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Title Page

Update on Gaseous Signaling Molecules Nitric Oxide and Hydrogen Sulfide: Strategies to Capture their Functional Activity for Human Therapeutics

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Running title: Nitric oxide and hydrogen sulfide

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Abbreviations:

NO – nitric oxide

H_2S – hydrogen sulfide

NOS – nitric oxide synthase

BH_4 – tetrahydrobiopterin

PPI – proton pump inhibitor

CIMT – carotid intima media thickness

CCO – cytochrome c oxidase

CA – carbonic anhydrase

sGC – soluble guanylyl cyclase

ASA - argininosuccinic aciduria

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CBS - cystathionine beta-synthase

CSE - cystathionine gamma-lyase

3-MST - 3-mercaptopyruvate sulfurtransferase

DATS - diallyl trisulfide

NSAIDS - non-steroidal anti-inflammatory drugs

Abstract:

The discovery of the production of gaseous molecules such as nitric oxide and hydrogen sulfide within the human body began a new concept in cellular signaling. Over the past 30 years, these molecules have been investigated and found to have extremely important beneficial effects in a number of chronic diseases. Gaseous signaling molecules that diffuse in three dimensions apparently contradict the selectivity and specificity afforded by normal ligand receptor binding and activation. This new concept has also created hurdles in the development of safe and efficacious drug therapy based on these molecules. Mechanisms involving formation of more stable intermediates and second messengers allows for new strategies for safe and effective delivery of these molecules for human disease. The purpose of this review is to highlight the biological effects of nitric oxide and hydrogen sulfide, their seemingly indistinguishable effects and how these molecules can be safely harnessed for drug development and pre-cursors or substrates administered for human consumption through applied physiology.

Key words: nitric oxide, hydrogen sulfide, gasotransmitter, nutrients, microbiome

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Introduction

Gaseous signaling molecules or more recently termed gasotransmitters are gaseous molecules that are either synthesized endogenously in the human body or are produced by organisms such as bacteria that live in and on the human body. These gases are then used to transmit chemical signals which induce certain and specific physiological or biochemical changes in the human host. The notion of a gas that diffuses in three dimensions created uncertainty on how these gases could selectively and specifically activate their targets for their intended physiological effects. The reactivity and relatively short biological half-life of these gases is thought to limit their biological activity. Although nitric oxide (NO) and hydrogen sulfide (H2S) have been known for many centuries, their role in human physiology is new. What is clear is the fundamental and necessary nature of these molecules for normal human health. Loss of production of these gaseous molecules is associated with many chronic diseases that plague the health care industry, mostly involving cardiovascular disorders including hypertension and chronic inflammation (Bryan 2011, Polhemus and Lefer 2014). A number of published studies in experimental animals and even humans reveal that delivery of bioactive NO, H2S and/or their more stable reaction intermediates improve or prevent many of these conditions and diseases. As a result, there have been enormous efforts to develop safe and effective activators and/or stimulators of the respective enzyme systems and even donor compounds that release the specific gasotransmitter. These efforts have largely been unsuccessful. Drug discovery programs typically rely on inhibitors that limit the production of a noxious or inflammatory molecule. There are very few conditions whereby endogenous overproduction of these gaseous molecules poses a problem to human disease management. Understanding the physiological basis for the production pathways for these molecules, both

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from human enzyme systems as well as from the microbiome may allow for better strategies for the development of safe and effective therapies. This review will highlight those production pathways for NO and H2S and review current and prospective technologies that may be able to harness their therapeutic activity. Signaling molecules or second messengers, by definition, should have limited half-life in order to avoid prolonged signal transduction. Considering this, NO and H2S share such characteristics and as such, strategies to produce physiological amounts of these molecules may be necessary and sufficient to initiate and recapitulate their functions. The principle of applied physiology, rather than applied pharmacology always prevails in terms of safety, efficacy and limiting unwanted side effects.

Nitric Oxide

Nitric oxide or NO is now considered one of the most important signaling molecules in human and mammalian physiology. Continuous and constitutive production of NO regulates blood pressure, oxygen delivery, immune and neuro-cognitive function, sexual function and many more fundamental physiological processes. Loss of the production of NO is consider to be one of the earliest events in the onset and progression of most if not all chronic diseases (Egashira, Inou et al. 1993). Therefore understanding how the body makes NO, what goes wrong in patients that can't make NO and then developing rationale therapies to restore the production of NO is considered the "Holy Grail" in medicine.

There are two predominant production pathways through which the human body produces NO. The first pathway to be discovered was through the 5-electron oxidation of the guanidine nitrogen of L-arginine to L-citrulline with NO as a by-product (Hibbs, Taintor et al. 1987). The second, more recently discovered pathway is through the serial reduction of inorganic nitrate to nitrite and to NO (Duncan, Dougall et al. 1995). The first pathway is through a mammalian

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enzyme system known as nitric oxide synthase (NOS). The NOS enzyme requires at least half a dozen substrates and co-factors, the most important being reduced tetrahydrobiopterin (BH4). Without sufficient BH4 or when BH4 becomes oxidized, the NOS enzyme uncouples and is no longer functional (Tayeh and Marletta 1989). Oxidation of BH4 is the rate limiting step in NO production from NOS. The two-electron reduction of nitrate to nitrite is dependent upon commensal bacteria that live in and on the human body (Lundberg, Weitzberg et al. 2004). Mammals lack a functional nitrate reductase enzyme system so this pathway is dependent upon the proper microbiome. Once nitrite is formed, either from reduction of nitrate or from oxidation of NO, there are mammalian systems that can reduce nitrite to NO, including hemoproteins, redox active metals, mitochondria and conditions of low pH and hypoxia (Kozlov, Staniek et al. 1999, Cosby, Partovi et al. 2003, Feelisch, Fernandez et al. 2008). A number of different bacteria have been identified in the oral cavity that contribute to nitrate reduction and nitrite/NO formation (Doel, Benjamin et al. 2005, Hyde, Andrade et al. 2014, Hyde, Luk et al. 2014). Eradicating oral bacteria with antiseptic mouthwash results in symptoms of NO deficiency including an increase in blood pressure (Kapil, Haydar et al. 2013, Woessner, Smoliga et al. 2016). When NO is produced, it is thought to act primarily through binding and activation of soluble guanylyl cyclase (sGC) and production of the second messenger cyclic guanosine monophosphate (cGMP) (Katsuki, Arnold et al. 1977). NO can also form other nitrogen oxides that can post-translationally modify critical cysteine residues on proteins affecting protein structure and function akin to phosphorylation (Lane, Hao et al. 2001). In this manner, NO regulates a host of physiological functions.

The fundamental question then becomes, how do you restore or recapitulate NO based signaling? Understanding both pathways for NO production allows one to focus on restoring

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these pathways. This includes strategies to preserve BH4 redox status to maintain NOS coupling and function as well as restoring the microbiome in the oral cavity, in the intestines and on the skin. Nitrite has been shown to prevent BH4 oxidation and maintain NOS function (Stokes, Dugas et al. 2009). Nitrite production in the oral cavity due to reduction of nitrate by the oral nitrate reducing bacteria is one way of restoring NO. This requires sufficient nitrate from the diet and the right oral bacteria. This pathways becomes disrupted when humans do not consume enough nitrate from vegetables and when the oral microbiome is disrupted due to antiseptic mouthwash or antibiotic use (Bryan 2018). Providing nitrite in the form a therapy is a logical, safe and effective strategy for restoring NO based signaling. In fact, nitrite has even been shown to be the endocrine mediator of NO based signaling (Elrod, Calvert et al. 2008). Recently many studies have been published demonstrating safety and efficacy of nitrite in humans within a large range of doses. Capsules containing sodium nitrite at doses of 160mg and 320mg have been used to investigate toxicity and pharmacokinetics. Nitrite even at high doses of 320mg did not show any clinical toxicity as measured by methemoglobinemia (<15%) (Greenway, Predmore et al. 2012). Therapy utilizing a 80 mg nitrite capsule caused a statistically significant drop in systolic blood pressure with no changes on diastolic blood pressures. Longer term studies using 80-160 mg nitrite capsules in a randomized, placebo control, double blind study over ten weeks led to an increase in plasma nitrite both acutely and chronically and was well tolerated (DeVan, Johnson et al. 2015). Endothelial function and carotid artery elasticity significantly improved (DeVan, Johnson et al. 2015). Other studies have shown significant improvements in measures of motor and cognitive outcomes in healthy middle aged and older adults using 80 and 160 mg nitrite (62 ± 7 years) (Justice, Johnson et al. 2015). Unlike nitrate, the effects of nitrite are not dependent upon or require the present of

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oral nitrate reducing bacteria. Doses of nitrite appear to be safe even at doses that far exceed daily human consumption.

The amounts of nitrite administered in the aforementioned studies are much higher than one would normally consume in an ordinary diet. This is due to the fact that nitrite is inefficiently reduced to NO at physiological concentrations of oxygen (Bryan, Fernandez et al. 2005, Feelisch, Fernandez et al. 2008). Therefore more is needed to get any appreciable amount of NO produced, especially in people that are NO deficient. We have discovered natural product chemistry that can reduce nitrite to NO even in the presence of physiological amounts of oxygen, thereby providing an exogenous source of NO in vivo(Tang, Garg et al. 2009). The premise of this technology is that if your body can't make NO due to endothelial dysfunction, lack of oral nitrate reducing bacteria, use of antiseptic mouthwash or proton pump inhibitors (PPIs), then this will provide an exogenous source of NO. These discoveries have been utilized to develop a commericial product (Neo40™, HumanN, Inc™) which uses 15-20 mg of supplemental sodium nitrite to account for differences in endogenous production along with the natural nitrite reductase in the form of an orally disintegrating tablet. Studies have shown that it could improve cardiovascular risk factors in older patients, significantly reduce triglycerides, and reduce blood pressure (Zand, Lanza et al. 2011). Single and acute administration of this lozenge leads to peak plasma levels of nitrite around 1.5μM after 20 minutes. In pediatric patients with a condition called argininosuccinic aciduria (ASA), which is an inborn error in metabolism that causes hyperammonemia along with hypertension, coagulopathy, renal and liver dysfunction, the nitrite lozenge led to a significant reduction in blood pressure when multiple classes of anti-hypertensive medications were ineffective. The lozenge also improved renal function, cognition and reversed cardiac hypertrophy (Nagamani, Campeau et al. 2012).

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In another randomized controlled study using the nitrite lozenge showed a significantly reduction in blood pressure, significant increase in blood vessel dilation and a significant improvement in endothelial function and arterial compliance. (Houston and Hays 2014). Furthermore in a study of pre-hypertensive patients (BP >120/80 < 139/89), administration of one lozenge twice daily for 30 days lowered both systolic and diastolic blood pressure by 12 mmHg and 6 mmHg respectively(Biswas, Gonzalez et al. 2014) along with improvements in functional capacity as measured by a 6 minute walk test. In an exercise study, the nitrite lozenge significantly improved exercise performance (Lee, Kim et al. 2015). Most recently, in subjects with stable carotid plaque, the NO lozenge led to a 11% reduction in carotid plaque as measured by carotid intima media thickness (CIMT) after 6 months (Lee 2016). To put this in perspective, treatment with statins (mean treatment duration of 25.6 months) reveal regression and slowing of progression of CIMT of approximately 2.7% (-0.04) after over 2 years (Bedi, Singh et al. 2010). Using the nitric oxide lozenge, CIMT decreased an average of 0.073 mm or 10.9% after 6 months (Lee 2016). Similarly, this same patented technology in the form of a concentrated beet root powder (Superbeets™, HumanN, Inc.™) has been shown to attenuate peripheral chemoreflex sensitivity without a concomitant change in spontaneous cardiovagal baroreflex sensitivity. The concentrated beet powder also reduces systemic blood pressure and mean arterial blood pressure in older adults (Bock, Ueda et al. 2017). These studies clearly demonstrate the safety and efficacy of low supplemental doses of nitrite. Furthermore providing an exogenous source of NO in humans appears to correct for any deficiencies from dietary exposure, pharmacological inhibition by antiseptics or PPIs.

Other studies in mice reveal that simply giving nitrate or nitrite in the drinking water can alter the oral microbiome to allow for colonization of more nitrate reducing bacteria (Hyde, Luk et al.

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2014). In this manner, nitrite therapy may provide the basis for restoring eNOS function and restoring the proper NO generating bacteria in the oral cavity.

Hydrogen Sulfide

Hydrogen sulfide (H2S) is a naturally occurring compound that is produced throughout the human body. Just like NO, H2S is acutely toxic at high concentrations The toxicity is attributed to inhibition of mitochondrial cytochrome c oxidase (CCO), carbonic anhydrase (CA), monoamine oxidase, Na+/K+-ATPase and cholinesterases (Beauchamp, Bus et al. 1984). H2S also reacts with the oxy forms of myoglobin and hemoglobin (FeII-O2) generating the sulfheme derivative thereby reducing oxygen binding and transport (Pietri, Roman-Morales et al. 2011). The modern history of H2S is mostly associated with its toxic effects. It was only recently that H2S may serve as an important biological mediator (Abe and Kimura 1996). Since then, studies have shown that H2S is involved in a number of biological signaling mechanism in numerous biological systems. Similar to sGC as a target for NO based signaling, Na/K-ATPase is a known physiological target for H2S (Xia, Chen et al. 2009). Also similar to post-translational modification of critical cysteine residues by nitrosation of NO based signaling, H2S can lead to persulfidation thereby affecting the structure and function of proteins (Paul and Snyder 2015). Among other biological effects, H2S has been reported to have anti-hypertensive and cytoprotective properties(Benavides, Squadrito et al. 2007, Polhemus and Lefer 2014), again similar to NO. Most of the research on H2S has focused mainly on the enzymatic production in the heart, kidneys, vasculature and the brain. The biological action of H2S produced by the gut microbiota has been recently examined . Given the prominent role of H2S in a number of diseases, many therapeutic targets have been discovered for H2S therapy. The molecular targets of H2S include proteins, enzymes,

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transcription factors, and membrane ion channels and other proteins. Cysteine is the major source and substrate of H2S in mammals. H2S is catalyzed by three enzymes: cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). The enzyme 3-MST is localized in mitochondria and CBS and CSE exist in the cytosol (Polhemus and Lefer 2014).

Similar to NO production, there are commensal bacteria that provide the human host with a source of hydrogen sulfide. Sulfate reducing bacteria (SRB) are ubiquitous and common to the mammalian colon (Tomasova, Konopelski et al. 2016). The dominant genera are Desulfovibrio (D. piger, D. desulfuricans), Desulfobacter, Desulfobulbus and Desulfotomaculum (Rabis, Venceslau et al. 2015). There are two substrates that are essential for SRB to produce H2S. Those are any form of sulfate and an electron donor for the sulfate reduction. A sulfate-rich diet has been shown to result in the increased growth of D. piger and increased H2S production in the colon of humans and mice (Gibson, Macfarland et al. 1988, Rey, Gonzalez et al. 2013). D. piger can also utilize sulfated glycans. Sulfate reducing bacteria therefore are a common source of H2S in the gut of mammals. Several anaerobic bacterial strains (Escherichia coli, Salmonella enterica, Clostridia and Enterobacter aerogenes) convert cysteine to H2S, pyruvate and ammonia by cysteine desulfhydrase (Kumagai, Sejima et al. 1975, Awano, Wada et al. 2005). In addition, gut bacteria can also produce H2S by sulfite reduction. Sulfite reductase activity is present in many species of bacteria such as E. coli, Salmonella, Enterobacter, Klebsiella, Bacillus, Staphylococcus, Corynebacterium, and Rhodococcus (Blauchier, Davila et al. 2010).

The total sulfide concentration produced in the large intestine has been quantified and reported to be in the range of 0.2–3.4 mmol/L in humans. However since the feces of humans and

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rodents have a large binding capacity for sulfur, it is estimated that less than 8% of total sulfide is in a free form (Jorgensen and Mortensen 2001, Levitt, Springfield et al. 2002). Colonic epithelial cells are more efficient in converting sulfide into thiosulfate than other tissues (Furne, Springfield et al. 2001). Infusion of radioactive H2S into the cecum of rats showed that all the absorbed H2S from the infusion was immediately oxidized to thiosulfate (Levitt, Furne et al. 1999). Flannigan et al. (Flannigan, McCoy et al. 2011). reported that fecal samples of germ-free mice contained 50% less H2S compared to feces of controls. Germ-free mice have significantly less free H2S in plasma and gastrointestinal tissues compared to control mice. CSE activity in tissue of germ-free mice is significantly reduced, whereas tissue cysteine levels appear to be significantly elevated compared to conventional mice. These data suggests that the microbiota profoundly regulates systemic bioavailability and metabolism of H2S (Shen, Carlstrom et al. 2013). Eliminated vitamin B6, a CSE and CBS cofactor, in the diet causes a 50% reduction of fecal H2S likely due to the reduction of enzymatic H2S synthesis in colonic tissues. Interestingly, after six weeks of the vitamin B6-deficient diet, the fecal H2S levels in mice were restored to normal. This suggests that the H2S generation in the gut of germ-free mice was shifted towards microbial production pathways by increasing the SRB activity (Flannigan, McCoy et al. 2011). Although Vitamin B6 deficiency is relatively rare, many people may still be deficient despite consuming the recommended daily allowance of Vitamin B6 (Morris, Picciano et al. 2008). Rats treated with neomycin show significantly lower levels of thiosulfate and sulfane sulfur in the portal vein blood but not in peripheral blood (Tomasova, Jurkowska et al. 2016). Overproduction of H2S in the colon has been implicated in colonic inflammation and cancer (Roediger, Duncan et al. 1993, Cao, Zhang et al. 2010). However other studies suggest that colonic epithelial cells are well-adapted to the H2S-rich

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environment, and that H2S plays a beneficial role in the protection of the gut brain barrier (GBB) (Goubern, Andriamihaja et al. 2007, Wallace 2010, Motta, Flannigan et al. 2015). H2S may serve as an energy source for colonic epithelial cells due to ATP formation from H2S oxidation(Goubern, Andriamihaja et al. 2007). H2S also promotes colonic mucus production thereby maintaining the integrity of bacterial biofilms (Motta, Flannigan et al. 2015). H2S protects and reverses damage induced by chronic administration of nonsteroidal anti-inflammatory drugs(Blackler, Motta et al. 2015).

The main dietary sources of exposure to sulfur compounds in the human diet are inorganic sulfates in drinking water and from sulfur containing amino acids in proteins derived from plants and animals. There are only two of the twenty amino acids that are sulfur-containing amino acids (SAAs), methionine and cysteine. Methionine is unable to be synthesized by the human body and must be consumed through the diet. Dietary methionine intake can increase cysteine levels Cysteine is known as a semi-essential amino acid since humans can produce it endogenously from methionine. However, the function of the enzymes required for the production of cysteine from methionine declines with age (Koc and Gladyshev 2007) and therefore with increasing age less cysteine is produced endogenously . Excess consumption cysteine and methionine from the diet is converted and stored as glutathione (GSH). Cysteine availability is the rate-limiting factor for GSH biosynthesis from glutamate, glycine, and cysteine.

You Can't Have One Without the Other

There is growing evidence that there is "crosstalk" between sulfide and NO. NO and H_2S elicit many of the same physiological actions in the cardiovascular system including: vasodilation, regulation of mitochondrial respiration, pro-angiogenic effects, inhibition of apoptosis, and

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antioxidant effects. Although H_2S and NO modulate independent signaling cascades, there is now strong evidence that these gaseous signaling molecules operate in a cooperative fashion to modulate a multitude of important actions (Cortese-Krott, Fernandez et al. 2015). eNOS function is regulated tightly by post-translational modifications (i.e., phosphorylation of amino acids Ser-1177 and Thr-495) that enhance or inhibit NO production by eNOS (Dimmeler, Fleming et al. 1999). In a pressure-overload murine heart failure, Kondo et al. (Kondo, Bhushan et al. 2013) reported that mice treated with an H_2S donor (i.e., SG1002) were protected against adverse cardiac remodeling and heart failure. Interestingly, the authors also reported a significant increase in the phosphorylation of the eNOS activation site, Ser-1177, in mice treated with SG1002 compared with the control group. This increase in eNOS phosphorylation was accompanied by increased NO production as demonstrated by increased circulating and tissue levels of NO metabolites. The authors suggest that the protective actions of H_2S donor therapy in heart failure is mediated in part via increased myocardial and circulating NO levels.

Furthermore, Predmore et al. (Predmore, Kondo et al. 2012) investigated the cardioprotective effects of the garlic-derived H_2S donor, diallyl trisulfide (DATS) in a murine model of myocardial ischemia and reperfusion. The authors reported that treatment with DATS significantly reduced the area of myocardial necrosis concomitant with marked increases in plasma nitrite, nitrate, and nitrosylated protein (RXNO) levels 30 minutes after injection. These data provide additional support for the concept of H_2S-NO cooperativity and clearly demonstrate that administration of exogenous H_2S thru the use of H_2S donors activates eNOS to augment NO bioavailability and protect the heart and circulation against cardiovascular diseases.

A recent study by King et al. (King, Polhemus et al. 2014) investigated the physiological regulation of eNOS-NO mediated cell signaling by endogenous H_2S generated from

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cystathionine gamma lyase (CSE) by using a CSE mutant mouse model. Interestingly, King (King, Polhemus et al. 2014) reported that basal circulating and myocardial tissue H_2S and NO levels were significantly decreased in CSE knockout mice as compared to wild-type mice. In this same study myocardial oxidative stress and eNOS uncoupling was significantly increased in the CSE knockout mouse. The authors treated the CSE deficient mice with H_2S donor therapy for 7 days and reported significant increases in circulating and tissue levels of NO that were similar to wild-type animals. Finally, the authors evaluated the effects of H2S donors on myocardial ischemia/reperfusion injury in both eNOS knockout and eNOS phosphomutants. The cardioprotective actions were completely abrogated in mice deficient in eNOS or mice with dysfunctional eNOS. This data very strongly suggest that endogenous H_2S derived from the H_2S-producing enzyme, CSE, attenuates endothelial oxidative stress resulting in improved eNOS activation status and the production of physiologically relevant NO levels. Thus, H_2S acts as a chaperone to preserve eNOS-NO signaling and normal cardiovascular homeostasis. Since sulfide is a strong nucleophile, it is also possible that the effects of hydrogen sulfide may be through protection of BH4 oxidation and maintaining the proper redox status for optimal NOS production of NO. Another potential mechanism is through H2S mediated S-sulfhydration of eNOS which promotes phosphorylation thereby inhibiting its S-nitrosylation, and increasing eNOS dimerization with the consequential improved NO production (Altaany, Ju et al. 2014) or a combination of all of the above.

Conclusions

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Despite the fact that these two gaseous signaling molecules are known to be cytoprotective and necessary for health and disease prevention, drug development around NO and H2S active therapies has been slow and unsuccessful. Perhaps the best approach to restoring production of these and other gaseous signals is to provide the body what it needs to produce these naturally and allow endogenous systems and nature to do their job. This will require at least 3 considerations: 1. optimizing the enzyme systems that produce both NO and H2S by providing the essential co-factors and substrates from the diet for enzymatic production i.e. tetrahydrobiopterin, Vitamin B6, glutathione, etc (proper nutrition); 2. Restoring normal microbiota and flora that are part of the nitrate and sulfate reducing bacteria (modifying diet, probiotics, prebiotics); 3. Reducing the use of antibiotics, antiseptic mouthwash, non-steroidal anti-inflammatory drugs (NSAIDS), proton pump inhibitors (PPIs), etc that interfere with NO and H2S production. Common practices such as rinsing with antiseptic mouthwash and overuse of antibiotics disrupt the bacterial production of NO and H2S. Diets that don't include sufficient nitrate and sulfate from foods will disrupt their production. Therapies or strategies that provide the human body with an exogenous source of NO or H2S may also be a viable approach. A NO generating lozenge has been shown to recapitulate NO based signaling in humans (Zand, Lanza et al. 2011, Nagamani, Campeau et al. 2012, Biswas, Gonzalez et al. 2014, Houston and Hays 2014, Lee, Kim et al. 2015, Lee 2016). This may be due to known endocrine or hormone like effects of NO (Elrod, Calvert et al. 2008). It is unclear at this time, if providing hydrogen sulfide gas directly to humans will have similar effects. The science clearly shows that providing nitrate or nitrite or sodium sulfide in the form of supplementation can restore and recapitulate NO and H2S based signaling. This provides an optimal opportunity to direct therapies of applied physiology.

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Figure Legend

There are two pathways for the production of NO and H2S in humans. The top left section of the figure illustrates commensal bacterial production of NO from nitrate reducing bacteria. The

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top right depicts H2S production from sulfate reducing bacteria. The bottom left illustrates the mechanism of eNOS production of NO and the required substrates and co-factors. The bottom right shows the three known enzymatic pathways for H2S production. Once produced the two molecules can react to form thionitrite to potentiate NO based signaling. Furthermore, H2S can protect from BH4 oxidation and/or S-sulfhydration of cysteine residues on eNOS to improve production of NO.