterle et al. 2009). Triciribine (5.0 μmol/L) was added in Krebs buffer 30 min before experiments. Cells were washed with PBS before and after addition of Triciribine.

Electron Paramagnetic Resonance Detection of Nitric Oxide

Spin-trapping measurements of NO were performed using a BrukerE-scan spectrometer (BrukerBioSpin Corporation, Billerica, MA USA) with the iron spin trapping complex N-methyl-D-glucaminedithiocarbamate (Fe-MGD) (Cardounel et al. 2002; Cardounel et al. 2007). For measurements of NO produced by BAECs, cells were cultured as described above and spin trapping was performed on cells grown in 6-well plates (1×10^6 cells/ well). In these studies, cells attached to the substratum were utilized since scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The medium from each well was removed and the cells were washed with PBS (phosphate-buffered saline, without $CaCl_2$ or $MgCl_2$). Next,

0.15 ml of Krebs buffer containing the NO spin trap FE-MGD (0.5 mmol/L Fe^{2+}, 5.0 mmol/L MGD), and calcium ionophore (A23187, 1 μmol/L) was added to each well and the plates were incubated at 37° C under a humidified environment containing 5% CO_2 /95% O_2 for 20 min (Cardounel et al. 2002; Cardounel et al. 2007). Following incubation, the medium from two wells was removed and pooled as one 0.3 ml sample, frozen in liquid nitrogen and stored at -80° C. This yielded three samples per plate. The frozen NO spin-trap samples are stable, and were later individually thawed, and trapped NO in the supernatants was quantified using the electron paramagnetic resonance technique. Spectra recorded were obtained using the following parameters: microwave power; 20 mW, modulation amplitude 3.16 G and modulation frequency; 100 kHz.

Western Blotting

BAECs from a 100 mm dish were scraped and suspended in 300 μl radioimmunoprecipitation assay (RIPA) buffer with Halt protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL USA), placed on ice, and sonicated to lyse the cells and suspend the protein. The suspension was centrifuged at 12000 × g for 20 min at 4° C and the supernatant removed, frozen in liquid nitrogen, and stored at -80° C. Western blotting was performed using commercially-available polyclonal antibodies for eNOS and Ser 1177 eNOS (BD Biosciences, San Jose, CA USA), monoclonal β-actin (Cell Signaling Technology, Danvers, MA USA) and secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich St. Louis, MO USA). Protein was separated using SDS-PAGE and transferred onto PVDF membrane (Immobilon P, Millipore, Billerica, MA USA) using a semidry blotter (BioRad, Hercules, CA USA). Using the Snap-ID system (Millipore, Billerica, MA USA), membranes were blocked in 0.05% non-fat milk in PBST (PBS with 0.05% Tween-20), washed in PBST and incubated 10 min with

appropriate primary antibodies (diluted 1:333 in PBST). Membranes were then washed in PBST four times and subsequently incubated 10 min with secondary antibody diluted 1:3,333 in blocking solution. Membranes were then washed two times in PBST, once in PBS, and once in Tris-HCl (100 mmol/L, pH 9.5), after which chemiluminescence substrate was added (DuoLux substrate, Vector Laboratories, Burlingame, CA USA). Images were captured with a digital imager (GeneSnap, Syngene,Frederick, MD USA) and were analyzed using commercial software (Quantity One,BioRad, Hercules, CA USA) to determine band intensity using local background subtraction.

Statistics

All data were analyzed using one-way ANOVA with Dunnett's post-hoc test for significant differences from a control, with alpha ≤ 0.05 considered significant (JMP 7.0, SAS Institute, Cary, NC USA).

Results

Initial experiments were conducted to establish the time course of hydrogen sulfide effects on endothelial NO production. BAECs were exposed to the hydrogen sulfide donor Na_2S (100 μmol/L) for 15, 30, 60, 120 and 240 min (Figure 3-1A). At each time point, endothelial-derived NO production was measured using EPR. Peak NO production was observed at 30 min post hydrogen sulfide treatment (ANOVA, $p < 0.001$); at which time mean NO production was increased by 87%. This effect was not observed at later time points, suggesting a transient activation of eNOS. To determine the dose-response to hydrogen sulfide, NO production was measured 30 min after the addition of 5-600 μmol/L Na_2S (Figure 3-1B). Mean NO production increased by 39-62% at Na_2S concentrations between 40-150 μmol/L (ANOVA, $p = 0.001$).

The transient nature of the hydrogen sulfide effects on endothelial NO production suggested a change in eNOS phosphorylation status. Therefore, western blotting was used to

determine the phosphorylation state of eNOS after addition of hydrogen sulfide at 15 and 30 min. Total eNOS expression was unchanged for all treatments (Figure 3-2A, ANOVA, $p = 0.6727$), but after 30 min of incubation in the presence of 150 μmol/L Na_2S, eNOS phosphorylation at Ser 1177 increased by 88% compared to control (Figure 3-2B, ANOVA, $p = 0.0031$). Furthermore, the ratio of phosphorylated Ser 1177 eNOS to total eNOS increased by 148% after 30 min compared to control (Figure 3-2C, ANOVA, $p = 0.0033$). To determine whether increased Ser 1177 phosphorylation was responsible for the augmented NO production, BAECs were pretreated for 30 min with the Akt inhibitor Triciribine (5 μmol/L), after which the cells were exposed to 150 μmol/L Na_2S. Triciribine prevented the augmentation of endothelial NO production (Figure 3-3, ANOVA, $p = 0.0038$). These results clearly indicate that hydrogen sulfide increases endothelial NO production through Akt activation and subsequent increased phosphorylation of eNOS at Ser 1177.

Discussion

Although an early study showed a synergistic effect of hydrogen sulfide on NO-induced relaxation of blood vessel rings (Hosoki et al. 1997), later studies showed that hydrogen sulfide inhibited eNOS-dependent NO production in aortic rings and cell culture, and from recombinant proteins (Geng et al. 2007; Kubo et al. 2007b; Kubo et al. 2007d). However, these later studies measured NO production 1-6 h after hydrogen sulfide addition. Since hydrogen sulfide is volatile and rapidly oxidizes in the presence of oxygen and free divalent metals (Tapley et al. 1999), we hypothesized that hydrogen sulfide acts within minutes of its application, and therefore that an hour or longer delay between hydrogen sulfide application and measurement of NO production could lead to a failure to detect an hydrogen sulfide effect. Here we demonstrate that hydrogen sulfide up-regulates NO production from eNOS within 30 min. While we assume hydrogen

sulfide is causing this action, hydrogen sulfide in solution exists as H_2S, HS^-, and S_2^-. With the extracellular ratio of H_2S/HS^- being between 1:3 and 1:5 and the intracellular ratio being approximately equal (Olson et al. 2009), it is equally likely that HS^- is causing the up-regulation. To our knowledge, no one has yet definitively demonstrated that only H_2S is causing the observed effects of hydrogen sulfide, as opposed to HS^-.

The comparatively rapid action led us to suspect that hydrogen sulfide was stimulating phosphorylation of eNOS at Ser 1177 (Mount et al. 2007). We investigated this using western blotting to detect total eNOS and phosphorylated eNOS. While total eNOS remained constant for all treatment groups, 30 min exposure to 150 μmol/L hydrogen sulfide resulted in a significant increase in both phosphorylated eNOS and the ratio of phosphorylated eNOS to total eNOS. To confirm that hydrogen sulfide-induced NO production was dependent on eNOS phosphorylation, we used Triciribine to block phosphorylation of eNOS at Ser 1177, thereby inhibiting Akt activation. This prevented the increase in NO production in cells exposed to hydrogen sulfide, but did not significantly affect NO production in control cells.

These data suggest a novel mechanism of endogenous hydrogen sulfide signaling: upregulation of NO production via Akt-dependent phosphorylation of eNOS at Ser1177. The mechanism by which hydrogen sulfide activates Akt remains unknown. However, upstream regulation of NO production by hydrogen sulfide could represent a novel and potentially important regulatory mechanism in the control of NO signaling pathways, and could further implicate defects of hydrogen sulfide signaling in cardiovascular pathologies.

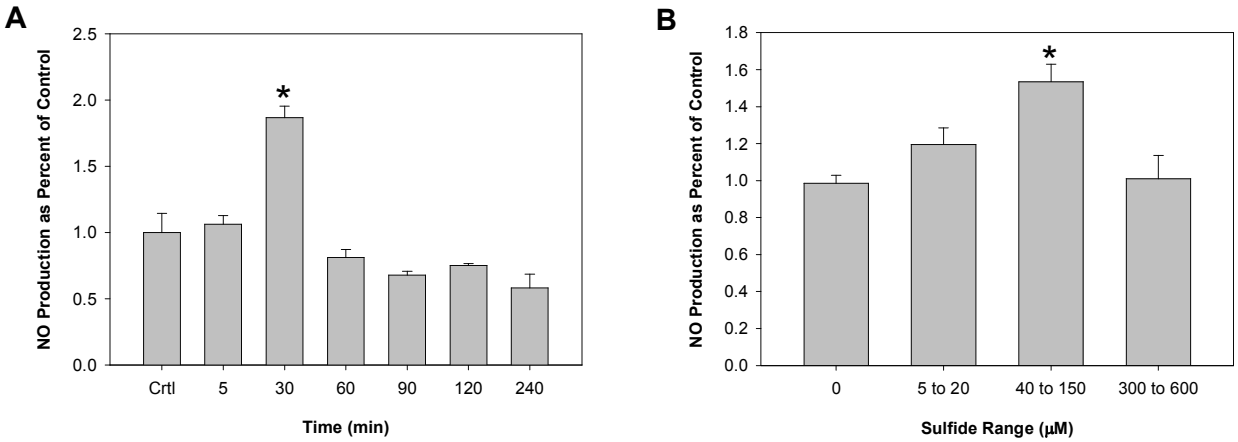

Figure 3-1. The effect of H_2S on NO production by BAECs. NO production was measured using EPR spectroscopy. NO values (y-axis) are presented in arbitrary units and have been normalized to control. Time (A) or hydrogen sulfide concentration range (B) are shown on the x-axis. Data are shown as mean ± SE. Statistical analysis was done using a one-way ANOVA ($p < 0.05$) with Dunnett's post-hoc. An asterisk denotes values significantly different from control.

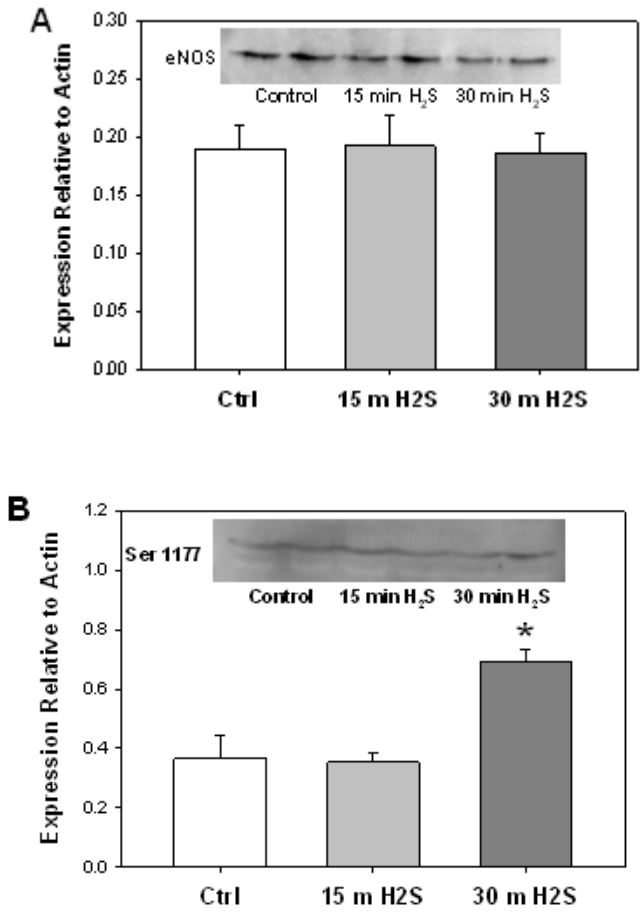

Figure 3-2. The effect of hydrogen sulfide on eNOS phosphorylation. Protein expression (y-axis, normalized to β-actin) was measured from BAECs untreated (Ctrl), or exposed to 15 or 30 min of hydrogen sulfide (x-axis). Using western blotting techniques, total eNOS expression (A), eNOS phosphorylated at Ser 1177 expression (B), and the ratio of Ser 1177 phosphorylated eNOS/total eNOS (C) were determined. Data are shown as mean ± SE. Statistical analysis was done using a one-way ANOVA ($p < 0.05$) with Dunnett's post-hoc. An asterisk denotes values significantly different from control.

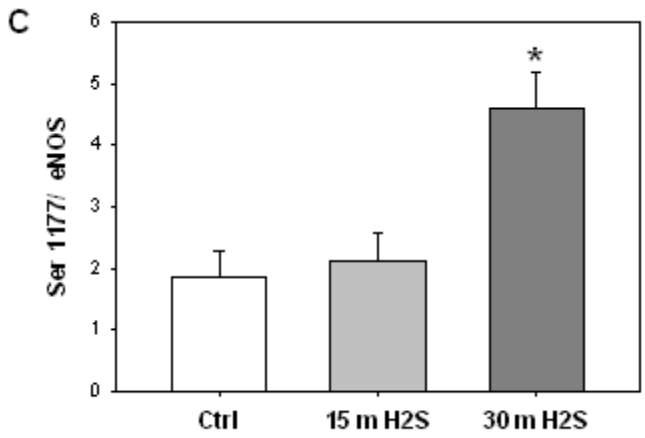

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Figure 3-2. Continued.

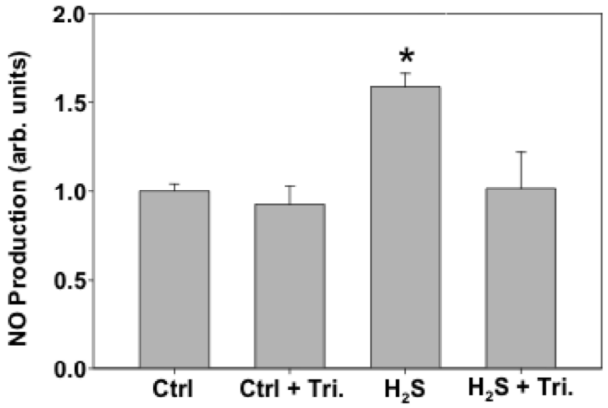

Figure 3-3. The effect of Akt inhibition on hydrogen sulfide-stimulated NO production. NO production was measured using EPR spectroscopy. NO values (y-axis) are presented in arbitrary units and have been normalized to control. NO was measured from untreated (Ctrl), Triciribine treated (Ctrl + Tri.), hydrogen sulfide treated (H_2S) and hydrogen sulfide treated with Triciribine (H_2S + Tri.). Data are shown as mean ± SE. Statistical analysis was done using a one-way ANOVA ($p < 0.05$) with Dunnett's post-hoc. An asterisk denotes values significantly different from control.

CHAPTER 4
THE HYDROGEN SULFIDE SIGNALING SYSTEM: CHANGES DURING AGING AND THE BENEFITS OF CALORIC RESTRICTION

Abstract

Hydrogen sulfide gas (H_2S) has been proposed as an important signaling molecule, but the effect of age on hydrogen sulfide signaling is unknown. Hydrogen sulfide causes diverse effects in mammalian tissues, including relaxation in mammalian systemic vessels, and increased perfusion and bicarbonate secretion in liver. Aging has negative impacts on the cardiovascular system, whereas the liver is more resilient with age. Caloric restriction (CR) attenuates affects of age in many tissues. This study investigates the effects of aging and CR on the hydrogen sulfide signaling system in the aorta and liver of rats by determining the contractile response of aortic rings to exogenous hydrogen sulfide, the protein and mRNA expression of the hydrogen sulfide producing enzymes cystathionine γ–lyase (CSE) and cystathionine β–synthase (CBS), and hydrogen sulfide production rates. Tissue was collected from Fisher 344 x Brown Norway rats from 8-38 months of age, maintained on an *ad libitum* (AL) or CR diet. Aortic rings exhibited a triphasic contraction-relaxation-contraction response to hydrogen sulfide. The hydrogen sulfide-sensitivity of the first phase contraction increased with age, while the magnitude of all three phases increased with CR compared to AL. In aorta, CSE protein expression increased with age on an AL diet, but this increase was attenuated with a CR diet. CBS expression showed the same trends as CSE, but with lower expression levels. In liver, both CSE and CBS protein expression remained relatively constant with age. In both aorta and liver, real-time PCR showed no changes in CSE or CBS mRNA expression with age, but mRNA expression in aorta was higher on a CR diet compared to AL. In both aorta and liver, hydrogen sulfide production did not change with age, but did increase with CR. Overall, these data show a significant effect of age and diet on the hydrogen sulfide signaling system in aorta, whereas liver was relatively unaffected. The

increased contractile sensitivity to hydrogen sulfide and increased protein expression of CSE and CBS in aorta point to a drop in hydrogen sulfide bioavailability with age. Additionally, CR seems to benefit CSE and CBS protein in both aorta and liver.

Introduction

Hydrogen sulfide (H_2S) functions as a gasotransmitter in the nervous, cardiovascular, and gastrointestinal systems. Abe and Kimura (Abe et al. 1996) first demonstrated that hydrogen sulfide is produced in the brain and that it increases N-methyl-D-aspartic acid receptor-mediated responses and facilitates hippocampal long-term potentiation. Since then a variety of physiological roles have been proposed for hydrogen sulfide (Lowicka et al. 2007; Szabo 2007), and at least some capacity for hydrogen sulfide production has been demonstrated in several vertebrate tissues (Zhao et al. 2003; Olson 2005; Dombkowski et al. 2006; Olson et al. 2006). When exogenously applied to isolated blood vessels of vertebrates, hydrogen sulfide elicits responses that range from a monophasic relaxation or contraction, to multiphasic contraction-relaxation-contraction responses, depending on the phylogenetic class and type of vessel examined (Dombkowski et al. 2005). In the liver, hydrogen sulfide has been proposed to regulate perfusion and maintain portal venous pressure, with deficiencies in hydrogen sulfide production pathways resulting in increases in intra-hepatic resistance (Fiorucci et al. 2005b). Hydrogen sulfide may also have a role in regulating biliary bicarbonate secretion in the liver (Fujii et al. 2005).

Hydrogen sulfide is endogenously produced from L-cysteine predominantly by the enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) (Kimura 2000; Julian et al. 2002; Wang 2002; Geng et al. 2004b; Fiorucci et al. 2005b; Gainey et al. 2005; Julian et al. 2005b; Kimura et al. 2005). In mammals, CSE and CBS are differentially expressed (Zhao et al.

2001; Zhao et al. 2003). CSE is the enzyme primarily expressed in the cardiovascular system, whereas CBS is the enzyme primarily expressed in the nervous system (Hosoki et al. 1997; Geng et al. 2004b; Ebrahimkhani et al. 2005; Kimura et al. 2005). In liver and kidney, CSE and CBS are both expressed in relatively high amounts compared to other tissues (Fujii et al. 2005; Lowicka et al. 2007).

Aging influences the action and/or efficacy of many signaling molecules, including norepinephrine and nitric oxide (NO) (Lakatta 1993; van der Loo et al. 2000; Tanaka et al. 2006) a gasotransmitter that is much better studied than hydrogen sulfide. A recent study of plasma hydrogen sulfide concentration in humans over 50-80 years of age reported that hydrogen sulfide levels may decline with age (Chen et al. 2005), but whether aging affects the hydrogen sulfide signaling system is unknown. Furthermore, any affect of age on the hydrogen sulfide signaling system may differ among tissues. For example, whereas the liver is relatively unaffected by age, substantial physiological changes occur with age in the cardiovascular system, including fibrosis of blood vessels leading to endothelial dysfunction (Castello et al. 2005), which affects regulation of blood vessel tone (Kitani 1991; Kitani 1994; Anantharaju et al. 2002).

Caloric restriction (CR) is an intervention that attenuates many effects of aging (Labinskyy et al. 2006; Leeuwenburgh et al. 2006), including cardiovascular morbidity (van der Loo et al. 2000), cross-linking of cardiac and skeletal muscle proteins (Leeuwenburgh et al. 1997), mitochondrial dysfunction (Aspnes et al. 1997; Payne et al. 2003), loss of skeletal muscle mass (van der Loo et al. 2000; Payne et al. 2003), and endothelial dysfunction (Gredilla et al. 2001; Barja 2002; Gredilla et al. 2004; Castello et al. 2005; Taddei et al. 2006). CR attenuates the age-related impairment of the NO signaling system (van der Loo et al. 2000; Marin et al. 2007) (Yang et al. 2004; Minamiyama et al. 2007; Sharifi et al. 2008), but whether CR affects the

hydrogen sulfide signaling system, and whether CR specifically attenuates any affects of age on the hydrogen sulfide signaling system, are all unknown.

In this study we investigated the effects of aging and CR on the hydrogen sulfide signaling system in liver and aorta in the Fisher 344 × Brown Norway hybrid rat. Liver and aorta tissue from rats of various ages were used to investigate CSE and CBS protein and mRNA expression and hydrogen sulfide production rates. Contraction of aorta smooth muscle was used as a functional indicator to investigate the effects of age and CR on functional responses to hydrogen sulfide.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specifically noted.

Animals

Fisher 344 × Brown Norway hybrid rats of 8, 14, 18, 23, 27, 34 and 38 months of age were singly housed under standard conditions and provided with food and water using either the NIH31 diet *ad libitum* (AL) or the NIH31-NIA fortified diet at 40% CR. Myography studies were performed on tissues from animals of 14, 27, and 34 months of age. All other studies were performed on tissues from animals of 8, 18, 29, and 38 months of age.

Western Blotting

Liver and aorta were removed within 5 min of euthanasia, frozen in liquid nitrogen and stored at -80° C. Samples were suspended in a lysis buffer consisting of 50 mmol/L Tris base, 2% (w/v) sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L dithiothreitol, 0.5 mmol/L sodium tetraborate, and 1% (v/v) Halt protease inhibitor cocktail (Thermo Fisher Scientific Rockford, IL USA). Western blotting was performed using

commercially available monoclonal antibodies for CSE and CBS (Abnova, Neihu District, Taipei City, Taiwan), a monoclonal antibody for β-actin (Cell Signaling Technology, Danvers, MA, USA) and a secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich St. Louis, MO, USA). Protein was separated using SDS-PAGE and transferred onto PVDF membrane (Immobilon P, Millipore, Billerica, MA USA) using a semidry blotter (BioRad, Hercules, CA USA). Using the Snap-ID system (Millipore, Billerica, MA USA) at room temperature, membranes were blocked in 0.05% non-fat milk in PBST (phosphate-buffered saline with 0.05% Tween-20), washed in PBST and incubated 10 min with appropriate primary antibodies (diluted 1:333 in PBST). Membranes were then washed in PBST four times and subsequently incubated 10 min with secondary antibody diluted 1:3,333 in blocking buffer. Membranes were washed two times in PBST, once in PBS, and once in Tris-HCl (100 mmol/L), pH 9.5. Chemiluminescent substrate (DuoLux, Vector Laboratories, Burlingame, CA USA) was used to generate a chemiluminescent signal, which was captured with a digital imager (GeneSnap, Syngene, Frederick, MD USA). Images were analyzed using commercial software (Quantity One, BioRad, Hercules, CA USA) to determine band intensity using local background subtraction.

RNA Extraction

Liver and aorta tissues were disrupted into powder under liquid nitrogen. All samples for each tissue were processed on the same day. For liver, total RNA was extracted using an Arum total RNA mini kit (BioRad, Hercules, CA USA). For aorta, total RNA was extracted using Tri reagent and a glass homogenizer (Kontes Glass, Vineland, NJ USA).

Real-Time PCR

Relative quantitative real time PCR was run in 96-well PCR plates in an Applied Biosystems 7300 Real-time PCR system (Life Technologies, Carlsbad, CA USA). Primers were

designed using commercial software (Premier Biosoft International, Palo Alto, CA USA) and synthesized by Integrated DNA Technologies (Coralville, IA USA). The primers used were as follows: CSE sense 5'- TGGGCTTAGTGTCTGTTAATTCC -3', CSE antisense 5'- GGCAGCAGAGGTAACAATCG – 3', CBS sense 5'- TGCCTGAGAAGATGAGTATG -3', CBS antisense 5'- GGTCCAGAATGTGAGAATTG – 3', Actin sense 5'- CTCCCAGCACACTTAACTTAG C -3', Actin antisense 5'- AAAGCC ACAAGAAACACTCAGG – 3', 18S rRNA sense 5'- CGAGGAATTCCCAGTAAGTGC -3', 18S rRNA antisense 5'- CCATCCAATCGCTAGTAGCG – 3'.For both liver and aorta plates, a standard curve for the housekeeping genes β-actin (run with CSE samples) or 18S rRNA (run with CBS samples) was run with a standard curve for the gene of interest (GOI), with all samples in triplicate. The remainder of the plate was filled with 8 random samples plus 1 calibrator sample for plate to plate corrections, loaded in triplicate for both the housekeeping gene and the GOI. Additional plates were run containing the calibrator sample on each plate until all samples had been processed. All plates for a given tissue-GOI combination were made and run on the same day, using the same real-time PCR machine.

Hydrogen Sulfide Production

Hydrogen sulfide production was measured essentially as previously described (Stipanuk et al. 1982; Julian et al. 2002; Dombkowski et al. 2006). Frozen tissues were ground into a fine powder under liquid nitrogen with mortar and pestle. For each sample, two small scoops (approx 50 mg wet weight) were added to 1.5 mL 100 mmol/L potassium phosphate buffer (pH 7.4) and homogenized with a Polytron homogenizer (Kinematica, Bohemia, NY USA) for 30–60 s until the solution was smooth. A 1 mL aliquot of this homogenate solution was then placed in the outer well of a 25 mL glass flask containing an additional plastic center well (Kontes Glass,

Vineland, NJ USA). The center well was filled with 0.5 mL 1% (w/v) zinc acetate, and a piece of chromatography paper (Whatman grade 3MM Chr, Maldstone England) cut 2.5 cm by 4.5 cm and folded into a fan shape was placed in the well to absorb the zinc acetate. The substrate L-cysteine (10 mmol/L) and the cofactor pyridoxal-5′-phosphate (PLP; 2 mmol/L) were added to the outer well, after which the flask was sealed with a septum stopper and flushed with N_2 gas for 30 s. Each flask was incubated on a shaker at 37 °C for 1.5 h for liver and 8 h for aorta. After incubation, 1 mL 50% (w/v) trichloroacetic acid was added to the outer well to stop enzyme activity and to convert all S^{2-} and HS^- to H_2S. The zinc acetate solution on the chromatography paper reacts with volatilized H_2S to form zinc sulfide, which is relatively stable. The flasks were then incubated for an additional hour to allow remaining hydrogen sulfide to volatilize and form zinc sulfide. The filter paper was then removed from the center well and placed in a test tube containing 3.5 mL of de-ionized water, 0.4 mL 20 mmol/L *N*,*N*-dimethyl-*p*-phenylenediamine oxalate in 7.2 mol/L HCl and 0.4 mL of 30 mmol/L $FeCl_3$ in 1.2 mol/L HCl. The test tubes were gently vortexed and incubated for 20 min at room temperature. Absorbance of the solution in the test tube was read at 670 nm with a plate reader (BioTek Instruments, Inc., Winooski, VT USA). Samples with an absorbance greater than 2 were diluted 10-fold with distilled water and the absorbance re-measured. Absorbance measurements were calibrated against a standard curve generated from NaHS in deoxygenated, de-ionized water added to the outside wells, incubated at 37 °C for 1.5 h, and assayed as above. To ensure that all measured hydrogen sulfide production is by tissue enzymes, several controls were run in addition to sample homogenates: a negative control of homogenate but no cofactors or substrate; and a blank of all cofactors and substrate in phosphate buffer, but without homogenate. Inhibitors of CSE (20 mmol/L propargylglycine, Pgly) and CBS (1 mmol/L aminooxyacetic acid, AOAA) were added to confirm the hydrogen

sulfide production was through these, and not alternate pathways (Julian et al. 2002). Protein concentration was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA USA), to calculate nmol hydrogen sulfide production per hour per mg protein.

Myography

The effect of hydrogen sulfide on vascular tone was measured in 5mm long aorta rings from 41 animals, with 5 to 8 rings tested for each age × diet treatment combination. To reduce artifact from anesthesia (Dombkowski et al. 2005), euthanasia was by guillotine under IACUC-approved methods. After dissection, aorta was cleaned of fat and connective tissue, and myography was performed essentially as previously described (Dombkowski et al. 2005). Rings were attached with stainless steel wire to force transducers and mounted in a 37°C tissue bath system (Radnoti Glass Technology, Monrovia, Ca USA) containing Kreb's bicarbonate buffer. Rings were allowed to equilibrate after mounting for a minimum of 1 h, and a baseline tension of 1.5 g was maintained throughout each experiment. At the start of an experiment, each ring was maximally contracted with two additions of 80 mmol/L KCl. After washing with Kreb's buffer and returning to baseline tension, the rings were incubated with 10 μmol/L propranolol (to block β-adrenergic receptor relaxation and maximize α-adrenergic receptor contraction), and precontracted with 1 μmol/L norepinephrine (NE). After the NE precontraction stabilized, rings were exposed to 100 μmol/L hydrogen sulfide. After 30-45 min, the tissue bath was drained and the rings were washed twice with Kreb's buffer and allowed to return to baseline tension. This sequence of propranolol, NE and hydrogen sulfide addition was repeated for 300, 600 and 900 μmol/L hydrogen sulfide. Data for hydrogen sulfide-induced contractions for each aortic ring were standardized to the weight of the ring.

Actual total hydrogen sulfide concentrations, as measured by a methylene blue assay (Gilboa-Garber 1971), were approximately 75% of the predicted value (presumably due mostly to oxidation). Note that in physiological saline at pH 7.4, the dissociation of hydrogen sulfide results in approximately 1/3 of total hydrogen sulfide as H_2S and 2/3 as HS^- (Beauchamp et al. 1984).

Statistics

All statistics were run using JMP statistical software, (JMP 7.0, SAS Institute, Cary, NC USA) with alpha ≤ 0.05 considered significant. Two-way ANOVAs were run with a Tukey post-hoc test, when possible. However, the functional response data were not always normally distributed, and therefore violated the assumptions of the ANOVA. In those cases, significant effects of age and diet were tested using the Kruskal-Wallis nonparametric ANOVA. When fewer than three groups were compared, pooled (two-tailed) or one-tailed t-tests were used. For real-time PCR, statistics were done on the average Ct of each sample, since the Cts are normally distributed (Wood et al. 2005). Statistical tests are listed throughout preceding their respective p-values.

Results

CSE and CBS Protein Expression

The relative expression of CSE and CBS protein differed between aorta and liver. In aorta, CSE protein expression (relative to β-actin) was 1.43-fold higher than CBS protein expression on average, whereas in liver, CBS protein expression (relative to β-actin) was 1.18-fold higher than CSE protein expression, on average.

In both aorta and liver, CSE protein expression was significantly affected by an interaction between age and diet (Figure 4-1A and 4-1B). In aorta, CSE protein expression

increased with age in AL animals, but it was unchanged with age in CR animals (Figure 4-1A, bars; 2-way ANOVA with Tukey post-hoc, $p < 0.0001$). At ages 18, 29, and 38 months CR CSE protein expression was significantly lower than AL at those ages, and was not significantly different than CSE expression at 8 months on either a CR or AL diet (Figure 4-1A, asterisks). In liver, CSE protein expression was unchanged with age in AL animals, but increased with age in CR animals (Fig 4-1B, bars; 2-way ANOVA with Tukey post-hoc, $p = 0.0041$). At 38 months of age AL CSE protein expression was significantly lower than CR (Figure 4-1B, asterisks).

In aorta but not liver, CBS expression was also significantly affected by an interaction between age and diet (Figure 4-1C and 4-1D). In aorta, there was significantly higher CBS protein expression in AL animals at 38 months compared to CR animals (Figure 4-1C, asterisks; 2-way ANOVA with Tukey post-hoc, $p = 0.0056$). In liver, there was no significant difference in CBS expression at any age, and there was no effect of CR (Figure 4-1D, 2-way ANOVA, $p >> 0.05$).

CSE and CBS mRNA Expression

There were no interactions between age and diet on the mRNA expression of CSE and CBS in aorta or liver (Figure 4-2). In aorta, the CR diet significantly increased expression of both CSE mRNA (Figure 4-2A, one-tailed t-test, $p = 0.0013$) and CBS mRNA (Figure 4-2C, one-tailed t-test, $p = 0.0258$) when compared to the AL diet. In liver, diet did not affect the expression of either CSE mRNA (Figure 4-2B, one-tailed t-test, $p >> 0.05$) or CBS mRNA (Figure 4-2D, one-tailed t-test, $p >> 0.05$).

Hydrogen Sulfide Production

There were no interactions between age and diet on capacity for hydrogen sulfide production in aorta or liver (Figure 4-3), but the capacity for hydrogen sulfide production was affected by diet. In aorta and liver hydrogen sulfide production was higher in tissues of CR

animals than AL animals (Figure 4-3A, one-tailed t-test, $p = 0.0407$ and Figure 4-3B, one-tailed t-test, $p = 0.0411$, respectively). Inhibitors of PLP-dependent enzymes were added to confirm hydrogen sulfide production was not occurring through PLP-independent mechanisms. Addition of the CSE inhibitor Pgly (20 mmol/L) decreased hydrogen sulfide production by 50% in aorta and by 94% in liver (data not shown), whereas addition of the CBS inhibitor AOAA (1 mmol/L) decreased hydrogen sulfide production by 57% in aorta and by 81% in liver (data not shown).

Contractile Response to Hydrogen Sulfide in Aorta

Application of hydrogen sulfide to isolated aorta rings typically elicited a triphasic, contraction-relaxation-contraction response (Figure 4-4). The contraction in the third phase was larger in magnitude and longer lasting than the contraction in the first phase, but at the lowest hydrogen sulfide concentration used (100 μmol/L) the response was typically very weak or absent.

Age did not affect the response to KCl or norepinephrine (Figure 4-5A and 4-5B, One-way ANOVA, $p >> 0.05$ for both), but age did have a significant effect on the first phase of the triphasic response to hydrogen sulfide. The first phase contraction was stronger in rings from 34 mo rats than from 14 mo rats after application of 100 μmol/L hydrogen sulfide (Figure 4-5C, one-way ANOVA with Tukey post-hoc, $p = 0.0062$). However, the first phase contraction to 300-900 μmol/L hydrogen sulfide did not significantly increase with age (Figure 4-5D, Kruskal-Wallis ANOVA, $p >> 0.05$ for all). The second phase relaxation showed a significant effect of age after application of 100 μmol/L hydrogen sulfide (data not shown, Kruskal-Wallis ANOVA, $p = 0.038$). However, post-hoc testing with a Wilcoxon non-parametric t-test between groups did not show significant differences between the ages ($p >> 0.05$ for all). The third phase contraction was not significantly affected by age (data not shown, Kruskal-Wallis ANOVA, $p >> 0.05$ for all).

Overall, CR had a significant effect on all phases of the contract-relax-contract response to hydrogen sulfide. Furthermore, the KCl and NE contractions were significantly stronger in rings from CR rats than from AL rats (Figure 4-6A and 4-6B, one-tailed t-test, $p = 0.0282$ and < 0.0001, respectively). Compared to rings from AL rats, CR increased the magnitude of the first phase contraction after application of 300 – 900 μmol/L hydrogen sulfide (Figure 4-7A, pooled t-test, $p = 0.0006$, $p = 0.0079$ and 0.0192, respectively), the magnitude of the second phase relaxation after application of 300 and 600 μmol/L hydrogen sulfide (Figure 4-7B, one-tailed t-test, $p = 0.0492$ and Wilcoxon test, $p = 0.0027$, respectively), and the magnitude of the third phase contraction after application of 300 - 900 μmol/L hydrogen sulfide (Figure 4-7C, pooled t-test, $p < 0.0001$, $p = 0.0025$, and $p = 0.0008$, respectively).

Discussion

CSE and CBS Protein Expression

In aorta, CSE expression increased with age on an AL diet, but remained constant on a CR diet. Aorta CBS expression showed a similar trend, but with lower expression levels. CBS expression was increased at age 38 months for AL animals, while CR animals had significantly lower CBS expression at 38 months. It is perhaps not surprising that changes with age were more evident for CSE than for CBS, since CSE is the expressed at higher levels in the endothelium and smooth muscle cells of aorta and is also believed to have the most functional contribution to regulating vascular tone (Hosoki et al. 1997; Zhao et al. 2001; Zhao et al. 2003; Wang et al. 2008). Therefore, CSE would be the more likely of the two enzymes to exhibit measurable effects of age and diet. We have been able to measure CBS protein in aorta even though previous researchers were only able to detect CSE mRNA and concluded there was no CBS in these tissues (Hosoki et al. 1997; Zhao et al. 2001). However, the protein expression level of CBS was

much less than that of CSE, consistent with the assumption that CBS has a less important role in the vasculature.

In aorta, the increase in CSE expression with age is similar to changes reported with age in eNOS expression in peripheral arteries (Cernadas et al. 1998; van der Loo et al. 2000; Goettsch et al. 2001; van der Loo et al. 2005), in which eNOS increased up to sevenfold with age. This was attributed to a homeostatic attempt to correct for a decrease in the bioavailability of NO caused by superoxide reacting with NO to form a peroxynitrite (van der Loo et al. 2000; van der Loo et al. 2005). Since NO and hydrogen sulfide have been reported to interact synergistically, changes in one system may impact the other (Hosoki et al. 1997; Wang et al. 2008). Additionally, hydrogen sulfide may also react with, and be quenched by, increasing peroxynitrite levels resulting from NO reacting with superoxide (Whiteman et al. 2004b). Furthermore, there is limited evidence that hydrogen sulfide reacts with superoxide, which may also directly impact hydrogen sulfide bioavailability (Geng et al. 2004a). CR has been hypothesized to prevent this age-related endothelial dysfunction by preventing increases in superoxide levels with age, thereby maintaining a more youthful state (Gredilla et al. 2001; Barja 2002; Gredilla et al. 2004; Castello et al. 2005; Taddei et al. 2006). Accordingly, CSE and, to a lesser extent, CBS expression in CR animals did not significantly increase with age and were more similar to 8 month old AL animals, consistent with a protective effect of CR in aorta maintaining a youthful state.

In liver, CSE expression increased with age in CR animals whereas CBS expression did not change with age. Since both CBS and CSE are involved in cysteine metabolism in addition to producing hydrogen sulfide (Zhao et al. 2001), they are present in relative abundance in the liver compared to other tissues. However, CBS is the primary hydrogen sulfide producing enzyme in

the liver (Zhao et al. 2003), and therefore the increase in CSE expression with age may not accurately reflect what is occurring to hydrogen sulfide levels as well as CBS expression does. Liver enzymatic capacity does not change substantially with age, and this also appears to be true for CBS (Kitani 1991; Kitani 1994; Anantharaju et al. 2002). Since the liver is functionally much different than aorta, it is perhaps not surprising that age and diet do not affect the liver to the same degree as the aorta.

The heterogeneity between tissue types observed between aorta and liver also occurs in the NO signaling system. Even when comparing peripheral vessels to heart tissue in the circulatory system, changes are seen in the vasculature even though no significant changes are observed in the heart (van der Loo et al. 2005). Therefore, the age and diet affects on the hydrogen sulfide signaling system may not be global events that occur during aging, but rather tissue-specific effects.

CSE and CBS mRNA Expression

There were complex and dramatic changes with age and diet in CSE protein expression in aorta and liver. There were no significant changes in the mRNA message for CSE or CBS with age, although CR did increase CSE and CBS mRNA levels in aorta. This seems to contradict the protein expression, in which CR reduced the expression of those proteins. Conversely, liver CSE and CBS mRNA message did not significantly change with age or diet, which was more consistent with the protein data. We cannot account for the discrepancy between mRNA and protein data, except to note that mRNA message does not always translate directly into protein (Gygi et al. 1999). We must conclude that while CR causes increases in mRNA for CSE and CBS in the vasculature, this does not translate into the synthesis of more new protein. Alternatively, CR may attenuate the need for new protein formation, but does not stop the synthesis of mRNA, leading to the observed increase in mRNA expression.

Hydrogen Sulfide Production

Extracellular concentrations of total hydrogen sulfide (representing the sum of H_2S, HS^- and S^{2-}) have been reported in the range of 50-160 μmol/L in the brain (Goodwin et al. 1989; Abe et al. 1996) and ~50 μmol/L in the plasma (Zhao et al. 2001). However, recent measurements in plasma seem to indicate that true hydrogen sulfide concentrations are much lower (<100 nmol/L) (Whitfield et al. 2008). This discrepancy is likely due to methodological differences in how free hydrogen sulfide is differentiated from that which is bound or "acid-labile" (Olson 2009).

Homogenates of aorta and liver both produced a consistent amount of hydrogen sulfide regardless of age, but CR samples consistently showed a higher hydrogen sulfide production rate. This is particularly interesting for the aorta, because our data indicate that it had lower levels of the hydrogen sulfide producing enzymes. Therefore, it can produce more hydrogen sulfide than the AL samples that have significantly more hydrogen sulfide producing enzymes. Note that because the data were calculated as nmol hydrogen sulfide/hr/*mg protein*, production rates for aorta and liver were very similar. When hydrogen sulfide production is instead calculated as nmol hydrogen sulfide/hr/*g tissue* or nmol hydrogen sulfide/hr/*ml of homogenate*, liver has a higher production rate than aorta.

Addition of AOAA and Pgly reduced hydrogen sulfide production in both aorta and liver, but did not completely block production. This indicates that both CSE and CBS contribute to hydrogen sulfide production in these tissues, but there may be a small contribution of "acid labile" hydrogen sulfide contributing to these production rate measurements. It is important to note that the recovery rate of hydrogen sulfide using the assay is not 100%, and is more likely 33-59% depending on the hydrogen sulfide production rate of the tissue (Julian et al. 2002).

Therefore, these rates should only be used as a comparison between the treatment groups in this study and not as an absolute rate of hydrogen sulfide production from aorta and liver in the rat.

Contractile Response to Hydrogen Sulfide in Aorta

Although triphasic responses to hydrogen sulfide application have been reported in rat pulmonary artery (Olson et al. 2006), previous studies of rat aorta have shown only a monophasic relaxation or contraction with hydrogen sulfide (Zhao et al. 2002; Dombkowski et al. 2005; Kubo et al. 2007b). We found that the development of all three phases typically required 15-30 min to develop after a bolus of hydrogen sulfide. Consequently, the triphasic response may have been masked in previous studies that did not allow sufficient time after hydrogen sulfide addition. Additional methodological variations, such as tissue preparation (ring vs. strip), method of euthanasia, and lag time between tissue dissection and hydrogen sulfide application, may have also contributed to differences in aorta response.

It has been suggested that high O_2 concentrations in the tissue bath may lead to formation of vasoactive hydrogen sulfide oxidation products (Koenitzer et al. 2007). Since our experiments were run at ambient O_2 concentrations, oxidation products may have contributed to the triphasic response. However, in dorsal aorta of cyclostomes hydrogen sulfide-induced contractions are enhanced at low O_2 concentrations, suggesting that hydrogen sulfide oxidation products are not the only cause of hydrogen sulfide-induced contractions (Olson et al. 2008). Additionally, in rat aorta rings we see the same triphasic response during hypoxia (Dissertation Chapter 2). This response to hypoxia is presumably mediated in part by hydrogen sulfide that is not readily oxidized due to the extremely low oxygen levels (Olson et al. 2006).

Our data indicate that the response of rat aortic rings to hydrogen sulfide increases in magnitude with age, with significant changes observed in the first phase contraction. The triphasic response in aorta has not been reported previously, and it is not known what causes the

initial contraction phase. A likely candidate is the reaction of hydrogen sulfide with NO to form a nitrosothiol (Whiteman et al. 2006), which has been suggested to reduce free NO and lead to contraction at low levels of hydrogen sulfide (Ali et al. 2006), whereas higher hydrogen sulfide concentrations may cause relaxation by activating K_{ATP} channels (Zhao et al. 2001). It is likely that after application of the hydrogen sulfide donor, NO reacts quickly with hydrogen sulfide causing contraction, which is followed by relaxation as K_{ATP} channels are activated. We have shown evidence of this in aorta during hypoxia-induced responses (Chapter 2). If indeed this NO-scavenging mechanism does mediate the first phase contraction, then the reduced bioavailability of NO observed with age (Cernadas et al. 1998; van der Loo et al. 2000; Goettsch et al. 2001; van der Loo et al. 2005) could cause the increase in sensitivity to hydrogen sulfide. That is, the less free NO is available, the larger the effect on vascular tone for a given amount of hydrogen sulfide.

Caloric restriction dramatically increased the response to hydrogen sulfide in all three phases, as well as the response to KCl. Therefore, rather than representing a global effect on hydrogen sulfide -signaling, the effect of CR may represent an overall enhancement of smooth muscle tissue health, as has been reported in skeletal muscle (Payne et al. 2003),.

Conclusions

Our data show that age affects the hydrogen sulfide signaling system in the aorta, on both the contractile response to hydrogen sulfide and the protein expression of CSE. Aorta responds to hydrogen sulfide with a triphasic contract-relax-contract response, which becomes sensitized to hydrogen sulfide with age. There was no age effect in the liver on the main hydrogen sulfide producing enzyme CBS. CR benefits the hydrogen sulfide signaling system in the aorta. All phases of the triphasic response were enhanced by CR, and CR maintained lower expression of CSE and CBS proteins, which could produce more hydrogen sulfide than AL animals. CR has no

significant affect on CBS protein expression in the liver. Overall, aorta shows the most impact of age and CR. While the effects of CR are likely due to maintenance of healthier tissue/proteins, the sensitization with age on hydrogen sulfide signaling in aorta may owe to impacts of age on NO. Therefore, the interactions of H_2S, NO and CO are key to fully elucidating the ramifications of these changes.

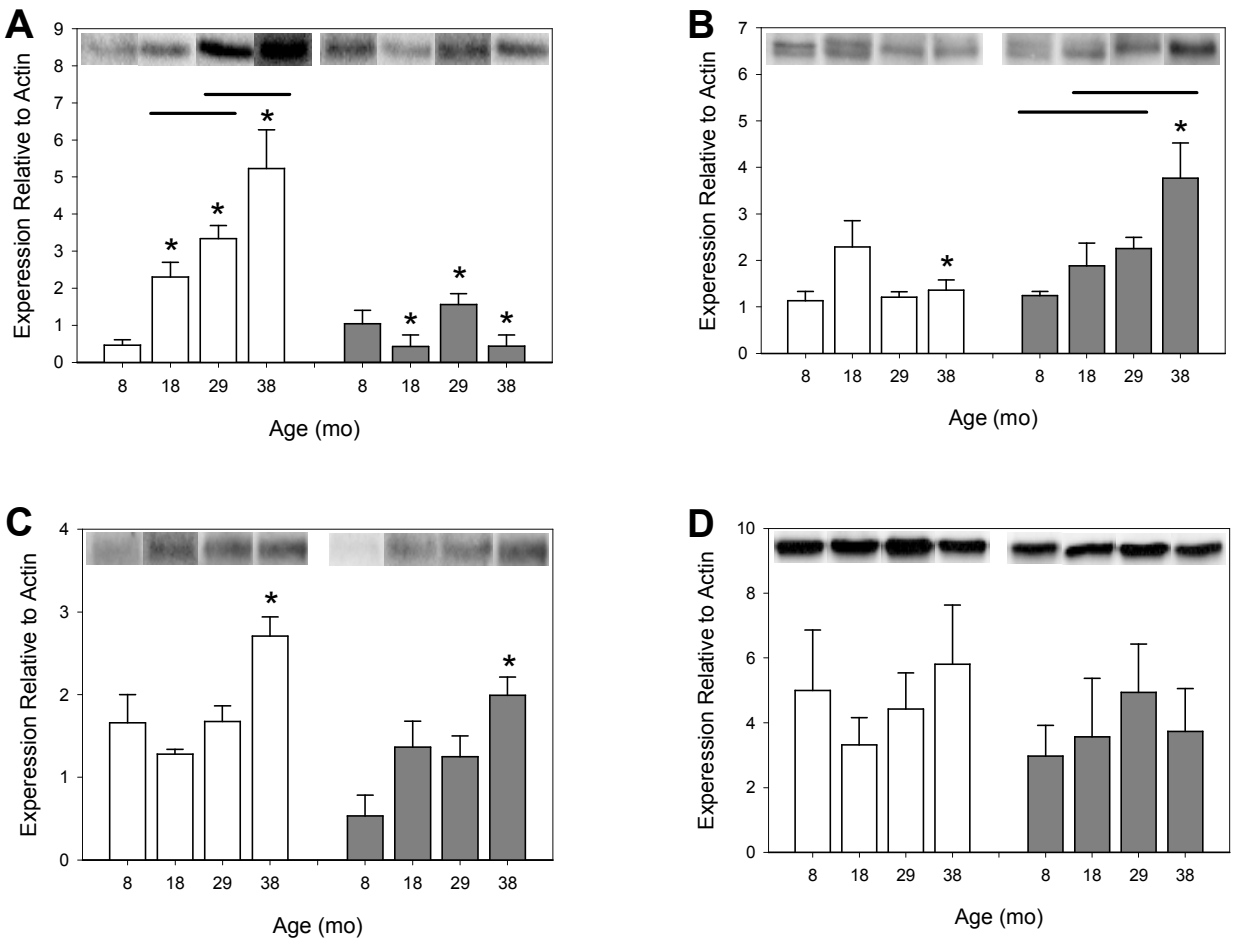

Figure 4-1. Effect of age and diet on CSE and CBS protein expression. CSE expression is shown in aorta (A) and liver (B). CBS expression is shown in aorta (C) and liver (D). The Y-axis shows CSE (A and B) or CBS (C and D) protein expression relative to β-actin. The X-axis shows the age in months (mo). AL data are in white columns and CR data are in grey columns. Representative blots are aligned along the top, above each column. Data are represented as mean ± SE and were analyzed by 2-way ANOVA with Tukey post-hoc tests. Means that are significantly different are indicated by different letters.

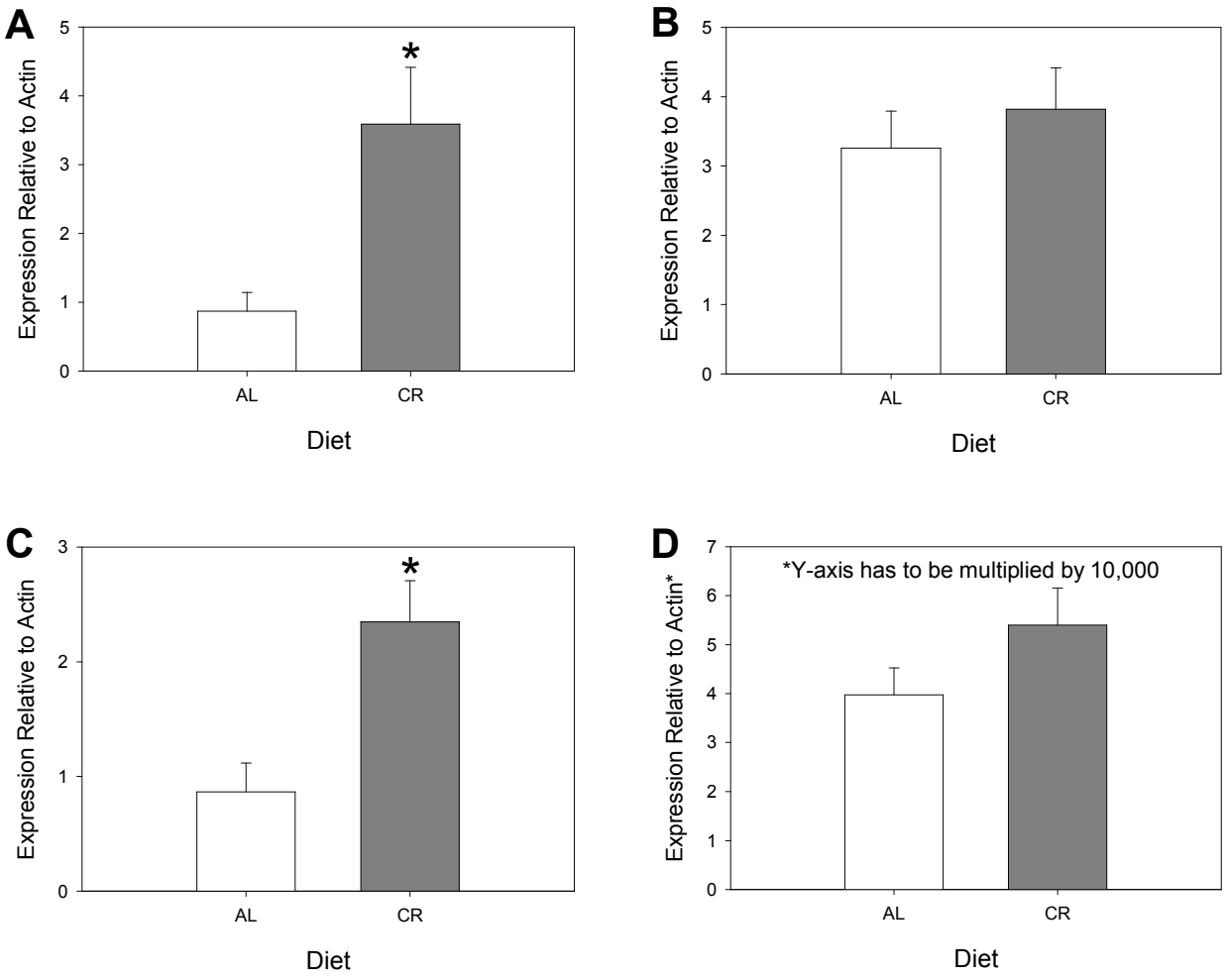

Figure 4-2. Effect of diet on CSE and CBS mRNA expression. CSE expression is shown in aorta (A) and liver (B). CBS expression is shown in aorta (C) and liver (D). The Y-axis shows CSE (A and B) protein expression relative to β-actin and CBS (C and D) protein expression relative to 18S rRNA. The X-axis shows the diet. AL data are in white columns and CR data are in grey columns. Data are represented as mean ± SE and were analyzed by one-tailed t-tests. Means that are significantly different are indicated by asterisks.

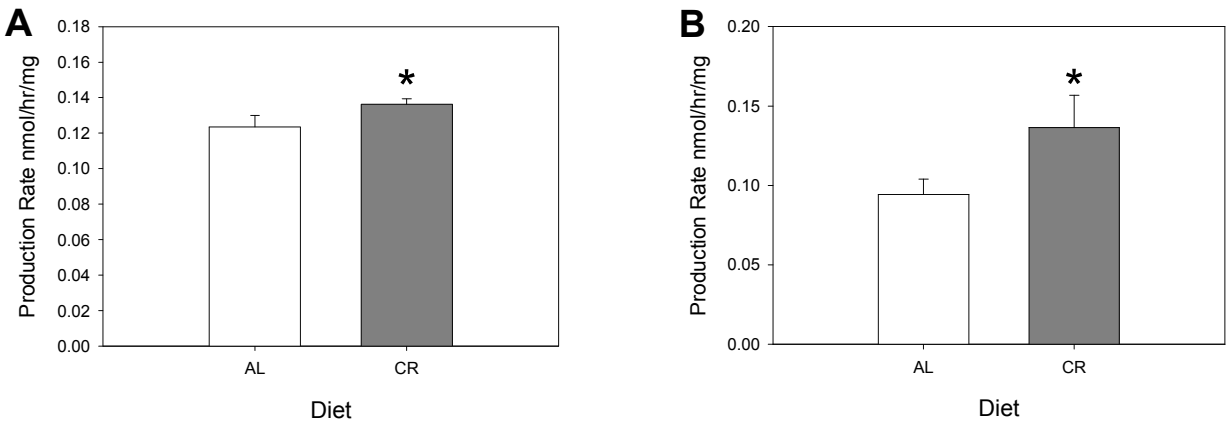

Figure 4-3. Effect of diet on hydrogen sulfide production. Hydrogen sulfide production rates are shown in aorta (A) and liver (B). The Y-axis shows production as nmol H_2S/hr/mg protein. The X-axis shows the diet. AL data are in white columns and CR data are in grey columns. Data are represented as mean ± SE and were analyzed by one-tailed t-tests. Means that are significantly different are indicated by asterisks.

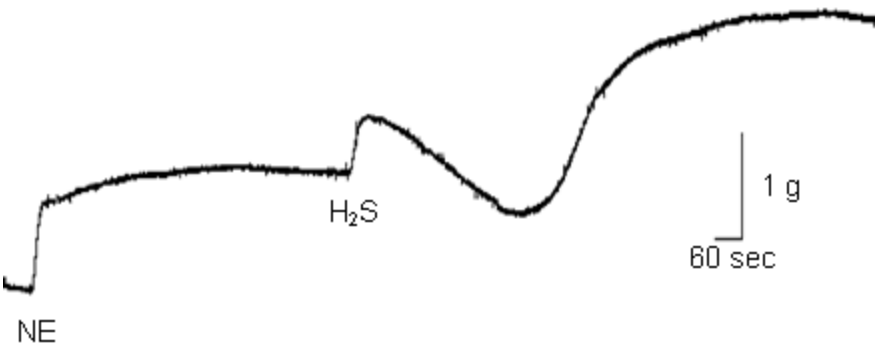

Figure 4-4. Representative tension tracing of a rat aorta ring pre-contracted with norepinephrine (NE) and exposed to hydrogen sulfide (H_2S, 300 μmol/L). Scale bar shows time in min and tension in grams.

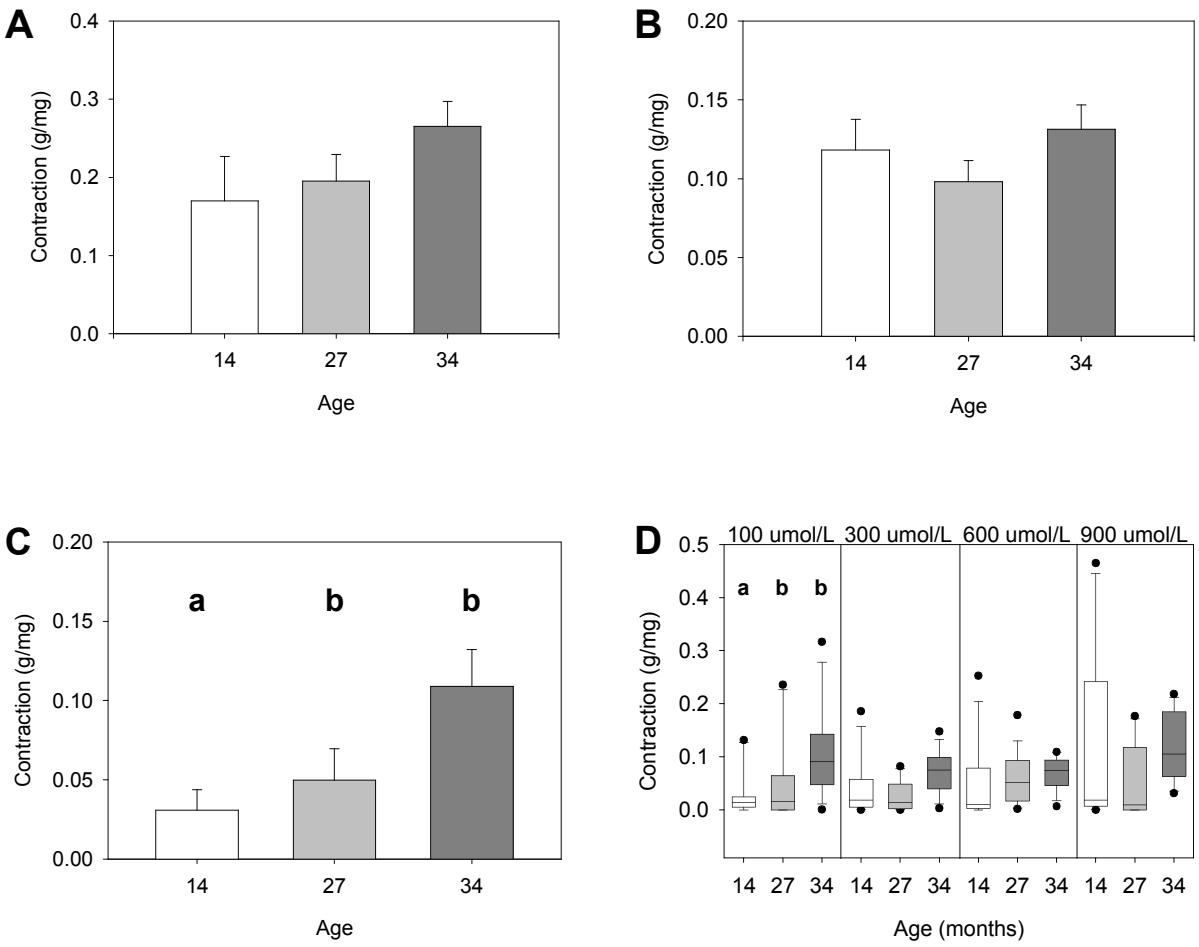

Figure 4-5. Effect of age on KCl- and norepinephrine (NE)-induced contractions and the first phase contraction to hydrogen sulfide. Age has no effect on KCl- (A) or NE-induced contractions (B). However, the first phase contraction to hydrogen sulfide (C) significantly increases with age at 100 µmol/L hydrogen sulfide addition. The first phase contractions did not significantly increase at 300-900 µmol/L hydrogen sulfide addition (D). The Y-axis represents the hydrogen sulfide-induced tension of aortic rings (g/mg). The X-axis shows the age (mo) (A-C) for each of the four hydrogen sulfide concentrations, divided into four panels (D). The top labels represent the concentration of hydrogen sulfide used for that panel (D). Data are presented as means ± SE (A-C) or as box plots showing the median, quartiles and outliers (D). Data were analyzed by one-way ANOVA with Tukey post-hoc test. Means that are significantly different are indicated by letters.

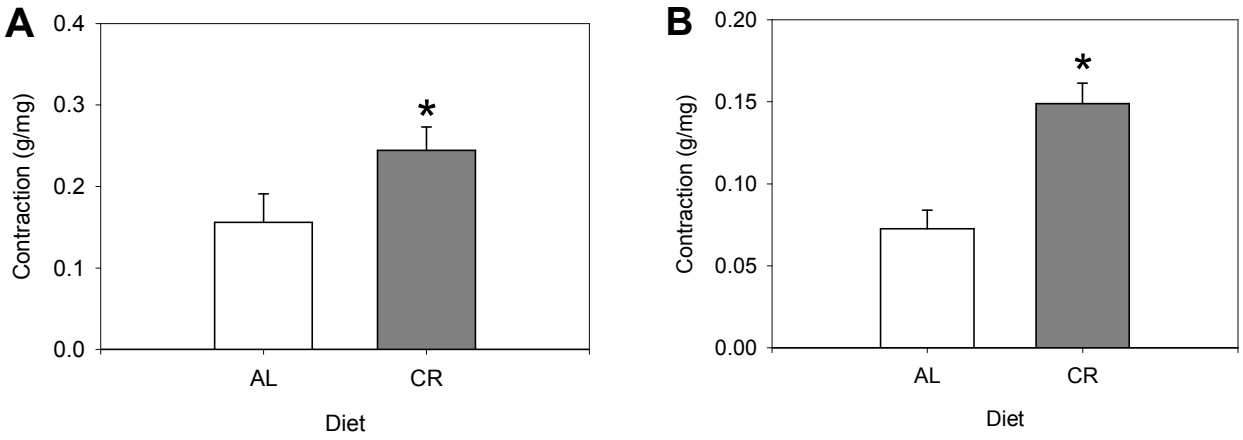

Figure 4-6. Effect of diet on KCl-and norepinephrine (NE)-induced contractions. CR causes an increase in contraction magnitude in response to KCl (**A**) and NE (**B**). The Y-axis represents the tension of aortic rings (g/mg). The X-axis represents age diet: *ad libitum* (AL) or caloric restricted (CR). AL data are in white columns and CR data are in grey columns. Data are represented as mean ± SE and were analyzed by one-tailed or pooled t-tests. Means that are significantly different are indicated by asterisks.

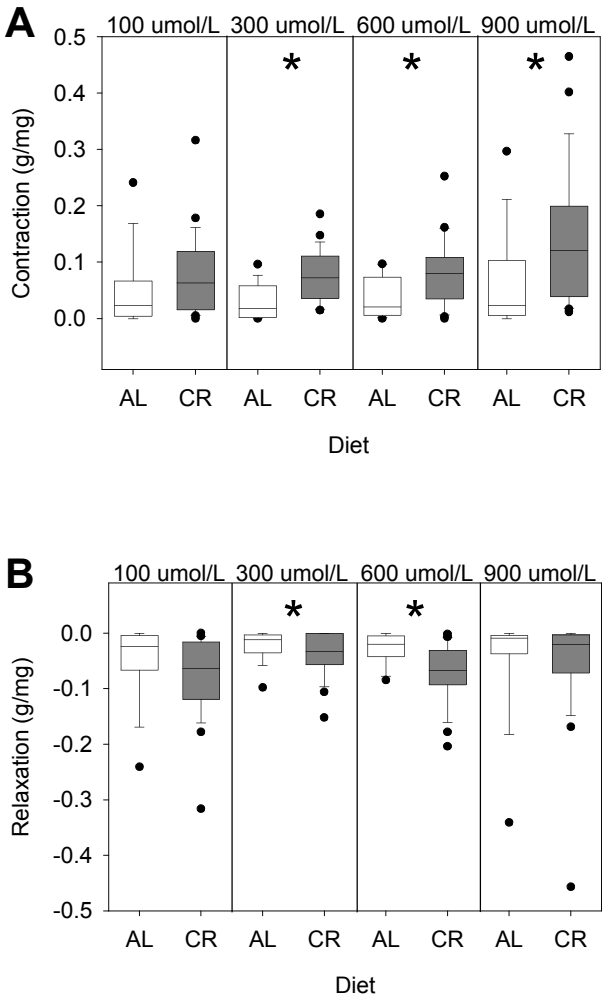

Figure 4-7. Effect of diet on hydrogen sulfide-induced contractions. CR causes an increase in contraction magnitude in response to hydrogen sulfide in the first phase contraction (A), second phase relaxation (B) and third phase contraction (C). The Y-axis represents the hydrogen sulfide-induced tension of aortic rings (g/mg). The X-axis represents diet: *ad libitum* (AL, white) or caloric restricted (CR, grey) for each of the four hydrogen sulfide concentrations, divided into four panels. The top labels represent the concentration of hydrogen sulfide used for that panel. Data are presented as medians, with error bars defining upper and lower quartiles. Data were analyzed by one-tailed or pooled t-tests. Medians that are significantly different are indicated by asterisks.

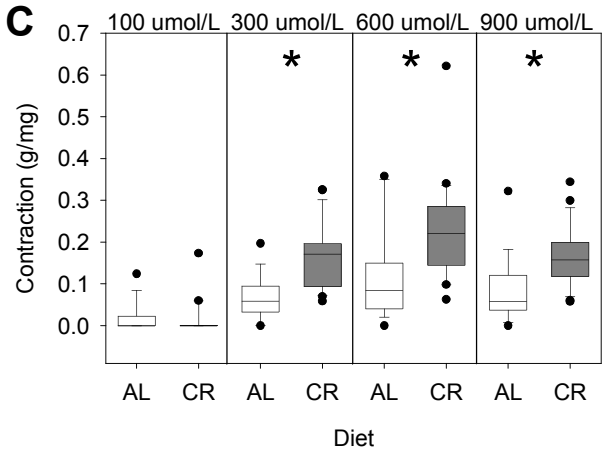

Figure 4-7. Continued.

CHAPTER 5
SYNTHESIS: UNDERSTANDING HYDROGEN SULFIDE SIGNALING IN VASCULAR SMOOTH MUSCLE AND THE TRIPHASIC RESPONSE

Introduction

Through this work on hydrogen sulfide signaling it has become clear that there are many pathways (potentially) involved in the vascular response. Some of these pathways have been elucidated, others have not. When investigating in vessel preparations, predominantly aorta in these studies, there is a distinct multiphasic contraction-relaxation-contraction response after application of hydrogen sulfide to the tissue bath that takes 2-3 times longer than a majority of the hydrogen sulfide actually remains in solution. Therefore hydrogen sulfide is most likely activating a variety of pathways initially, which in turn modulate the phases after hydrogen sulfide has been oxidized in the solution.

We also observe a similar contraction-relaxation-contraction response pattern in aorta rings exposed to hypoxia. It has been hypothesized that hydrogen sulfide is mediating the hypoxia response because hypoxia will allow the accumulation of hydrogen sulfide in tissues since there is little oxygen to oxidize it. However, the time course is much slower in hypoxia responses, and the mechanisms may not be the same.

Hydrogen sulfide also has a demonstrated effect on nitric oxide (NO) production from endothelial nitric oxide synthase (eNOS). Within 30 min of application, hydrogen sulfide causes an Akt-dependent increase in NO production via phosphorylation of Ser 1177 on eNOS. This finding fits within a developing theme in the literature on gasotransmitters; that hydrogen sulfide and NO having a synergistic relationship (Hosoki et al. 1997; Kimura 2002; Julian et al. 2005b; Jeong et al. 2006). NO has also been demonstrated to increase hydrogen sulfide production from cystathionineγ-lyase (Zhao et al. 2001; Zhao et al. 2003). Therefore, another signal must be present to quench their production, such as a feedback inhibition, or carbon monoxide (CO),

which can inhibit iNOS (Oh et al. 2006). However, hydrogen sulfide and NO can also chemically interact to form a nitrosothiol, so they may quench each other's signal as a form of regulation.

Age does change the initial contraction phase of the triphasic response to hydrogen sulfide, and this is likely due to this contraction being partially mediated by the reaction of hydrogen sulfide with NO, reducing free NO levels. The mechanisms underlying the initial contraction phase are discussed further below. Caloric restriction (CR) is an intervention that appears to reduce the increase in oxidative stress that occurs with age, which includes increased superoxide production. For the aging studies, we hypothesized that CR would ameliorate any changes we saw with age. While this was true in the case of cystathionineγ-lyase (CSE) and cystathionineβ-sythase (CBS) protein expression, it was not true of the functional response measurements (smooth muscle tone) in aorta rings. Here we observed an increase in responsiveness to all stimuli: KCl, norepinephrine, and hydrogen sulfide. It is believed that CR enhances the contractile function of smooth muscle, as is the case in skeletal muscle (Payne et al. 2003), and that this explains the increased general responsiveness. Therefore it is not a hydrogen sulfide-specific effect, but rather a global benefit of the CR intervention.

What is Modulating Phase 1 of the Triphasic Response?

The chemical reaction between NO and hydrogen sulfide likely contributes to the initial contraction phase by quickly reducing NO bioavailability (Whiteman et al. 2006; Whiteman et al. 2009). However, since removal of the endothelium from the aorta rings does not completely block the initial contraction phase from occurring, loss of NO bioavailability does not entirely explain what happens at this initial contraction. If this phase were completely NO-dependent, then removal of the endothelium, eNOS would be removed with the endothelium, and therefore the initial contraction should be completely abolished because there should be no NO to

scavenge. This also assumes that there would be no iNOS activity in smooth muscle. Application of the eNOS inhibitor L-NAME produced mixed results, depending on how it was added experimentally. Adding L-NAME prior to the PE precontraction and adjusting the tension after its application (to maintain 1.5 g tension) does not significantly reduce the initial contraction phase to hydrogen sulfide, further supporting the proposition that the reaction with NO is not solely responsible for causing this initial contraction. However, adding L-NAME after the PE precontraction and allowing the ring to further constrict (but not beyond the maximum tension developed by KCl) did significantly reduce the initial contraction when hydrogen sulfide was added. L-NAME also significantly reduced the initial contraction phase to hypoxia when added before the PE precontraction. This suggests that the initial contraction to hypoxia may be more dependent on NO/NOS than hydrogen sulfide, and that hydrogen sulfide may not play a large role in hypoxia signaling during this initial contraction phase.

The partial dependence of hydrogen sulfide on NO during the initial contraction is consistent with the observation that the initial contraction increases in sensitivity with age. Bioavailable NO declines with age due to increases in formation of superoxide, which can scavenge NO to form a peroxynitrite (van der Loo et al. 2000). If hydrogen sulfide is reducing bioavailable NO as well as by chemically reacting with it, then more hydrogen sulfide would be needed to reduce the NO pool in younger animals compared to older animals. Looking at this another way, comparing small and large NO pools, if one were to add the same amount of hydrogen sulfide to both pools, the smaller pool will show a larger effect than the larger pool because the proportion of NO removed would be greater.

What is Modulating Phase 2 of the Triphasic Response?

The relaxation of blood vessels and smooth muscle in general, to hydrogen sulfide has an accepted mechanism. Patch-clamping studies show that hydrogen sulfide activates ATP-sensitive

potassium channels (K_{ATP}) (Zhao et al. 2001; Zhao et al. 2002). Although it remains to be determined how this activation occurs, it is most likely a direct interaction with the channels rather than changing the ADP/ATP status (Whiteman et al. 2009). Using glibenclamide, a K_{ATP} inhibitor, we have not been able to statistically show that these channels are causing the relaxation in our observed triphasic response. It is clearly not endothelium-dependent, since removal of the endothelium does not affect the relaxation phase, and neither does adding L-NAME before addition of hydrogen sulfide. Other investigators have been unable to satisfactorily show the action of K_{ATP} channels in the relaxation caused by hydrogen sulfide, and others have proposed that K_{ATP} only partially mediates the relaxation (Kubo et al. 2007b).

We initially assumed that the discrepancy is related to dosage of glibenclimide. Our initial studies used glibenclimide dosages that were comparatively high, which may affect not only K_{ATP}, but chloride channels as well. Interestingly, this same dose did significantly reduce the relaxation observed during hypoxia responses, while only causing a small, non-significant decrease in the hydrogen sulfide treatments. This raises the question of what concentrations of hydrogen sulfide are present during hypoxia, if at all? While a hydrogen sulfide-sensitive electrode exists, no researchers have yet been able to perform these measurements. Therefore, the true mechanism of the relaxation phase remains unknown, but it is likely at least partially through K_{ATP}.

Recently however, a new mechanism of relaxation induced by hydrogen sulfide has been uncovered: the Cl^-/HCO_3^- anion exchanger (Kiss et al. 2008). I performed preliminary experiments with an anion channel blocker, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonate (DIDS), to inhibit this exchanger. When adding DIDS, there was a complete blockade of the relaxation phase in the response to hydrogen sulfide. Therefore, it is quite likely that these anion

exchangers are activated by hydrogen sulfide. This activation would lead to a reduction in intracellular pH (Lee et al. 2007), which in itself will cause relaxation of the smooth muscle and may also activate K_{ATP} channels leading to the observed relaxation phase to hydrogen sulfide (Kiss et al. 2008).

What is Modulating Phase 3 of the Triphasic Response?

The third phase contraction is almost certainly not directly mediated by hydrogen sulfide, and is likely endothelially-derived. This conclusion is drawn simply from the oxidation rate of hydrogen sulfide from the tissue bath compared to the time this contraction phase starts. Hydrogen sulfide will be at least 50% oxidized by ten minutes after application. By fifteen minutes, which is the point at which the third phase contraction usually begins, most of the hydrogen sulfide will have disappeared. Additionally, removal of the endothelium significantly reduces the third phase contraction. It is likely, then, that hydrogen sulfide is causing a change in the concentration of another vasoactive agent produced in the endothelial cells. When we investigated the contribution of arachadonic acid metabolites (AAM) by inhibition of cyclooxygenase, lypoxygenase, and cytochrome P-450, we saw a significant reduction in the third phase contraction when all three enzymes were inhibited. Which AAM is causing this contraction remains to be elucidated, but it is likely a combination since inhibition of cyclooxygenase, lypoxygenase and cytochrome P-450 individually did not significantly reduce the third phase contraction. Interestingly in this case, the third phase contraction to hypoxia was not reduced with inhibition of AAM, suggesting another difference between the two signaling events.

A Proposed Time-course and Mechanism of the Triphasic Response

We can now attempt to piece together a model of the chain of events that occurs in a blood vessel (in this case a precontracted ring of rat thoracic aorta) following the addition of

hydrogen sulfide. Upon addition of Na_2S, it rapidly dissociates into H_2S gas and HS^-. The H_2S diffuses into the aorta ring and the vascular smooth muscle cells (VSMC). Here H_2S comes into contact with free NO, and reacts to form a nitrosothiol (a vasoactive molecule in itself which may go on to cause downstream events (Henderson et al. 2005; Que et al. 2005)). This reduces NO bioavalability, which causes the VSMC to constrict, thereby starting the first phase contraction. At the same time this contraction begins, H_2S starts to activate K_{ATP} channels (or the Cl^-/HCO_3^- anion exchanger), causing the VSMC to hyperpolarize and begin to relax. This relaxation continues for several minutes. Up to this point, the hydrogen sulfide has been oxidizing steadily, and is by now at least half gone. However, from the very start, H_2S has caused a conformational change in cyclooxygenase, lipoxygenase and cytochrome P-450, potentially by interacting with heme groups present in some of these enzymes, or by modifying and/or making new thiol groups, causing conformational changes. This stimulates the production of vasoconstricting AAMs, such as thromboxanes, leukotrienes, and prostaglandins ($PGF_{2\alpha}$). As these AAMs accumulate, they override the K_{ATP} channel (or Cl^-/HCO_3^- anion exchanger)-induced relaxation and start the third phase contraction, which eventually peaks, and comes back to resting tension. This decrease to resting tension may also be aided by hydrogen sulfide initiating the activation of eNOS via phosphorylation, which peaks at around 30 minutes. This increases basal NO production, replenishing the NO that hydrogen sulfide initially scavenged, thereby decreasing vascular tone.

Conclusions and Future Directions

While we have made a good start at understanding what is modulating the vascular response to hydrogen sulfide, there is much to be learned. The triphasic response is likely the end result of a multitude of changes resulting from the addition of hydrogen sulfide. Given the

extremely reactive nature of hydrogen sulfide, it is possible that many pathways are activated or deactivated. This makes it difficult to isolate individual contributors within a given contraction or relaxation phase. Even removal of the endothelium does not completely block any phase, despite the majority of the response probably being endothelium-derived. Hydrogen sulfide likely acts directly on the VSMCs as well. We also have not been able to completely block the relaxation phase with K_{ATP} inhibitors, and therefore an additional mechanism probably contributes to the relaxation phase.

Future work will be required to fully elucidate the mechanisms behind hydrogen sulfide signaling in the cardiovascular system. A systematic, whole-animal approach would be helpful to test the physiological relevance of the triphasic response. There is a consistent finding of a decrease in blood pressure when hydrogen sulfide donors are injected into whole animals (Zhao et al. 2001; Zhong et al. 2003; Wang et al. 2008; Webb et al. 2008; Yang et al. 2008; Zhao et al. 2008). However, one group has observed a biphasic response in blood pressure to hydrogen sulfide injection, with blood pressure showing an initial decrease followed by a transient increase (Kubo et al. 2007c). This is consistent with our observed triphasic response, which would initially cause a drop in blood pressure (assuming the initial contraction is too short and small in magnitude to be detected) and then a rise in blood pressure as the second contraction phase begins. Whole animal studies may also be useful for resolving the seemingly contradictory data that hydrogen sulfide activates eNOS, but causes contraction in aorta. The activation of eNOS does not occur until 30 minutes after hydrogen sulfide addition, and therefore it may contribute to the attenuation of the third phase contraction, and thus recovery of the vessel to base tension. This activation should be tested in vivo to determine whether application of hydrogen sulfide

after 30 minutes significantly alters blood pressure or enhances the response to acetylcholine compared to controls.

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BIOGRAPHICAL SKETCH

Benjamin Predmore was born in Allentown, Pennsylvania in 1983. An only child, he grew up in the rural area outside of Bangor, Pennsylvania, graduating from Bangor Area Senior High School in 2001. He earned his bachelor's degree in Biology from the Pennsylvania State University (PSU) in 2005. While at PSU, he was active in research working in the lab of Dr. Charles Fisher beginning in 2002.

While in the Fisher lab he participated in community ecology studies of the hydrocarbon seep communities in the Gulf of Mexico. This afforded him the opportunity to participate in two research cruises to the Gulf of Mexico during the summers of 2003 and 2004 onboard the R/V Seward Johnson II. During these cruises he was able to make a total of dives on a variety of hydrocarbon seep sites onboard the DSV Johnson Sea-link I. In addition to community ecology work, he was also responsible for sampling the hydrogen sulfide concentrations around the water column of these sites. Due to the volatility of hydrogen sulfide and it's rapid oxidation in seawater, this assay required strict attention to detail and had to be performed in very in a nitrogen-filled glove bag in oxygen-free conditions. This experience primed him for his doctoral work with hydrogen sulfide, and also allowed him to meet with Dr. David Julian of the University of Florida who also worked extensively with hydrogen sulfide and gave Ben some key troubleshooting techniques for his assay.

In 2005, Ben joined the Doctor of Philosophy program in the Department of Zoology at the University of Florida under the supervision of Dr. Julian working on the mechanisms of hydrogen sulfide signaling in mammals. Upon completion of his doctorate, Ben will work as a post-doctoral member in the laboratory of Dr. David Lefer at Emory University. There he will learn new surgical techniques on murine, and porcine models, continuing investigation of hydrogen sulfide signaling.