A PHYSIOLOGICAL ROLE FOR HYDROGEN SULFIDE IN THE HUMAN CUTANEOUS CIRCULATION

A Dissertation in
Kinesiology
by
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ABSTRACT

Hydrogen sulfide (H$_2$S), once considered a malodorous and toxic gas, is now recognized as a third endogenous gas-transmitter, in addition to nitric oxide (NO) and carbon monoxide. H$_2$S has significant vasoactive properties and plays a role in the modulation of vascular function. The purpose of this series of studies was to examine the vasodilatory role of H$_2$S in the cutaneous circulation of healthy young adults and subsequently determine alterations in H$_2$S-dependent vasodilation that occur with hypertension. In the first series of studies we hypothesized that (1) the H$_2$S generating enzymes, cystathionine-γ-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (3-MPST), would be expressed in the cutaneous circulation, and (2) that H$_2$S-donors would elicit dose-dependent increases in cutaneous vasodilation through potassium ATP channels. We further hypothesized that both NO and by-products of cyclooxygenase (COX) metabolism would contribute, at least in part, to exogenous H$_2$S-induced cutaneous vasodilation. The novel findings from the first series of studies were (1) CSE and 3-MPST were expressed in the human cutaneous microvasculature, (2) H$_2$S donors elicited vasodilation in a dose-dependent manner in the cutaneous circulation of healthy young adults, (3) exogenous H$_2$S-induced cutaneous vasodilation was mediated, in part, by calcium-dependent potassium channels, and (4) NO and COX inhibition attenuated exogenous H$_2$S-induced cutaneous vasodilation. The results of these studies confirm the presence of H$_2$S-generating enzymes in the cutaneous circulation as well as vasodilator responsiveness to a H$_2$S donor. Additionally, H$_2$S-mediated vasodilation occurred through downstream potassium channels and via interaction with the NO and COX vasodilatory pathways.

H$_2$S production and function are altered in the presence of hypertension in animal models. In the second series of studies, we hypothesized that endothelium-dependent (ACh, acetylcholine) vasodilation would be blunted in pre/stage 1 hypertensive adults compared to
normotensive controls, due to blunted NO- and H₂S-dependent vasodilation. Additionally, we hypothesized that the end-organ responsiveness to exogenous H₂S would be preserved in pre/stage 1 hypertensive adults but that vasodilatory interaction with NOS and COX signaling pathways would be diminished compared to normotensive age matched controls. Our data demonstrated blunted vasodilation to a cholinergic stimulus in pre/stage 1 hypertensives, in part, due to a diminished H₂S-dependent vasodilation. Additionally, our data demonstrated preserved end-organ responsiveness to exogenous H₂S in pre/stage 1 hypertensive adults, despite a loss of vasodilatory interaction with NOS and COX vasodilatory pathways.

Finally, we used reactive hyperemia to non-invasively assess microvascular function in pre/stage 1 hypertensive adults and normotensive age-matched controls. We sought to determine the role of H₂S to the overall total hyperemic response (THR). Inhibition of endogenous H₂S production blunted the THR in pre/stage 1 hypertensive adults, but not in normotensive adults; however this blunted response was not significantly different from the control site. Additionally, we found that NO does not mediate the reactive hyperemic response in normotensive or pre/stage 1 hypertensive adults. Also, the THR was related to both the H₂S- and NO-dependent ACh-mediated vasodilation. These data support the use of cutaneous reactive hyperemia to assess generalized microvascular function; however, we were unable to detect statically significant differences in vascular function in a group of pre/stage 1 hypertensive adults.

In summary, these data suggest that H₂S contributes to vasodilation in the cutaneous microcirculation in humans. H₂S donors caused robust vasodilation through calcium-dependent potassium channels and interaction with NOS and COX vasodilatory pathways. Pre/stage 1 hypertensive adults had diminished H₂S-dependent vasodilation in response to a cholinergic stimulus; however, end-organ sensitivity to exogenous H₂S was preserved despite a lack of vasodilatory interaction with NOS and COX pathway. Finally, H₂S may modulate a portion of the
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LIST OF ABBREVIATIONS

3-mercaptopyruvate sulfurtransferase 3-MPST
Acetylcholine ACh
Aminooxyacetic acid AOAA
Big calcium-dependent potassium channel BK$_{Ca}$
Bisulfide HS$^-$
Carbon monoxide CO
Cardiovascular disease CVD
Cutaneous vascular conductance CVC
Cyclic adenosine monophosphate cAMP
Cyclic guanosine monophosphate cGMP
Cyclooxygenase COX
Cystathionine-β-synthase CBS
Cystathionine-γ-lyase CSE
Diastolic blood pressure DBP
Endothelial derived hyperpolarizing factor EDHF
eNOS: endothelial nitric oxide synthase eNOS
Epoxyeicosatrienoic acids EETS
Glybenclamide GLY
Hydrogen sulfide H$_2$S
Intermediate calcium-dependent potassium channel IK$_{Ca}$
Ketorolac KETO
Mean arterial pressure MAP
Nitric oxide NO
Nitric oxide synthase NOS
N-Nitro-L-Arginine Methyl Ester L-NAME
Potassium ATP K$_{ATP}$
Prostacyclin PGI$_2$
Senicapoc SENI
Small calcium-dependent potassium channel SK$_{Ca}$
Sodium hydrosulfide NaHS
Sodium sulfide Na$_2$S
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<td>Tetraethylammonium</td>
<td>TEA</td>
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<td>Total hyperemic response</td>
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<td>Vascular smooth muscle</td>
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Chapter 1
INTRODUCTION

Background and Significance

Hydrogen sulfide (H$_2$S) is the third gasotransmitter, along with nitric oxide (NO) and
carbon monoxide, which has recently emerged as having physiological relevance in vascular
function. H$_2$S is an endothelium derived hyperpolarizing factor (EDHF) with potassium channel
targets on the endothelium and vascular smooth muscle (VSM) that lead to hyperpolarization and
subsequent vasodilation of blood vessels *in vitro* and *in vivo* in animal models (Zhao *et al.*, 2001).
A loss of enzymatic sources of H$_2$S leads to profound hypertension while administration of
exogenous H$_2$S can reduce blood pressure in hypertensive rodent models (Yang *et al.*, 2008). To
date, the studies examining the role of H$_2$S in vascular function have been primarily conducted in
animal models. Investigation into the basic mechanisms of H$_2$S in vascular function in humans
and how that changes with disease is important in basic vascular biology and for the development
of target therapeutics for the treatment of cardiovascular disease.

Modulation of Vascular Function by H$_2$S

Vasorelaxation of the VSM is mediated by a number of vasoactive molecules synthesized
in the endothelium. NO was the first gasotransmitter to be systematically characterized. NO is a
ubiquitous vasoprotective molecule that is enzymatically synthesized from the amino acid L-
arginine and induces vasodilation through cyclic guanosine monophosphate-dependent
mechanisms (Schultz et al., 1977; Förstermann et al., 1994). In addition to NO, enzymatic production of vasodilators also occurs through the cyclooxygenase (COX) pathway that induces vasodilation in the VSM through cyclic adenosine-monophosphate-dependent pathways (Narumiya et al., 1999; Funk, 2001). An additional endothelial vasodilatory pathway exists, independent of cyclic guanosine monophosphate and cyclic adenosine monophosphate pathways, which leads to the hyperpolarization and subsequent relaxation of VSM cells. Endothelial-derived hyperpolarizing factors (EDHFs), a term coined in the late 80’s, induce vasodilation that is endothelium-dependent but NO- & COX-independent.

In the past 20 years, H$_2$S has emerged as an EDHF that is synthesized enzymatically by cystathionine-$\gamma$-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MPST) in the vascular endothelium. H$_2$S-mediated vasodilation is variable throughout the vascular tree, with increased importance in smaller resistance vessels (Hosoki et al., 1997; Zhao et al., 2001).

Upon stimulation of the vascular endothelium (cholinergic), H$_2$S is synthesized in the endothelium and passively diffuses to the VSM (Mustafa et al., 2011; Wang, 2012a) where it induces vasodilation through activation of potassium ATP (K$_{ATP}$) channels and subsequent membrane hyperpolarization (Zhao et al., 2001; Distrutti et al., 2006; Gallego et al., 2008; Liang et al., 2011; Fitzgerald et al., 2014). Recently, small, intermediate, and big calcium-dependent potassium channels (SK$_{Ca}$, IK$_{Ca}$ and BK$_{Ca}$, respectively) have emerged as additional mechanisms on the endothelium and VSM by which H$_2$S mediates vasodilation, specifically in the smaller resistance vessels (Mustafa et al., 2011; Jackson-Weaver et al., 2013).

While NO, COX-derived products and EDHFs all contribute directly to VSM relaxation, the EDHF-dependent contributions likely make up a large proportion, especially under pathological conditions where NOS and COX-dependent mechanisms may be compromised (Mustafa et al., 2011). Early in the pathogenesis of vascular disease NO synthase (NOS) function is lost and COX begins synthesizing vasoconstrictors instead of vasodilators (Moncada & Vane,
There is strong evidence in rodent models suggesting that H$_2$S is a primary mechanism mediating vasodilation at the start of hypertensive vascular disease (Mustafa et al., 2011). Collectively, these data suggest that H$_2$S is an important signaling modulator and may be a putative molecular target, especially early on in the pathogenesis of vascular dysfunction associated with cardiovascular disease. However, to date, the investigation of basic H$_2$S signaling and downstream targets have been almost exclusively explored in animal models.

**Skin as a Model Circulation**

There are several non-invasive methods used to evaluate vascular function in humans. However, many of these measures only evaluate the conduit arteries and do not allow for the mechanistic interrogation of basic vascular signaling. These techniques include: flow-mediated dilation, pulse wave velocity and carotid intima media thickness (Thom et al., 2006). While these techniques are useful for monitoring gross disease progression and efficacy of treatment modalities they are limited in that they only functionally assess the magnitude of changes in the conduit vessels and do not identify the mechanisms by which systemic vascular dysfunction occurs (Holowatz et al., 2008).

The cutaneous circulation is an accessible circulation to examine mechanisms underlying microvascular function. Deficits in cutaneous vascular signaling are evident in human skin prior to, and are predictive of, the onset of conduit artery disease (IJzerman et al., 2003; Holowatz et al., 2008). This is especially useful for examining EDHF-dependent mechanisms because EDHFs are vital for health and function of small resistance vessels compared to the conduit vasculature (Vicaut, 1992; Struijker Boudier et al., 1992).

Laser-Doppler flowmetry is a minimally invasive technique that allows for the direct measurement of dynamic fluctuations in blood flow in a small area of skin. This technique can be
paired with vasoreactive stimuli to systematically evaluate vascular signaling in health and disease. Vasoactive stimuli used in the cutaneous circulation include: post-occlusive reactive hyperemia, whole body heating and cooling, local heating and cooling, and vasoactive drug delivery via iontophoresis and intradermal microdialysis. Laser-Doppler flowmetry coupled with microdialysis is a powerful and useful technique to dissect vascular signaling pathways in physiological and pathological conditions utilizing the cutaneous circulation of humans.

The cutaneous circulation is a representative vascular bed to investigate the mechanisms of microvascular dysfunction that parallels generalized systemic vascular dysfunction in other vascular beds (IJzerman et al., 2003; Abularrage et al., 2005; Rossi et al., 2008). Pathology induced vascular dysfunction is apparent in the cutaneous circulation and has been explored in hypertension (Levy et al., 2001; Rizzoni et al., 2003; Lindstedt et al., 2006; Holowatz & Kenney, 2007), hypercholesterolemia (Khan et al., 1999; Holowatz & Kenney, 2011; Alexander et al., 2013), renal disease (Stewart et al., 2004), peripheral vascular disease (Rossi & Carpi, 2004), atherosclerotic coronary artery disease (Shamim-Uzzaman et al., 2002), primary aging (Lang et al., 2010; Stanhewicz et al., 2012), heart failure (Cui et al., 2005; Green et al., 2006), type II diabetes (Sokolnicki et al., 2007), and systemic sclerosis (Della Rossa et al., 2013).

The potential role of EDHFs in vascular function has been investigated in the cutaneous microcirculation. Specifically, epoxyeicosatrienoic acids (EETs), an arachidonic acid metabolite, and the downstream EDHF target, calcium-dependent potassium (K_{Ca}) channels, have been examined (Brunt & Minson, 2012) in response to the vasodilator stimuli of local skin heating and reactive hyperemia. Brunt et al.’s data suggest that EDHFs are responsible for ~40% of local heating-induced hyperemia and specifically that EETs are one EDHF accounting for the remaining hyperemia, though they do not account for all of the remaining EDHF component (Brunt & Minson, 2012). Collectively these data suggest that EDHFs are significant modulators
of microvascular tone and that other putative EDHFs, including \( \text{H}_2\text{S} \), contribute to vascular control in the cutaneous circulation of humans.

**Hypertension and Microvascular Dysfunction**

Microvascular dysfunction is one manifestation of hypertensive vascular disease. Primary vascular alterations include 1) endothelial dysfunction and 2) increased VSM activity and over time results in 3) structural changes in the vasculature (Levy et al., 2001). These vascular alterations are linked to accelerated vascular aging (Brunner et al., 2005). There are several different classes of medication for the treatment of essential hypertension to lower arterial blood pressure, however a reduction in pressure does not necessarily result in reduced end-organ damage (Levy et al., 2001). The microcirculation (vessel diameter <150 µm) is where target organ damage first occurs with hypertensive vascular pathology (Levy et al., 2001).

Various alterations in cutaneous microvascular control have been observed in hypertensive humans. The maximal vasodilator capacity in the cutaneous circulation of the forearm (Schulte et al., 1988; Carberry et al., 1992; Smith et al., 2011) and hand (Sivertsson, 1970; Horwitz & Patel, 1985) is blunted in hypertension. This has been interpreted as a structural limitation of the microvessels ability to vasodilate. This measure has been used as an index of structural vascular remodeling with hypertension.

In addition to structural vascular limitations, Levy et al, described three mechanisms by which the microcirculation can become compromised with hypertension (Levy et al., 2001). First, increased vasoconstrictor tone or decreased vasodilator capacity that limit the range of vasoregulation (Zhang et al., 2004; Zhao et al., 2008; Smith et al., 2011). Second, anatomical changes occur over time and result in a narrowing of the wall-to-lumen ratio (Struijker Boudier et al., 1992; Rizzoni et al., 1994). Finally, rarefaction, which is defined as a reduction in the number
of perfused arterioles and capillaries within a vascular bed, due to sustained high pressure (le Noble et al., 1990; Struijker Boudier et al., 1992).

Microdialysis coupled with laser-Doppler flowmetry in the cutaneous circulation is a novel technique to meticulously investigate endothelial dysfunction, due to decreased vasodilator capacity to vasoactive molecules, in the pathogenesis of hypertension. The primary pathological vascular alterations due to hypertension, studied to date, are a reduction in NO- and COX-dependent vasodilatory pathways (Minuz et al., 1990; Raij, 2006; Smith et al., 2011). However, little is known about the EDHF-dependent mechanisms, specifically H₂S-dependent vasodilation, in the cutaneous circulation of humans.

**Alterations in H₂S physiology in Hypertension**

H₂S plays a prominent role in the development of essential hypertension. Genetic deletion of the enzymes that produce H₂S leads to pronounced hypertension (Yang et al., 2008). Moreover, spontaneously hypertensive rats have lower plasma H₂S concentrations and pronounced endothelial dysfunction (Zhao et al., 2008). In a hypertensive mouse model, treatment with a H₂S donor reduced blood pressure, and improved endothelial function through a reduction in oxidative stress mechanisms (Al-Magableh et al., 2015). These data in the animal models suggest diminished bioavailability of H₂S leads to the development of hypertension and supplementation with exogenous H₂S was an effective treatment to correct hypertensive-vascular pathology. However, the specific mechanisms by which this occurs and the translation to human hypertensive vascular pathology remains unknown.

To date, no studies have systematically examined the role of H₂S in vascular function *in vivo* in humans. Indirect measures in humans indicate that plasma concentrations of H₂S are negatively correlated with systolic and diastolic blood pressure (Whiteman et al., 2010a).
Collectively, the animal data and indirect human data suggest that \( \text{H}_2\text{S} \) signaling may be a key mechanism underlying hypertensive vascular pathology in humans, and may be a viable pathway for the development of targeted therapeutics.

**Summary**

Four studies utilizing three different *in vivo* protocols and one *in vitro* protocol were conducted to characterize the role of \( \text{H}_2\text{S} \) in the cutaneous circulation of humans. The aim of the *in vitro* study was to determine the presence of \( \text{H}_2\text{S} \) generating enzymes in the human cutaneous circulation. The first series of *in vivo* microdialysis studies examined (1) exogenous \( \text{H}_2\text{S} \)-mediated vasodilation, (2) possible interactions with NO and products of COX, and (3) the downstream potassium channels activated to elicit vasodilation in healthy young adults. The second set of *in vivo* microdialysis studies examined \( \text{H}_2\text{S} \)-mediated vasodilation in pre/stage 1 hypertensive adults. The final study utilized the technique of reactive hyperemia to assess EDHF-dependent vasodilation in normotensive and pre/stage 1 hypertensive adults and the possible contribution of \( \text{H}_2\text{S} \) as an EDHF to this response.

**Specific Aims and Hypotheses**

**Specific Aim 1:** The purpose of the study “Evidence for a functional vasodilatory role for hydrogen sulfide in the human cutaneous microvasculature” was to (1) determine the presence of enzymatic sources of \( \text{H}_2\text{S} \) in human cutaneous microvasculature, (2) characterize the vasodilator responsiveness to exogenous \( \text{H}_2\text{S} \), and (3) determine downstream potassium channel targets of \( \text{H}_2\text{S} \).
Hypothesis 1a: The enzymes CSE and 3-MPST would be present in the cutaneous circulation of young healthy adults.

Hypothesis 1b: Exogenous donors of H$_2$S would elicit vasodilation in a dose-dependent manner.

Hypothesis 1c: H$_2$S-induced vasodilation would be mediated by potassium ATP channels.

Hypothesis 1d: Functional vasodilatory interaction would exist between H$_2$S and other vasodilatory pathways, NO and COX products, to mediate vasodilation in the cutaneous circulation of humans.

Specific Aim 2: The purpose of the study “Altered vasodilatory properties of hydrogen sulfide in the human cutaneous microvasculature in pre/stage 1 hypertensive humans” was to examine alterations in H$_2$S-mediated vasodilation in pre/stage 1 hypertensive adults. We evaluated the contribution of endogenous H$_2$S and its interaction with the NO-dependent signaling pathway during cholinergic-mediated (endothelium-dependent) vasodilation. Additionally, we aimed to determine if there was reduced end-organ sensitivity to exogenous H$_2$S in the cutaneous circulation in pre/stage 1 hypertensive adults.

Hypothesis 2a: Inhibition of endogenous H$_2$S production would attenuate ACh-mediated vasodilation in normotensive age-matched adults but not in pre/stage 1 hypertensive adults.

Hypothesis 2b: Inhibition of endogenous NO production would attenuate ACh-mediated vasodilation to a greater extent in normotensive age-matched adults compared to pre/stage 1 hypertensive adults.
Hypothesis 2c: Pre/stage 1 hypertensive adults would have a similar vasodilatory response to exogenous H\textsubscript{2}S compared to normotensive age-matched adults.

Hypothesis 2d: In pre/stage 1 hypertensive adults, H\textsubscript{2}S-mediated vasodilation would not be partially mediated through vasodilatory interaction with NO or COX vasodilatory pathways, while in normotensive adults this functional vasodilatory interaction would be preserved.

Specific Aim 3: The purpose of the study “The contribution of H\textsubscript{2}S to the reactive hyperemic response in the cutaneous circulation of pre/stage 1 hypertensive and normotensive adults” was to explore the role of H\textsubscript{2}S and NO in the overall reactive hyperemic response in the cutaneous circulation of pre/stage 1 hypertensive and age-matched normotensive adults.

Hypothesis 3a: Inhibition of CSE would blunt the hyperemic response in pre/stage 1 hypertensive adults to a greater extent compared with normotensive age-matched adults.

Hypothesis 3b: Inhibition of NOS would not affect the hyperemic response in pre/stage 1 hypertensive adults or normotensive age-matched adults.

Hypothesis 3c: Measurements of reactive hyperemia would be related to functional measures of ACh-mediated vasodilation.
Chapter 2
REVIEW OF LITERATURE

Hydrogen Sulfide

Hydrogen sulfide (H$_2$S), once known for its potent scent of rotten eggs and toxicity, has recently emerged as a viable target for therapeutic intervention for many cardiovascular diseases in humans. H$_2$S is a colorless, odoriferous gaso-transmitter (along with nitric oxide (NO) and carbon monoxide (CO)) with a diverse physiologic profile (Zhao et al., 2003). H$_2$S can be smelled at concentrations less than 1 ppm, results in headaches at 4 ppm and becomes lethal at very high concentrations (Reiffenstein et al., 1992). However, under physiological conditions, H$_2$S does not accumulate nor is it toxic to cells due to balanced cellular metabolism (Furne et al., 2001). Additionally, H$_2$S has significant vasoactive properties and plays a critical role in the modulation of vascular function (Zhao et al., 2001; Yang et al., 2008; Papapetropoulos et al., 2014); however, little is known about H$_2$S physiology in humans.

A gaso-transmitter

H$_2$S is the most recent gas molecule to be dubbed a gaso-transmitter with physiological relevance. In order to meet this classification a gas molecule must meet five primary criteria that have clearly been defined previously (Wang, 2002). First, the gas molecule must be small. H$_2$S has a molecular weight of 34.08 g/mol. Second, the gas molecule must be freely permeable to membranes and not be dependent on membrane receptors. H$_2$S freely passes through plasma membranes with solubility in lipophilic solvents that is approximately fivefold greater than in
water (Wang, 2002). Third, the gas molecule must be produced endogenously via enzyme(s) that regulate production. H$_2$S is enzymatically produced by several enzymes that are tissue specific and ubiquitously expressed throughout the human body (Lu et al., 1992; Lefer, 2007; Li et al., 2011a; Wang, 2012a; Yang et al., 2013). Fourth, gaso-transmitters must have distinct functions at physiologically relevant concentrations. Though the role of H$_2$S in various species, tissues and concentrations remains to be explored, there is a clear functional role for H$_2$S at physiological concentrations in vivo (Zhao et al., 2001; Liang et al., 2011; Mustafa et al., 2011). Fifth, the cellular effects of the gas molecule may or may not be facilitated by second messengers; however, they must have cellular or molecular targets. Specific to the vasculature, H$_2$S activates multiple potassium channels (K$_{ATP}$, SK$_{Ca}$, IK$_{Ca}$ and BK$_{Ca}$ channels) on the endothelium and vascular smooth muscle (VSM) leading to vasodilation (Zhao et al., 2001; Gallego et al., 2008; Liang et al., 2011; Mustafa et al., 2011; Jackson-Weaver et al., 2013; Fitzgerald et al., 2014). Less often, H$_2$S causes vasoconstriction in select tissues and species, and the mechanisms behind this functional difference is likely due to alterations in the interactions with NO and cyclooxygenase (COX) pathways (Ping et al., 2015). Though research continues to elucidate the specific vascular signaling mechanisms of H$_2$S in vivo, it is clear that H$_2$S conforms to the requirements to be a gaso-transmitter of physiological relevance along with NO and CO.

**Production & Excretion**

It is important to note that the term “hydrogen sulfide” is ubiquitously used to refer to H$_2$S, bisulfide (HS$^-$) and sulfide (S$^{2-}$). H$_2$S easily dissolves in water and is dissociated into H$^+$, HS$^-$ and S$^{2-}$ (Figure 2-1). Under physiological conditions, approximately 20% exists as H$_2$S while 80% exists as HS$^-$ (Warenycia et al., 1989; Goodwin et al., 1989; Savage & Gould, 1990). H$_2$S is produced endogenously through enzymatic activity, non-enzymatic pathways, and through
intracellular sulfur stores (Li et al., 2011). \( \text{H}_2\text{S} \) is principally synthesized enzymatically via the pyridoxal phosphate-dependent enzymes cystathionine-\( \gamma \)-lyase (CSE) and cystathionine-\( \beta \)-synthase (CBS), and independently by 3-mercaptoppyruvate sulfurtransferase (3-MPST). Both CSE and CBS are pyridoxal-5’-phosphate-dependent (vitamin B6) enzymes that use amino acids L-cysteine, L-homocysteine and L-cystathionine to produce \( \text{H}_2\text{S} \) (Wang, 2012a). CSE and CBS are tissue specific; CSE is primarily expressed in the vasculature (Webb et al., 2008), liver (Lefer, 2007) and kidney (Yang et al., 2013), while CBS functions mainly in the central nervous system (Lu et al., 1992; Hosoki et al., 1997; Levonen et al., 2000; Meier et al., 2001). 3-MPST generates \( \text{H}_2\text{S} \) from 3-mercaptoppyruvate sulfurtransferase, which is produced by cysteine aminotransferase from L-cysteine and \( \alpha \)-ketoglutarate in the presence of the cofactors thioredoxin and dihydrolipoic acid (Shibuya et al., 2009b, 2009a; Mikami et al., 2011a). 3-MPST is expressed in a variety of tissues including the brain and peripheral vasculature (Shibuya et al., 2009b; Bucci et al., 2014). Non-enzymatic production of \( \text{H}_2\text{S} \) via reduction of elemental sulfur using reducing equivalents obtained from oxidation of glucose also occurs but is less important to the overall pool of available endogenous \( \text{H}_2\text{S} \) (Searcy & Lee, 1998). \( \text{H}_2\text{S} \) is metabolized by oxidation in the mitochondria or through methylation in the cytosol. In the mitochondria, \( \text{H}_2\text{S} \) is oxidized by sulfide quinone oxidoreductase to produce persulfide. Persulfide is then oxidized by sulfur dioxygenase to sulfite. Finally, sulfite is metabolized by rhodeonese to produce thiosulfate which can be excreted by the kidney (Beauchamp et al., 1984).

\[
\begin{align*}
\text{H}_2\text{S}_{(g)} + \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{S}_{(aq)} \rightleftharpoons \text{H}^+ + \text{HS}^- \rightleftharpoons \text{H}^+ + \text{S}^{2-}
\end{align*}
\]

**Figure 2-1:** Chemistry of \( \text{H}_2\text{S} \) gas as it dissolves into an aqueous solution.
H$_2$S elicits vasodilation in the peripheral vasculature making CSE and 3-MPST of primary interest because both enzymes are expressed in the endothelium and are likely the primary enzymatic sources of H$_2$S in blood vessels (Hosoki et al., 1997; Webb et al., 2008; Yang et al., 2008). CSE and 3-MPST are both regulated by Ca$^{2+}$/calmodulin stimulation in the endothelial cell (Mikami et al., 2011b). The localization of CSE in the human mammary arteries further validates the ubiquitous expression of CSE in human vasculature and the importance of H$_2$S to contribute to vascular homeostasis in healthy humans (Papapetropoulos et al., 2014).

A balance of endogenous production and metabolism determines the physiological concentrations of H$_2$S. A critical question that remains, with regard to H$_2$S production and function, is what constitutes biologically significant concentrations of H$_2$S in vivo. The current literature suggests differences in H$_2$S concentration that vary by over a 105-fold concentration range. The variability in measured H$_2$S concentration is due to limitations in the technology available to measure H$_2$S in vivo (Kabil & Banerjee, 2010), thus the physiologically relevant concentration of H$_2$S remains elusive at this time.

**Exogenous Vasodilation & Potassium channels**

Exogenous administration of H$_2$S elicits vasodilation via hyperpolarization of the VSM in both in vitro and in vivo preparations in rodent models (Zhao et al., 2001; Li et al., 2008; Sun et al., 2011; Tian et al., 2012; Chitnis et al., 2013). This has been substantiated in isolated human internal mammary arteries (Webb et al., 2008). It is important to note that the degree of H$_2$S-mediated vasodilation is variable throughout the vascular tree (Hosoki et al., 1997; Zhao et al., 2001). The increased importance of H$_2$S-induced vasodilation in smaller resistance vessels lends support to H$_2$S as an endothelial derived hyperpolarizing factor (EDHF). In the animal literature,
the primary mechanism by which H$_2$S induces vasodilation is through activation of potassium ATP (K$_{ATP}$) channels and subsequent membrane hyperpolarization while there is minimal evidence in human models (Zhao et al., 2001; Distrutti et al., 2006; Gallego et al., 2008; Liang et al., 2011; Fitzgerald et al., 2014). Similar vasodilatory properties have been demonstrated for pinacidil (a K$_{ATP}$ channel activator), sodium sulfide (Na$_2$S), and sodium hydrosulfide (NaHS) in piglet cerebral arteriole smooth muscle cells. Glybenclamide (a selective K$_{ATP}$ channel inhibitor) fully abolished the pinacidil-induced vasodilation and partially blunted (58%) the vasodilation induced by the H$_2$S donors in vitro (Liang et al., 2011). Thus, there are additional potassium channels that mediate H$_2$S-induced vasodilation.

More recently, small, intermediate, and big calcium-dependent potassium channels (SK$_{Ca}$, IK$_{Ca}$ and BK$_{Ca}$, respectively) have emerged as additional pathways by which H$_2$S mediates vasodilation in resistance vessels (Mustafa et al., 2011; Jackson-Weaver et al., 2013). This is contrary to the primary target most commonly reported in the animal literature, K$_{ATP}$ channels (Zhao et al., 2001). Figure 2-2 is a putative schematic of H$_2$S signaling in the vasculature. Collectively, evidence from studies in rodents, and limited studies in vitro in human tissues, suggest that H$_2$S is an EDHF that activates downstream potassium channels with significant vasoactive properties.
Figure 2-2: Putative schematic of hydrogen sulfide signaling in the vasculature.

**Endothelial Derived Hyperpolarizing Factor**

In 1988, Taylor and Weston conceived the term “hyperpolarizing factor” as an explanation for the endothelium-dependent but NO-independent vasodilation that occurs in response to bradykinin or acetylcholine (Taylor & Weston, 1988). The term now widely used is EDHF in reference to this component of endothelium-dependent vasodilation. There are three main elements that constitute an EDHF: it must (1) be produced by the endothelium, (2) be released in response to vasoactive stimuli (bradykinin or acetylcholine) and (3) elicit vasodilation in response to $K_{Ca}$ channel stimulation (Quilley et al., 1997). EDHF is not a single molecule but instead a group of molecules that may serve as a redundant pathway, each contributing to vasodilation in different proportions dependent upon species and the tissue being studied. Arachidonic acid metabolites, hydrogen peroxide, carbon monoxide, lipoxygenases and cytochrome P450 pathways and $H_2S$ are all supported in the vascular literature as likely EDHFs.
Though NO has the capacity to hyperpolarize the vascular smooth muscle, both directly and indirectly, it is not the sole hyperpolarizing factor given hyperpolarization still persists following NO blockade (Bény & Brunet, 1988; Robertson et al., 1993; Bolotina et al., 1994; Miyoshi & Nakaya, 1994).

H₂S meets all three criteria to be considered an EDHF. It is produced in the vascular endothelium by both CSE and 3-MPST and is released from the endothelium via cholinergic stimulation (Mustafa et al., 2011; Wang, 2012a). Finally, the primary mechanism by which H₂S induces vasodilation, in animals, is through activation of K_{ATP} channels (Zhao et al., 2001; Distrutti et al., 2006; Gallego et al., 2008; Liang et al., 2011).

**Interaction with NO and COX**

NO- and COX-dependent vasodilatory pathways induce VSM relaxation through activation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), respectively. H₂S-induced vasodilation occurs independent from cGMP and cAMP mechanisms and instead mediates direct hyperpolarization of the VSM by activation of potassium channels (Zhao et al., 2001). However, the signaling mechanisms of H₂S are made more complex by its interaction with both NO and products of COX in the modulation of vascular tone (Hu et al., 2008; Minamishima et al., 2009).

Several studies have demonstrated significant interaction between H₂S-donors and NO signaling pathways. Findings from studies examining this molecular interaction are complex, with reports of both a positive (Cai et al., 2007; Yong et al., 2008; Whiteman & Moore, 2009; Lei et al., 2010; Wang et al., 2010; Kondo et al., 2013) and negative interactions (Oh et al., 2006; Li et al., 2006; Geng et al., 2007; Kubo et al., 2007; Prathapasinghe et al., 2008). For example, mice treated orally with H₂S demonstrated an up-regulation of endothelial NO synthase (eNOS) and
increased NO bioavailability (Kondo et al., 2013). In contrast, H$_2$S has been demonstrated to inhibit eNOS activity via alterations in the co-factor tetrahydrobiopterin (Kubo et al., 2007).

Limited data exist regarding the signaling interaction between H$_2$S and COX-derived by-products. H$_2$S has been shown to up-regulate the COX-2/prostaglandin pathway, creating a pro-vasodilatory environment, in isolated pulmonary artery smooth muscle cells (Li et al., 2014). Alternatively, in pre-constricted bovine mesenteric vessels, the use of a COX inhibitor (flurbiprofen) in combination with a H$_2$S donor resulted in augmented vasodilation (Chitnis et al., 2013). The reasons for these differing results may be associated with methodological differences, including the choice of COX inhibitor, the type of H$_2$S donor, and the vascular bed examined.

It is probable that H$_2$S contributes to vasodilation independently and through interaction with NO and products of COX. This makes H$_2$S an important target in clinical populations such as humans with essential hypertension.

**Clinical Relevance in Hypertension**

A healthy endothelium is required in the vascular system in order to maintain the ability to vasodilate, inhibit adhesion and migration of leukocytes, suppress smooth muscle cell proliferation and inhibit platelet aggregation and clotting. In essential hypertension, as well as other cardiovascular diseases, the endothelium is damaged and no longer functions to fully vasodilate to a given stimuli (Triggle et al., 2012). The systemic endothelial dysfunction present in hypertension contributes to promote increased arterial blood pressure (Lüscher, 1994). Reduced bioavailability of NO is one of the central mechanisms responsible for endothelial dysfunction.

As mentioned previously EDHFs, more specifically H$_2$S, likely serve as backup endothelium-dependent vasodilator mechanisms for the primary NO-dependent mechanism.
However, dysregulated production of \( \text{H}_2\text{S} \) is apparent in multiple hypertensive models. CSE knockout mice present with severe hypertension as well as an abolished endothelium-dependent vasodilation in the mesenteric artery in response to an endothelium-dependent agonist (methacholine) (Yang et al., 2008). Spontaneously hypertensive rats have significantly lower plasma \( \text{H}_2\text{S} \) concentrations and lower vascular production of \( \text{H}_2\text{S} \) compared to normotensive control rats, due to down regulation of the CSE-L-cysteine pathway (Zhao et al., 2008).

Similarly in humans, \( \text{H}_2\text{S} \) concentrations were lower in a study of hypertensive children that had higher systolic blood pressure compared to normotensive age-matched controls (Chen et al., 2007). Furthermore, in a study of adult males, plasma concentrations of \( \text{H}_2\text{S} \) were negatively correlated with systolic and diastolic blood pressure (Whiteman et al., 2010a). In hypertension, when NO bioavailability is clearly diminished and the endothelium is compromised, there is a compounding issue that one of the proposed backup mechanisms, \( \text{H}_2\text{S} \)-mediated vasodilation, is also compromised leaving a minimal functional vascular reserve. However, this information highlights a potential for therapeutic intervention with a focus on enzymatic \( \text{H}_2\text{S} \) production or supplementation.

**Delivery Strategies**

Sulfide salts are the most common \( \text{H}_2\text{S} \) donors utilized to examine the role of \( \text{H}_2\text{S} \) in health and disease. \( \text{NaHS} \) and \( \text{Na}_2\text{S} \), have been widely used in rodent models to examine \( \text{H}_2\text{S} \)-mediated vascular function (Zhao et al., 2001; Wang et al., 2010; Papapetropoulos et al., 2014). Both donors are inorganic salts that quickly dissolve in solution, resulting in the immediate formation of \( \text{H}_2\text{S} \) in a pH-dependent manner and are thus considered “fast \( \text{H}_2\text{S} \) generators.” The use of this class of “fast \( \text{H}_2\text{S} \) generators” *in vivo* in animals results in significant dose-dependent vasodilation in the vasculature (Zhao et al., 2001; Tian et al., 2012). Upon being dissolved in an
aqueous solution, sulfide salts instantaneously release a bolus amount of \( \text{H}_2\text{S} \) gas that dissipates within seconds (Li et al., 2006; Whiteman et al., 2010b). It is clear that the bolus release of \( \text{H}_2\text{S} \) does not mimic the slow and sustained release that is more representative of \( \text{H}_2\text{S} \) release \textit{in vivo}. A more stable, pH neutral, injectable formulation of \( \text{Na}_2\text{S} \) is IK-1001 (Szabó, 2007; Rose et al., 2015). The functional \textit{in vivo} effects of exogenous \( \text{H}_2\text{S} \) in this formulation have been studied in animals and humans (Insko et al., 2009; Toombs et al., 2010). Utilizing a bolus intravenous infusion in healthy human adults, IK-1001 did not elicit alterations in systemic blood pressure; however, no direct assessment of conduit or microvessel reactivity was performed (Toombs et al., 2010). With careful timing of procedures there is utility in the use of “fast generators” to elucidate signaling mechanisms of \( \text{H}_2\text{S} \), but this sheds limited light to the chronic influences of \( \text{H}_2\text{S} \) on the vasculature.

To mimic the physiological release of \( \text{H}_2\text{S} \), modified pharmacological compounds have been designed for a slow, sustained release that is more similar to \textit{in vivo} \( \text{H}_2\text{S} \) production. Non-steroidal anti-inflammatory drugs (NASIDs) have been combined with ADT-OH \([5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione]\) which releases \( \text{H}_2\text{S} \) for \textit{in vivo} use at a low and sustained rate. Although the use of these drugs has been efficacious to treat inflammatory bowel disease, acute inflammation, edema, and endotoxic shock, the effects of these drugs on the vasculature are unknown (Fiorucci et al., 2005; Li et al., 2009; Whiteman & Winyard, 2011; Fiorucci & Santucci, 2011; Geng et al., 2015). A significant drawback to this class of drugs is the inability to decipher their precise mechanism of action. ADT-OH derivatives have biological effects that are separate from the actions of released \( \text{H}_2\text{S} \). For example, ADT-OH can active Nrf-2-dependent phase II enzymes, which leads to up regulation of various antioxidant pathways that could account for improved vascular function independent of \( \text{H}_2\text{S} \) (Whiteman & Winyard, 2011; Fiorucci, 2011).
Recent developments have led to the synthesis of an individual molecule GYY4137 (not a structurally modified established drug), which is a slow-releasing H$_2$S compound. GYY4137 donates two molecules of H$_2$S for every molecule of GYY4137 (Li et al., 2008, 2009; Whiteman et al., 2010b). The advantage of this molecule is that its components are inactive (unlike ADT-OH), thus the direct mechanisms of slow H$_2$S release can be evaluated (Jiang et al., 2005). In vivo testing demonstrates that GYY4137 elicits endothelium-dependent vasodilation via K$_{ATP}$ channels in addition to anti-inflammatory effects on the vasculature in rats (Li et al., 2008). Metabolic and pharmacokinetic profiles for these slow releasing H$_2$S molecules are unknown.

**Conclusion and Future Perspectives**

H$_2$S is gaso-transmitter with a diverse and clinically relevant physiologic profile (Zhao et al., 2003). Irrefutably, H$_2$S has vasoactive properties and likely plays a critical role in the modulation of vascular function in health and disease (Zhao et al., 2001; Yang et al., 2008; Papapetropoulos et al., 2014). Following the identification of H$_2$S as an endogenous mediator of vascular function nearly two decades ago by Abe and Kimura, the vasodilatory and blood pressure mechanisms of H$_2$S have been extensively explored in animal models (Abe & Kimura, 1996; Zhao et al., 2001; Yan et al., 2004; Yang et al., 2008). Due to signaling differences between tissues and species, research is necessary in humans to establish the enzymatic sources of H$_2$S as well as the mechanisms of H$_2$S-mediated vasodilation. Additionally, it is necessary to fully elucidate the role of H$_2$S in health and disease. Despite a delay to understand the mechanisms of H$_2$S in the human vasculature, H$_2$S based pharmacologies have positive and clinically significant outcomes (Bucci et al., 2014) and need to be more widely explored in humans.
Chapter 3

EVIDENCE FOR A FUNCTIONAL VASODILATORY ROLE FOR HYDROGEN SULFIDE IN THE HUMAN CUTANEOUS MICROVASCULATURE

Introduction

Hydrogen sulfide (H\textsubscript{2}S), once considered a malodorous toxic gas, is now recognized as a third endogenous gaso-transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO). H\textsubscript{2}S has significant vasoactive properties and plays a role in the modulation of vascular function (Zhao et al., 2001; Yang et al., 2008; Mani et al., 2013; Papapetropoulos et al., 2014). H\textsubscript{2}S is produced endogenously through enzymatic activity, non-enzymatic pathways, and intracellular sulfur stores (Li et al., 2011b).

In peripheral vascular tissue, H\textsubscript{2}S is synthesized via the pyridoxal phosphate-dependent enzyme cystathionine-\(\gamma\)-lyase (CSE) and by 3-mercaptoppyruvate sulfurtransferase (3-MPST), and its release is dependent upon cholinergic stimulation (Mustafa et al., 2011). In aorta of healthy rats, inhibition of endogenous H\textsubscript{2}S production blunted acetylcholine-dependent vasodilation (Paredes et al., 2012). Further, CSE knockout mice exhibit decreased endogenous H\textsubscript{2}S synthesis, resulting in significant impairments in both endothelium-dependent vasodilation and hyperpolarization and also the development of pronounced hypertension (Yang et al., 2008; Mustafa et al., 2011). Collectively, these studies suggest an important functional role for H\textsubscript{2}S as an endothelium-derived hyperpolarizing factor (EDHF) in the healthy vasculature. The expression of CSE, the primary enzymatic source of H\textsubscript{2}S in the endothelium (Hosoki et al., 1997; Webb et al., 2008; Yang et al., 2008), has been demonstrated in isolated human mammary
arteries (Webb et al., 2008); however, the specific mechanistic role of H$_2$S in the microvasculature of healthy humans remains unclear.

Exogenous administration of H$_2$S elicits vascular relaxation in both in vitro and in vivo animal preparations (Zhao et al., 2001; Sun et al., 2011; Tian et al., 2012; Chitnis et al., 2013). In these models, H$_2$S-induced vascular smooth muscle relaxation occurs predominantly through activation of potassium ATP (K$_{ATP}$) channels and subsequent membrane hyperpolarization (Zhao et al., 2001). Recently, small, intermediate, and large conductance calcium-dependent potassium channels (SK$_{Ca}$, IK$_{Ca}$ and BK$_{Ca}$, respectively) have emerged as additional signaling pathways by which H$_2$S mediates vasodilation in resistance vessels (Mustafa et al., 2011; Jackson-Weaver et al., 2013). In contrast to both the NO- and cyclooxygenase (COX)-dependent pathways, which result in cyclic guanosine monophosphate (cGMP)-induced vascular smooth muscle relaxation, H$_2$S-induced vasodilation occurs independent from cGMP, inducing direct hyperpolarization of the vascular smooth muscle (Zhao et al., 2001). In addition to its direct hyperpolarizing effects, murine studies suggest that H$_2$S also interacts with both NO and by-products of COX metabolism to modulate vascular tone (Hu et al., 2008; Minamishima et al., 2009). However, to date, no studies have examined H$_2$S-induced vasodilation or potential interactions with the NO and COX signaling pathways in vivo in the healthy human vasculature.

The human cutaneous circulation is an accessible regional vascular bed that allows for the in vivo investigation of the specific molecular mechanisms mediating the regulation of vascular function in healthy, preclinical, and diseased adults (Abularrage et al., 2005; Rossi et al., 2006; Holowatz et al., 2008). Deficits in cutaneous vascular function are highly correlated with, and predictive of, vascular dysfunction evident in both the coronary and renal circulations (Khan et al., 2008; Coulon et al., 2012). Therefore, the cutaneous circulation has significant utility for examining the mechanisms underlying microvascular signaling and potential functional alterations with cardiovascular pathology (Abularrage et al., 2005; Rossi et al., 2006).
As a necessary first step to understand the functional role of H₂S signaling in health and disease, the purpose of this series of studies was to identify the presence of CSE and 3-MPST in the microcirculation and to examine the mechanisms of H₂S-induced vasodilation in young healthy humans. We hypothesized that CSE and 3-MPST would be expressed in the microvessels of the cutaneous circulation, and functionally, that H₂S-donors would elicit dose-dependent increases in cutaneous vasodilation predominately through K<sub>ATP</sub> channels. We further hypothesized that both NO and by-products of COX metabolism contribute, in part, to exogenous H₂S-induced cutaneous vasodilation.

**Materials and Methods**

**Subjects**

All experimental procedures were approved by the Institutional Review Board at The Pennsylvania State University. Verbal and written consent were obtained voluntarily from all subjects prior to participation according to guidelines set forth by the Declaration of Helsinki. Six adults participated in Protocol 1, and subjects for Protocols 2 and 3 were from a pool of ten adults. All subjects underwent a complete medical screening including a resting 12-lead electrocardiogram, physical examination, and 12-h fasting blood chemistry (Quest Diagnostics, Pittsburgh, PA). All subjects were normotensive, normocholesterolemic, non-obese, normally active, without dermatological disease, and not taking any medications. Women were tested during the early follicular phase of their menstrual cycle or during the placebo phase if taking oral contraceptives.
**In Vitro Microvascular Biochemical Analysis**

On a separate day from the *in vivo* studies, forearm skin samples were obtained via punch biopsy in five subjects (24±3 yrs), as previously described (Smith *et al.*, 2011). Skin samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Homogenates were centrifuged twice at 15000g at 4°C for 20 minutes. For western blot analysis, 1 µg total protein were resolved by SDS PAGE and electrotransferred to nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences). Antibodies used were rabbit-polyclonal anti-CSE antibody (1:1000; Protein Tech), rabbit polyclonal anti-3-MPST antibody (1:1000, Atlas), and mouse monoclonal anti-GAPDH antibody (1:5000, Novus Biologicals). Rat liver homogenate was used as a positive control for CSE expression and rat carotid artery homogenate was used as a positive control for 3-MPST. Densitometry analysis was performed using ImageJ software (NIH).

**In Vivo Vascular Function Analysis**

Protocols were performed in a thermoneutral (20-22°C) laboratory with the subject semi supine and the experimental arm at heart level. Integrated laser-Doppler flowmetry was used to measure red cell flux, an index of skin blood flow. Local skin temperature was controlled using a local heater (clamped at 33°C) placed directly above each microdialysis membrane (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). Laser-Doppler probes were secured in each local heater and used to continuously measure skin blood flow over each microdialysis fiber. An automated brachial cuff (Cardiocap 5; GE Healthcare) was used to measure arterial blood pressure on the contralateral arm every 5 minutes throughout the protocol. Procedures for preparation of pharmacological agents were held to a rigorous time schedule to ensure consistency between subjects and among experimental visits.
Protocol 1

Two intradermal microdialysis fibers (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) were inserted into the forearm for local delivery of pharmacological agents as previously described (FDA IND 105 572)(Smith et al., 2011). All pharmacological solutions were mixed in lactated Ringer’s solution immediately before use and filtered using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI)(Smith et al., 2011). The pH of each solution was 7.0 (Sigma-aldrich; Z113425-EA) and solutions were wrapped in foil to prevent photodegradation. Microdialysis sites were perfused with sodium sulfide (Na$_2$S) or sodium hydrogen sulfide (NaHS), both of which act as fast releasing H$_2$S donors (Papapetropoulos et al., 2014), at a rate of 2 µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems). Following resolution of the insertion trauma hyperemia (~60-90 min) and a 20-minute stable baseline period, increasing concentrations of Na$_2$S or NaHS were progressively perfused through the microdialysis fiber (0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM). Pilot work in our laboratory indicated that each donor elicited maximal H$_2$S-induced vasodilation at a concentration of ~60 mM in healthy adults; therefore, a purposefully wide range of doses was used in the current study. After the dose-response protocol, all sites were flushed with Ringer’s solution before 28 mM sodium nitroprusside was perfused at 4 µl/min and temperature of the local heaters was increased to 43°C to elicit maximal cutaneous vascular conductance (CVC$_{\text{max}}$).

Protocol 2

Five intradermal microdialysis probes were inserted for local delivery of the pharmacological agents: lactated Ringer’s solution (control), 5 mM glybenclamide (GLY; $K_{\text{ATP}}$ channel inhibitor), 0.1 mM senicapoc (SENI; $I_{\text{KCa}}$ channel inhibitor), 50 mM tetraethylammonium (TEA; non-specific $K_{\text{Ca}}$ channel inhibitor), or GLY+SENI+TEA (Zhao et
Based on extensive pilot work in our laboratory, as well as the results from Protocol 1, 5 mM Na$_2$S was used to elicit cutaneous vasodilation, a dosage that approximates the half maximal concentration (EC$_{50}$). Thus, following a 20-minute stable baseline, 5 mM Na$_2$S was co-perfused with the site-specific pharmacological agent until a stable plateau in skin blood flow was obtained and CVC$_{\text{max}}$ was then elicited as described above.

**Protocol 3**

Three intradermal microdialysis probes were inserted for local delivery of the pharmacological agents: lactated Ringer’s solution (control), 20 mM NG-nitro-L-arginine methyl ester (L-NAME) to inhibit NO production via nonspecific NO synthase inhibition, or 10 mM ketorolac (KETO) to inhibit downstream vasodilator products of COX. In a subset of subjects (n=6), a fourth microdialysis probe was perfused with 20 mM L-NAME + 10 mM KETO to inhibit both NO and COX vasodilatory pathways concurrently. Pilot testing in our laboratory indicates that maximal NO synthase and COX inhibition occur with these concentrations of L-NAME and ketorolac (Holowatz et al., 2005). Following a 20 minute stable baseline, Na$_2$S was co-perfused with the site specific pharmacological agent in progressively increasing concentrations (0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM), and a stable plateau in skin blood flow was obtained at each dose. Following the dose-response protocol, CVC$_{\text{max}}$ was elicited.

**Data and Statistical Analysis**

Data were collected continuously at 40 Hz and stored for offline analysis (Windaq, DataQ Instruments). Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP). Data were normalized and expressed as a percentage of
maximal CVC (%CVC \textsubscript{max}). CVC was averaged during 5 minutes of baseline, during the plateau (~3 minutes) of each NaHS/Na\textsubscript{2}S dose (Protocols 1 and 3), and during 5 minutes of Na\textsubscript{2}S-induced vasodilation (Protocol 2).

For Protocol 1, NaHS/Na\textsubscript{2}S doses were transformed to logarithmic concentrations and normalized with the lowest value of the data set at 0% and the highest value of the data set at 100%. In addition, constraints were set for the top (100) and bottom (0) to best fit parameters of the model, which allows for the comparison of the dose-response curves on a similar scale and is useful when comparing curve position among the same subjects (Cook & Bielkiewicz, 1984). For Protocol 3, Na\textsubscript{2}S doses were transformed to logarithmic concentrations, but because of differences in baseline CVC between pharmacological agents, no constraints were imposed. Sigmoidal dose-response curves with variable slope were generated using a four-parameter nonlinear regression modeling (Prism, GraphPad, San Diego CA) (Wenner \textit{et al.}, 2011; Greaney \textit{et al.}, 2014). NaHS/Na\textsubscript{2}S-induced cutaneous vasodilation was compared between microdialysis sites in each protocol by the effective concentration causing 50% of the maximal response (logEC\textsubscript{50}), as previously described (Wenner \textit{et al.}, 2011; Greaney \textit{et al.}, 2014). The differences between treatments were analyzed using an \textit{F} test for repeated measures comparisons (Prism v5.0), which takes into account all points over the entire curve as opposed to each specific dose (Wenner \textit{et al.}, 2011). A one-way repeated-measure ANOVA (SigmaPlot 12.5) was used to determine differences in the increase in cutaneous vasodilation from baseline for each drug treatment (Protocol 2). Results are reported as means ± SE, and the alpha level was set at P<0.05.
Results

Sample western blots for CSE and 3-MPST expression in the human microvasculature are presented in Figure 3-1. All cutaneous samples tested (4 men, 1 woman; 24±3 yrs; resting MAP 88±3 mmHg; body mass index 26±6 kg/m²) expressed both CSE and 3-MPST, confirming their presence in the human cutaneous microvasculature.

Subject characteristics for the in vivo functional protocols are presented in Table 3-1. NaHS and Na₂S dose-response curves are illustrated in Figure 3-2. Both H₂S donors elicited dose-dependent vasodilation. There were no differences in cutaneous vascular responsiveness, assessed as the logEC₅₀, between the H₂S donors (NaHS: 0.83 ± 0.12 v. Na₂S: 0.55 ± 0.17; P=0.19). Because there were no differences in the cutaneous vasodilatory response to either H₂S donor, Na₂S was utilized in subsequent protocols.

As expected, Na₂S induced cutaneous vasodilation above baseline. TEA and GLY+SENI+TEA significantly reduced Na₂S-induced cutaneous vasodilation, whereas GLY or SENI alone had no effect (Fig. 3-3).

Na₂S dose-response curves with concomitant pharmacological inhibition of NO and COX are depicted in Figure 3-4. There was a difference in baseline CVC between treatments (Ringers: 7.9±1.1; KETO: 16.5±3.1; L-NAME: 6.1±0.8; KETO+L-NAME: 10.8±2.2 %CVC₅₀; P<0.01). Perfusion of KETO or L-NAME alone blunted cutaneous H₂S-induced vasodilation, as evidenced by the rightward shift in the dose-response curve (Fig. 3-4A, B) and increased logEC₅₀ (Ringers: 0.90 ± 0.18; KETO: 1.45 ± 0.12; L-NAME: 1.50 ± 0.07; P<0.01 for both). Combined inhibition further increased the logEC₅₀ from control (L-NAME + KETO: 1.72 ± 0.10; P<0.01); however, the attenuation in cutaneous vasodilation during combined inhibition was not statistically different from that elicited by either treatment alone (Fig. 3-4C).
Discussion

The principal new findings of the current study were: 1) CSE and 3-MPST are expressed in the human cutaneous microvasculature, 2) H$_2$S donors elicited dose-dependent vasodilation in the cutaneous circulation of healthy young adults, 3) exogenous H$_2$S-induced cutaneous vasodilation is mediated, in part, by K$_{ca}$ channels, and 4) NO and COX inhibition attenuated exogenous H$_2$S-induced cutaneous vasodilation. Taken together, these *in vitro* molecular findings suggest that the enzymes that produce H$_2$S are expressed in the microvasculature, and coupled with the *in vivo* functional findings, further suggest that H$_2$S, via K$_{ca}$ channels, may participate as a vasoactive molecule in the human cutaneous vasculature. These data confirm several lines of evidence in rodent models regarding H$_2$S-induced vasodilation and suggest a potential functional interaction between H$_2$S and both NO and by-products of COX metabolism in the control of microvascular function in healthy humans.

These findings demonstrate that CSE and 3-MPST, enzymes responsible for endogenous endothelial-derived H$_2$S production, are expressed in the cutaneous circulation in humans (Hosoki *et al.*, 1997; Shibuya *et al.*, 2009a). The localization of CSE and 3-MPST to the human cutaneous microvasculature further validates the use of intradermal microdialysis as an *in vivo* experimental technique to pharmaco-dissect the role of H$_2$S in contributing to vascular homeostasis in healthy humans. The skin is an accessible and representative vascular bed for the study of the vascular signaling mechanisms (Abularrage *et al.*, 2005; Rossi *et al.*, 2006; Holowatz *et al.*, 2008). CSE has been detected in isolated human mammary arteries (Webb *et al.*, 2008), and when considered together with the results of the present investigation, provides further support for the ubiquitous expression of CSE, and subsequent endogenous production of H$_2$S, in human vasculature. A secondary enzymatic source of H$_2$S, 3-MPST, was also detected. 3-MPST has been reported to
localize to both the vascular endothelium and smooth muscle (Shibuya et al., 2009a; Yadav et al., 2013), and likely represents an additional source of H$_2$S in the cutaneous circulation.

The H$_2$S donor molecules used in the present study, NaHS and Na$_2$S, have been widely used in rodent models to examine H$_2$S-mediated vascular function (Siebert et al., 2008; Wang et al., 2010; Liu et al., 2012; Chen et al., 2013). Both donors are inorganic salts that quickly dissolve in solution, resulting in the immediate formation of H$_2$S in a pH-dependent manner, and are therefore considered “fast H$_2$S generators” (Papapetropoulos et al., 2014). The use of these “fast H$_2$S generators” in the present study resulted in significant dose-dependent vasodilation in the cutaneous circulation. Further, there was no difference in cutaneous vascular responsiveness between donors. To date, only one other study has examined the functional in vivo effects of exogenous H$_2$S in humans; that study utilized a bolus intravenous infusion of the H$_2$S donor IK-1001 in healthy adults (Toombs et al., 2010). IK-1001 did not elicit alterations in systemic blood pressure; however, no direct assessment of conduit or microvessel H$_2$S-mediated vasoreactivity was performed (Toombs et al., 2010). Future studies measuring H$_2$S production and utilizing direct pharmacological inhibition of endogenous H$_2$S are needed to confirm the physiological function of H$_2$S in the human vasculature.

Rodent studies suggests that H$_2$S elicits vasodilation predominantly through activation of K$_{ATP}$ channels and subsequent membrane hyperpolarization (Zhao et al., 2001; Cheng et al., 2004; Szabó, 2007; Webb et al., 2008; Tay et al., 2010; Kimura, 2011). More recently, SK$_{Ca}$, IK$_{Ca}$ and BK$_{Ca}$ have emerged as additional pathways by which H$_2$S mediates vasodilation in resistance vessels (Mustafa et al., 2011; Jackson-Weaver et al., 2013). In the present investigation, K$_{ATP}$ channel inhibition with glybenclamide did not significantly affect the vasodilatory response to exogenous Na$_2$S. Contrary to our initial hypothesis, our data suggest that K$_{ATP}$ channels do not appear to mediate exogenous H$_2$S-induced cutaneous vasodilation. Instead, H$_2$S-induced cutaneous vasodilation is mediated, at least in part, by TEA-sensitive K$_{Ca}$ channels.
The differences between the findings of the present investigation regarding the specific channels mediating H$_2$S-induced vasodilation and those reported using murine models may be a result of the use of *in vivo* versus *in vitro* assessment of vascular signaling mechanisms, as well as the concentrations of H$_2$S delivered to the tissue. In addition, very few selective pharmacological SK$_{Ca}$, IK$_{Ca}$, or BK$_{Ca}$ channel inhibitors are currently approved for use in humans (Ataga *et al.*, 2008; Brunt & Minson, 2012), making it challenging to precisely examine these mechanisms in an *in vivo* experimental paradigm. However, senicapoc, a specific antagonist for IK$_{Ca3.1}$ channels (Ataga *et al.*, 2008), did not affect the cutaneous vasodilation in response to exogenous H$_2$S, suggesting that this mechanism does not contribute to the cutaneous vascular responses to H$_2$S in humans.

In the current study, we also investigated potential cross-talk that may exist *in vivo* between H$_2$S signaling and the endothelial-derived signaling molecules NO and downstream by-products of COX metabolism. Our data suggest that both NO and COX-derived metabolic by-products are required for full expression of exogenous H$_2$S-induced cutaneous vasodilation. The literature regarding the interaction between H$_2$S and NO is equivocal (Li *et al.*, 2006; Cai *et al.*, 2007; Kubo *et al.*, 2007; Wang *et al.*, 2010; Kondo *et al.*, 2013). For example, mice treated orally with H$_2$S have an up-regulation of endothelial NO synthase and increased NO bioavailability (Kondo *et al.*, 2013). In contrast, H$_2$S has also been reported to inhibit endothelial NO synthase activity via alterations in the co-factor tetrahydrobiopterin (Kubo *et al.*, 2007). The present findings suggest a synergistic interaction between exogenous H$_2$S and NO in mediating cutaneous vasodilation in young adults, evidenced by attenuated H$_2$S-induced cutaneous vasodilation during NO synthase inhibition with L-NAME. Limited data exist regarding the signaling interaction between H$_2$S and COX-derived by-products. H$_2$S upregulates the COX-2/PGI$_2$ pathway in isolated human pulmonary artery smooth muscle cells, creating a pro-vasodilatory environment (Li *et al.*, 2014). We found a significant interaction, albeit weaker, between H$_2$S and COX by-
products, evidenced by the reduced vascular sensitivity to exogenous H$_2$S during concurrent COX inhibition. In contrast to the blunted vasodilatory response during COX inhibition in the current study, the use of the COX inhibitor flurbiprofen during H$_2$S-induced vasodilation with a slow-release donor resulted in augmented vasodilation in pre-constricted bovine conduit vessels (Chitnis et al., 2013). The reasons for these differing conclusions are unclear but may be related to the choice of COX inhibitor, the type of H$_2$S donor, the vascular bed examined, or the difference between *in vitro* and *in vivo* investigation of signaling mechanisms. Surprisingly, concurrent inhibition of both NO and COX signaling pathways did not further attenuate cutaneous vasodilation in response to exogenous H$_2$S. However, when considered collectively, the findings of the current investigation indicate that exogenous H$_2$S interacts with both NO and COX-derived metabolic byproducts to mediate cutaneous vasodilation in healthy humans.

As a necessary first step, the present study utilized exogenous sources of H$_2$S to characterize cutaneous vascular end-organ responsiveness and the mechanisms by which these responses occur. We did not specifically test the functional role of H$_2$S in the control of cutaneous blood flow during physiological stimuli (i.e., local skin and reflex heating). However, given the extensive work that has been done to determine the mechanisms underlying the control of skin blood flow during these physiological stimuli, we speculate that H$_2$S may contribute as a putative EDHF during local heating-induced and reflex-mediated cutaneous vasodilation. Local heating-induced increases in cutaneous blood flow are primarily mediated by endothelial NO synthase (Bruning et al., 2012) and epoxyeicossatrienioc acid-dependent mechanisms (Brunt & Minson, 2012); however, as an EDHF, H$_2$S may also contributing to local thermal hyperemia. During reflex cutaneous vasodilation, both NO and COX-derived vasodilator products, along with EDHFs, contribute to the full expression of this response (McCord et al., 2006). Given the role of cholinergic nerves (Kellogg et al., 1995) and the downstream interactions of these pathways in
reflex cutaneous vasodilation (McCord et al., 2006), it seems plausible that H₂S may also play a role as a putative EDHF during reflex vasodilation.

These studies served to characterize the mechanisms mediating the functional vasodilatory role of H₂S in the healthy human vasculature. We demonstrated the presence of the enzymes responsible for endogenous H₂S production in the cutaneous microvasculature and suggest a role for H₂S in mediating cutaneous vascular relaxation. These findings have potential implications for an important functional role for H₂S in vivo. Future studies designed to delineate the physiological role of H₂S in modulating vascular function in humans are warranted, particularly studies aimed at measuring H₂S production and utilizing direct pharmacological inhibition of endogenous H₂S. There is substantial evidence from rodent studies suggesting that dysregulation of H₂S likely plays an important role in the pathogenesis of hypertension-associated vascular dysfunction via its effects on blood pressure regulation, vessel function and remodeling, and inflammatory processes (Yan et al., 2004; Jiang et al., 2005; Liu et al., 2010; Taniguchi et al., 2011). Future investigation of the potential role for H₂S in the modulation of vascular regulation in humans may therefore have important clinical implications for understanding the mechanisms underlying the vascular dysfunction characteristic of multiple cardiovascular pathologies.
Table 3-1: Subject characteristics.

<table>
<thead>
<tr>
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<th>Protocol 1</th>
<th>Protocols 2/3</th>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>4M, 2W</td>
<td>5M, 5W</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>25 ± 3</td>
<td>24 ± 3</td>
</tr>
<tr>
<td><strong>BMI (kg/m^2)</strong></td>
<td>25 ± 1</td>
<td>25 ± 1</td>
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<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>86 ± 4</td>
<td>86 ± 8</td>
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<tr>
<td><strong>LDL (mg/dL)</strong></td>
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<td>67 ± 16</td>
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<tr>
<td><strong>HDL (mg/dL)</strong></td>
<td>55 ± 13</td>
<td>56 ± 12</td>
</tr>
<tr>
<td><strong>Total CHO (mg/dL)</strong></td>
<td>140 ± 31</td>
<td>143 ± 8</td>
</tr>
<tr>
<td><strong>HbA1C (%)</strong></td>
<td>5 ± 0.2</td>
<td>5 ± 0.2</td>
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Data are expressed as mean ± SE. BMI, body mass index; MAP, mean arterial pressure; LDL, low-density lipoproteins; HDL, high density lipoproteins; TOTAL CHO, total cholesterol; HbA1C, glycated hemoglobin.
Figure 3-1: Representative western blots depicting cystathionine-$\gamma$-lyase (CSE; Panel A) and 3-mercaptopuruvate sulfurtransferase (3-MPST; Panel B) protein expression in human cutaneous biopsy samples. All human whole skin homogenates expressed CSE and 3-MPST.
Figure 3-2: Exogenous H$_2$S dose-response curves. Cutaneous vasodilation during perfusion of the H$_2$S donors: sodium hydrogen sulfide (NaHS) and sodium sulfide (Na$_2$S).
Figure 3-3: Exogenous H$_2$S with K$^+$ channel inhibition. Summary data, expressed as the difference between baseline skin blood flow and the cutaneous vasodilation elicited by perfusion of 5 mM Na$_2$S ($\Delta_{\text{base}}$) for each pharmacological treatment site. TEA and GLY+SENI+TEA resulted in blunted vasodilation in response to Na$_2$S. *P<0.05 v. Na$_2$S.
Figure 3: H2S interactions with NO and COX. Summary data for exogenous H2S-induced cutaneous vasodilation during concurrent KETO (Panel A), L-NAME (Panel B), and KETO+L-NAME (Panel C) administration. NO and COX inhibition, separately and combined, blunted exogenous H2S-induced cutaneous vasodilation. *P<0.05 v. CONTROL.
Chapter 4

ALTERED HYDROGEN SULFIDE-MEDIATED VASODIALTION IN THE HUMAN CUTANEOUS MICROVASCULATURE IN PRE/STAGE 1 HYPERTENSIVES

Introduction

Hydrogen sulfide (H$_2$S) is a vasoactive gaso-transmitter (Hosoki et al., 1997) that functionally contributes to vascular homeostasis (Wang, 2012b). In rodent models, dysregulation of the enzymatic production and function of H$_2$S plays an important role in the pathogenesis of hypertension-associated vascular dysfunction (Yan et al., 2004; Jiang et al., 2005; Liu et al., 2010; Taniguchi et al., 2011). Mice lacking endogenous H$_2$S synthesizing enzymes develop pronounced hypertension, secondary to significant impairments in both endothelium-dependent vasodilation and vascular smooth muscle hyperpolarization (Yang et al., 2008; Mustafa et al., 2011). Further, spontaneously hypertensive rats have lower serum concentrations of H$_2$S compared with healthy wild type rats (Yan et al., 2004), and inhibition of endogenous H$_2$S production results in attenuated vasodilation in response to a cholinergic stimulus. Despite a reduction in endogenous H$_2$S production, vascular sensitivity to exogenous H$_2$S is not impaired (Yang et al., 2008). Collectively, these rodent studies suggest hypertension-associated alterations in both H$_2$S production and potentially function.

In humans, lower serum H$_2$S concentrations have been documented in various pathologies, including coronary heart disease (Jiang et al., 2005), chronic obstructive pulmonary disease (Chen et al., 2005) and chronic smokers (Chen et al., 2005). Moreover, plasma concentrations of H$_2$S are negatively correlated with blood pressure (Whiteman et al., 2010a). Even children with essential hypertension (~11 years) exhibit lower serum concentrations of H$_2$S
compared to normotensive children (Chen et al., 2007). These descriptive studies suggest that altered H$_2$S function may be characteristic of hypertensive pathology; however, to date, no studies have mechanistically examined the functional role of H$_2$S in the vasculature of hypertensive adults.

In the peripheral vasculature, H$_2$S elicits vasodilation via activation of small, intermediate, and big calcium-dependent potassium channels, in addition to potassium ATP channels, leading to subsequent membrane hyperpolarization and vascular smooth muscle cell relaxation (Zhao et al., 2001; Distrutti et al., 2006; Gallego et al., 2008; Liang et al., 2011). In addition to its direct vasodilatory properties, H$_2$S also induces vasodilation through nitric oxide (NO) and cyclooxygenase (COX)-dependent mechanisms in healthy young adults (Kutz et al., 2015). In humans, hypertension-induced vascular dysfunction is characterized by reductions in both NO- and COX-mediated vasodilation (Minuz et al., 1990; Nakamura et al., 2001; Cooke, 2004; Gluais et al., 2005, 2006; Giles, 2006; Raij, 2006; Smith et al., 2011). To date, no study has assessed the relative contribution and potential alterations in the signaling between H$_2$S and NO- and COX-dependent pathways in vivo in humans. This is clinically important because H$_2$S has been proposed as a secondary redundant mechanism to elicit vasodilation when NO and COX pathways are compromised.

Therefore, the aim of the present study was to examine the functional role of H$_2$S in the cutaneous microvasculature of pre/stage 1 hypertensive adults. We hypothesized that endothelium-dependent (acetylcholine; ACh) vasodilation would be attenuated in pre/stage 1 hypertensive adults and that this would be mediated by a reduction in H$_2$S-mediated vasodilation. We further hypothesize that end-organ responsiveness to exogenous H$_2$S would be preserved, but that functional vasodilatory interactions with both NO and COX signaling pathways would be diminished, in pre/stage 1 hypertensive adults.
Methods

Subjects

The Institutional Review Board at The Pennsylvania State University approved all experimental procedures. Verbal and written consent were obtained voluntarily from all subjects prior to participation according to guidelines set forth by the Declaration of Helsinki. 15 adults participated in protocol 1 and 14 adults participated in protocol 2. All subjects underwent a complete medical screening including a resting 12-lead electrocardiogram, physical examination, 24-hour blood pressure monitor (Mortara Instruments) and 12-h fasting blood chemistry (Quest Diagnostics, Pittsburgh, PA). Age-matched subjects were normotensive, normcholesterolemic, non-obese, normally active, without dermatological disease, and not taking any medications. Women were tested during the early follicular phase of their menstrual cycle or during the placebo phase if taking oral contraceptives. The same was true for all pre/stage 1 hypertensive subjects, except they were classified as having high blood pressure following the measurement of 2 resting screening blood pressures (on separate days) and a 24 ambulatory blood pressure monitor test. Pre-hypertension was defined as an average 24 hour blood pressure >130/80 or daytime pressures > 135/85 (Grossman, 2013). Stage 1 hypertension was defined as systolic blood pressure greater than 140 and/or diastolic blood pressure greater than 90 (Chobanian et al., 2003).

In Vivo Vascular Function Analysis

Protocols were performed in a thermoneutral (20-22°C) laboratory with the subject semi supine and the experimental arm at heart level. Integrated laser-Doppler flowmetry was used to measure red blood cell flux, an index of skin blood flow. Local skin temperature was controlled using a local heater (clamped at 33°C) placed directly above each microdialysis fiber (MoorLAB,
Temperature Monitor SH02, Moor Instruments, Devon, UK). Laser-Doppler probes were secured in each local heater and used to continuously measure skin blood flow over each microdialysis fiber. An automated brachial cuff (Cardiocap 5; GE Healthcare) was used to measure arterial blood pressure on the contralateral arm every 5 minutes throughout the protocol. Maximal blood flow was calculated as an average of 10 min during a stable plateau after locally heating the skin to 43°C and infusion of sodium nitroprusside. Procedures for preparation of pharmacological agents were held to a rigorous time schedule to ensure consistency between subjects and among experimental visits.

Protocol 1

Four intradermal microdialysis probes were inserted for local delivery of the pharmacological agents: lactated Ringer’s solution (control), 20 mM NG-nitro-L-arginine methyl ester (L-NAME) to inhibit NO production via inhibition of NO synthase (NOS), 0.5 mM aminoxyacetic acid (AOAA) to inhibit H₂S production via inhibition of CSE, or 20 mM L-NAME + 0.5 mM AOAA to inhibit both NO and H₂S vasodilatory pathways concurrently. Pilot work was conducted using an in vitro preparation to determine the efficacy of AOAA at inhibiting H₂S production. Following a 20-minute stable baseline, ACh was co-perfused with the site-specific pharmacological agent in progressively increasing concentrations (0.001 mM, 0.01 mM, 0.1 mM, 1 mM). Each dose was perfused until a stable plateau in skin blood flow was obtained (~5 to 10 minutes). Following the dose-response protocol, the local heaters were increased to 43 degrees Celsius and 28mM sodium nitroprusside was perfused to elicit maximum vasodilation (CVC_max).
Protocol 2

Four intradermal microdialysis probes were inserted for local delivery of the pharmacological agents: lactated Ringer’s solution (control), 20 mM NG-nitro-L-arginine methyl ester (L-NAME) to inhibit NO production via nonspecific NOS inhibition, 10 mM ketorolac (KETO) to inhibit downstream vasodilator products of COX, or 20 mM L-NAME + 10 mM KETO to inhibit both NOS and COX vasodilatory pathways concurrently. Pilot testing in our laboratory indicates that maximal NOS and COX inhibition occur with these concentrations of L-NAME and ketorolac (Holowatz et al., 2005). Following a 20 minute stable baseline, sodium sulfide (Na₂S) was co-perfused with the site specific pharmacological agent in progressively increasing concentrations (0.01 mM, 0.1 mM, 1 mM, 10 mM, 100 mM), and a stable plateau in skin blood flow was obtained at each dose (Kutz et al., 2015). Following the dose-response protocol, the local heaters were increased to 43 degrees Celsius and 28mM sodium nitroprusside was perfused to elicit maximum vasodilation (CVC_{max}).

Data and Statistical Analysis

Data were collected continuously at 40 Hz and stored for offline analysis (Windaq, DataQ Instruments). Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP). Data were normalized and expressed as a percentage of maximal CVC (%CVC_{max}). CVC was averaged during 5 minutes of baseline, during the plateau (~3 minutes) of each Na₂S dose (Protocol 1), and during the plateau (~1 min) of each ACh dose (Protocol 2).

For Protocol 1 data were graphed in Prism (GraphPad, San Diego CA). %CVC_{max} were analyzed using a three way, mixed model, repeated measures ANOVA (group * pharmacological site * ACh dose; proxmix SAS 9.3). Specific planned comparisons were performed when
appropriate to determine where difference between groups and pharmacological sites occurred with appropriate Bonferroni corrections.

For Protocol 2, (Na2S) doses were transformed to logarithmic concentrations and normalized with the lowest value of the data set at 0%. Sigmoidal dose-response curves with variable slope were generated using a four-parameter nonlinear regression modeling (Prism, GraphPad, San Diego CA) (Wenner et al., 2011; Greaney et al., 2014). Na2S-induced cutaneous vasodilation was compared between microdialysis sites by the effective concentration causing 50% of the maximal response (logEC50), as previously described (Wenner et al., 2011; Greaney et al., 2014). The differences between treatments were analyzed using an F test for repeated measures comparisons (Prism v5.0), which takes into account all points over the entire curve as opposed to each specific dose (Wenner et al., 2011).

For Protocol 1 & 2, one-way repeated-measure ANOVA (SigmaPlot 12.5) was used to determine differences in baseline and CVCmax for each drug treatment. Results are reported as means ± SE, and the alpha level was set at P<0.05.

**Results**

The physical characteristics of the subjects are presented in Table 4-1. Subjects were matched for age, body mass index, total cholesterol, and high and low-density lipoproteins. Pre/stage 1 hypertensive subjects had a significantly higher 24-hour and daytime systolic and diastolic pressure compared to normotensive controls (p<0.001).

Figure 4-1 shows the mean ACh dose-response curves at the control site and CSE inhibited site (AOAA) for normotensive: pre/stage 1 hypertensive adults. Inhibition of CSE significantly attenuated the response to exogenous ACh at all doses (5A: 0.001mM, p=0.008; 0.01mM, p=0.001; 0.1 mM, p= 0.002; 1mM, p= 0.002) compared to the control site within the
normotensive group (p< 0.0001). In pre/stage 1 hypertensive adults there was no difference between the control site and CSE inhibited site at any ACh.

Figure 4-2 shows the mean ACh dose response curves at the CONTROL site and NOS inhibited site (L-NAME) for normotensive: pre/stage 1 hypertensive adults. Inhibition of NOS attenuated the response to exogenous ACh at all doses compared to the control site in the normotensive group (4A: p<0.0001). In contrast, there was no difference between the control site and NOS inhibited site at any ACh dose in the pre/stage 1 hypertensive group.

Figure 4-3 shows the mean ACh dose response curves at the control site and NOS + CSE inhibited site (COMBO) for normotensive: pre/stage 1 hypertensive adults. Inhibition of NOS + CSE attenuated the response to exogenous ACh at all doses compared to the control site within the normotensive group (6A p<0.001), but there was no difference compared to NOS inhibition alone. In pre/stage 1 hypertensive adults there was no difference between the control site and NOS + CSE inhibited site at any ACh dose.

Figure 4-4 shows the mean ACh dose response curves at the control site for normotensive and pre/stage 1 hypertensives. Pre/stage 1 hypertensives had a blunted response to exogenous ACh compared to normotensive adults at all ACh doses (p<0.0001).

Figure 4-5 illustrates the vasodilation induced by exogenous Na₂S in a subgroup of healthy normotensive adults (avg 24 daytime BP: SBP 110 ± 4, DBP 74 ± 2) and pre/stage 1 hypertensive adults (avg 24 daytime BP: SBP 140 ± 4, DBP 88 ± 4). There was no difference in the vasodilatory response to exogenous H₂S between normotensive (EC₅₀: 1.37 ± 0.12; R²= 0.78) and pre/stage 1 hypertensive adults (EC₅₀: 1.57 ± 0.13; R²= 0.75).

Figure 4-6A illustrates the Na₂S dose response curve for normotensive adults at the CONTROL site, lactated ringers; L-NAME site, NOS inhibited; KETO site, COX inhibited and L-NAME + KETO site, NOS + COX inhibited. There was a significant difference between the CONTROL site (EC₅₀: 1.37 ± 0.12; R²= 0.78) and the L-NAME site (EC₅₀: 1.83 ± 0.07; R²=
0.89), KETO site (EC\textsubscript{50}: 2.07 ± 0.18; R\textsuperscript{2}= 0.62) and L-NAME + KETO site (EC\textsubscript{50}: 2.33 ± 0.13; R\textsuperscript{2}= 0.83) demonstrated by a shift to the right of the dose-response curves and an increase in the EC\textsubscript{50} values (p<0.001). Figure 4-6B illustrates the Na\textsubscript{2}S dose response curve for pre/stage 1 hypertensive adults at the CONTROL site (EC\textsubscript{50}: 1.57 ± 0.13; R\textsuperscript{2}= 0.75), L-NAME site (EC\textsubscript{50}: 1.64 ± 0.06; R\textsuperscript{2}= 0.78), KETO site (EC\textsubscript{50}: 2.05 ± 0.20; R\textsuperscript{2}= 0.69) and the L-NAME + KETO site (EC\textsubscript{50}: 2.25 ± 0.20; R\textsuperscript{2}= 0.66). There is no difference between the CONTROL site and the L-NAME or KETO sites. However, there was a rightward shift in the dose-response curve for the L-NAME + KETO inhibited site (EC\textsubscript{50}: 2.25 ± 0.20, p= 0.001).

**Discussion**

The novel findings of these two studies are that in pre/stage 1 hypertensive adults (1) endogenous H\textsubscript{2}S-mediated vasodilation to a cholinergic stimulus is blunted and (2) that end-organ responsiveness to exogenous H\textsubscript{2}S is preserved, despite a diminished contribution of the NOS and COX vasodilatory pathways. We also demonstrated that pre/stage 1 hypertensive adults have an attenuated NO-dependent response to a cholinergic stimulus. Collectively, reduced H\textsubscript{2}S- and NO-dependent vasodilation resulted in an overall attenuated vasodilatory response to a cholinergic stimulus in pre/stage 1 hypertensive adults compared to normotensive healthy adults.

In the present study, our *in vivo* data demonstrate that a reduction in endogenous H\textsubscript{2}S production contributes to attenuated ACh-induced vasodilation in pre/stage 1 hypertensive adults. These findings are consistent with studies in rodent models of hypertension (Yang *et al.*, 2008; Bucci *et al.*, 2014). CSE inhibition (Figure 4-1) attenuated ACh-induced vasodilation in normotensive adults by approximately 10-15 %; however, in pre/stage 1 hypertensives, CSE inhibition with AOAA had no effect on the ACh-induced vasodilation. These data suggest that impairments in H\textsubscript{2}S function in pre/stage 1 hypertensive humans contribute to vascular
endothelial dysfunction. While plasma H$_2$S concentration are negatively correlated with systolic and diastolic blood pressures in both adults and children (Chen et al., 2007; Whiteman et al., 2010a), the precise cause and effect relating impairments in H$_2$S signaling and increases in systemic blood pressure remain to be determined.

In the present study, inhibition of NOS (Figure 4-2) reduced the vasodilatory response to ACh in normotensive adults by approximately 40 %, while there was no significant functional contribution of NO to ACh-induced vasodilation in pre/stage 1 hypertensive adults. This finding is consistent across the literature demonstrating a loss of NO-dependent vasodilation in hypertensive populations (Nakamura et al., 2001; Cooke, 2004; Giles, 2006; Raij, 2006; Smith et al., 2011). Additionally, combined inhibition of both H$_2$S and NO generating enzymes (Figure 4-3) did not result in a further reduction in vasodilation compared to NOS inhibition alone in either blood pressure group. The results of the current study suggest that both H$_2$S- and NO-dependent vasodilation are significantly reduced in pre/stage 1 hypertensive adults and likely do not contribute substantially to ACh-induced vasodilation. However, in both normotensive and pre/stage 1 hypertensive subjects, a portion of the cholinergic-mediated vasodilation remained despite dual inhibition of both NOS and CSE. Thus, other vascular signaling pathways contribute to the full expression of vasodilation in response to cholinergic stimulation. Likely vasodilatory molecules include prostanoids and other potential EDHF molecules, including epoxyeicosatrienoic acids, hydrogen peroxide, and potassium ions.

While functional vasodilation to endogenous enzymatic production of H$_2$S was reduced in the pre/stage 1 hypertensive group we found no difference in end-organ responsiveness to exogenous administration of H$_2$S. This finding is consistent with the literature in rodent models (Yang et al., 2008), suggesting that vascular responsiveness to exogenous H$_2$S is preserved early in the pathogenesis of hypertension-induced vascular dysfunction. This has potentially important
clinical implications given the current development of novel pharmaceuticals with slow release H$_2$S moieties for the treatment of hypertension (Bucci et al., 2014).

We previously demonstrated that a functional interaction exists between H$_2$S and NO and by products of COX in mediating H$_2$S-induced vasodilation in the cutaneous circulation of healthy young adults (Kutz et al., 2015). In the present study we showed that the independent contribution of both NOS and COX signaling pathways to H$_2$S-induced vasodilation are reduced in pre/stage I hypertensive adults. However, when both NOS and COX are inhibited the dose-response curve to exogenous H$_2$S is shifted to the right in pre/stage 1 hypertensives. These data suggest that there is a limited functional interaction between these vasoactive molecules in contributing to H$_2$S-induced vasodilation in pre/stage 1 hypertensive adults. Despite some differences in downstream signaling between NOS and COX vasodilator pathways in the pre/stage 1 hypertensive group, vascular responsiveness to exogenous H$_2$S-induced vasodilation was preserved.

A potential limitation to our study was the use of the drug AOAA to inhibit endogenous production of H$_2$S. Currently, AOAA is the only CSE inhibitor available for use in humans. AOAA was first thought to be specific for cystathionine-β-synthase (CBS: neuronal enzymatic source of H$_2$S). However, recently AOAA has been found to be a more specific inhibitor for CSE than CBS in vitro in the vasculature (Asimakopoulou et al., 2013). We have previously shown that another enzymatic source for H$_2$S, 3-mercaptopropionate sulfurtransferase (3-MPST), is expressed in the cutaneous microvasculature (Kutz et al., 2015). AOAA may exert additional inhibitory effects on H$_2$S production through the indirect inhibition of 3-MPST via inhibition of cysteine aminotransferase (Hellmich et al., 2015). However, AOAA is not an established inhibitor of 3-MPST. Thus, how much 3-MPST actually contributes to H$_2$S generation in the vasculature and to what extent AOAA inhibits 3-MPST generation remains unclear.
In conclusion, the principle findings of the present study was that attenuated vasodilation in response to a cholinergic stimulus was mediated, at least in part, by reductions in both H$_2$S- and NO-dependent vasodilation in the cutaneous microvasculature of pre/stage 1 hypertensives. Secondly, vascular end-organ responsiveness to exogenous H$_2$S was preserved in this population despite a lack of a functional vasodilatory interaction with either NOS or COX vasodilating pathways. Collectively, these data suggest that alterations in H$_2$S function may contribute to the vascular impairments characteristic of human hypertension. H$_2$S may be a viable interventional target in the treatment of hypertension.

**Perspectives**

One is every three adults in the United States has high blood pressure, 50 % of which are uncontrolled (Nwankwo *et al.*, 2013). In 2013, more than 360,000 Americans died (~1,000 per day), with high blood pressure as a primary or contributing factor (Mozaffarian *et al.*, 2015). It is projected that by 2030, more than 40 % of Americans will suffer from hypertension. Thus, additional studies are warranted to study the systemic effects of H$_2$S in targeted pharmacotherapy for hypertensive vascular pathology. Stage I clinical trials indicate that anti-hypertensive medications with H$_2$S releasing moieties lead to better clinical outcomes in patients (Bucci *et al.*, 2014). However, whether this is due to the independent vasodilatory effect of H$_2$S or alterations in the enzymatic production of other endothelium-dependent signaling molecules within the vasculature (e.g., NO or COX products) remains to be determined.
Table 4-1: Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Protocol 1 Normotensives</th>
<th>Protocol 1 Pre/stage 1 Normotensives</th>
<th>Protocols 2 Normotensives</th>
<th>Protocol 2 Pre/stage 1 Normotensives</th>
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<tbody>
<tr>
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<td>3M, 3W</td>
<td>2M, 5W</td>
<td>2M, 5W</td>
</tr>
<tr>
<td>Age (yrs)</td>
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<td>59 ± 4</td>
<td>56 ± 1</td>
<td>60 ± 3</td>
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<td>BMI (kg/m²)</td>
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<td>30 ± 1</td>
<td>26 ± 1</td>
<td>28 ± 2</td>
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<td>24 hr daytime systolic (mmHg)</td>
<td>110 ± 4</td>
<td>140 ± 4*</td>
<td>110 ± 4</td>
<td>144 ± 5*</td>
</tr>
<tr>
<td>24 hr daytime diastolic (mmHg)</td>
<td>74 ± 2</td>
<td>88 ± 4*</td>
<td>73 ± 2</td>
<td>86 ± 3*</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>113 ± 15</td>
<td>98 ± 8</td>
<td>104 ± 11</td>
<td>110 ± 10</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>73 ± 7</td>
<td>51 ± 5</td>
<td>73 ± 6</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Total CHO (mg/dL)</td>
<td>196 ± 17</td>
<td>173 ± 12</td>
<td>192 ± 13</td>
<td>183 ± 12</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5.7 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. BMI, body mass index; MAP, mean arterial pressure; LDL, low-density lipoproteins; HDL, high density lipoproteins; TOTAL CHO, total cholesterol; HbA1C, glycated hemoglobin.
Figure 4: Exogenous ACh dose-response curves. Cutaneous vasodilation during an ACh dose response at the control site (closed circles) and CSE inhibiting site (open circles). In normotensive adults (Panel A) and pre/ stage 1 hypertensive adults (Panel B). * = p < 0.05. ACh, acetylcholine.
Figure 4-2: Exogenous ACh dose-response curves. Cutaneous vasodilatation during an ACh dose response at the control site (closed circles) and NOS inhibited site (open circles) in normotensive adults (Panel A) and pre-stage I hypertensive adults (Panel B). * = p < 0.05. ACh, acetylcholine.
Figure 4-3. Exogenous ACh dose-response curves. Cutaneous vasodilation during an ACh dose response at the control site (closed circles) and NOS inhibited + CSE inhibited site (open circles) in normotensive adults (Panel A) and pre-stage 1 hypertensive adults (Panel B). * = p < 0.05.
Figure 4-4: Exogenous ACh dose-response curves. Cutaneous vasodilation during an ACh dose response (0.001mM – 1mM) at the control site (lactated ringers) in normotensive adults (closed circles) and pre/ stage 1 hypertensive adults (open circles). * = p <0.05 ACh, acetylcholine.
Figure 4-5: Exogenous H₂S dose-response curves control site. Cutaneous vasodilation induced by exogenous sodium sulfide (Na₂S) infusion in normotensive (closed circles) and pre/stage 1 hypertensives (open circles).
Figure 4: Exogenous H2S dose-response curves. Cumulative vasodilation during perfusion of the control site, lactated Ringer’s (closed circle); COX-inhibited site, KETO (open circles); NOS-inhibited site, L-NAME (open triangle) and COX & NOS inhibited site, KETO + L-NAME (open diamond). In normotensive adults (Panel A), KETO, L-NAME and KETO + L-NAME are significantly shifted to the right * = p<0.0001. In pre/stage 1 hypertensive adults (Panel B), only the KETO + L-NAME site is significantly shifted to the right ( # = p=0.001).

Panel A: Normotensive subjects (Panel A) in normotensive subjects (Panel A), only the KETO + L-NAME site is significantly shifted to the right ( # = p=0.001).
Chapter 5

THE CONTRIBUTION OF HYDROGEN SULFIDE TO THE REACTIVE HYPEREMIC RESPONSE IN THE CUTANEOUS CIRCULATION.

Introduction

Reactive hyperemia is a pronounced vasodilation and a transient increase in blood flow following release of upstream arterial occlusion (Patterson, 1956; Blair et al., 1959; Kilbom & Wennmalm, 1976). This marked increase in blood flow is a result of myogenic relaxation as well as local release of mediators and metabolites from the ischemic tissue (Patterson, 1956; Kilbom & Wennmalm, 1976; Nowak & Wennmalm, 1979). Reactive hyperemia is used as a non-invasive test to examine cutaneous microvascular function (Stewart et al., 2004; McCord et al., 2006; Lorenzo & Minson, 2007; Cracowski et al., 2013).

There is controversy as to the precise mechanisms mediating reactive hyperemia in the cutaneous circulation. Each of the following mechanisms has been shown to modulate a portion of the reactive hyperemic response: sensory nerves (Larkin & Williams, 1993; Lorenzo & Minson, 2007; Cracowski et al., 2013), nitric oxide (NO) (Binggeli et al., 2003; Wong et al., 2003; Zhao et al., 2004; Medow et al., 2007; Lorenzo & Minson, 2007), products of cyclooxygenase (COX) (Binggeli et al., 2003; Dalle-Ave et al., 2004; Medow et al., 2007; Dahmus et al., 2013), epoxyeicosatrienoic acids (EETs) (Cracowski et al., 2013), and big calcium-dependent potassium channels (K_{Ca}) (Lorenzo & Minson, 2007). Of these mechanisms, only NO has been shown not to contribute directly to the overall hyperemic response (Wong et al., 2003).

Considering that NO does not directly contribute to the reactive hyperemia response in the cutaneous circulation (Wong et al., 2003; Zhao et al., 2004; Lorenzo & Minson, 2007) most
studies suggest a substantial role for EDHFs in reactive hyperemia. The hyperemic response is augmented with COX inhibition and data suggests that metabolites of COX may inhibit EDHFs (Lorenzo & Minson, 2007). Other EDHFs may independently mediated vasodilation (Nelson & Quayle, 1995; Christ et al., 1996; Bauersachs et al., 1996). The identity of EDHFs are not clear but they appear to induce vasodilation through K$_{Ca}$ channels as blockade reduced the hyperemic response by $\sim 50\%$ (Lorenzo & Minson, 2007). EETs also modulate $\sim 50\%$ of the hyperemic response (Cracowski et al., 2013). Thus, there is clear evidence that the EDHF pathway plays a prominent role in cutaneous reactive hyperemia; however the contributions of EDHFs, and specifically H$_2$S, have not been examined.

Reactive hyperemia may be a physiological stimulus to examine alterations in EDHF-dependent vasodilation. Reactive hyperemia is often used clinically as a gross assessment of vasodilatory magnitude. In hypertensive animal and human models EHDF-mediated vasodilation is reduced (Yang et al., 2008; Mustafa et al., 2011). Considering that H$_2$S is a putative EDHF and that the endogenous production of H$_2$S is reduced in the vasculature of hypertensive adults (Whiteman et al., 2010a), alterations in reactive hyperemia may reflect H$_2$S impairments in hypertensive vascular pathology.

Therefore, the purpose of this study was to investigate the specific contribution of H$_2$S in the reactive hyperemia response in the cutaneous circulation of pre/stage 1 hypertensive adults and normotensive controls. Our secondary objective was to determine the relation between physiologically induced cutaneous vasodilation (reactive hyperemia) and acetycholine (ACh)-mediated vasodilation across a wide range of blood pressures.
Methods

Subjects

All experimental procedures were approved by the Institutional Review Board at The Pennsylvania State University. Verbal and written consent were obtained voluntarily from all subjects prior to participation according to guidelines set forth by the Declaration of Helsinki. Table 5-1 is a composition of the pre/stage 1 hypertensive and age-matched normotensive subject characteristics. All subjects were normocholesterolemic, non-obese, normally active, without dermatological disease and not taking any medications. All pre/stage 1 hypertensive subjects were classified using a 24 hour blood pressure monitor (Mortara Instruments). Pre-hypertension was defined as an average 24 hour blood pressure >130/80 or daytime pressures > 135/85 (Grossman, 2013). Stage 1 hypertension was defined as systolic blood pressure greater than 140 and/or diastolic blood pressure greater than 90 (Chobanian et al., 2003). All subjects underwent a complete medical screening including a resting 12-lead electrocardiogram, physical examination, and 12-h fasting blood chemistry (Quest Diagnostics, Pittsburgh, PA).

Vascular Function Analysis

Protocols were performed in a thermoneutral (20-22°C) laboratory with the subject semi supine and the experimental arm at heart level. Integrated laser-Doppler flowmetry was used to measure red cell flux, an index of skin blood flow. Local skin temperature was controlled using a local heater (clamped at 33°C) placed directly above each microdialysis membrane (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). Laser-Doppler probes were secured in each local heater and used to continuously measure skin blood flow over each microdialysis fiber. An automated brachial cuff (Cardiocap 5; GE Healthcare) was used to measure arterial blood pressure on the contralateral arm every 5 minutes throughout the protocol.
**Protocol**

Four intradermal microdialysis probes (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) were inserted into the forearm for local delivery of pharmacological agents as previously described (FDA IND 105 572) (Smith et al., 2011) at a rate of 2 µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems). The microdialysis sites contained the following pharmacological agents: 1) lactated Ringer’s solution (control), 2) 20 mM L-NAME (non-specific NOS inhibitor), 3) 0.5 mM aminooxyacetic acid (AOAA: CSE inhibitor), or 4) L-NAME + AOAA (double blockade). Pilot work was conducted using an in vitro preparation to determine the efficacy of AOAA as an inhibitor of H₂S production. All pharmacological solutions were mixed in lactated Ringer’s solution immediately before use and filtered using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI). Following resolution of the insertion trauma hyperemia (~60-90 min) a blood pressure cuff was placed on the upper arm, above the positioning of the microdialysis sites. A 20-minute stable baseline period was recorded followed by two reactive hyperemia periods. Each reactive hyperemia consisted of a 5-min arterial occlusion in which a blood pressure cuff on the upper arm was inflated to suprasystolic pressure (~200 mmHg), an immediate cuff release, and then a 15-min recovery period following the occlusion release. Once a final steady state of laser-Doppler flux was reached following the second occlusion, local skin temperature was increased by 0.5°C/5 s to 43°C and 28 mM sodium nitroprusside was perfused at 4 µl/min at all microdialysis sites to elicit maximal cutaneous vascular conductance (CVC\textsubscript{max}).

**Data and Statistical Analysis**

Data were collected continuously at 40 Hz and stored for offline analysis (Windaq, DataQ Instruments). Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux...
divided by mean arterial pressure (MAP). Data were normalized and expressed as a percentage of maximal CVC (%CVC\textsubscript{max}). Maximal CVC was calculated as an average of 10 min during a stable plateau after locally heating the skin to 43°C and infusion of sodium nitroprusside. The area under the curve (AUC) was calculated by determining the area under the reactive hyperemia response curve (from time of release of occlusion until flux returned to a steady state) as previously described (Wong et al., 2003). Total hyperemic response (THR) was calculated [i.e., THR = AUC – (baseline skin blood flow as %CVC\textsubscript{max} x duration of hyperemic response in s)]. The two reactive hyperemic responses were averaged for each subject and were included in the subsequent statistical analyses. If there was inconsistency in a hyperemic response, the atypical response was dropped and only one reactive hyperemia was analyzed.

A three-way mixed-model ANOVA with repeated measures were performed to examine the effect of subject group (pre/stage 1 hypertensive & age-match control) and local pharmacological treatment (i.e., L-NAME, AOAA, Ringer’s) on the parameters of the reactive hyperemic response (time to peak, peak, THR, tau).

A linear regression was conducted to assess a relation between the THR at the CONTROL site verses the acetylcholine-mediated vasodilation due to H\textsubscript{2}S and verses the acetylcholine-mediated vasodilation due to NO (SigmaPlot, 13.0).

**Results**

Table 5-1 contains the subject characteristics of the pre/stage 1 hypertensive adults and healthy age matched controls. Data are presented as mean ± standard error. The pre/stage 1 hypertensive adults had a significantly higher mean 24-awake blood pressure (SBP: 141 ± 4, DBP: 85 ± 3) compared to the normotensive controls (SBP: 113 ± 2, DBP: 71 ± 2) p< 0.05.
Reactive Hyperemia

Figure 5-1 illustrates the THR in normotensive (Panel A) and pre/stage 1 hypertensive (Panel B) adults. There was no significant difference in baseline for any microdialysis treatment site. There was no difference in the THR at the CONTROL site between the normotensive (2621 ± 512) and pre/stage 1 hypertensive adults (2393 ± 264). In the normotensive adults there was no difference between the CONTROL site and the L-NAME (3540 ± 500), AOAA (3280 ± 768) or COMBO (3258 ± 224) site. The pre/stage 1 hypertensive also demonstrated no difference in the L-NAME (3034 ± 621) or COMBO site (3142 ± 227); however, there was a trend for a blunted hyperemic response at the AOAA site (1521 ± 93, p= 0.08)

Reactive Hyperemia & Acetylcholine Dose-Response Correlation

Figure 5-2 illustrates the correlation between the THR and acetylcholine (ACh)-mediated vasodilation due to H$_2$S in normotensive and pre/stage 1 hypertensive adults (Panel A) and the correlation between the THR and ACh-mediated vasodilation due to NO (Panel B) (ACh-mediated vasodilation due to NO and H$_2$S data are from Chapter 5). There was a relation between the THR and the component of ACh-mediated vasodilation due to H$_2$S ($R^2= 0.58; F= 15.1; p = 0.003$). There was also a weaker correlation between the THR and the component of ACh-mediated vasodilation due to NO ($R^2= 0.38; F= 6.6; p = 0.026$).

Discussion

The novel findings of the present study are that (1) there was no difference in the THR at the control site between normotensive and pre/stage 1 hypertensive adults, (2) H$_2$S appears to contribute to the reactive hyperemic response in pre/stage 1 hypertensive adults but not in the
age-matched normotensive adults and (3) there is a relation between the portion of ACh-mediated vasodilation due to H₂S and due to NO and the THR in the cutaneous circulation.

Our data are consistent with the hypothesis that EDHF-dependent mechanisms are involved in cutaneous reactive hyperemia (Lorenzo & Minson, 2007; Cracowski et al., 2013). In healthy normotensive adults, CSE inhibition did not affect the THR, which is likely due to a preservation of signaling mechanisms in the endothelium of healthy individuals. Inhibition of EETs blunts approximately half of the THR response, thus H₂S likely contributes less to the pool of EDHFs and its contribution is only unmasked in the presence of vascular dysfunction using this technique. Interestingly, dual inhibition of CSE and NOS did not augment the THR, a response that we and others have observed with dual inhibition of COX and NOS. This lends support to the idea that removal of vasoconstrictor COX metabolites may unmask the EDHF pathways during reactive hyperemia that are otherwise blunted.

Our data in a pre/stage 1 hypertensive adults indicate that the THR is attenuated (p= 0.08) during inhibition of cystathionine-γ-lyase (CSE) with AOAA. This is interesting because this is a vascular disease where signaling pathways including NO and vasodilator COX-products are lost (Minuz et al., 1990; Nakamura et al., 2001; Cooke, 2004; Gluais et al., 2005, 2006; Raij, 2006; Smith et al., 2011; Giles et al., 2012).

Reactive hyperemia is used as a clinical tool in various patient groups with cardiovascular disease risk factors to assess micro and macrovascular function (Dakak et al., 1998; Vuilleumier et al., 2002). A significant concern with the use of reactive hyperemia is the significant inter and intra variability that exists even when reactive hyperemia is repeated during a single study day (Wong et al., 2003). Controversy exists as to whether this pronounced variability would limit the utility of reactive hyperemia to assess vascular differences that are physiological versus pathological. The clamping of skin temperature (Abraham et al., 2013), consistency in drug dosage (Medow et al., 2007), consistency of occlusion time and expression of data as
%CVCmax with subtraction of baseline values (Wong et al., 2003) all limit variability and provide more consistent comparisons between subjects and between studies. In the present study we followed this protocol to maximize accuracy and limit variability within subjects in order to capture any differences that may exist between clinical populations. Despite strict adherence to these techniques to limit variability in our data, our intra subject coefficient of variation ranged from 2% to 50% and our inter subject coefficient of variation ranged from 30% to 60%. The coefficient of variations from this study are similar to what has been observed previously utilizing this technique (Minson & Wong, 2004; Harris et al., 2006).

Finally, reactive hyperemia has been suggested to be a tool to be used in parallel with another measure of vascular function. Due to the variability of the measurement, reactive hyperemia is not considered a robust enough measure to assess vascular function independently. The subjects from this study previously participated in an ACh dose-response microdialysis protocol. We calculated the H$_2$S- and NO-dependent ACh-mediated dilation from a previous study and we regressed these calculations against the reactive hyperemia control site to assess potential links between reactive hyperemia and an established pharmacological measure of vascular function. The THR was positively correlated with H$_2$S-dependent ACh-mediated vasodilation ($R^2$ = 0.58; $p = 0.003$). Also, NO-dependent ACh-mediated vasodilation was positively correlated with the THR ($R^2$ = 0.38; $p = 0.026$) in a population of subjects with a range of NO-dependent vasodilation (thus a range of vascular function). Additionally, even though NO does not directly contribute to cutaneous reactive hyperemia and H$_2$S appears to play a minimal role in healthy adults, these data do support the use of reactive hyperemia to detect microvascular function albeit not the specific mechanisms mediating the response.
**Perspectives**

We were able to correlate a well-established measurement of vascular function (NO-mediated ACh-dependent vasodilation) with cutaneous reactive hyperemia in a population with a range of vascular function. However, because H₂S appears to play a minimal role in cutaneous reactive hyperemia in healthy adults and NO does not directly contribute to this response, studying a generalized mechanism of EDHF's would be beneficial to better understand the role of EDHF's to basic vascular control. Our study was limited by the use of AOAA as an inhibitor of H₂S production because we are unable to know the extent to which we were able to successfully inhibit the endogenous production of H₂S. Because BK_{Ca} channels, the downstream target of EDHF's, play a significant role in reactive hyperemia (Lorenzo & Minson, 2007) combining additional measure of the contribution of BK_{Ca} channels to ACh-mediated dilation or local heating might provide important information to understand the role of EDHF's in vascular function.
Table 5-1: Subject characteristics for pre/stage 1 hypertensives and normotensive age-matched controls.

<table>
<thead>
<tr>
<th></th>
<th>Protocol 1 &amp; 2 Normotensives</th>
<th>Protocol 1 &amp; 2 Hypertensives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>3M, 6W</td>
<td>5M, 4W</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>56 ± 1</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>24 hr daytime systolic (mmHg)</td>
<td>115 ± 4</td>
<td>138 ± 3</td>
</tr>
<tr>
<td>24 hr daytime diastolic (mmHg)</td>
<td>75 ± 6</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>110 ± 13</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>74 ± 6</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>Total CHO (mg/dL)</td>
<td>195 ± 14</td>
<td>174 ± 8</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6 ± 0.1</td>
<td>6 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. BMI, body mass index; MAP, mean arterial pressure; LDL, low-density lipoproteins; HDL, high density lipoproteins; TOTAL CHO, total cholesterol; HbA1C, glycated hemoglobin.
Figure 5-1: Total hyperemic response. Summary data, expressed as the total hyperemic response in the cutaneous circulation of normotensives (Panel A) and pre/stage 1 hypertensives (Panel B) adults following 5 minutes of occlusion. Total hyperemic response is calculated as the area under the curve (%CVC_max*sec) minus baseline. CONTROL, Ringer’s; AOAA, CSE inhibited; L-NAME, NOS inhibited; COMBO, CSE+ NOS inhibited.
Figure 5-2: Correlation Graphs. Panel A, H$_2$S-dependent ACh-mediated vasodilation versus the control site THR ($R^2 = 0.58$, $p= 0.003$). Panel B, NO-dependent ACh-mediated vasodilation versus the control site THR ($R^2 = 0.38$, $p= 0.026$). The concentration of ACh used was 0.1 mM. H$_2$S, hydrogen sulfide; ACh, acetylcholine; THR, total hyperemic response.
Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

The studies that comprise this dissertation were performed to investigate the functional role of hydrogen sulfide (H$_2$S) in the cutaneous circulation of healthy adults and potential functional signaling alterations that occur in adults with pre/stage 1 hypertension. Specifically these studies investigated (1) the presence of H$_2$S generating enzymes in the cutaneous circulation, (2) the vasodilatory properties to exogenous H$_2$S, (3) the downstream potassium channel targets of H$_2$S, (4) the interaction of H$_2$S with other endothelium-dependent vasodilatory pathways, (5) the contribution of H$_2$S to cholinergic-mediated vasodilation and (6) the contribution of H$_2$S to reactive hyperemia. Collectively, these studies suggest a functional endogenous vasodilatory role of H$_2$S in the cutaneous circulation that is blunted in pre/stage 1 hypertensive adults; however, this is not due to impaired vascular sensitivity to exogenous H$_2$S.

The purpose of this chapter is to summarize the results of these studies and discuss future directions for research to clarify the mechanisms of H$_2$S-mediated vasodilation. Finally, H$_2$S will be addressed as a mechanistic target for therapeutic intervention in hypertensive adults.

Evidence for a functional vasodilatory role for hydrogen sulfide in the human cutaneous microvasculature.

The first finding in this set of studies confirmed the presence of cystathionine-$\gamma$-lyase (CSE) and 3-mercaptopuruvate sulfurtransferase (3-MPST) in the cutaneous circulation of healthy young adults. The presence of these enzymes confirms that the cutaneous circulation of humans, in conjunction with microdialysis, is an appropriate vascular bed to study the vascular signaling mechanisms of H$_2$S. Next, these studies demonstrate that both sodium sulfide (Na$_2$S) and sodium hydrosulfide (NaHS), H$_2$S donors, elicit vasodilation in a dose-dependent manner in
the cutaneous circulation of adults. The results of these studies also confirm that H₂S-induced vasodilation is mediated, in part, by calcium-dependent potassium channels that are sensitive to tetraethylammonium. Finally, inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) vasodilatory pathways blunted the vasodilatory response to exogenous H₂S. This suggests that interaction exists between H₂S-, NOS- and COX-dependent vasodilatory pathways to elicit full expression of vasodilation to an exogenous H₂S donor.

These findings have several implications for the role of H₂S to modulate vascular function in humans. CSE is known to be ubiquitously expressed in the cardiovascular system, specifically the aorta (Yang et al., 2006), mammary arteries (Webb et al., 2008; Papapetropoulos et al., 2014) and umbilical vein (Yang et al., 2008). The in vitro data from this series of studies furthers these finding in the cutaneous microcirculation of healthy young adults for both CSE and 3-MPST. Complementary in vivo studies utilized Na₂S concentrations ranging from 0.01mM to 100mM. Endogenous H₂S concentrations in humans have been measured from 0.01mM to 0.06 mM (Chen et al., 2007; Whiteman et al., 2010a). During the process to dissolve Na₂S into solution, a bolus unknown amount of H₂S gas is evaporated into the air. Thus, the quantity of Na₂S that reaches the cutaneous circulation during microdialysis is likely less than what was originally calculated. This suggests our delivery of Na₂S successfully matched endogenous concentrations of H₂S that have been measured previously; however, we are unable to know which dose on the dose-response curve is most representative of endogenous H₂S concentration.

H₂S and the donor NaHS cause vasodilation in vitro in rat mesenteric arteries. K_ATP channels, in part, mediate the vasodilatory response however; the contribution of small, intermediate and large calcium-dependent potassium channels has since been demonstrated by others using rodent models (Mustafa et al., 2011; Jackson-Weaver et al., 2013). These data suggest that H₂S-mediated vasodilation is induced through multiple downstream potassium channel targets that may differ in large conduit vessels compared to smaller resistance vessels.
Our data extend these findings in the human cutaneous circulation. While $K_{\text{ATP}}$ channels are the primary downstream targets in animal models, our data suggest a more significant role for tetraethylammonium-sensitive calcium-dependent potassium channels in humans.

Whether $H_2S$ and nitric oxide (NO) interact in a synergistic or inhibitory fashion in the vasculature is unclear (Li et al., 2006; Cai et al., 2007; Kubo et al., 2007; Wang et al., 2010; Kondo et al., 2013). Data in humans regarding the interaction of $H_2S$, NO and by products of COX are sparse and limited to in vitro studies. In human umbilical vein endothelial cells, $H_2S$ inhibits L-arginine catalytic velocity, thereby inhibiting the NO pathway (Geng et al., 2007).

Additionally, exogenous $H_2S$ administration induced an up-regulation of COX-2/PGI$_2$ pathway in isolated human pulmonary leading to a shift toward a pro-vasodilatory environment (Li et al., 2014). Our data support a positive vasodilatory interaction between $H_2S$ and NO and COX by products, which is contradictory to the previous findings for NO in humans. Specifically, inhibition of either NO or by products of COX in the cutaneous vasculature caused a shift to the right in the dose-response curves to exogenous $H_2S$. These data imply that both of these vasodilatory pathways contribute to $H_2S$-mediated vasodilation.

**Future Directions**

1.) Because CSE and 3-MPST are clearly expressed in the cutaneous circulation; future mechanistic studies utilizing the cutaneous circulation to examine $H_2S$-dependent vascular signaling are justified.

2.) Development of $H_2S$ probes that can utilize a very small quantity of solution and quickly measure $H_2S$ concentration are necessary to elucidate accurate concentration of $H_2S$. The development of such probes will also allow for the measurement of dialysate from microdialysis fibers. Measurement of $H_2S$ from dialysate would provide an index of the concentration of $H_2S$
delivered to the cutaneous circulation via H$_2$S donors and in addition determine the efficacy of endogenous enzymatic H$_2$S inhibitors.

3.) Due to the difficult solubility of many of the potassium channel inhibitors as well as a lack of inhibitors that are specific to one type of potassium channel, future studies are warranted to confirm our finding that calcium-dependent potassium channels are the primary downstream target of H$_2$S. It is important to know the precise downstream target of H$_2$S-mediated vasodilation so that there can be future development of pharmacological agonists for a specific target in the development of hypertensive therapies.

4.) Our data suggests a positive interaction between H$_2$S and NO and COX vasodilatory pathways. Future studies are necessary to determine differences in interaction between these vasodilatory pathways when H$_2$S is produced endogenously versus given exogenously. Additionally, alterations in the interaction of these molecules with the onset of vascular pathology, when the homeostatic balance of vasoactive molecules becomes altered, has yet to be explored.

**Altered hydrogen sulfide-mediated vasodilation in the human cutaneous microvasculature in pre/stage 1 hypertensives.**

The primary findings from these studies were that pre/stage 1 hypertensive adults had a blunted vasodilatory response to a cholinergic stimulus that was mediated, in part, by reductions in both H$_2$S- and NO-dependent vasodilation in the cutaneous microvasculature. Additionally, vascular end-organ responsiveness to exogenous H$_2$S is preserved in this population despite a lack of a functional vasodilatory interaction with either NOS or COX vasodilating pathways.
Rodent studies suggest hypertension-associated alterations in both H$_2$S production and potentially function. Dysregulation in the production and function of H$_2$S likely plays an important role in the pathogenesis of hypertension-associated vascular dysfunction via its effects on blood pressure regulation, vessel function and remodeling, and inflammatory processes (Yan et al., 2004; Jiang et al., 2005; Liu et al., 2010; Taniguchi et al., 2011). Additionally, spontaneously hypertensive rats have lower serum concentrations of H$_2$S compared with healthy wild type rats (Yan et al., 2004). Despite a reduction in endogenous H$_2$S production vascular sensitivity to exogenous H$_2$S is not impaired (Yang et al., 2008).

We found that pre/stage 1 hypertensive adults had a blunted vasodilatory response to a cholinergic stimulus that was mediated, in part, by reductions in both H$_2$S- and NO-dependent vasodilation. This is the first study in humans to demonstrate that attenuated H$_2$S-dependent vasodilation contributes to vascular dysfunction and aligns with previous in vitro and in vivo animal studies. H$_2$S plasma concentrations in humans are negatively correlated with systolic and diastolic blood pressure in humans early in the pathogenesis of hypertension-induced vascular dysfunction (Chen et al., 2007 p.2; Whiteman et al., 2010a); suggesting altered H$_2$S physiology at the onset of hypertension. Though we did not measure endogenous H$_2$S production, we hypothesize that a reduction in H$_2$S-mediated vasodilation is due to a blunted endogenous production of H$_2$S in the vascular endothelium. Interestingly, in the pre/stage 1 hypertensive adults we found no difference in end-organ responsiveness to exogenous administration of H$_2$S; however, the mechanism by which H$_2$S-dependent vasodilation is elicited was altered. In humans, hypertension-induced vascular dysfunction is characterized by reductions in both NO- and COX-mediated vasodilation (Minuz et al., 1990; Nakamura et al., 2001; Cooke, 2004; Gluais et al., 2005, 2006; Giles, 2006; Raji, 2006; Smith et al., 2011). Parallel to those findings, we were not surprised that the contribution of NO and COX to H$_2$S-induced vasodilation was reduced in pre/stage 1 hypertensive adults.
**Future Directions:**

1.) Our data suggest that pre/stage 1 hypertensive adults have blunted H$_2$S-dependent vasodilation that contributes to generalized microvascular dysfunction. These data suggest that H$_2$S and its downstream potassium channels are viable targets in the pharmacological treatment of hypertension and should be explored. This assumption is based on rodent data that suggests H$_2$S-induced vascular dysfunction contributes early in the development of hypertension.

2.) End-organ responsiveness to exogenous H$_2$S was not blunted in pre/stage 1 hypertensive adults. Pharmacological agents with slow releasing H$_2$S moieties are used in the treatment of gastric inflammation; however, a slow releasing H$_2$S drugs, specific to cardiovascular dysfunction, have potential to improve H$_2$S-induced vasodilation. In a clinical trial, zofenapril, an ACE-inhibitor with a slow releasing H$_2$S moiety, proved more efficacious in both blood pressure control and adverse event profiles when compared with enalapril. Future investigation into the mechanisms through which zofenapril improves blood pressure control, specifically examining whether H$_2$S-dependent endothelial function is restored and through what mechanism(s), would clarify the potential benefits of this drug and influence future hypertensive treatment.

The contribution of hydrogen sulfide to the reactive hyperemic response in the cutaneous circulation.

This principle finding from this study was that H$_2$S-dependent vasodilation does not contribute to the total hyperemic response in normotensive adults; however, it appears to contribute to the response in pre/stage 1 hypertensive adults. Additionally, these data coupled with previously collected acetylcholine dose-response data, show a correlation between both H$_2$S- and NO-dependent cholinergic-mediated vasodilation with reactive hyperemia measures. These
data support the use of reactive hyperemia to assess vascular function when paired with other measures of microvascular function.

Reactive hyperemia is a non-invasive test to examine cutaneous microvascular function in humans (Stewart et al., 2004; McCord et al., 2006; Lorenzo & Minson, 2007; Cracowski et al., 2013). The exact mechanisms mediating reactive hyperemia in the cutaneous circulation is not clear. Consistently, NO has been shown to not directly mediate the reactive hyperemic response in the cutaneous circulation (Wong et al., 2003; Medow et al., 2007; Lorenzo & Minson, 2007), while the contribution of by-products of COX have previously been unclear as to whether or not they unmask an NO-dependent augmentation in the hyperemic response (Binggeli et al., 2003; Dalle-Ave et al., 2004; Medow et al., 2007; Dahmus et al., 2013).

The contribution of EDHFs to reactive hyperemia has been limited to EETs and the downstream target BKCa. Our data extends these findings and suggest a role for H2S-dependent vasodilation early in the pathogenesis of hypertension-induced vascular dysfunction to reactive hyperemia stimuli. We hypothesized that this is due to a loss of redundant signaling mechanisms in pre/stage 1 hypertensive adults. Additionally, few have attempted to link the total hyperemia response (a measure of microvascular function) with another well-established vascular function measurement. We were able to correlate the total hyperemic response with NO-dependent acetylcholine-mediated vasodilation in a population of subjects with a range of NO-dependent vasodilation (thus a range of vascular function). H2S-dependent acetylcholine-mediated vasodilation was also positively correlated with the total hyperemic response. These data support the use of reactive hyperemia to detect microvascular function.

**Future Directions**

1.) Our data suggests a role for H2S-dependent vasodilation in reactive hyperemia in the presence of hypertension-induced vascular dysfunction, when redundant signaling mechanisms are
compromised. Future studies that examine multiple EDHF molecules as well as all potential
downstream molecule targets of EDHFs are warranted to determine the mechanisms that mediate
the reactive hyperemic response in the cutaneous circulation.

2.) Reactive hyperemia is considered a non-invasive test to examine cutaneous microvascular
function but has profound variability in its measurement. Thus, linking this measure to another
established measurement of vascular function will provide assurance in the ability to measure
vascular function as well as detect alterations in the mechanisms that control vascular function in
physiological and pathological conditions.

Future Research Directions

This dissertation contains the first studies to mechanistically examine the vascular effect
of H₂S in vivo in humans. Though important information can be taken from this collection of
studies, limitations in the pharmacology available to be used in humans constrained our ability to
determine the exact quantities of H₂S reaching the cutaneous circulation, endogenous production
of H₂S and the conclusive involvement of specific potassium channels mediating H₂S-induced
vasodilation. The identification of a stable end product of H₂S biosynthesis or the development of
a measurement technique with high sensitivity at low and high values, real time measurement and
the ability to use unaltered samples will greatly aid in the ability to determine concentrations of
H₂S produced in vivo as well as mixed solutions used in experimental protocols. Additionally, the
advancement of pharmacology approved for human use that specifically and fully inhibits
potassium channels and enzymatic sources of H₂S will allow for the identification of the precise
mechanisms of H₂S-mediated vasodilation that will build upon the generalized findings from this
dissertation.
This dissertation demonstrates attenuated functional endogenous production of H$_2$S in pre/stage 1 hypertensive adults; however, the profound vasodilatory effects of exogenous H$_2$S are similar to those of normotensive adults despite different downstream mechanisms. This knowledge, coupled with known anti-oxidant properties of this molecule, makes it an ideal target for pharmacological intervention in humans with vascular dysfunction, specifically hypertension. Zofenopril, an ACE-inhibitor with a H$_2$S releasing moiety, is currently available on the market in Europe for the treatment of high blood pressure. Zofenopril has both positive and clinically significant outcomes compared to traditional ACE-inhibitors in the treatment of hypertension and prevention of mortality (Bucci et al., 2014). Zofenopril is an ideal candidate to study the systemic vascular effects of H$_2$S. Future studies should utilize Zofenopril, or another slow releasing H$_2$S drug, because systemic delivery of H$_2$S via Zofenopril would mimic endogenous production of H$_2$S, allowing for an accurate assessment of this molecule in the contribution of vascular function and potential for clinical intervention.


METHODOLOGICAL ISSUES USING H$_2$S DONORS IN VIVO IN HUMANS

Hydrogen sulfide (H$_2$S) is the newest member of the gaso-transmitter family, along with nitric oxide (NO) and carbon monoxide (CO). H$_2$S was first identified in 1996 for its role in neuronal activity (Abe & Kimura, 1996) and a year later for its contribution to functional vasoactivity (Hosoki et al., 1997). Today H$_2$S is known for implications in physiological and pathophysiological processes. In humans, H$_2$S is an endothelial derived hyperpolarizing factor that is produced by cystathionine-γ-lyase and 3-mercaptoppyruvate sulfurtransferase in the vascular endothelium. Cholinergic stimulation leads to the release of H$_2$S from the endothelium (Mustafa et al., 2011; Wang, 2012a) where H$_2$S acts on downstream potassium channels to hyperpolarize the VSM cells leading to vasodilation (Zhao et al., 2001; Distrutti et al., 2006; Gallego et al., 2008; Liang et al., 2011).

The effects of H$_2$S on the vasculature has become a topic of interest in cardiovascular physiology due to its potent vasodilatory properties (Zhao et al., 2001). To date, the majority of research, studying the signaling mechanisms of H$_2$S, has been conducted using animal models or human tissues in vitro (Zhao et al., 2003; Yang et al., 2006, 2008; Gallego et al., 2008; Liang et al., 2011; Mustafa et al., 2011). The purpose of this dissertation was to translate current physiological understanding of H$_2$S in the vasculature of animal models (primarily rodent) to an in vivo human model.

The novel technique of this dissertation was Laser-Doppler flowmetry coupled with intradermal microdialysis in the cutaneous circulation of humans. The cutaneous circulation is an accessible circulation to examine mechanisms underlying microvascular function. Deficits in cutaneous vascular signaling are evident in human skin prior to, and are predictive of the onset of
conduit artery disease (IJzerman et al., 2003; Holowatz et al., 2008). Additionally, EDHFs are more important in the health and function of small resistance vessels compared to the conduit vasculature (Vicaut, 1992; Struijker Boudier et al., 1992).

Laser-Doppler flowmetry is a technique that is minimally invasive for the direct measurement of dynamic fluctuations that occur in a small area of the cutaneous circulation in response to vasoreactive stimuli. Laser-Doppler flowmetry coupled with microdialysis is a powerful and useful technique to dissect vascular signaling pathways in the cutaneous circulation of humans. For the studies included in this dissertation, intradermal drug delivery by microdialysis was the primary stimulus.

The major methodological challenge of this series of studies was the delivery of H₂S donor through microdialysis probes to the cutaneous circulation. Sulfide salts are the most common H₂S donors utilized to examine the role of H₂S in health and disease. Sodium hydrosulfide (NaHS) and sodium sulfide (Na₂S), have been widely used in rodent models to examine H₂S-mediated vascular function (Zhao et al., 2001; Wang et al., 2010; Papapetropoulos et al., 2014). Both donors are inorganic salts that quickly dissolve in solution, resulting in the immediate formation of H₂S in a pH-dependent manner and are thus considered “fast H₂S generators”. Upon being dissolved in an aqueous solution, sulfide salts instantaneously release a bolus amount of H₂S gas that dissipates within seconds (Li et al., 2006; Whiteman et al., 2010a).

In solution H₂S is in equilibrium. However sulfide salts that are mixed with lactated Ringer’s, to be used for intradermal microdialysis, become severely basic (pH= 10-11).

![Chemistry of H₂S gas as it dissolves into an aqueous solution.](image)

**Figure A-1:** Chemistry of H₂S gas as it dissolves into an aqueous solution.
The pKa₁ for H₂S is ~7.0 and is largely temperature sensitive. For the protocols in this series of studies, all solutions where mixed in a thermoneutral environment (20 – 21°C). The most problematic issue is that NaHS and Na₂S utilize H⁺ to reestablish equilibrium once they have been dissolved in a solution. Olson et al, demonstrated the changes in pH that occur observing a range of mM concentrations. They showed that dissolving 1mM of Na₂S (the same sulfide salt used for the majority of this dissertation) increased the pH of the solution from 7.4 to 7.8. There was an exponential increase in pH for a given rise in mM concentration of Na₂S (Olson et al., 2014). In this dissertation 40 mg of Na₂S was dissolved in 1.7 ml of Ringer’s solution for a final solution that was 100 mM Na₂S. Based on the findings by Olson et al we should have observed a pH around 10. Indeed upon mixture with solely Ringer’s solution to the desired mM concentration the solution had a measured pH of slightly higher than 10. It is not feasible to perfuse a severely basic solution through a microdialysis fiber as this will cause a severe burning sensation and will lead to discomfort of the subject.

In order to combat the severely basic solution that occurred once Na₂S was dissolved into a 100mM solution, we used highly pure hydrochloric acid in order to pH the solution as it was dissolved. The following is the procedure that was used to mix each solution of Na₂S to be perfused through a microdialysis fiber:

Step 1: Calibrate H₂S analyzer (for this dissertation we used a micro pH combination electrode (Sigma-Aldrich) with a JENCO 6230 pH/mV/TEMP meter).

Step 2: Weigh 40 mg of Na₂S salt, cover in tinfoil and store in beaker.

Step 3: Fill a 1 mL syringe with HCl (set aside).

Step 4: Fill a 10 mL syringe with lactated Ringer’s or the desired inhibitor solution.

Step 5: Remove tinfoil from beaker containing Na₂S and add 1 mL of lactated Ringer’s or inhibitor solution.

Step 6: Measure pH using H₂S probe (pH should measure slightly over 10).
Step 7: Quickly add 0.3 mL of HCl to the beaker, stir the solution and then measure pH (should measure around 8-9).

Step 8: Titrate the solution using drops of HCl and lactated Ringer’s (or inhibitor solution) until final volume is 1.7 ml, a 100 mM Na₂S solution with a pH of 7.

NOTE: ~ 4 drops of solution using a 19 gauge needle is the equivalent of 0.1mL. Always add a small amount of HCl compared to neutral pH solution and continually measure pH to observe its drift toward 7. If one is too generous with the HCl the solution will become white in color, acidic in pH and is not able to be restored with the addition of a neutral pH solution. Hence, use caution to proceed drop by drop for the final 0.4mL while titrating to a pH of 7. The final solution should have a clear yellow color.

Step 9: Filter the solution into a 1 mL syringe using an Acrodisc syringe filter (Life Sciences) or equivalent.

Step 10: Solution can now be used or diluted for a dose-response protocol.

NOTE: The solution, despite a current pH of 7, will continue to alkalinize as the H₂S gas volatilizes from the solution (consuming 2 moles of H⁺ for every mole of sulfur lost to the atmosphere). To prevent evaporation of gas, cover each syringe tip with foil in addition to the foil that is already wrapped around the syringe to prevent photo-degradation.

CAUTION: If one is mixing multiple samples and any sample after the first sample does not pH properly, consider reweighing the Na₂S and starting all the dilutions over. To limit the amount of time that the solution is able to alkalinize, a time from of 1 hr 30 mins from the start of mixing the drugs is appropriate based on pilot data collected in our lab.
Appendix B

MECHANISMS OF CUTANEOUS REACTIVE HYPEREMIA IN HEALTHY YOUNG ADULTS

Introduction

Reactive hyperemia is a pronounced vasodilation and transient increase in blood flow that occurs in response to the release of an upstream arterial occlusion (Patterson, 1956; Blair et al., 1959; Kilbom & Wennmalm, 1976). Reactive hyperemia is a technique that is increasingly used as a test of microvascular function in different clinical populations (Dakak et al., 1998; Vuilleumier et al., 2002). However, there is debate as to the exact mechanisms mediating reactive hyperemia in the cutaneous circulation in healthy young adults. The mechanisms that have been explored to date include: sensory nerves (Larkin & Williams, 1993; Lorenzo & Minson, 2007; Cracowski et al., 2013), nitric oxide (NO) (Binggeli et al., 2003; Wong et al., 2003; Zhao et al., 2004; Medow et al., 2007; Lorenzo & Minson, 2007), products of cyclooxygenase (COX) (Binggeli et al., 2003; Dalle-Ave et al., 2004; Medow et al., 2007; Dahmus et al., 2013), epoxyeicosatrienoic acids (EETs) (Cracowski et al., 2013), and big calcium-dependent potassium channels (K_{Ca}) (Lorenzo & Minson, 2007).

NO and metabolites of COX contribute to physiological vasodilation in the cutaneous circulation, however their role in reactive hyperemia is unclear. During shear stress, endothelial cells are stimulated resulting in the production of NO, making NO a logical metabolite involved in reactive hyperemia. However, studies utilizing microdialysis and laser-Doppler flowmetry suggest that NO does not directly contribute to the reactive hyperemic response in the cutaneous circulation (Wong et al., 2003; Zhao et al., 2004; Lorenzo & Minson, 2007).
Interestingly, while there is no direct role for NO, it may play a modulatory role for COX-derived vasoactive products. When COX is inhibited with either ketorolac or indomethacin, the reactive hyperemic response (THR) is augmented. A main area of controversy is whether this augmentation in the THR during cyclooxygenase (COX) inhibition is due to (1) an unmasking of NO-dependent vasodilation (Medow et al., 2007), (2) a removal of vasoconstricting metabolic by products of COX (Lorenzo & Minson, 2007) or (3) potentially a combination of the two.

The purpose of this study was to determine whether COX-inhibition augments the THR through NO-dependent mechanisms by testing a cohort of young healthy adults, using the same techniques and pharmacological sites as the two previous conflicting studies (Medow et al., 2007; Lorenzo & Minson, 2007). We used caution to implement all known techniques to limit variability in our data which are outlined in the discussion of chapter 5 of this dissertation (Wong et al., 2003; Medow et al., 2007; Abraham et al., 2013). We hypothesized that the augmentation in the THR would not be NO-dependent and instead it is due to the removal of vasoconstricting COX metabolites thus unmasking endothelium-dependent hyperpolarizing factor(s) (EDHF) mediated vasodilation.

**Methods**

**Subjects**

All experimental procedures were approved by the Institutional Review Board at The Pennsylvania State University. Verbal and written consent were obtained voluntarily from all subjects prior to participation according to guidelines set forth by the Declaration of Helsinki. Table B-1 is a composition of the healthy young subject characteristics. All subjects were normotensive, normocholesterolemic, non-obese, normally active, without dermatological disease
and not taking any medications. Women were tested during the early follicular phase of their menstrual cycle or during the placebo phase if taking oral contraceptives. All subjects underwent a complete medical screening including a resting 12-lead electrocardiogram, physical examination, and 12-h fasting blood chemistry (Quest Diagnostics, Pittsburgh, PA).

**Vascular Function Analysis**

Protocols were performed in a thermoneutral (20-22°C) laboratory with the subject semi-supine and the experimental arm at heart level. Integrated laser-Doppler flowmetry was used to measure red cell flux, an index of skin blood flow. Local skin temperature was controlled using a local heater (clamped at 33°C) placed directly above each microdialysis membrane (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). Laser-Doppler probes were secured in each local heater and used to continuously measure skin blood flow over each microdialysis fiber. An automated brachial cuff (Cardiocap 5; GE Healthcare) was used to measure arterial blood pressure on the contralateral arm every 5 minutes throughout the protocol.

**Protocol: NO & COX (young healthy adults)**

Four intradermal microdialysis probes (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) were inserted into the forearm for local delivery of pharmacological agents as previously described (FDA IND 105 572) at a rate of 2 µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems). The microdialysis sites contained the following pharmacological agents: 1) lactated Ringer’s solution (control), 2) 20 mM L-NAME (non-specific NOS inhibitor), 3) 10 mM ketorolac (KETO: non-specific COX inhibitor), or 4) L-NAME + KETO (double blockade). All pharmacological solutions were mixed in lactated Ringer’s solution immediately before use and filtered using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI). Following resolution of the insertion trauma hyperemia (~60-90
a blood pressure cuff was placed on the upper arm, above the positioning of the microdialysis sites. A 20-minute stable baseline period was recorded followed by two reactive hyperemia periods. Each reactive hyperemia consisted of a 5-min arterial occlusion in which a blood pressure cuff on the upper arm was inflated to suprasystolic pressure (~200 mmHg), an immediate cuff release, and then a 15-min recovery period following the occlusion release. Once a final steady state of laser-Doppler flux was reached following the second occlusion, local skin temperature was increased by 0.5°C/5 s to 43°C and 28 mM sodium nitroprusside was perfused at 4 µl/min at all microdialysis sites to elicit maximal cutaneous vascular conductance (CVC_max).

Data and Statistical Analysis

Data were collected continuously at 40 Hz and stored for offline analysis (Windaq, DataQ Instruments). Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP). Data were normalized and expressed as a percentage of maximal CVC (%CVC_max). The peak was assessed as the highest point after the rapid release of the occlusion cuff. Maximal CVC was calculated as an average of 10 min during a stable plateau after locally heating the skin to 43°C and infusion of sodium nitroprusside. The area under the curve (AUC) was calculated by determining the area under the reactive hyperemia response curve (from time of release of occlusion until flux returned to a steady state) as described by Wong et al (Wong et al., 2003). Total hyperemic response (THR) was calculated [i.e., THR = AUC – (baseline skin blood flow as %CVC_max x duration of hyperemic response in s)] The decay constant (τ) provided an index of the time that it takes for one-third of the reactive hyperemic response to resolve. This measure is calculated by taking the amount of seconds it takes to go from peak blood flow to a return to steady-state baseline and then dividing by three.
The two reactive hyperemic responses were averaged for each subject and were included in the subsequent statistical analyses. If there was inconsistency in a reactive hyperemia response, the atypical response was dropped and only one reactive hyperemia was analyzed.

A two-way mixed-model ANOVA with repeated measures were performed to examine the effect of local pharmacological treatment (i.e., L-NAME, KETO, Ringer’s) on the parameters of the reactive hyperemic response (time to peak (TTP), peak, THR, tau).

**Results**

Table 1 contains the subject characteristics for the healthy young adults from study 1. Data are presented as mean ± standard error.

There was no significant difference in baseline among any of the microdialysis treatment sites. The THR was augmented in both sites treated with the COX-inhibitor ketorolac (KETO and COMBO) compared to the CONTROL site (KETO: 5623 ± 2308, COMBO: 8326 ± 2578, p<0.0001 vs. CONTROL: 2559 ± 987 %CVC_{max}*sec, both p<0.001). The COMBO site had a greater peak (COMBO: 63 ± 9 vs. CONTROL: 41 ± 15 %CVC_{max}; p = 0.009) decay constant (COMBO: 195 ± 74 vs. CONTROL: 68 ± 24 sec; p<0.0001) and a longer TTP (COMBO: 56 ± 14 vs. CONTROL: 31 ± 8 sec; p=0.002) compared with the CONTROL site, all of which contributed to the augmented THR.
Discussion

The principle finding of this study was that the augmented THR, in the presence of COX inhibition, was not NO-mediated. Additionally, our data support previous findings that NO does not directly contribute to reactive hyperemia in the cutaneous circulation of healthy young adults. These data suggest that the augmented THR due to COX inhibition must be a result of (1) the removal of vasoconstricting COX metabolites, (2) unmasked EDHF-dependent vasodilation, or (3) a combination of both.

Naylor et al (1999) were the first to demonstrate that COX inhibition, with oral indomethacin, lead to an augmented reactive hyperemic response (Naylor et al., 1999). Indomethacin and ketorolac are non-specific COX-inhibitos that result in an inhibition of the synthesis of both COX-derived vasodilators and vasoconstrictors, prostacyclin and thromboxane, respectively (Moncada & Vane, 1978). Medow et al (2008) suggest that vasoconstrictor, not vasodilator, COX-derived products dominate the homeostatic balance of prostaglandin synthesis at room temperature (Medow et al., 2008). Thus, vasodilation resulting from non-specific COX inhibition may be due to a removal of vasoconstrictor prostaglandins (thromboxanes). This is a plausible mechanism to mediate the augmented hyperemic response during COX-inhibition; however, there are additional downstream interactions between COX inhibition and the production of EETs that may influence the reactive hyperemia response in the cutaneous circulation.

EDHFs contribute independently to the THR in the cutaneous circulation. The identity of EDHFs in specific vascular beds are not clear but they appear to induce vasodilation through K\textsubscript{Ca} channels (Lorenzo & Minson, 2007). In the cutaneous circulation, K\textsubscript{Ca} blockade reduced the hyperemic response by ~ 50 % (Lorenzo & Minson, 2007). Specific EET inhibition, an established EDHF molecule, also reduce the hyperemic response by ~50 %.
The production of COX metabolites and EETs share a common metabolic pathway (Narumiya et al., 1999; Zordoky & El-Kadi, 2010). It is possible that inhibition of COX leads to augmented production of EETs and an increase in vasodilation, which may or may not be further facilitated by the removal of vasoconstricting prostaglandins. Fatty acids are converted to arachidonic acid that can then be metabolized through three pathways: (1) lipoxygenase (LOX), (2) cyclooxygenase and (COX) (3) cytochrome P450 (CYTP). Activation of the LOX pathway leads to the production of leukotrienes that act as inflammatory mediators (Tang et al., 2006; Osher et al., 2006). Activation of the COX pathway, also involved in inflammation, leads to the production of vasodilating prostaglandins and vasoconstricting thromboxanes (Moncada & Vane, 1978). Finally, activation of the CYTP pathway results in the production of EETs, which act as autocrine and paracrine mediators with robust vasodilating and anti-inflammatory properties (Inceoglu et al., 2007). Low-dose non-steroidal anti-inflammatory drugs (NSAIDs), a COX inhibitor, increase the biological effects of EETs by shuttling the arachidonic acid precursors into the CYTP branch of the cascade. This finding is not limited to NSAIDs and has also been observed with the use of specific COX-1, COX-2 and non-selective COX inhibitors. Inhibition of EETs with fluconazole induced a 50% reduction in the total hyperemic response in young healthy adults (Cracowski et al., 2013). However, in that study they examined the interaction of EETs and NO but not COX metabolites. Interestingly, dual inhibition of NO and EETs removed the inhibitory effect of fluconazole on the reactive hyperemic response (Cracowski et al., 2013). Thus, a more complex interaction exists between NO and EETs in reactive hyperemia and remains to be explored.

In summary, the primary finding of this study was that augmented THR in the presence of COX inhibition was not NO-mediated. Our results corroborate previous findings (Wong et al., 2003; Lorenzo & Minson, 2007; Cracowski et al., 2013) that NO does not contribute to reactive hyperemia in the cutaneous circulation of healthy young adults. Finally, we proposed that the
augmented THR, due to COX inhibition, is likely a combination of the removal of vasoconstricting COX metabolites and unmasked EDHF-dependent vasodilation. Future studies are warranted to investigate the complex interaction between EDHFs, metabolites of COX and NO in the reactive hyperemic response in the cutaneous circulation.
Table B-1: Subject characteristics for healthy young adults.

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Sex</td>
<td>5M, 5W</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>24 ± 0.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Total CHO (mg/dL)</td>
<td>143 ± 8</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. BMI, body mass index; MAP, mean arterial pressure; LDL, low-density lipoproteins; HDL, high density lipoproteins; TOTAL CHO, total cholesterol; HbA1C, glycated hemoglobin.
Figure B-1: Total hyperemic response. Summary data, expressed as the total hyperemic response in the cutaneous circulation of healthy young adults following 5 minutes of occlusion. Total hyperemic response is calculated as the area under the curve (%CVCmax * sec) minus baseline. CONTROL, Ringer’s; KETO, COX inhibited; L-NAME, NO synthase inhibited; COMBO, L-NAME & COX inhibited. The KETO and COMBO site are both significantly greater than the CONTROL site, p < 0.001 for both.
INFORMED CONSENT FORMS

Version #1

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY

The Pennsylvania State University

Title of Project: Cutaneous vascular effects of Hydrogen Sulfide

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This is to certify that I, ______________________ have been given the following information with respect to my participation as a volunteer in a program of investigation.

1. Purpose of the study:
   The human body controls the amount of blood flowing through healthy blood vessels. It does this by changing their size. The way the body controls blood flow can be different with age and the presence of some diseases. One of the first signs of a disease that affects blood vessels is a change in the function of the very small blood vessels of the skin.

   The skin has a web of small blood vessels that is easy to access and study. The health of
the skin’s blood vessels can mirror the health of the body’s other blood vessels. Studying blood flow control in the skin’s blood vessels helps researchers to learn about the onset, effects, and treatment of blood vessel diseases. Our lab has studied the body’s control skin blood flow in young and older humans. We have also studied humans with high blood pressure (hypertension) and high cholesterol for the past ten years. We have seen that aging and disease can damage the control of blood vessels. Our current research explores the reasons for that impairment.

Hydrogen sulfide is a natural gas made in your body. It helps your blood vessels to get bigger and increase blood flow. The goal of this study is to look at the role of hydrogen sulfide in blood vessels in healthy young subjects, older subjects, and subjects with high blood pressure. This research may lead to future treatments that improve blood vessel health. The goal of this study is to look at the role of hydrogen sulfide in blood vessels in healthy young subjects, older subjects and subjects with high blood pressure.

In these studies, the researchers use “microdialysis” (MD). This technique involves placing very thin plastic tubing between the layers of the skin. The largest part of the tubing is about 6x the diameter of a human hair. They pump fluid like that found in the body’s tissues through the tubing. The tubing acts like very small blood vessels in the skin by allowing some substances to pass between the fluid in the tubing and the fluid in the skin. During the experiment, they will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm² (0.4 inch²), nickel-sized area of skin at each tube. Some of these substances are like natural chemicals found in the body. Some of these substances block the actions of natural chemicals found in the body.

The substances used for these experiments are:

1. NaHS – (Sodium Hydrogen Sulfide) – a substance that makes hydrogen sulfide.
2. Na₂S – (Sodium Sulfide) – a substance that makes hydrogen sulfide.
3. L-NAME (N⁶-nitro-L-arginine methyl ester) – keeps blood vessels from getting bigger.
4. SNP (sodium nitroprusside) – causes blood vessels to get as big as they can.
6. TEA (Tetraethylammonium chloride) Blocks the action of some chemicals made by your body and keeps blood vessels from getting bigger.
7. Senicapoc (ICA-17043) – Keeps blood vessels from getting bigger.
8. Ketorolac (5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid) - Keeps blood vessels from getting bigger.
9. AOAA (aminoxyacetic acid) – Keeps blood vessels from getting bigger.
10. ACh (acetylcholine) – A substance made by your body to make your blood vessels get bigger.
11. Lactated Ringers – a fluid like that which bathes the tissues in your body.

This research study has 1 biopsy and 3 MD experiments. In the MD experiments, researchers use weak laser light to measure blood flow in small vessels in the skin. For the biopsy, the researchers take two small pieces of skin from your arm.
2. **Procedures:** Please read the descriptions of the days. Then write your initials by the days.

You could be asked to repeat a trial, procedure, or test. This could include blood draws with your OK. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

_____ **initial Screening Day:** Drink only water and do not eat after 10 PM the evening before your visit. Go to the Noll Lab for your appointment. The research and/or Clinical Research Center staff performs the screening. When you arrive, the nurse draws about 30 ml (2 Tbsp) of blood from a vein in your arm. During the screening, the staff measures blood pressure, heart rate, weight, height, and waist circumference. They also take a medical history and 12-lead resting ECG. They send the blood to labs that test it for blood cells, fats in the blood, blood chemistry, and proteins in which they are interested. If you take thyroid hormone, they draw an extra 3.5 ml (0.2 Tbsp) to check the level of thyroid hormone. They may test the blood for other substances of interest. They do not perform genetic tests on the blood. They do not test the blood for the presence of disease (e.g. HIV). Women who are not post-menopausal will submit urine samples for pregnancy tests.

_____ **initial Visit 2 Biopsy experiment:** The researcher takes two small pieces of skin from your arm (skin biopsy) using the following method. First, you wash your arm with soap and warm water. The researcher cleans the top of the lidocaine-vial with alcohol. An approved clinician wipes your skin with alcohol and injects lidocaine into the skin of your arm at the biopsy sites to numb them. The researcher will wait a couple of minutes after injecting the lidocaine to give the drug time to work. The researcher will clean the biopsy site 3 times with an orange cleanser (povidone iodine) and an alcohol pad. If you are allergic to iodine, the researchers will use only alcohol. The researcher will gently touch the site with the tip of a needle to see if you can feel anything. You may feel the slight pain of the pin-prick or only pressure. If you can feel pain, the researcher will wait a little longer or the approved clinician will add more lidocaine into the skin. When the site is numb, the researcher will place a sterile drape over your arm. The biopsy sites are located in an opening in the middle of the drape. The researcher uses a punch-tool that looks like a screwdriver that has a round, hollow tip. The tip is 3mm (0.12 in) in diameter. The hollow tip acts like a cookie cutter. The researcher places the tip of the punch against the skin at the biopsy site and applies mild pressure. You will feel the pressure. The tip of the punch will go about 3 mm (0.12 in) into your skin. The punch collects a small piece of skin about 3mm x 2mm (0.12 in x 0.08 in). The researcher holds sterile gauze on the site to stop any bleeding. The researcher places the piece of skin into a small container. The researcher uses the punch to remove the second piece of skin in the same way. A sterile bandage will be applied to your arm. The researcher will give you instructions about how to take care of the biopsy site.

_____ **initial Visit 3 Dose Response experiment:**

**Microdialysis (MD):** The researchers place a tight band around your upper arm so they can easily see your veins. For each MD site, the researchers make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. They remove the tight band after the pen marks are made. The MD tubing will enter and exit your skin at the marks. The researchers clean your arm with an orange-colored povidone iodine fluid and alcohol. They place an ice bag on your arm for 5 minutes to numb your skin. Then the researchers insert a thin needle into your skin at each entry
mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin at the matching exit mark. The researchers thread the tubing through the needle. Next, they withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes. The treatments at the three MD sites are: (6.4)

1. Lactated Ringer’s
2. Lactated Ringer’s + NaHS or Na$_2$S
3. Lactated Ringer’s + NaHS or Na$_2$S + Glibenclamide

The researchers tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow with a weak laser light. The researchers can control the temperature of the holders. The holders will start at 33°C (91.4°F). During the experiment, the researchers measure blood pressure with a cuff that inflates on your upper arm. The researchers place 3 sticky tabs on your chest to which they attach the wires of an ECG machine that measures your heart rate. Throughout the experiment, they measure skin blood flow and skin temperature at the MD sites.

**Dose Response:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record baseline skin blood flow for about 20 minutes. They increase the concentration of NaHS or Na$_2$S every 10 to 15 minutes. They do this until skin blood flow becomes stable at each concentration. There are 7 different concentrations of NaHS or Na$_2$S. Following the last concentration, all sites are flushed with lactated Ringers. After flushing of sites for 10 minutes, lactated Ringers + SNP are perfused at all sites. Also, the researcher increases the local heat to 43°C (109°F) for about 20 minutes. This makes your blood vessels as big as possible. After 30 minutes, the researchers remove the local heaters and the MD probes. They apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

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**initial Visits 4 Mechanism of Action Experiment (maximum 6 hours):**

**Microdialysis (MD):** The researchers insert the MD tubing in the same manner described above.

The treatments at the four MD sites are:

1. Lactated Ringer’s + NaHS or Na$_2$S
2. Lactated Ringer’s + NaHS or Na$_2$S + Glibenclamide
3. Lactated Ringer’s + NaHS or Na$_2$S + TEA
4. Lactated Ringer’s + NaHS or Na$_2$S + Senicapoc

The researcher tapes down and attached the local heaters and weak lasers in the same manner as described above.

**Drug protocol:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. Only lactated Ringers flows through the tubing during the 20-minute baseline. Each site then has NaHS or Na$_2$S perfused for 20 minutes. This is done until skin blood flow becomes stable. Next, the researchers add glibenclamide, TEA, or Senicapoc to the sites as shown above. Then they flush all sites with lactated Ringers. Then lactated Ringers + SNP are perfused at all sites. Also, the researcher increases the local heat to 43°C (109°F) for about 20 minutes. This makes your blood vessels as big as possible. After 30 minutes, the
researchers remove the local heaters and the MD probes, and apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

**Initial Visit 5 Acetylcholine Dose Response (maximum 6 hours):**

Microdialysis (MD): The researchers insert the MD tubing in the same manner described above.

The treatments at the five MD sites are:
1. Lactated Ringer’s
2. Lactated Ringer’s + L-NAME
3. Lactated Ringer’s + Ketorolac
4. Lactated Ringer’s + AOAA
5. Lactated Ringer’s + AOAA + L-NAME + Ketorolac

**Dose Response:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. Then they perfuse each drug in an MD site for 60 to 90 minutes. They do this until skin blood flow becomes stable. This level of skin blood flow is the “drug baseline.” Each site then receives an acetylcholine concentration. These acetylcholine concentrations are perfused until blood flow returns to drug baseline. The same concentration of acetylcholine is perfused again until blood flow returns to drug baseline. Next there is an ACh dose response with 5 concentrations; each is perfused for 10 to 15 minutes. SNP is then perfused at all sites while the researcher increases the local heat to 43°C (109°F) for about 20 minutes to make the blood vessels as big as possible. After 30 minutes, the researchers remove the local heaters and the MD probes. They apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

3. Discomforts and risks:

**Skin Biopsy:** You may stop the procedure at any time. Trained staff will perform the biopsy. You may lie on a bed during the biopsy, if you wish. The researcher will make sure that you are informed and ready. You may still be nervous enough to feel dizzy, sick to your stomach, and/or you could faint. The lidocaine will numb the site so that you feel very little or no pain during the biopsy. You will feel the pressure of the biopsy tool on your skin. As with any event that breaks the skin, you could get an infection. Trained staff use sterile techniques to keep the risk of infection very small. The skin biopsy may cause some pain, swelling, bleeding, and bruising. Gauze pressed onto the site stops bleeding. The researcher places a sterile bandage on the site. You will be given instructions about caring for the biopsy site. Most people with have a small scar. The skin of some people overreacts to injury. If you are one of these, your skin may produce a scar that is larger and easier to see. There may be some minor pain for a couple of days when the lidocaine wears off. The pain would be like that felt after some blood draws.

**Microdialysis (MD):** The risks are less than that for a blood draw because MD uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. MD is likely to cause some pain and bruising like that of a blood draw. However, the researchers use ice to numb the arm before they insert the tubing. Also, the small needle helps to reduce pain. Most people do not feel pain after the tubing is in place. You may feel a little pain when they remove the tubing at the end of the experiment. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick
to your stomach, or may faint. Sometimes the tubing can break during removal from the skin. Then the researchers remove the tubing by pulling on the other end of it. This produces no added risk for you. The tubing could break so that a small piece is left under the skin. This has not occurred in any of the studies in this lab. If this happened, they would treat any tubing remaining in the skin like a splinter. In this case, they would cut the thin layer of skin over the tubing to remove the tubing. Mild pressure with sterile gauze stops any slight bleeding that may occur. Aseptic technique and sterile supplies like those used in hospitals keep the risk of infection minimal. Infection has not occurred with MD in this lab or others that the researchers know of. They apply a sterile bandage after the experiment. They tell you how to take care of the site.

**Fluid flowing through the tubing:** The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. If a bad reaction should occur, medical help is summoned right away.

**Lactated Ringer’s Solution:** This fluid is similar to the natural fluids in the skin. This fluid contains salt, potassium, lactate, and chloride. The acid content is like that of the body’s fluids. A bad reaction to this fluid is highly unlikely.

**L-NAME, ACh, Glibenclamide, NaHS, Na₂S, AOAA, Ketorolac, TEA, ICA-17043 and SNP:** Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. These and other researchers have used the substances in humans before. There have been no reports of bad reactions.

**Blood Draw:** Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. Using the same techniques used in hospitals keeps the chance of infection minimal. Do not exercise hard for 24 hours before a blood draw.

**ECG:** This machine measures the electrical activity of the heart. The researchers tape 3-12 wires from the machine to spots on your body. There have been no adverse effects. The tape may irritate the skin.

**Blood Pressure (manual, CardioCap):** The researchers measure blood pressure using the method common in a doctor’s office or with a machine. A cuff inflates on the upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in the elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly. The cuff could cause mild bruising.

**Medical Screening:** You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. If you request someone of the same sex to conduct the screening, the researchers will make their best effort to provide one.

**Phone screening form:** Only the researcher uses this form. They use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions.
You may request someone of the same sex to ask you the questions. They collect the information in a private and professional manner. The completed form is kept confidential and secure.

**Laser Doppler Flowmetry:** Weak lasers can hurt your eye if you stared into the light for a long time. The researchers do not turn on the laser until the probes are taped to a surface. The tape may irritate the skin.

**Local Heating:** The researchers measure the temperature of the skin under the holders. The skin feels very warm but does not hurt. The heating makes the skin of the arm under the holders red like when you take a hot bath. The redness will not last more than several hours. Some people may be more sensitive to the heating than others. If your arm feels too hot, you tell the researchers, and they reduce or stop the heating.

**Povidine Iodine:** Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to povidone iodine if you are allergic to iodine. You inform the researchers if you have this allergy so that they use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or blood pressure change and/or fainting.

**Latex:** Some gloves and medical materials are made of latex rubber. Inform the researchers if you are allergic to latex and decline to participate in the study.

4. **a. Benefits to me:** You will receive a medical screening that could inform you about your health. You will learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we will advise you to work with a health care provider to keep your blood pressure controlled.

**b. Potential benefits to society:** With aging and diseases (high blood pressure, high cholesterol, diabetes, etc.) blood vessel changes occur during which blood vessels rely of different methods to make vessels bigger. Hydrogen sulfide is a substance that is used as a “backup” system when blood vessels change and cannot rely on their normal system. Little is known about how hydrogen sulfide functions in human skin. This project can determine the role hydrogen sulfide plays in making skin blood vessels bigger and how it works. This could lead to new targets to treat with drugs in aging and diseases to improve health. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

5. **Time duration of the procedures and study:** The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

- ____initial Day 1 Screening: typically about ½ hour, no longer than hour
- ____initial Day 2 (Biopsy): 1 hour
- ____initial Days 3 - 5 (MD Experiment): typically about 5 ½ hours, no longer than 6 hours

6. **Statement of confidentiality:** Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation
in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personally identifiable information is disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project. Your confidentiality will be kept to the degree permitted by the technology used. No guarantees can be made regarding the interception of data sent via the Internet by any third parties.

7. **Right to ask questions:** Please contact Jessica Kutz (W: 814-865-2432, M: 570-490-1426), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints or concerns about this research. You can also call these numbers if you feel this study has harmed you. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the research team.

8. **Compensation:**

   a. **Biopsy Experiment:** You will receive $50.00 for each of the biopsies (maximum $100.00).

   b. **Microdialysis Experiments:** $15.00 for each MD probe inserted in the arm. $40 more for completing each experiment.

\[
\begin{align*}
3 \text{ probe MD experiment: } & 3 \text{ probes } \times 15.00 = 45.00 \\
\rightarrow & + $40 \text{ completing study } = 85.00 \text{ (total for experiment 3 probes)} \\
4 \text{ Probe MD experiment: } & 4 \text{ probes } \times 15.00 = 60.00 \\
\rightarrow & + $40 \text{ completing study } = 100.00 \text{ (total for experiment 4 probes)} \\
5 \text{ probe MD experiment: } & 5 \text{ probes } \times 15.00 = 75.00 \\
\rightarrow & + $40 \text{ completing study } = 115.00 \text{ (total for experiment 5 probes)} \\
\end{align*}
\]

**Total if you complete all possible experiments:** \(85.00 + 100.00 + 115.00 + 100 = 400\)

In addition, you may choose one of the following: lab T-shirt, bag, or other item we may offer.

For each trial, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of the 3-probe MD trial you will be paid for each probe that was inserted (3 x $15.00 = $45.00) plus $20.00 for that trial. This is because $20.00 is one-half of $40.00. You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.
9. **Injury Clause:** In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Jessica Kutz (W: 814-865-2432, M: 570-490-1426), Susan Slimak (W: 814-863-8556, H: 814-237-4618), and Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

10. **Voluntary participation:** Your being in this study is voluntary. You may withdraw from this study at any time by telling the researcher. If you decide to withdraw, you will not have a penalty or loss of benefits you would receive otherwise. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures. The researcher may end your role in the study without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You have been given an opportunity to ask any questions you may have, and all such questions or inquiries have been answered to your satisfaction.

11. In the event that abnormal test results are obtained, you will be apprised of the results immediately and advised to contact a health care provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

______________________________________________  ____________________________
Volunteer                                                                 Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

______________________________________________  ____________________________
Investigator                                                                             Date
Version #2

INFORMED CONSENT FORM FOR CLINICAL RESEARCH STUDY
The Pennsylvania State University

Title of Project: Cutaneous vascular effects of Hydrogen Sulfide

Principal Investigator: Jessica Kutz
Address: 132 Noll Laboratory
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Phone: 814-863-8556

Advisor: Lacy Alexander, Ph.D.
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Advisor: W. Larry Kenney, Ph.D.
Address: 102 Noll Laboratory
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Research Assistants: Susan Slimak, RN
Phone: 814-863-8556

Jane Pierzga, M.S., Research Assistant
Phone: 814-865-1236

This is to certify that I, ______________________ have been given the following information with respect to my participation as a volunteer in a program of investigation.

1. Purpose of the study:
The human body controls the amount of blood flowing through healthy blood vessels. It does this by changing their size. The way the body controls blood flow can be different with age and the presence of some diseases. One of the first signs of a disease that affects blood vessels is a change in the function of the very small blood vessels of the skin.

The skin has a web of small blood vessels that is easy to access and study. The health of the skin’s blood vessels can mirror the health of the body’s other blood vessels. Studying blood flow control in the skin’s blood vessels helps researchers to learn about the onset, effects, and treatment of blood vessel diseases. Our lab has studied the body’s control skin blood flow in young and older humans. We have also studied humans with high blood pressure (hypertension) and high cholesterol for the past ten years. We have seen that aging and disease can damage the control of blood vessels. Our current research explores the reasons for that impairment.

Hydrogen sulfide is a natural gas made in your body. It helps your blood vessels to get
bigger and increase blood flow. The goal of this study is to look at the role of hydrogen sulfide in blood vessels in healthy young subjects, older subjects, and subjects with high blood pressure. This research may lead to future treatments that improve blood vessel health. The goal of this study is to look at the role of hydrogen sulfide in blood vessels in healthy young subjects, older subjects, and subjects with high blood pressure.

In these studies, the researchers use “microdialysis” (MD). This technique involves placing very thin plastic tubing between the layers of the skin. The largest part of the tubing is about 6x the diameter of a human hair. They pump fluid like that found in the body’s tissues through the tubing. The tubing acts like very small blood vessels in the skin by allowing some substances to pass between the fluid in the tubing and the fluid in the skin. During the experiment, they will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm² (0.4 inch²), nickel-sized area of skin at each tube. Some of these substances are like natural chemicals found in the body. Some of these substances block the actions of natural chemicals found in the body. Some of these substances are like natural chemicals found in the body.

The substances used for these experiments are:

1. NaHS – (sodium hydrogen sulfide) - a substance that makes hydrogen sulfide.
2. Na₂S – (Sodium Sulfide) - a substance that makes hydrogen sulfide.
3. L-NAME (N²-nitro-L-arginine methyl ester) – keeps blood vessels from getting bigger.
4. SNP (sodium nitroprusside) – causes blood vessels to get as big as they can.
6. TEA (Tetraethylammonium chloride) Blocks the action of some chemicals made by your body and keeps blood vessels from getting bigger.
7. Senicapoc (ICA-17043) – Keeps blood vessels from getting bigger.
8. Ketorolac (5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid) - Keeps blood vessels from getting bigger.
9. AOAA (aminooxyacetic acid) – Keeps blood vessels from getting bigger.
10. ACh (acetylcholine) – A substance made by your body to make your blood vessels get bigger.
11. Lactated Ringers – a fluid like that which baths the tissues in your body.

This research study has 1 biopsy and 3 MD experiments. In the MD experiments, researchers use weak laser light to measure blood flow in small vessels in the skin. For the biopsy, the researchers take two small pieces of skin from your arm.

2. Procedures: Please read the descriptions of the days. Then write your initials by the days.

You could be asked to repeat a trial, procedure, or test. This could include blood draws with your OK. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

______ initial Visits: Blood Pressure Visits If we think that you have high blood pressure, we will measure your blood pressure on three occasions within a 2-week period. This will make
sure that you belong in the high blood pressure group. We make these readings on 2 separate visits to the Noll Lab and then during your screening visit. Also, you wear a monitor to record your blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist or on your shoulder.

________ initial Screening Day: Drink only water and do not eat after 10 PM the evening before your visit. Go to the Noll Lab for your appointment. The research and/or Clinical Research Center staff performs the screening. When you arrive, the nurse draws about 30 ml (2 Tbsp) of blood from a vein in your arm. During the screening, the staff measures blood pressure, heart rate, weight, height, and waist circumference. They also take a medical history and 12-lead resting ECG. They send the blood to labs that test it for blood cells, fats in the blood, blood chemistry, and proteins in which they are interested. If you take thyroid hormone, they draw an extra 3.5 ml (0.2 Tbsp) to check the level of thyroid hormone. They may test the blood for other substances of interest. They do not perform genetic tests on the blood. They do not test the blood for the presence of disease (e.g. HIV). Women who are not post-menopausal will submit urine samples for pregnancy tests.

________ initial Visit 4 Biopsy experiment: The researcher takes two small pieces of skin from your arm (skin biopsy) using the following method. First, you wash your arm with soap and warm water. The researcher cleans the top of the lidocaine-vial with alcohol. An approved clinician wipes your skin with alcohol and injects lidocaine into the skin of your arm at the biopsy sites to numb them. The researcher will wait a couple of minutes after injecting the lidocaine to give the drug time to work. The researcher will clean the biopsy site 3 times with an orange cleanser (povidone iodine) and an alcohol pad. If you are allergic to iodine, the researchers will use only alcohol. The researcher will gently touch the site with the tip of a needle to see if you can feel anything. You may feel the slight pain of the pin-prick or only pressure. If you can feel pain, the researcher will wait a little longer or the approved clinician will add more lidocaine into the skin. When the site is numb, the researcher will place a sterile drape over your arm. The biopsy sites are located in an opening in the middle of the drape. The researcher uses a punch-tool that looks like a screwdriver that has a round, hollow tip. The tip is 3mm (0.12 in) in diameter. The hollow tip acts like a cookie cutter. The researcher places the tip of the punch against the skin at the biopsy site and applies mild pressure. You will feel the pressure. The tip of the punch will go about 3 mm (0.12 in) into your skin. The punch collects a small piece of skin about 3mm x 2mm (0.12 in x 0.08 in). The researcher holds sterile gauze on the site to stop any bleeding. The researcher places the piece of skin into a small container. The researcher uses the punch to remove the second piece of skin in the same way. A sterile bandage will be applied to your arm. The researcher will give you instructions about how to take care of the biopsy site.

________ initial Visit 5 Dose Response experiment:

Microdialysis (MD): The researchers place a tight band around your upper arm so they can easily see your veins. For each MD site, the researchers make pairs of pen-marks on your arm 2.5 cm (1 inch) apart and away from veins. They remove the tight band after the pen marks are made. The MD tubing will enter and exit your skin at the marks. The researchers clean your arm with an orange-colored povidone iodine fluid and alcohol. They place an ice bag on your arm for 5 minutes to numb your skin. Then the researchers insert a thin needle into your skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch) and leaves your skin
at the matching exit mark. The researchers thread the tubing through the needle. Next, they withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes. The treatments at the four MD sites are: (6.4)

1. Lactated Ringer’s
2. Lactated Ringer’s + Na₂S + L-NAME
3. Lactated Ringer’s + Na₂S + KETO
4. Lactated Ringer’s + Na₂S + L-NAME + KETO

The researchers tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow with a weak laser light. The researchers can control the temperature of the holders. The holders will start at 33°C (91.4°F). During the experiment, the researchers measure blood pressure with a cuff that inflates on your upper arm. The researchers place 3 sticky tabs on your chest to which they attach the wires of an ECG machine that measures your heart rate. Throughout the experiment, they measure skin blood flow and skin temperature at the MD sites.

**Dose Response:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. They record baseline skin blood flow for about 20 minutes. They increase the concentration of Na₂S every 10 to 15 minutes. They do this until skin blood flow becomes stable at each concentration. There are 7 different concentrations of Na₂S. Following the last concentration, all sites are flushed with lactated Ringers. After flushing of sites for 10 minutes, lactated Ringers + SNP are perfused at all sites. Also, the researcher increases the local heat to 43°C (109°F) for about 20 minutes. This makes your blood vessels as big as possible. After 30 minutes, the researchers remove the local heaters and the MD probes. They apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

**initial Visits 6 Mechanism of Action Experiment (maximum 6 hours):**

**Microdialysis (MD):** The researchers insert the MD tubing in the same manner described above. The treatments at the four MD sites are:

1. Lactated Ringer’s + Na₂S
2. Lactated Ringer’s + Na₂S + Glibenclamide
3. Lactated Ringer’s + Na₂S + TEA
4. Lactated Ringer’s + Na₂S + Senicapoc

The researcher tapes down and attached the local heaters and weak lasers in the same manner as described above.

**Drug protocol:** The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. Only lactated Ringers flows through the tubing during the 20-minute baseline. Each site then has Na₂S perfused for 20 minutes. This is done until skin blood flow becomes stable. Next, the researchers add glibenclamide, TEA, or Senicapoc to the sites as shown above. Then they flush all sites with lactated Ringers. Then lactated Ringers + SNP are perfused at all sites. Also, the researcher increases the local heat to 43°C (109°F) for about 20 minutes. This makes your blood vessels as big as possible. After 30 minutes, the
researchers remove the local heaters and the MD probes, and apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

initial Visit 7 Acetylcholine Dose Response (maximum 6 hours):

Microdialysis (MD): The researchers insert the MD tubing in the same manner described above. The treatments at the five MD sites are:

1. Lactated Ringer’s
2. Lactated Ringer’s + L-NAME
3. Lactated Ringer’s + Ketorolac
4. Lactated Ringer’s + AOAA
5. Lactated Ringer’s + AOAA + L-NAME + Ketorolac

Dose Response: The researchers continue to record blood pressure every 5-7 minutes on the arm that does not have the local heaters. Then they perfuse each drug in an MD site for 60 to 90 minutes. They do this until skin blood flow becomes stable. This level of skin blood flow is the “drug baseline.” Each site then receives an acetylcholine concentration. These acetylcholine concentrations are perfused until blood flow returns to drug baseline. The same concentration of acetylcholine is perfused again until blood flow returns to drug baseline. Next there is an ACh dose response with 5 concentrations; each is perfused for 10 to 15 minutes. SNP is then perfused at all sites while the researcher increases the local heat to 43°C (109°F) for about 20 minutes to make the blood vessels as big as possible. After 30 minutes, the researchers remove the local heaters and the MD probes. They apply a sterile dressing over the sites. They measure blood pressure and heart rate before you leave.

3. Discomforts and risks:

Skin Biopsy: You may stop the procedure at any time. Trained staff will perform the biopsy. You may lie on a bed during the biopsy, if you wish. The researcher will make sure that you are informed and ready. You may still be nervous enough to feel dizzy, sick to your stomach, and/or you could faint. The lidocaine will numb the site so that you feel very little or no pain during the biopsy. You will feel the pressure of the biopsy tool on your skin. As with any event that breaks the skin, you could get an infection. Trained staff use sterile techniques to keep the risk of infection very small. The skin biopsy may cause some pain, swelling, bleeding, and bruising. Gauze pressed onto the site stops bleeding. The researcher places a sterile bandage on the site. You will be given instructions about caring for the biopsy site. Most people with have a small scar. The skin of some people overreacts to injury. If you are one of these, your skin may produce a scar that is larger and easier to see. There may be some minor pain for a couple of days when the lidocaine wears off. The pain would be like that felt after some blood draws.

Microdialysis (MD): The risks are less than that for a blood draw because MD uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. MD is likely to cause some pain and bruising like that of a blood draw. However, the researchers use ice to numb the arm before they insert the tubing. Also, the small needle helps to reduce pain. Most people do not feel pain after the tubing is in place. You may feel a little pain when they remove the tubing at the end of the experiment. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick
to your stomach, or may faint. Sometimes the tubing can break during removal from the skin. Then the researchers remove the tubing by pulling on the other end of it. This produces no added risk for you. The tubing could break so that a small piece is left under the skin. This has not occurred in any of the studies in this lab. If this happened, they would treat any tubing remaining in the skin like a splinter. In this case, they would cut the thin layer of skin over the tubing to remove the tubing. Mild pressure with sterile gauze stops any slight bleeding that may occur. Aseptic technique and sterile supplies like those used in hospitals keep the risk of infection minimal. Infection has not occurred with MD in this lab or others that the researchers know of. They apply a sterile bandage after the experiment. They tell you how to take care of the site.

Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. If a bad reaction should occur, medical help is summoned right away.

Lactated Ringer’s Solution: This fluid is similar to the natural fluids in the skin. This fluid contains salt, potassium, lactate, and chloride. The acid content is like that of the body’s fluids. A bad reaction to this fluid is highly unlikely.

L-NAME, ACh, Glibenclamide, Na₂S, AOAA, Ketorolac, TEA, ICA-17043 and SNP: Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. These and other researchers have used the substances in humans before. There have been no reports of bad reactions.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. Using the same techniques used in hospitals keeps the chance of infection minimal. Do not exercise hard for 24 hours before a blood draw.

ECG: This machine measures the electrical activity of the heart. The researchers tape 3-12 wires from the machine to spots on your body. There have been no adverse effects. The tape may irritate the skin.

Blood Pressure (manual, CardioCap): The researchers measure blood pressure using the method common in a doctor’s office or with a machine. A cuff inflates on the upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in the elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly. The cuff could cause mild bruising.

Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. If you request someone of the same sex to conduct the screening, the researchers will make their best effort to provide one.

Phone screening form: Only the researcher uses this form. They use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions.
You may request someone of the same sex to ask you the questions. They collect the information in a private and professional manner. The completed form is kept confidential and secure.

**Laser Doppler Flowmetry:** Weak lasers can hurt your eye if you stared into the light for a long time. The researchers do not turn on the laser until the probes are taped to a surface. The tape may irritate the skin.

**Local Heating:** The researchers measure the temperature of the skin under the holders. The skin feels very warm but does not hurt. The heating makes the skin of the arm under the holders red like when you take a hot bath. The redness will not last more than several hours. Some people may be more sensitive to the heating than others. If your arm feels too hot, you tell the researchers, and they reduce or stop the heating.

**Povidine Iodine:** Hospitals and researchers use this orange-colored fluid to clean and sterilize the skin. You could have a bad reaction to povidone iodine if you are allergic to iodine. You inform the researchers if you have this allergy so that they use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or blood pressure change and/or fainting.

**Latex:** Some gloves and medical materials are made of latex rubber. Inform the researchers if you are allergic to latex and decline to participate in the study.

**Blood Pressure Monitor:** You wear the device for 24 hours to collect blood pressure. The device uses AA or rechargeable batteries for power. We place a cuff on your upper arm. We attach the control unit to the cuff. We hang the unit from strap on your shoulder or around your waist. The controller takes a measure once every hour by making the cuff inflate, taking a reading, and deflating the cuff. You need to stay as still as possible and hold your arm slightly away from your body while the cuff is inflated. You need to keep the system dry and properly attached for the whole 24-hours. This could be a bother to you. The unit may interfere with daily routines such as showering and sleeping. The control unit prevents the cuff from inflating too high (more than 300 mmHg) or too long (more than 180 seconds). A safety release valve allows you to leave the air out of the cuff if the batteries should fail while the cuff is inflated. You could have an allergic reaction in the area of the cuff caused by the cuff’s fabric. The reaction could include itching, rash, and/or swelling. The pressure of the cuff on your skin could cause one or several small reddish or purplish spots to form in the skin. Usually these are harmless and will disappear in a few days. However, the spots could lead to a sudden, longer lasting bruising or inflammation of a vein. The pressure of the cuff could cause bruising. You will tell us whether you bruise easily. If you bruise easily, you will not use the monitor. We will give you verbal and written instructions. If we accept you into the study, you perform the 24-hour monitoring twice.

4. **a. Benefits to me:** You will receive a medical screening that could inform you about your health. You will learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we will advise you to work with a health care provider to keep your blood pressure controlled.

**b. Potential benefits to society:** With aging and diseases (high blood pressure, high cholesterol,
diabetes, etc.) blood vessel changes occur during which blood vessels rely of different methods to make vessels bigger. Hydrogen sulfide is a substance that is used as a “backup” system when blood vessels change and cannot rely on their normal system. Little is known about how hydrogen sulfide functions in human skin. This project can determine the role hydrogen sulfide plays in making skin blood vessels bigger and how it works. This could lead to new targets to treat with drugs in aging and diseases to improve health. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

5. **Time duration of the procedures and study:** The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

   _____ initial Day 1-2 Blood pressure: Typically about ½ hour each visit, 24 hr at home
   _____ initial Day 3 Screening: 1 hour typically about ½ hour, no longer than 1 hour
   _____ initial Day 4 (Biopsy): Typically about ½ hour, no longer than 1 hour
   _____ initial Days 5 - 7 (MD Experiment): Typically about 5 ½ hours, no longer than 6 hours

6. **Statement of confidentiality:** Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personally identifiable information is disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project. Your confidentiality will be kept to the degree permitted by the technology used. No guarantees can be made regarding the interception of data sent via the Internet by any third parties.

7. **Right to ask questions:** Please contact Jessica Kutz (W: 814-865-2432, M: 570-490-1426), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints or concerns about this research. You can also call these numbers if you feel this study has harmed you. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the research team.

8. **Compensation:**

   c. **Biopsy Experiment:** You will receive $50.00 for each of the biopsies (maximum $100.00).
   d. **Microdialysis Experiments:** $15.00 for each MD probe inserted in the arm. $40 more for completing each experiment.
4 Probe MD experiment: 4 probes x $15.00 = $60.00
   → + $40 completing study = $100.00 (total for experiment 4 probes)
5 probe MD experiment: 5 probes x $15.00 = $75.00
   → + $40 completing study = $115.00 (total for experiment 5 probes)

Total if you complete all possible experiments: 85.00 + $100.00 + $115.00 + $100 = $400

In addition, you may choose one of the following: lab T-shirt, bag, or other item we may offer.

For each trial, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of the 3-probe MD trial you will be paid for each probe that was inserted (3 x $15.00 = $45.00) plus $20.00 for that trial. This is because $20.00 is one-half of $40.00. You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

9. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Jessica Kutz (W: 814-865-2432, M: 570-490-1426), Susan Slimak (W: 814-863-8556, H: 814-237-4618), and Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

10. Voluntary participation: Your being in this study is voluntary. You may withdraw from this study at any time by telling the researcher. If you decide to withdraw, you will not have a penalty or loss of benefits you would receive otherwise. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures. The researcher may end your role in the study without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You have been given an opportunity to ask any questions you may have, and all such questions or inquiries have been answered to your satisfaction.

11. In the event that abnormal test results are obtained, you will be apprised of the results immediately and advised to contact a health care provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.
I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

______________________________  Volunteer  
I, the undersigned, have defined and explained the studies involved to the above volunteer.

______________________________  Investigator  

Date  
Date
Appendix D

TETRAHYDROBIOPTERIN INCREASES NO-DEPENDENT VASODILATION IN HYPERCHOLESTEROLEMIC HUMAN SKIN THROUGH ENOS-COUPLING MECHANISMS

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16802
Abstract

Localized exogenous R-BH₄ corrects the deficit in local heat-induced vasodilation (VD) in hypercholesterolemic (HC) human skin through one of two plausible mechanisms: by serving as an essential cofactor to stabilizing endothelial nitric oxide synthase (eNOS) or through generalized antioxidant effects. We used the stereoisomer S-BH₄, which has the same antioxidant properties but does not function as an essential NOS cofactor, to elucidate the mechanism by which R-BH₄ restores cutaneous VD in hypercholesterolemic humans. Intradermal microdialysis fibers were placed in 20 normocholesterolemic (NC), 13 mid-range cholesterolemic (MC) and 18 hypercholesterolemic (LDL: 94±3, 124±3 and 179±6 mg/dl, respectively) men and women to perfuse Ringers (control site) and R-BH₄. In 10 NC, 13 MC and 9 HC subjects (LDL: 94±3, 124±3, 180±10 mg/dl), S-BH₄ was perfused at a third microdialysis site. Skin blood flow was measured during a standardized local heating protocol to elicit eNOS-dependent VD. After cutaneous vascular conductance (CVC = LDF/ MAP) plateaued, NO-dependent VD was quantified by perfusing L-NAME. Data were normalized as %CVCₘₐₓ. Fully expressed VD (NC: 97.9±2.3 vs. MC: 85.4±5.4 & HC: 79.9±4.2%CVCₘₐₓ) and the NO-dependent portion (NC: 62.1±3 vs. MC: 45.8±3.9 & HC: 35.7±2.8%CVCₘₐₓ) were reduced in HC (both p<0.01 vs. NC), but only the fully expressed VD was reduced in MC (p<0.01 vs. NC). R-BH₄ increased the fully expressed (93.9±3.4%CVCₘₐₓ; p<0.01) and NO-dependent VD (52.1±5.1%CVCₘₐₓ; p<0.01) in HC but not in NC or MC. S-BH₄ increased full-expressed VD in HC (p<0.01) but did not affect NO-dependent VD in HC or MC. In contrast S-BH₄ attenuated NO-dependent VD in NC (control: 62.1±3 vs. S-BH₄: 41.6±7 %CVCₘₐₓ; p<0.001). Exogenous R-BH₄ restores NO-dependent VD in hypercholesterolemic human skin predominantly through NOS coupling mechanisms but increases full expression of the local heating response through generalized antioxidant properties.
Introduction

Hypercholesterolemia, specifically elevated low-density lipoproteins, is a major risk factor for the development of atherosclerotic vascular disease (25, 26). One early indicator of functionally manifested hypercholesterolemia-induced vascular disease is a loss of the vasoprotective molecule nitric oxide (NO) in the microcirculation. Microvascular dysfunction is detectable prior to the onset of atherosclerotic plaque formation in the conduit arteries and may contribute to the development of conduit by inducing retrograde blood flow patterns which could cause damaging shear stress (5). The human cutaneous circulation is an accessible regional circulation to assess microvascular dysfunction and mechanisms contributing to vascular disease with hypercholesterolemia (13-15). Impairments in cutaneous microvascular function are related to end-organ damage and indices of vascular function in the coronary and renal vascular beds (5, 19).

We previously showed that cutaneous NO-dependent vasodilation in response to an eNOS-specific local skin heating stimulus is attenuated in human subjects with clinically defined hypercholesterolemia (LDL>160mg/dl) (13-15). The reduction in NO-dependent vasodilation is mediated by: (1) upregulated arginase activity which limits substrate availability through eNOS (15), (2) an increase in ascorbate-sensitive oxidant stress mechanisms (12), and (3) possibly a reduction in the essential eNOS cofactor tetrahydrobiopterin (R-BH₄) (11). Ascorbate improves NO bioavailability through several different mechanisms including scavenging radicals which oxidize NO and through stabilizing and resynthesizing R-BH₄ from BH₂(21). Furthermore, R-BH₄ has both eNOS cofactor properties and generalized antioxidant properties primarily through quenching superoxide (20). It is unclear if the restoration in cutaneous NO-dependent vasodilation to local heating with localized R-BH₄ in hypercholesterolemic humans is due eNOS recoupling mechanisms or generalized antioxidant effects preventing the oxidation of NO.
The purpose of this study was to differentiate how R-BH₄ augments NO-dependent vasodilation during local heating of hypercholesterolemic human skin. We used the stereoisomer S-BH₄, which has the same antioxidant properties but does not function as an eNOS cofactor (17, 22), to elucidate the mechanism by which R-BH₄ restores cutaneous vasodilation in this population. We hypothesized that local administration of R-BH₄ restores cutaneous NO-dependent vasodilation during local heating in the skin of hypercholesterolemics via stabilization of eNOS and not through generalized antioxidant mechanisms.

**Methods**

**Subjects**

All experimental procedures were approved by the Institutional Review Board at The Pennsylvania State University which follows the guidelines set forth by the Declaration of Helsinki. Twenty healthy normocholesterolemic, thirteen mid-range cholesterolemic and eighteen hypercholesterolemic men and women participated in the study after giving written and oral consent (Table D-1). The age of the subjects ranged from 40 to 69 years old and the groups (normocholesterolemic, mid-range cholesterolemic and hypercholesterolemic) were age-matched to account for any age-related differences in local heating response. The subjects were normally active, not currently taking statins or other medications including low-dose aspirin, vitamins, antioxidants, or hormone replacement.

During the protocol, subjects were in a thermoneutral laboratory in a semi-supine position with their arm positioned at the level of the heart. Four intradermal microdialysis probes were inserted into the dermal layer of the ventral left forearm for local delivery of pharmacological agents (16). Microdialysis sites were perfused with: (1) 20 mM N⁶-nitro-L-arginine (L-NAME) to inhibit NO production via a non-specific NO synthase inhibition during
local heating; (2) 10 mM R-BH$_4$ to locally supplement the essential cofactor for eNOS (Sigma-Aldrich, St Louis, MO, USA) (14); (3) 10 mM S-BH$_4$, to have the same antioxidant properties of R-BH$_4$ but without the cofactor properties for eNOS (22); and (4) lactated Ringer to serve as control. The S-BH$_4$ site was utilized in eleven of the normocholesterolemic, thirteen mid-range cholesterolemic and nine of the hypercholesterolemic subjects. Pharmacological solutions were mixed in lactated Ringer solution immediately prior to use and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI, USA). Solutions were wrapped immediately in foil to prevent photodegradation.

Skin blood flow was measured using integrated laser-Doppler flowmeter. Local temperature was controlled using a local heater placed directly above each microdialysis membrane (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). The laser Doppler probe was secured in the local heater and monitored blood flow over each microdialysis fiber. An automated brachial cuff (Cardiocap) measured arterial blood pressure every 5 minutes on the right arm. Cutaneous vascular conductance (CVC) was calculated as laser-Doppler flux divided by mean arterial pressure (MAP). Data were normalized as %CVC$_{\text{max}}$.

*Local heating protocol*

The insertion of the microdialysis fibers results in a transient period of hyperemia. Sixty to ninety minutes were allowed for hyperemia to cease prior to a standard local heating protocol to induce eNOS-dependent vasodilation (1, 24). Local heater temperature was increased from the baseline clamped temperature of 33°C to 42°C at a rate of 0.1°C every second and then clamped at 42°C for the remainder of the heating protocol. After approximately 30 to 40 minutes, when skin blood flow reached an established plateau, 20 mM L-NAME was perfused to quantify NO-dependent vasodilation at all sites. The phases of the local heating response include the initial peak and nadir (small NO contribution) followed by a predominately NO-dependent plateau.
Figure C-1 is a representative tracing of a local heating response in a hypercholesterolemic subject at their R-BH₄ and S-BH₄ sites, the control site has been omitted for clarity. Following infusion of L-NAME and subsequent stabilization of a post L-NAME plateau in skin blood flow, 28 mM sodium nitroprusside (SNP) was perfused and local temperature increased to 43°C to elicit maximal cutaneous vasodilation (CVCₘₐₓ)(18).

Data and Statistical Analysis

Data were collected continuously at 40 Hz and stored for offline analysis with signal-processing software (Windaq, DATAQ Instruments). All skin blood flow data (CVC) were averaged for a stable 5 minutes of baseline, local heating plateau, post L-NAME plateau and maximal vasodilation and normalized to percent of maximal CVC (% CVCₘₐₓ). The NO-dependent vasodilation during local heating was calculated by the difference between the local heating plateau and the post L-NAME plateau. A three way mixed model ANOVA was conducted to distinguish differences in %CVCₘₐₓ between subject groups, local heating phase and pharmacological treatment sites (SAS, version 9.1). A separate two way ANOVA was conducted to examine differences between groups and localized pharmacological treatments for the vasodilation due to NO. A priori specific planned comparisons with Bonferroni correction were performed when appropriate to determine where differences between groups and localized drug treatments existed. The level of significance was set at α = 0.05. Values are presented as means ± SE unless specified otherwise.
Results

Subject characteristics are presented in Table D-1. The subject groups differed in total cholesterol, low-density lipoproteins (LDL) and oxidized-LDL, by design but there were no differences among groups for high density lipoprotein. Otherwise the subjects were well matched for age, BMI, fasting blood glucose, and mean arterial pressure.

Figure D-1 illustrates time course representative tracings at the R-BH$_4$ and S-BH$_4$ sites for a hypercholesterolemic subject. Full expression of the plateau of local heating-induced vasodilation, and the post-L-NAME plateau are labeled. The difference between the plateau and the post-L-NAME plateau is calculated as functional NO-dependent vasodilation (13-15).

The group means %CVC$_{max}$ for the local heating-induced plateau and the post-L-NAME plateau are illustrated in figure C-2. At the control sites full expression of the local heating response was attenuated in the mid-range cholesterolemic and hypercholesterolemic groups compared to the normocholesterolemic group (both $P < 0.01$). The post L-NAME plateau in the hypercholesterolemic group was elevated compared to the normocholesterolemic group ($P < 0.05$) (Figure D-2A), but there was no difference between the mid-range and normocholesterolemic groups. Localized R-BH$_4$ administration increased the local heating plateau in hypercholesterolemic subjects, compared to their control site ($P < 0.01$) (Figure D-2B). S-BH$_4$ augmented the full expression of the local heating plateau in the hypercholesterolemic group compared to their control site ($P < 0.01$), but the post L-NAME plateau remained elevated.

Further, S-BH$_4$ had no effect on the full local heating response in the normocholesterolemic or mid-range cholesterolemic group, but the post L-NAME plateau was elevated compared to the control site in the normocholesterolemic group (Figure D-2C). There was no difference at the L-NAME site throughout the heating protocol among groups (Figure D-2D).
Figure D-3 illustrates the decrease in skin blood flow following NOS inhibition within each site for all of the cholesterol groups. NO-dependent vasodilation was attenuated in the hypercholesterolemic and the mid-range cholesterolemic group compared to the normocholesterolemic group \((P < 0.01)\). Localized R-BH_4 treatment augmented NO-dependent vasodilation in the hypercholesterolemic group \((P < 0.01)\) with no change in the mid-range cholesterolemic group. There was no difference in NO-dependent vasodilation with local S-BH_4 treatment for the mid-range and hypercholesterolemic groups compared to their control sites. In contrast, in the normocholesterolemic group local S-BH_4 treatment reduced NO-dependent vasodilation compared with their control sites \((P < 0.01)\).

Absolute maximal CVC (flux/mmHg) values for each site are presented in Table D-2. There were no differences in absolute maximal CVC among localized microdialysis treatment sites or cholesterol groups.

**Discussion**

The major new finding of this study is that supplementation with the essential NOS cofactor BH_4 augments NO-dependent vasodilation during local heating of the skin predominantly by improving NOS coupling mechanisms. Local S-BH_4 administration modestly increased full expression of the local heating plateau in hypercholesteroleemics but did not improve NO-dependent vasodilation in any of the cholesterol groups, suggesting that the generalized antioxidant effects of BH_4 may increase non-NO-dependent mechanisms. These results suggest that the reduced NO bioavailability associated with hypercholesterolemic-induced microvascular dysfunction is mediated in part through a reduction in the eNOS essential cofactor BH_4 leading to NOS uncoupling, and localized administration of R-BH_4 likely augments NO-dependent vasodilation through recoupling eNOS. We also found that vasodilation during local
heating was reduced in a cohort of subjects with moderately elevated LDL cholesterol (midrange), but there was not a detectable difference in NO-dependent vasodilation with localized R-BH₄ administration in this group. Finally, a secondary unexpected finding was that S-BH₄ treatment increased the post-L-NAME plateau in the normcholesterolemic group and reduced NO-dependent vasodilation, suggesting that supplementing antioxidants to healthy vasculature may decrease functional NO bioavailability.

We have previously demonstrated that the hypercholesterolemia-induced microvascular dysfunction is mediated by a variety of mechanisms contributing to eNOS uncoupling where the eNOS dimer uncouples to produce superoxide instead of functional NO(7). These mechanisms including 1) increased arginase activity which limits L-arginine availability for eNOS (15), 2) through ascorbate sensitive oxidant stress mechanisms (13), and 3) through a reduction in the essential eNOS cofactor BH₄ (14). In these prior experiments it was not possible to determine if localized ascorbate or BH₄ supplementation improved NO-dependent mechanisms through eNOS coupling mechanisms or through acting as an antioxidant. This was in part because ascorbate is a powerful generalized antioxidant capable of quenching hydroxyl, alkoxy, preoxyl, thiol, and toopheroxyl radicals (6) and stabilizing/resynthesizing BH₄ from the salvage pathway (21). Further, BH₄ independently possesses both eNOS coupling actions through acting as an essential co-factor and weak generalized antioxidant properties. The antioxidant effects are primarily a result of the rapid reaction of superoxide radical reducing BH₄ to BH₃(21), in essence quenching superoxide. At present, there has been no direct examination of the antioxidant capacity between ascorbate and BH₄, however superoxide reacts with BH₄ 6-10 times faster than it reacts with ascorbate(21). In the present study we utilized the (S) enantiomer of the functional eNOS cofactor R-BH₄. We found that providing a BH₄ compound with the same antioxidant properties but without functional co-factor properties did not affect NO-dependent vasodilation in our hypercholesterolemic subjects. This is in contrast to R-BH₄, which significantly augmented NO-
dependent vasodilation in the hypercholesterolemic. Based on these new data it is likely that localized BH₄ supplementation increases functional NO-dependent vasodilation predominantly through acting as an essential cofactor to recouple eNOS.

In this study we designed our experiments to provide equamolar concentrations (10mM) of both BH₄ enantiomers. We observed a positive effect of R-BH₄ on NO-dependent vasodilation in our hypercholesterolemic subjects that was not observed with S-BH₄. Similar results have also been seen chronic smokers with pre-existing endothelial dysfunction(10). However, other studies in humans using similar methodology and relative concentrations of these drugs have found that both enantiomers are equally effective at augmenting endothelium-dependent vasodilation after ischemia-reperfusion injury (22). Together, these results suggest in models of chronic endothelial dysfunction (i.e. smoking, hypercholesterolemia) the principle target BH₄ results in increased NO-dependent vasodilation through the reversal of NOS uncoupling, whereas in acute cases of endothelial injury, where there is an increase in superoxide, BH₄ may be beneficial as a generalized antioxidant.

In our previous studies examining hypercholesterolemia-induced microvascular dysfunction we have focused on two distinct but well matched groups to draw comparisons based on different cholesterol concentrations. In this study we have also tested an intermediary “midrange” cholesterol group. Using our standardized local heating protocol to induce eNOS-dependent vasodilation the midrange cholesterol group demonstrated an attenuated plateau response (Figure D-2A) due to a reduction in NO-dependent vasodilatory mechanisms (Figure D-3A). With R-BH₄ administration there was no longer a difference the in plateau of the local heating response (Figure D-2B) or vasodilation due to the production of NO (Figure D-3B), however there was not a significant improvement in NO-dependent vasodilation within the group after the localized R-BH₄ treatment. This is likely because of the small decrement in cutaneous vascular function observed in the midrange subject group. Our study was powered to detect a
meaningful physiological difference 15%CVC$_{max}$ difference due to group and localized microdialysis treatment. While we saw group differences in the control site there was no difference within group due to localized R-BH4 treatment.

One unexpected finding in the present study was that localized supplementation of S-BH$_4$ increased in NO-independent vasodilatory mechanisms in both hyper and normocholesterolemsics and there was a reduction in functional NO-dependent vasodilation in the normocholesterolemic group. Vascular studies conducted in healthy (i.e. no evidence of endothelial dysfunction) models have shown that supplementing supraphysiological doses of antioxidants can alter redox potential and decrease NO bioavailability (8). Using a similar local heating protocol in young healthy subjects, Stewart et al. have shown that H$_2$O$_2$ can increase NO-independent vasodilatory mechanisms (23). There is also increasing evidence that ascorbate and BH$_4$ can potentiate endothelium derived hyperpolarizing factors (EDHF) through H$_2$O$_2$. Because the NO-independent portion of the local heating response is in part mediated by EDHFs (2), it is possible that S-BH$_4$ is increasing EDHFs through altering the redox potential within the vessel. We have observed a similar phenomenon in young healthy human subject with the local administration of ascorbate (16). However, it remains unclear why we do not observe a similar increase in NO-independent vasodilation with R-BH$_4$ or ascorbate in our healthy middle-aged cohort of subjects.

**Perspectives**

In the current study we utilized supraphysiological concentrations of both BH$_4$ enantiomers to examine eNOS coupling mechanisms in humans with hypercholesterolemia. One criticism of these localized infusion studies is the potential for non-specific effects of the highly concentrated drugs on the vessel wall. In this study we specifically chose a compound (S-BH$_4$) to examine the potential non-specific antioxidant effects delivered at equimolar concentrations. A different approach that others have used examining the mechanisms of BH$_4$ on
hypercholesterolemia-induced microvascular dysfunction is a chronic oral BH₄ intervention. Similar to the results from this study with localized administration of BH₄, monotherapy with chronic dosing of oral BH₄ in hypercholesterolemic humans reverses endothelial dysfunction and decrease systemic markers of oxidant stress (3). While treatment of hypercholesterolemia-induced vascular dysfunction with BH₄ appears to be efficacious, it is cost prohibitive and the long term repercussions are unclear. For example, recent studies in populations with more significant atherosclerotic vascular disease indicate that high doses of oral BH₄ oxidize rapidly adversely affecting the BH₄/BH₂ ratio and resulting in increased oxidant stress through inducing eNOS uncoupling (4).

We have explored the effects of an oral atorvastatin intervention on hypercholesterolemia-induced cutaneous microvascular dysfunction. In this series of studies we consistently observed an improvement in NO-dependent vasodilation after twelve weeks on an oral atorvastatin intervention, with no further improvements from the local delivery of arginase inhibitors, non-specific antioxidants (ascorbate), or BH₄ (13-15). Furthermore, our biochemical studies support that oral atorvastatin at relatively low clinical doses (10mg) decreases arginase activity and restored endothelium-dependent vasodilatory function. Because statins have many “pleiotropic” effects unrelated to their direct action of decreasing LDL, including acting as a generalized antioxidant (27) and increasing intracellular BH₄ content (9), this multipronged approach to treating hypercholesterolemia-induced vasodilatory dysfunction in this preclinical model appears efficacious. However, the effects of systemic statin therapy on microvascular pro-constrictor pathways and atherosclerotic vascular remodeling detectable in the cutaneous circulation remain unclear.
Summary

In summary supplementation with the essential NOS cofactor R-BH₄ augments NO-dependent vasodilation, and S-BH₄ modestly increased NO-independent vasodilation during local heating in subjects with hypercholesterolemia-induced cutaneous microvascular dysfunction. Local administration of the S-BH₄ enantiomer, which has the same antioxidant properties as R-BH₄ but does not serve as a NOS cofactor, did not improve NO-dependent vasodilation suggesting that the reduction in NO bioavailability is predominantly mediated through NOS uncoupling mechanisms. Vasodilation to local heating was reduced in a cohort of subjects with moderately elevated LDL cholesterol (midrange), but there was not a detectable difference in NO-dependent vasodilation with localized R-BH₄ administration in this group. Finally, the BH₄ pathway through the use of an oral intervention or statin therapy is a viable molecular target for the treatment of hypercholesterolemia-induced microvascular dysfunction.

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Author contributions

L.M.A.: study design, data analysis, interpretation, and manuscript preparation; J.L.K.: data collection, analysis, and manuscript preparation; W.L.K.: data interpretation and manuscript
preparation. All studies took place at PSU. All authors approved the final version of the manuscript.
Table D-1: Subject characteristics.

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<th>Mid-range cholesterolemic</th>
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<td>49 ± 1.6</td>
<td>54 ± 1.8</td>
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<tr>
<td>Total Cholesterol (mg/dl)</td>
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<td>194 ± 4.6</td>
<td>262 ± 6.6*†</td>
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<tr>
<td>HDL (mg/dl)</td>
<td>55 ± 3.0</td>
<td>53 ± 3.8</td>
<td>53 ± 4.5</td>
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<tr>
<td>LDL (mg/dl)</td>
<td>94 ± 2.5</td>
<td>124 ± 3.0*</td>
<td>179 ± 5.6*†</td>
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<td>oXLDL (U/L)</td>
<td>43 ± 2.1</td>
<td>59 ± 2.1*</td>
<td>88 ± 4.2*†</td>
</tr>
<tr>
<td>Trig (mg/dl)</td>
<td>89 ± 7.6</td>
<td>87 ± 11.0</td>
<td>154 ± 14.9*†</td>
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<td>MAP (mmHg)</td>
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<td>90 ± 2</td>
<td>89 ± 2</td>
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<tr>
<td>Heart Rate (beats/min)</td>
<td>62 ± 5</td>
<td>67 ± 4</td>
<td>67 ± 3</td>
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Subject characteristics. mean ± SEM * p < 0.001 vs. normocholesterolemic subject group ; †p < 0.001 vs. mid-range cholesterolemic subject group. HDL, high density lipoprotein; LDL, low density lipoprotein; oXLDL, oxidized low density lipoprotein; Trig, triglyceride; MAP, mean arterial pressure.
Table D-2: Absolute cutaneous vascular conductance.

<table>
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<th>Mid-range cholesterolemic</th>
<th>Hypercholesterolemic</th>
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<td>1.7 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>R-BH4</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.3</td>
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<tr>
<td>S-BH4</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.3</td>
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Absolute cutaneous vascular conducatance in all microdialysis treatment sites for the normocholesterolemic, mid-range cholesterolemic and hypercholesterolemic groups. There was no significant difference due to localized drug treatment or between groups.
Figure D-1. Representative Tracing. The time course of cutaneous vascular conductance ($\%CVC_{\text{max}}$) throughout the local heating response in a hypercholesterolemic subject for the R-$\text{BH}_4$ (●) and S-$\text{BH}_4$ (+) sites. The nitric oxide-dependent plateau and the post L-NAME plateau are labeled. The difference between the plateau and the post L-NAME plateau is the decrease with nitric oxide synthase (NOS) inhibition represented by the arrows for each site. The control site was omitted for clarity.
Figure D-2. Mean skin blood flow responses for the plateau and the post L-NAME plateau. Cutaneous vascular conductance (CVC) represented at percent maximum at the plateau in skin blood flow as a result of local heating and after NOS inhibition with L-NAME in normocholesterolemic (NC) control subjects, mid-range cholesterolemic (MC) subjects and hypercholesterolemic (HC) subjects. Each panel illustrates a different localized microdialysis treatment site: (A) control site, (B) R-BH4 site, (C) S-BH4 site and (D) L-NAME throughout local heating. * p < 0.01 different vs. normocholesterolemic group, † p < 0.05 vs. normocholesterolemic control site. δ p< 0.001 vs. hypercholesterolemic control site.
**Figure D-3.** Within site nitric oxide-dependent vasodilation during local heating. The decrease in cutaneous vascular conductance with NOS inhibition in normocholesterolemic (NC) control subjects, mid-range cholesterolemic (MC) subjects and hypercholesterolemic (HC) subjects. Each panel illustrates a different localized microdialysis treatment site (A) control site, (B) R-BH4 site and (C) S-BH4 site * p < 0.001 vs. normocholesterolemic control site, † p = 0.005 vs. hypercholesterolemic control site
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