

## MINIREVIEW

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

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### ABSTRACT

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 numerous 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 allow for new strategies for safe and effective delivery of these molecules for human disease. The purpose of this review is to highlight the biologic effects of nitric oxide and hydrogen sulfide, their seemingly indistinguishable effects, and how these molecules can be safely harnessed for drug development and precursors or substrates administered for human consumption through applied physiology.

### Introduction

Gaseous signaling molecules, 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 that induce certain and specific physiologic or biochemical changes in the human host. The notion of a gas that diffuses in three dimensions created uncertainty about how these gases could selectively and specifically activate their targets for their intended physiologic effects. The reactivity and relatively short biologic half-life of these gases are thought to limit their biologic activity. Although nitric oxide (NO) and hydrogen sulfide (H<sub>2</sub>S) have been known for many centuries, their role in human physiology is new knowledge. The fundamental and necessary nature of these molecules for normal human health is known. The 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). Numerous published studies in experimental animals and even humans have revealed that delivery of bioactive NO, H<sub>2</sub>S, and/or their more stable reaction intermediates improve or prevent many of these conditions and diseases. As a result, enormous efforts have been made 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 physiologic basis for the production pathways for these molecules, both from human enzyme systems and from the microbiome, may allow for better strategies for the development of safe and effective therapies. This review highlights those production pathways for NO and H<sub>2</sub>S and reviews 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 to avoid prolonged signal transduction. Therefore, NO and H<sub>2</sub>S share such characteristics,

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**ABBREVIATIONS:** 3-MST, 3-mercaptopyruvate sulfurtransferase; ASA, argininosuccinic aciduria; BH<sub>4</sub>, tetrahydrobiopterin; CA, carbonic anhydrase; CBS, cystathionine β-synthase; CIMT, carotid intima media thickness; CSE, cystathionine γ-lyase; H<sub>2</sub>S, hydrogen sulfide; NO, nitric oxide; NOS, nitric oxide synthase; PPI, proton pump inhibitor; sGC, soluble guanylyl cyclase; SRB, sulfate-reducing bacteria.

and strategies to produce physiologic 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 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 neurocognitive function, sexual function, and many more fundamental physiologic processes. Loss of the production of NO is considered one of the earliest events in the onset and progression of most, if not all, chronic diseases (Egashira et al., 1993). Therefore, understanding how the body makes NO, what goes wrong in patients who cannot 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 five-electron oxidation of the guanidine nitrogen of L-arginine to L-citrulline with NO as a by-product (Hibbs et al., 1987). The second, more recently discovered pathway is through the serial reduction of inorganic nitrate to nitrite and to NO (Duncan et al., 1995). The first pathway is through a mammalian enzyme system known as nitric oxide synthase (NOS). The NOS enzyme requires at least half a dozen substrates and cofactors, 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 on commensal bacteria that live in and on the human body (Lundberg et al., 2004). Mammals lack a functional nitrate reductase enzyme system, so this pathway is dependent on 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 et al., 1999; Cosby et al., 2003; Feelisch 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 et al., 2005; Hyde et al., 2014a,b). Eradicating oral bacteria with antiseptic mouthwash results in symptoms of NO deficiency including an increase in blood pressure (Kapil et al., 2013; Woessner 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 (Katsuki 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 et al., 2001). In this manner, NO regulates a host of physiologic functions.

The fundamental question then becomes how to restore or recapitulate NO-based signaling. Understanding both pathways for NO production allows one to focus on restoring these pathways, including 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 prevents BH4 oxidation and maintains NOS function (Stokes et al., 2009). Nitrite production in the

oral cavity resulting from reduction of nitrate by the oral nitrate-reducing bacteria is one way of restoring NO. This method requires sufficient nitrate from the diet and the right oral bacteria. This pathway becomes disrupted when humans do not consume enough nitrate from vegetables and when the oral microbiome is disrupted by antiseptic mouthwash or antibiotic use (Bryan, 2018). Providing nitrite in the form of 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 et al., 2008). Many studies have been published that describe demonstrating the safety and efficacy of nitrite in humans within a large range of doses. Capsules containing sodium nitrite at doses of 160 and 320 mg have been used to investigate toxicity and pharmacokinetics. Nitrite, even at high doses of 320 mg, did not show any clinical toxicity as measured by methemoglobinemia (<15%) (Greenway et al., 2012). Therapy using 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 of nitrite capsules in a randomized, placebo control, double-blind study over 10 weeks led to an increase in plasma nitrite both acutely and chronically and was well tolerated (DeVan et al., 2016). Endothelial function and carotid artery elasticity significantly improved (DeVan et al., 2016). 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 of nitrite ( $62 \pm 7$  years) (Justice et al., 2015). Unlike nitrate, the effects of nitrite are not dependent on or require the present of 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 because nitrite is inefficiently reduced to NO at physiologic concentrations of oxygen (Bryan et al., 2005; Feelisch et al., 2008). Therefore, more is needed to get any appreciable amount of NO produced, especially in people who are NO-deficient. We have discovered natural product chemistry that can reduce nitrite to NO even in the presence of physiologic amounts of oxygen, thereby providing an exogenous source of NO in vivo (Tang et al., 2009). The premise of this technology is that if your body cannot make NO owing to endothelial dysfunction, lack of oral nitrate-reducing bacteria, use of antiseptic mouthwash or proton pump inhibitors (PPIs), then this therapy will provide an exogenous source of NO. These discoveries have been used to develop a commercial 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 et al., 2011). Single and acute administration of this lozenge leads to peak plasma levels of nitrite around  $1.5 \mu\text{M}$  after 20 minutes. In pediatric patients with a condition called arginininosuccinic 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 antihypertensive medications were ineffective. The lozenge also improved renal

function and cognition and reversed cardiac hypertrophy (Nagamani et al., 2012). In another randomized, controlled study using the nitrite lozenge showed a significantly reduction in blood pressure, a 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 prehypertensive patients (blood pressure  $> 120/80 < 139/89$ ), administration of one lozenge twice daily for 30 days lowered both systolic and diastolic blood pressure levels by 12 and 6 mm Hg, respectively (Biswas et al., 2015), 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 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) reveals regression and slowing of progression of CIMT of approximately 2.7% ( $-0.04$ ) after more than 2 years (Bedi 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 cardiovascular baroreflex sensitivity. The concentrated beet powder also reduces systemic blood pressure and mean arterial blood pressure in older adults (Bock et al., 2018). 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, pharmacologic 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 et al., 2014b). 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 ( $H_2S$ ) is a naturally occurring compound that is produced throughout the human body. Just like NO,  $H_2S$  is acutely toxic at high concentrations. This toxicity is attributed to inhibition of mitochondrial cytochrome c oxidase, carbonic anhydrase, monoamine oxidase,  $Na^+/K^+$ -ATPase, and cholinesterases (Beauchamp et al., 1984).  $H_2S$  also reacts with the oxy forms of myoglobin and hemoglobin ( $FeII-O_2$ ) generating the sulfheme derivative, thereby reducing oxygen binding and transport (Pietri et al., 2011). The modern history of  $H_2S$  is mostly associated with its toxic effects. It was only recently that  $H_2S$  may serve as an important biologic mediator (Abe and Kimura, 1996). Since then, studies have shown that  $H_2S$  is involved in a number of biologic signaling mechanisms in numerous biologic systems. Similar to sGC, as a target for NO-based signaling,  $Na/K$ -ATPase is a known physiologic target for  $H_2S$  (Xia et al., 2009). Also, similar to post-translational modification of critical cysteine residues by nitrosation of NO-based signaling,  $H_2S$  can lead to persulfidation, thereby affecting the structure and function of proteins (Paul and Snyder, 2015). Among other biologic effects,  $H_2S$  has been reported to have antihypertensive and cytoprotective properties (Benavides et al., 2007; Polhemus and Lefer, 2014), again like NO. Most of the research on  $H_2S$

has focused mainly on the enzymatic production in the heart, kidneys, vasculature, and brain. The biologic action of  $H_2S$  produced by the gut microbiota has been recently examined. Given the prominent role of  $H_2S$  in numerous diseases, many therapeutic targets have been discovered for  $H_2S$  therapy. The molecular targets of  $H_2S$  include proteins, enzymes, transcription factors, and membrane ion channels and other proteins. Cysteine is the major source and substrate of  $H_2S$  in mammals.  $H_2S$  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).

Like in NO production, commensal bacteria provide the human host with a source of hydrogen sulfide. Sulfate-reducing bacteria (SRB) are ubiquitous and common to the mammalian colon (Tomasova et al., 2016b). The dominant genera are *Desulfovibrio* (*D. piger*, *D. desulfuricans*), *Desulfobacter*, *Desulfobulbus* and *Desulfotomaculum* (Rabus et al., 2015). There are two substrates that are essential for SRB to produce  $H_2S$ . 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  $H_2S$  production in the colon of humans and mice (Gibson et al., 1988; Rey et al., 2013). *D. piger* can also use sulfated glycans. Sulfate-reducing bacteria therefore are a common source of  $H_2S$  in the gut of mammals. Several anaerobic bacterial strains (*Escherichia coli*, *Salmonella enterica*, *Clostridia*, and *Enterobacter aerogenes*) convert cysteine to  $H_2S$ , pyruvate and ammonia by cysteine desulfhydrase (Kumagai et al., 1975; Awano et al., 2005). In addition, gut bacteria can also produce  $H_2S$  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* spp. (Blachier et al., 2010).

The total sulfide concentration produced in the large intestine has been quantified to be in the range of 0.2–3.4 mmol/liter in humans; however, since the feces of humans and rodents have a large binding capacity for sulfur, it is estimated that less than 8% of total sulfide is in a free form (Jørgensen and Mortensen, 2001; Levitt et al., 2002). Colonic epithelial cells are more efficient in converting sulfide into thiosulfate than other tissues (Furne et al., 2001). Infusion of radioactive  $H_2S$  into the cecum of rats showed that all the absorbed  $H_2S$  from the infusion was immediately oxidized to thiosulfate (Levitt et al., 1999). Flannigan et al. (2011) reported that fecal samples of germ-free mice contained 50% less  $H_2S$  compared with feces of controls. Germ-free mice have significantly less free  $H_2S$  in plasma and gastrointestinal tissues compared with control mice. CSE activity in tissue of germ-free mice is significantly reduced, whereas tissue cysteine levels appear to be significantly elevated compared with conventional mice. These data suggest that the microbiota profoundly regulates systemic bioavailability and metabolism of  $H_2S$  (Shen et al., 2013). Eliminated vitamin B6, a CSE and CBS cofactor, in the diet causes a 50% reduction of fecal  $H_2S$ , likely owing to the reduction of enzymatic  $H_2S$  synthesis in colonic tissues. Interestingly, after 6 weeks of the vitamin B6-deficient diet, the fecal  $H_2S$  levels in mice were restored to normal, suggesting that the  $H_2S$  generation in the gut of germ-free mice was shifted toward microbial production pathways by increasing

the SRB activity (Flannigan 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 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 et al., 2016a). Overproduction of H<sub>2</sub>S in the colon has been implicated in colonic inflammation and cancer (Roediger et al., 1993; Cao et al., 2010); however, other studies suggest that colonic epithelial cells are well adapted to the H<sub>2</sub>S-rich environment, and that H<sub>2</sub>S plays a beneficial role in the protection of the gut brain barrier (Goubern et al., 2007; Wallace, 2010; Motta et al., 2015). H<sub>2</sub>S may serve as an energy source for colonic epithelial cells as a result of ATP formation from H<sub>2</sub>S oxidation (Goubern et al., 2007). H<sub>2</sub>S also promotes colonic mucus production, thereby maintaining the integrity of bacterial biofilms (Motta et al., 2015). H<sub>2</sub>S protects and reverses damage induced by chronic administration of nonsteroidal anti-inflammatory drugs (Blackler et al., 2015).

The main dietary sources of exposure to sulfur compounds in the human diet are inorganic sulfates in drinking water and sulfur-containing amino acids in proteins derived from plants and animals. Only two of the 20 amino acids are sulfur-containing amino acids: 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 semiessential 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); therefore, with increasing age, less cysteine is produced endogenously. Excess consumption of cysteine and methionine from the diet is converted and stored as glutathione. Cysteine availability is the rate-limiting factor for glutathione biosynthesis from glutamate, glycine, and cysteine.

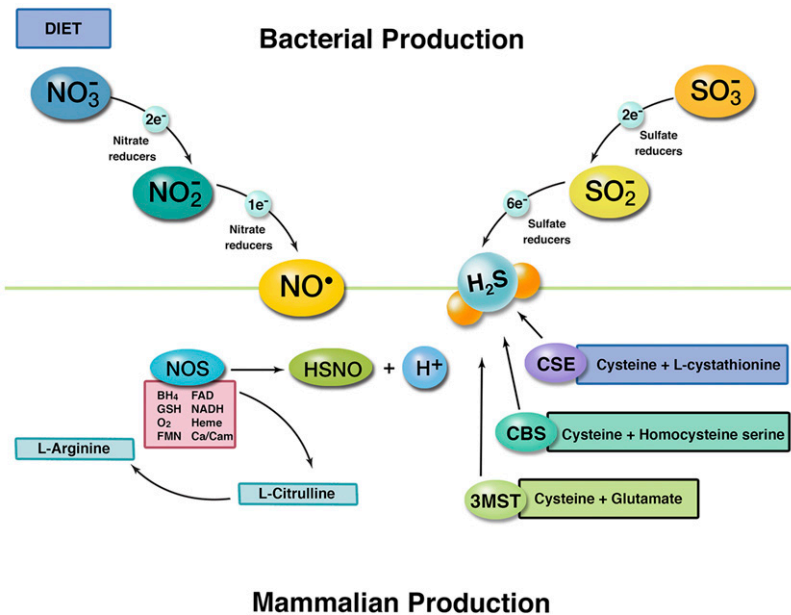
**You Cannot Have One without the Other.** Growing evidence shows that there is “crosstalk” between sulfide and NO. NO and H<sub>2</sub>S elicit many of the same physiologic actions in the cardiovascular system, including: vasodilation, regulation of mitochondrial respiration, proangiogenic effects, inhibition of apoptosis, and antioxidant effects. Although H<sub>2</sub>S 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 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 et al., 1999). In a pressure-overload murine heart failure, Kondo et al. (2013) reported that mice treated with an H<sub>2</sub>S donor (i.e., SG1002) were protected against adverse cardiac remodeling and heart failure. Interestingly, the authors also reported a significant increase in 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<sub>2</sub>S donor therapy in heart failure is mediated in part via increased myocardial and circulating NO levels.

Furthermore, Predmore et al. (2012) investigated the cardioprotective effects of the garlic-derived H<sub>2</sub>S donor diallyl trisulfide in a murine model of myocardial ischemia and reperfusion. The authors reported that treatment with diallyl trisulfide significantly reduced the area of myocardial necrosis concomitant with marked increases in plasma nitrite, nitrate, and nitrosylated protein levels 30 minutes after injection. These data provide additional support for the concept of H<sub>2</sub>S-NO cooperativity and clearly demonstrate that administration of exogenous H<sub>2</sub>S by the use of H<sub>2</sub>S donors activates eNOS to augment NO bioavailability and protect the heart and circulation against cardiovascular diseases.

A recent study by King et al. (2014) investigated the physiologic regulation of eNOS-NO mediated cell signaling by endogenous H<sub>2</sub>S generated from cystathionine  $\gamma$  lyase (CSE) by using a CSE mutant mouse model. Interestingly, King et al. (2014) reported that basal circulating and myocardial tissue H<sub>2</sub>S and NO levels were significantly decreased in CSE knockout mice compared with wild-type mice. In this same study, myocardial oxidative stress and eNOS uncoupling were significantly increased in the CSE knockout mouse. The authors treated the CSE-deficient mice with H<sub>2</sub>S donor therapy for 7 days and reported significant increases in circulating and tissue levels of NO that were similar to those of wild-type animals. Finally, the authors evaluated the effects of H<sub>2</sub>S 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. These data very strongly suggest that endogenous H<sub>2</sub>S derived from the H<sub>2</sub>S-producing enzyme CSE attenuates endothelial oxidative stress, resulting in improved eNOS activation status and the production of physiologically relevant NO levels. Thus, H<sub>2</sub>S 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 BH<sub>4</sub> oxidation and maintaining the proper redox status for optimal NOS production of NO. Another potential mechanism is through H<sub>2</sub>S-mediated S-sulfhydration of eNOS, which promotes phosphorylation, thereby inhibiting its S-nitrosylation and increasing eNOS dimerization with the consequential improved NO production (Altaany et al., 2014) or a combination of all the above.

## Conclusions

Despite these two gaseous signaling molecules being known to be cytoprotective and necessary for health and disease prevention, drug development around NO and H<sub>2</sub>S-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 three considerations: 1) optimizing the enzyme systems that produce both NO and H<sub>2</sub>S by providing the essential cofactors and substrates from the diet for enzymatic production (i.e., tetrahydrobiopterin, vitamin B6, glutathione, and such for 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,



**Fig. 1.** The two pathways for production of NO and H<sub>2</sub>S in humans. The top left section illustrates commensal bacterial production of NO from nitrate-reducing bacteria. The top right depicts H<sub>2</sub>S production from sulfate-reducing bacteria. Bottom left illustrates the mechanism of eNOS production of NO and the required substrates and cofactors. Bottom right shows the three known enzymatic pathways for H<sub>2</sub>S production. Once produced, the two molecules can react to form thionitrite to potentiate NO-based signaling. Furthermore, H<sub>2</sub>S can protect from BH<sub>4</sub> oxidation and/or S-sulfhydration of cysteine residues on eNOS to improve production of NO.

antiseptic mouthwash, nonsteroidal anti-inflammatory drugs, PPIs, and such that interfere with NO and H<sub>2</sub>S production. Common practices such as rinsing with antiseptic mouthwash and overuse of antibiotics disrupt the bacterial production of NO and H<sub>2</sub>S. Diets that do not include sufficient nitrate and sulfate from foods will disrupt their production. This concept is illustrated in Fig. 1. Therapies or strategies that provide the human body with an exogenous source of NO or H<sub>2</sub>S may also be a viable approach. An NO-generating lozenge has been shown to recapitulate NO-based signaling in humans (Zand et al., 2011; Nagamani et al., 2012; Houston and Hays, 2014; Biswas et al., 2015; Lee et al., 2015; Lee, 2016), possibly owing to known endocrine or hormone-like effects of NO (Elrod et al., 2008). It is unclear at this time whether 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 H<sub>2</sub>S-based signaling, which provides an optimal opportunity to direct therapies of applied physiology.

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#### Authorship Contributions

Wrote or contributed to the writing of the manuscript: Bryan, Lefer.

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