1521-0111/96/1/109–114$35.00
MOLECULAR PHARMACOLOGY

https://doi.org/10.1124/mol.118.113910
Mol Pharmacol 96:109–114, July 2019

MOLECULAR PHARMACOLOGY

MINIREVIEW

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

Nathan S. Bryan and David J. Lefer

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas (N.S.B.); and Louisiana State University School of Medicine, New Orleans, Louisiana (D.J.L.)

Received August 15, 2018; accepted May 2, 2019

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_2S) 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_2S, 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_2S 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_2S share such characteristics,

This work is supported by the National Institutes of Health [Grants 1R44 HL139195-01, 1R44 HL139161-01, 1R01 HL146098-01, and 1R44 HL136233-01 (to DJL)].
https://doi.org/10.1124/mol.118.113910.

ABBREVIATIONS: 3-MST, 3-mercaptopyruvate sulfurtransferase; ASA, argininosuccinic aciduria; BH_4, tetrahydrobiopterin; CA, carbonic anhydrase; CBS, cystathionine β-synthase; CIMT, carotid intima media thickness; CSE, cystathionine γ-lyase; H_2S, 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 ± 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 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 et al., 2011). Single and acute administration of this lozenge leads to peak plasma levels of nitrite around 1.5 μM 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 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 motr 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 cardiovagal 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-O2) 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 β-synthase (CBS), cystathionine γ-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_2S 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_2S-rich environment, and that H_2S plays a beneficial role in the protection of the gut brain barrier (Goubern et al., 2007; Wallace, 2010; Motta et al., 2015). H_2S may serve as an energy source for colonic epithelial cells as a result of ATP formation from H_2S oxidation (Goubern et al., 2007). H_2S also promotes colonic mucus production, thereby maintaining the integrity of bacterial biofilms (Motta et al., 2015). H_2S 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_2S 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_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 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_2S 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_2S 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_2S 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_2S-NO cooperativity and clearly demonstrate that administration of exogenous H_2S by 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. (2014) investigated the physiologic regulation of eNOS-NO mediated cell signaling by endogenous H_2S generated from cystathionine γ lyase (CSE) by using a CSE mutant mouse model. Interestingly, King et al. (2014) reported that basal circulating and myocardial tissue H_2S 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_2S 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_2S 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_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 H_2S-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_2S-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_2S 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,

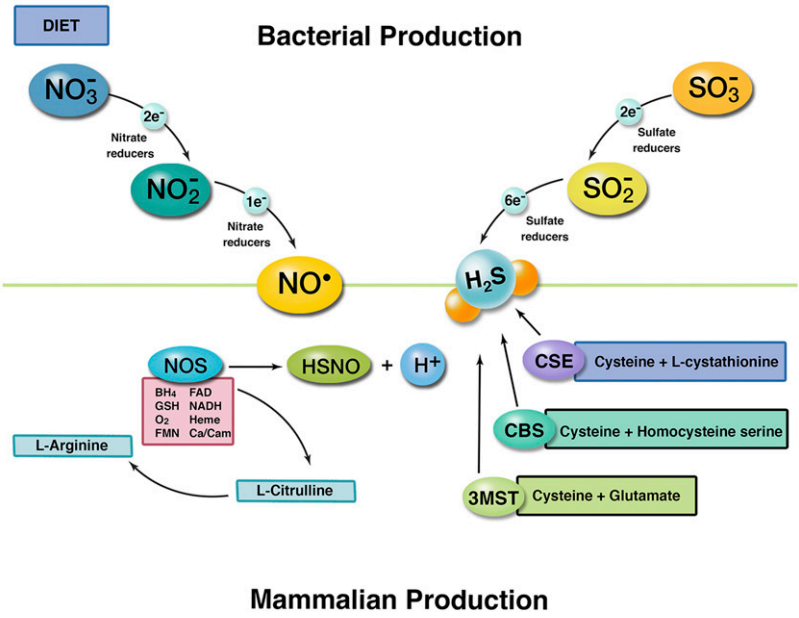

Fig. 1. The two pathways for production of NO and H_2S in humans. The top left section illustrates commensal bacterial production of NO from nitrate-reducing bacteria. The top right depicts H_2S production from sulfate-reducing bacteria. Bottom left illustrates the mechanism of eNOS production of NO and the required substrates and cofactors. Bottom right shows the three known enzymatic pathways for H_2S production. Once produced, the two molecules can react to form thionitrite to potentiate NO-based signaling. Furthermore, H_2S can protect from BH4 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 H2S production. Common practices such as rinsing with antiseptic mouthwash and overuse of antibiotics disrupt the bacterial production of NO and H_2S. 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_2S 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 H2S-based signaling, which provides an optimal opportunity to direct therapies of applied physiology.

Acknowledgments

N.S.B. is a founder and shareholder, HumanN, Inc.; a consultant and shareholder, SAJE Pharma; and receives royalties from patents from the University of Texas Health Science Center at Houston.

Authorship Contributions

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

References

Abe K and Kimura H (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* **16**:1066–1071.
Altaany Z, Ju Y, Yang G, and Wang R (2014) The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Sci Signal* **7**:ra87.
Awano N, Wada M, Mori H, Nakamori S, and Takagi H (2005) Identification and functional analysis of Escherichia coli cysteine desulfhydrases. *Appl Environ Microbiol* **71**:4149–4152.
Beauchamp RO Jr, Bus JS, Popp JA, Boreiko CJ, and Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. *Crit Rev Toxicol* **13**: 25–97.
Bedi US, Singh M, Singh PP, Bhuriya R, Bahekar A, Molnar J, Khosla S, and Arora R (2010) Effects of statins on progression of carotid atherosclerosis as measured by carotid intimal—medial thickness: a meta-analysis of randomized controlled trials. *J Cardiovasc Pharmacol Ther* **15**:268–273.
Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, and Kraus DW (2007) Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci USA* **104**:17977–17982.
Biswas OS, Gonzalez VR, and Schwarz ER (2015) Effects of an oral nitric oxide supplement on functional capacity and blood pressure in adults with pre-hypertension. *J Cardiovasc Pharmacol Ther* **20**:52–58.
Blachier F, Davila AM, Mimoun S, Benetti PH, Atanasiu C, Andriamihaja M, Benamouzig R, Bouillaud F, and Tomé D (2010) Luminal sulfide and large intestine mucosa: friend or foe?. *Amino Acids* **39**:335–347.
Blackler RW, Motta JP, Manko A, Workentine M, Bercik P, Surette MG, and Wallace JL (2015) Hydrogen sulphide protects against NSAID-enteropathy through modulation of bile and the microbiota. *Br J Pharmacol* **172**:992–1004.
Bock JM, Ueda K, Schneider AC, Hughes WE, Limberg JK, Bryan NS, and Casey DP (2018) Inorganic nitrate supplementation attenuates peripheral chemoreflex sensitivity but does not improve cardiovagal baroreflex sensitivity in older adults. *Am J Physiol Heart Circ Physiol* **314**:H45–H51.
Bryan NS (2011) Application of nitric oxide in drug discovery and development. *Expert Opin Drug Discov* **6**:1139–1154.
Bryan NS (2018) Functional nitric oxide nutrition to combat cardiovascular disease. *Curr Atheroscler Rep* **20**:21.
Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, and Feelisch M (2005) Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol* **1**: 290–297.
Cao Q, Zhang L, Yang G, Xu C, and Wang R (2010) Butyrate-stimulated H2S production in colon cancer cells. *Antioxid Redox Signal* **12**:1101–1109.
Cortese-Krott MM, Fernandez BO, Kelm M, Butler AR, and Feelisch M (2015) On the chemical biology of the nitrite/sulfide interaction. *Nitric Oxide* **46**: 14–24.
Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, et al. (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* **9**: 1498–1505.
DeVan AE, Johnson LC, Brooks FA, Evans TD, Justice JN, Cruickshank-Quinn C, Reisdorph N, Bryan NS, McQueen MB, Santos-Parker JR, et al. (2016) Effects of sodium nitrite supplementation on vascular function and related small metabolite signatures in middle-aged and older adults. *J Appl Physiol (1985)* **120**:416–425.
Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**:601–605.
Doel JJ, Benjamin N, Hector MP, Rogers M, and Allaker RP (2005) Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci* **113**:14–19.
Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, and Benjamin N (1995) Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* **1**:546–551.
Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, and Takeshita A (1993) Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation* **88**:77–81.
Elrod JW, Calvert JW, Gundewar S, Bryan NS, and Lefer DJ (2008) Nitric oxide promotes distant organ protection: evidence for an endocrine role of nitric oxide. *Proc Natl Acad Sci USA* **105**:11430–11435.
Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, and Ashrafian H (2008) Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. *J Biol Chem* **283**:33927–33934.

Flannigan KL, McCoy KD, and Wallace JL (2011) Eukaryotic and prokaryotic contributions to colonic hydrogen sulfide synthesis. *Am J Physiol Gastrointest Liver Physiol* **301**:G188–G193.
Furne J, Springfield J, Koenig T, DeMaster E, and Levitt MD (2001) Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. *Biochem Pharmacol* **62**:255–259.
Gibson GR, Macfarlane GT, and Cummings JH (1988) Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *J Appl Bacteriol* **65**:103–111.
Goubern M, Andriamihaja M, Nübel T, Blachier F, and Bouillaud F (2007) Sulfide, the first inorganic substrate for human cells. *FASEB J* **21**:1699–1706.
Greenway FL, Predmore BL, Flanagan DR, Giordano T, Qiu Y, Brandon A, Lefer DJ, Patel RP, and Kevil CG (2012) Single-dose pharmacokinetics of different oral sodium nitrite formulations in diabetes patients. *Diabetes Technol Ther* **14**:552–560.
Hibbs JB Jr, Taintor RR, and Vavrin Z (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235**:473–476.
Houston M and Hays L (2014) Acute effects of an oral nitric oxide supplement on blood pressure, endothelial function, and vascular compliance in hypertensive patients. *J Clin Hypertens (Greenwich)* **16**:524–529.
Hyde ER, Andrade F, Vaksman Z, Parthasarathy K, Jiang H, Parthasarathy DK, Torregrossa AC, Tribble G, Kaplan HB, Petrosino JF, et al. (2014a) Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. *PLoS One* **9**:e88645.
Hyde ER, Luk B, Cron S, Kusic L, McCue T, Bauch T, Kaplan H, Tribble G, Petrosino JF, and Bryan NS (2014b) Characterization of the rat oral microbiome and the effects of dietary nitrate. *Free Radic Biol Med* **77**:249–257.
Jørgensen J and Mortensen PB (2001) Hydrogen sulfide and colonic epithelial metabolism: implications for ulcerative colitis. *Dig Dis Sci* **46**:1722–1732.
Justice JN, Johnson LC, DeVan AE, Cruickshank-Quinn C, Reisdorph N, Bassett CJ, Evans TD, Brooks FA, Bryan NS, Chonchol MB, et al. (2015) Improved motor and cognitive performance with sodium nitrite supplementation is related to small metabolite signatures: a pilot trial in middle-aged and older adults. *Aging (Albany NY)* **7**:1004–1021.
Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, and Ahluwalia A (2013) Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic Biol Med* **55**:93–100.
Katsuki S, Arnold W, Mittal C, and Murad F (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res* **3**:23–35.
King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, Bradley JM, Islam KN, Calvert JW, Tao YX, et al. (2014) Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci USA* **111**:3182–3187.
Koc A and Gladyshev VN (2007) Methionine sulfoxide reduction and the aging process. *Ann N Y Acad Sci* **1100**:383–386.
Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G Sr, Gojon G Jr, et al. (2013) H_2S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. *Circulation* **127**:1116–1127.
Kozlov AV, Staniek K, and Nohl H (1999) Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett* **454**:127–130.
Kumagai H, Sejima S, Choi Y, Tanaka H, and Yamada H (1975) Crystallization and properties of cysteine desulfhydrase from Aerobacter aerogenes. *FEBS Lett* **52**:304–307.
Lane P, Hao G, and Gross SS (2001) S-nitrosylation is emerging as a specific and fundamental posttranslational protein modification: head-to-head comparison with O-phosphorylation. *Sci STKE* **2001**:re1.
Lee E (2016) Effects of nitric oxide on carotid intima media thickness: a pilot study. *Altern Ther Health Med* **22**(Suppl 2):32–34.
Lee J, Kim HT, Solares GJ, Kim K, Ding Z, and Ivy JL (2015) Caffeinated nitric oxide-releasing lozenge improves cycling time trial performance. *Int J Sports Med* **36**:107–112.
Levitt MD, Furne J, Springfield J, Suarez F, and DeMaster E (1999) Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *J Clin Invest* **104**:1107–1114.
Levitt MD, Springfield J, Furne J, Koenig T, and Suarez FL (2002) Physiology of sulfide in the rat colon: use of bismuth to assess colonic sulfide production. *J Appl Physiol (1985)* **92**:1655–1660.
Lundberg JO, Weitzberg E, Cole JA, and Benjamin N (2004) Nitrate, bacteria and human health. *Nat Rev Microbiol* **2**:593–602.
Morris MS, Picciano MF, Jacques PF, and Selhub J (2008) Plasma pyridoxal 5′-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. *Am J Clin Nutr* **87**:1446–1454.
Motta JP, Flannigan KL, Agbor TA, Beatty JK, Blackler RW, Workentine ML, Da Silva GJ, Wang R, Buret AG, and Wallace JL (2015) Hydrogen sulfide protects from colitis and restores intestinal microbiota biofilm and mucus production. *Inflamm Bowel Dis* **21**:1006–1017.
Nagamani SC, Campeau PM, Shchelochkov OA, Premkumar MH, Guse K, Brunetti-Pierri N, Chen Y, Sun Q, Tang Y, Palmer D, et al. (2012) Nitric-oxide supplementation for treatment of long-term complications in argininosuccinic aciduria. *Am J Hum Genet* **90**:836–846.
Paul BD and Snyder SH (2015) H2S: a novel gasotransmitter that signals by sulfhydration. *Trends Biochem Sci* **40**:687–700.
Pietri R, Román-Morales E, and López-Garriga J (2011) Hydrogen sulfide and hemeproteins: knowledge and mysteries. *Antioxid Redox Signal* **15**:393–404.
Polhemus DJ and Lefer DJ (2014) Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. *Circ Res* **114**:730–737.
Predmore BL, Kondo K, Bhushan S, Zlatopolsky MA, King AL, Aragon JP, Grinsfelder DB, Condit ME, and Lefer DJ (2012) The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. *Am J Physiol Heart Circ Physiol* **302**:H2410–H2418.
Rabus R, Venceslau SS, Wöhlbrand L, Voordouw G, Wall JD, and Pereira IA (2015) A post-genomic view of the ecophysiology, catabolism and biotechnological relevance of sulphate-reducing prokaryotes. *Adv Microb Physiol* **66**:55–321.
Rey FE, Gonzalez MD, Cheng J, Wu M, Ahern PP, and Gordon JI (2013) Metabolic niche of a prominent sulfate-reducing human gut bacterium. *Proc Natl Acad Sci USA* **110**:13582–13587.
Roediger WEW, Duncan A, Kapaniris O, and Millard S (1993) Sulphide impairment of substrate oxidation in rat colonocytes: a biochemical basis for ulcerative colitis?. *Clin Sci (Lond)* **85**:623–627.
Shen X, Carlström M, Borniquel S, Jädert C, Kevil CG, and Lundberg JO (2013) Microbial regulation of host hydrogen sulfide bioavailability and metabolism. *Free Radic Biol Med* **60**:195–200.
Stokes KY, Dugas TR, Tang Y, Garg H, Guidry E, and Bryan NS (2009) Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *Am J Physiol Heart Circ Physiol* **296**:H1281–H1288.
Tang Y, Garg H, Geng YJ, and Bryan NS (2009) Nitric oxide bioactivity of traditional Chinese medicines used for cardiovascular indications. *Free Radic Biol Med* **47**:835–840.
Tayeh MA and Marletta MA (1989) Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate: tetrahydrobiopterin is required as a cofactor. *J Biol Chem* **264**:19654–19658.
Tomasova L, Dobrowolski L, Jurkowska H, Wróbel M, Huc T, Ondrias K, Ostaszewski R, and Ufnal M (2016a) Intracolonic hydrogen sulfide lowers blood pressure in rats. *Nitric Oxide* **60**:50–58.
Tomasova L, Konopelski P, and Ufnal M (2016b) Gut bacteria and hydrogen sulfide: the new old players in circulatory system homeostasis. *Molecules* **21**:1–18.
Wallace JL (2010) Physiological and pathophysiological roles of hydrogen sulfide in the gastrointestinal tract. *Antioxid Redox Signal* **12**:1125–1133.
Woessner M, Smoliga JM, Tarzia B, Stabler T, Van Bruggen M, and Allen JD (2016) A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. *Nitric Oxide* **54**:1–7.
Xia M, Chen L, Muh RW, Li PL, and Li N (2009) Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. *J Pharmacol Exp Ther* **329**:1056–1062.
Zand J, Lanza F, Garg HK, and Bryan NS (2011) All-natural nitrite and nitrate containing dietary supplement promotes nitric oxide production and reduces triglycerides in humans. *Nutr Res* **31**:262–269.

Address correspondence to: Dr. Nathan S. Bryan, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Alkek Building for Biomedical Research R-850, Houston, TX 77030. E-mail: Nathan.bryan@bcm.edu