Opinion

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Gasotransmitters: growing pains and joys

Rui Wang

Department of Biology, Cardiovascular and Metabolic Research Unit, Lakehead University, Thunder Bay, Ontario, Canada

Gasotransmitters are endogenously generated molecules of gas. Over the past decade we have come to realize that these gaseous signaling molecules are crucially important, being irreplaceable in wide biological applications. However, there are still many challenges for future gasotransmitter research to tackle. These include clarifying the interactions among gasotransmitters; understanding the significance of the cellular gasotransmitter signaling network; and adding new members to the modern family of gasotransmitters in addition to nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). Ammonia fulfills all criteria for being a gasotransmitter, and methane is another conceivable candidate. Following the original article postulating the concept of multiple gasotransmitters over a decade ago, this sequel article aims to further inspire interest and exploration into gasotransmitter research.

Appraisal of the known gasotransmitters

Gasotransmitters are endogenously generated gaseous signaling molecules (Box 1) [1,2]. Nitric oxide (NO) was the first identified gasotransmitter. Using L-arginine as the substrate, various isoforms of NO synthase catalyze NO production in different types of mammalian cells. The discovery of mammalian NO metabolism catalyzed a true revolution, as it showed that a molecule of gas can carry important signals for regulating cellular functions. Among its numerous biological implications, NO is best known as an endothelium-derived relaxing factor (EDRF) [3].

A product of heme metabolism [4], carbon monoxide (CO) is another gasotransmitter. Two major enzymes are involved in CO production: heme oxygenase-1 (HO-1) is an inducible enzyme and HO-2 is constitutively expressed. CO functions similar to NO in many ways [5]; for instance, among other functions, CO relaxes vascular tissues, lowers blood pressure, and protects the heart from ischemia/reperfusion damage.

Hydrogen sulfide (H_2S) was the third gasotransmitter to be discovered after NO and CO. This 'rotten-egg gas' is produced in mammalian cells via the enzymatic actions of cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopyruvate sulfurtransferase. L-Cysteine and homocysteine or their derivatives are the common substrates of these H_2S-generating enzymes [6]. H_2S plays an important role in regulating the physiological functions of many, if not all, systems and organs in the body [7]. H_2S is a signaling molecule for neurotransmission and neuromodulation, and is involved in learning, memory, and nociception. Oxygen-sensitive fluctuation of endogenous H_2S levels in intracellular organelles triggers compensative changes in bioenergy production and cellular functions [8,9]. In the context of an EDRF role for NO, H_2S is an endothelium-derived hyperpolarizing factor (EDHF) [10,11].

The research on NO biology and physiology gained momentum from 1984 to 1993. Thousands of papers involving CO and H_2S were published during the same period, but these studies were related mostly, if not all, to toxicological and environmental concerns. Whereas NO research bloomed in the following decade (1994–2003), biological and physiological research on CO and H_2S started to bud (Figure 1). The term 'gasotransmitter' was coined in 2002 [1], inspiring a field of research into novel cellular signaling mechanisms. Over the past 10 years (2004–2013), scientists have uncovered the wide applications and crucial physiological importance of gasotransmitters to the human body.

The surge in H_2S research and continued interest in NO and CO are just some of the signs that the high tides of gasotransmitter research have arrived (Figure 1). Hundreds of papers and books with the keyword 'gasotransmitters' have been published [12–15]. New graduate training programs, such as the Gasotransmitter REsearch And Training (GREAT) Program in Canada (http://www.usask.ca/healthsci/cardiovascular/gasotransmitters/gasotransmitters1.htm), and courses on gasotransmitters have been integrated into the undergraduate and graduate curricula of several universities. Most recently, the European Network on Gasotransmitters was established in 2012 (http://gasotransmitters.eu/), and numerous new research teams and laboratories have joined in conducting gasotransmitter research worldwide.

Each step of progress in gasotransmitter research has come with its fair share of excitement and eagerness, as well as wears and woes. Here, I discuss the promises and challenges of gasotransmitter research along three emerging lines of inquiry.

Advocacy of gasotransmitters as favored signaling molecules for eukaryotes

Gasotransmitters are evolutionally conserved from bacteria to plant and mammalian cells. The abbreviated term 'SAVE'

Corresponding author: Wang, R. (rwang@lakeheadu.ca).
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Box 1. Classification and criteria for gasotransmitters [1,2]

1. They are small molecules of gas, dissolved in biological milieu or not.
2. They are freely permeable to membrane. As such, their intracellular and intercellular movements do not exclusively rely on cognate membrane receptors or other transportation machineries.
3. They are endogenously generated in mammalian cells with specific substrates and enzymes; more than the products of metabolism, their production is regulated to fulfill signaling messenger functions.
4. They have well-defined specific functions at physiologically relevant concentrations.
5. Functions of endogenous gases can be mimicked by their exogenously applied counterparts.
6. They are involved in signal transduction and have specific cellular and molecular targets.

summarizes the four aspects that make gasotransmitters biologically irreplaceable.

Simplicity

Gasotransmitters are small molecules with the simplest molecular composition and structure. Their production does not rely on complicated chemical processes or on supplies from multiple substrates. Furthermore, the simplicity of gasotransmitters allows them to travel intracellularly and intercellularly quickly and on short notice. In the circulatory system, gasotransmitters can be carried to different destinations by short-lived covalent reactions with certain proteins.

Availability

Gasotransmitters are present in all organs, cells, and many intracellular organelles in significant abundance. Gasotransmitters can thus form multiple signaling webs, interweaving all cellular organelles to each other, and to all of the cells inside the body.

Volatility

Gasotransmitters are chemically volatile by nature. The easy removal of gasotransmitters from their production sites serves as a rapid way to turn off related signaling pathways. In comparison, many non-gaseous endogenous signaling molecules require enzymatic actions for their removal, which takes both time and energy.

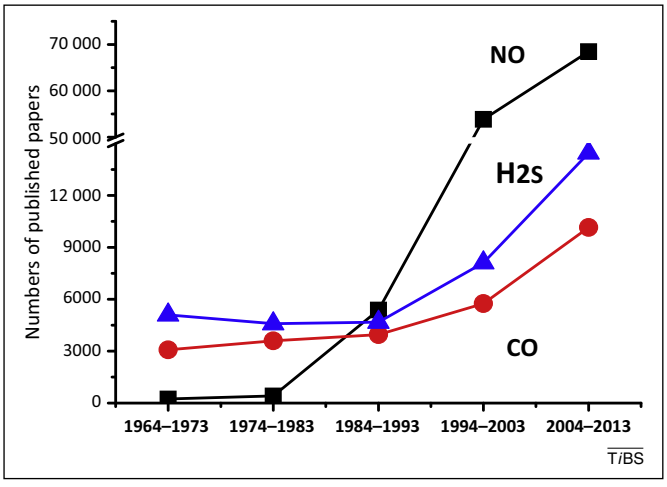

Figure 1. Publication trends on gasotransmitter research. A PubMed search is conducted based on three keywords: nitric oxide, carbon monoxide, and hydrogen sulfide. The number of published papers with the related keyword over each decade was pooled.

Effectiveness

Gasotransmitters are not only the products of metabolism. Their production is also regulated to fulfill their signaling messenger functions. Whereas the endogenous levels of gasotransmitters in the circulation or in tissues are relatively low, the molecular and cellular effects of these molecules are profound and extremely widespread. Can one find a cellular event or an organ function that is not affected by gasotransmitters in one way or another?

Ambiguity of the interactions among gasotransmitters and the significance of their crosstalk

Gasotransmitters share many common molecular targets but modulate their activities through different mechanisms [6,16,17]. For instance, both NO and CO activate BK_{Ca} channels, but whereas NO acts on the β subunit, CO acts on the α subunit of the BK_{Ca} channel complex [18].

Gasotransmitters may also work on different targets but eventually affect the same outcome. For example, NO binds to the heme group of soluble guanylate cyclase (sGC) to increase cGMP production. CO does the same to sGC but with a much weaker affinity. Intriguingly, CO does not stimulate sGC unless the tissue level of NO is low [5]. Although H_2S can be scavenged by certain heme proteins, it does not directly interact with sGC. Instead, H_2S decreases cGMP degradation by inhibiting phosphodiesterase [19,20]. In this example, we can see how three gasotransmitters converge their cellular impacts to affect cGMP level, but the outcome will vary depending on the availability and concentrations of each gasotransmitter and their interactions.

Another example comes from looking at how NO and H_2S both modify sulfhydryl groups of given proteins, but often generate opposing effects. H_2S interacts with low molecular weight thiols, such as glutathione, or protein cysteine residues (RSH) to form persulfide (RSSH) [21,22]. This S-sulfhydration increases the activities of modified proteins [21]. By contrast, the NO-dependent reaction with RSH that leads to an S-nitrosothiol (RSNO), called S-nitrosylation [23], appears to decrease the functions of the modified proteins [24]. It is a formidable challenge to elucidate the conditions and mechanisms that govern and coordinate the procession of S-sulfhydration or S-nitrosylation of proteins, individually or collectively. S-Nitrosylation and S-sulfhydration most likely occur in cysteine residues with low pK_a, which possess chiefly thiolate anion (S^-) in the physiological pH range. S-Nitrosylation may occur faster, but be less stable than S-sulfhydration [22] because the steady-state kinetics of chemical reactions between H_2S/NO and their targets are different [25] and the chemical strength of the S-nitrosylated bond (S-NO) is weaker (12–20 kcal/mol) than that of S-sulfhydrated bond (S-SH) (60 kcal/mol). This consideration leads to another prediction: that S-sulfhydration would inhibit the subsequent S-nitrosylation of the same protein. Indeed, it has been observed that decreased S-sulfhydration of nuclear

factor (NF)-κB p65 is linked to increased p65 S-nitrosylation [21].

Additions to the gasotransmitter family

Do our bodies possess gasotransmitters beyond NO, CO, and H_2S? Here, I evaluate ammonia, methane, and hydrogen for their candidacies of gasotransmitters.

Ammonia (NH_3)

This colorless basic gas is essential for all life forms as the primary source of nitrogen. Physiological concentrations of ammonia are <40 μM in human circulation and <80 μM in rat blood [26]. In the brain, it can be as high as 300 μM [27]. Many enzymes are involved in ammonia production (Figure 2). For example, glutamate dehydrogenase transforms glutamate to ammonia in astrocytes. Primarily in neurons, phosphate-activated glutaminase catalyzes ammonia production from glutamine. The breakdown of adenosine monophosphate to inosine monophosphate in the purine nucleotide cycle releases free ammonia [27].

Free ammonia can be removed from the cell or the body in multiple ways. In the transamination pathways, ammonia is disposed of by incorporation into various nonessential amino acids. Ammonia can also be converted to carbamoyl phosphate in mammalian livers, entering the urea cycle [28]. Glutamine synthetase condenses ammonia and glutamate into glutamine in astrocytes [29], whereas glutamate dehydrogenase combines ammonia and α-ketoglutarate to make glutamate in neurons [26]. Ammonia can be directly excreted from the urine, diffusing across renal tubules. Similar to other gasotransmitters, ammonia can passively diffuse through plasma membranes [27]. The cross-membrane movement of ammonia gas is often linked to that of ammonium ions, a process affected by local pH.

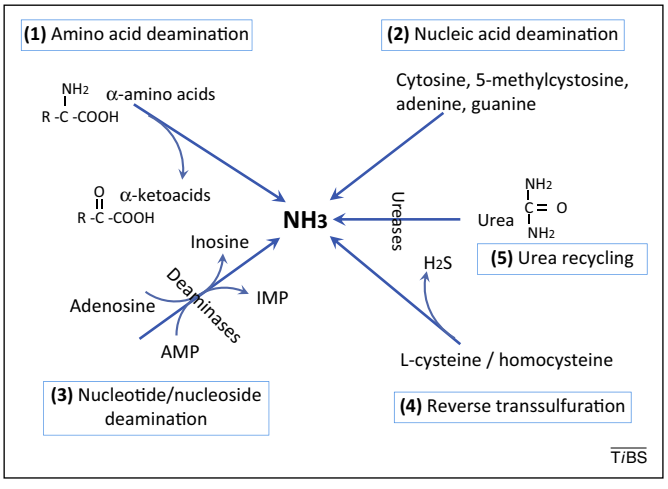

Figure 2. Ammonia generation in mammalian cells. **(1)** Amino acid deamination. Different enzymes are involved in this pathway including, but not limited to, glutamate dehydrogenase, serine deaminase, and glycine oxidase. **(2)** Nucleic acid deamination. In this process, the corresponding nucleic acids are deaminated to uracil, thymine, hypoxanthine, and xanthine, and release ammonia. **(3)** Nucleotide and nucleoside deamination. Adenosine monophosphate (AMP) or adenosine is deaminated to inosine monophosphate (IMP) or inosine, respectively, and releases ammonia. **(4)** Reverse-transsulfuration pathway. The production of ammonia is synchronized with the generation of H_2S via the enzymes cystathionine γ-lyase and/or cystathionine β-synthase. **(5)** Urea recycling.

NH_3 has one lone pair of electrons with its nitrogen atom. Similar to H_2O, H_2S has two lone pairs of electrons. NO and CO have three and two lone pairs of electrons, respectively (Figure 3). The lone pairs of electrons confer chemical reactivity to these gasotransmitters, a trait NO, CO, H_2S, and NH_3 have in common. Ammonia reacts with strong acids to form stable ammonium salts and reacts with Lewis acids (electron acceptors). Ammonia performs three fundamental functions in the body: it provides usable forms of nitrogen for the synthesis of DNA, RNA, and proteins; is involved in the redox balance; and regulates acid-base balance. For example, deamination of α-amino acids generates ammonium together with α-keto acids, such as α-ketoglutarate. The latter can be oxidized to two molecules of bicarbonate, providing buffering capacity for acidosis. During a chronic metabolic acidosis, renal excretion of ammonium can be significantly increased as a mechanism for renal acid-base regulation. An imbalance in endogenous ammonia metabolism has significant pathophysiological implications. Hyperammonemia, a condition characterized by high concentrations of ammonia in the blood, may result from liver diseases (such as cirrhosis) or from inborn errors of the urea cycle and may cause neurological diseases and hepatic encephalopathy.

The liver, kidney, gut, skeletal muscles, and brain are the most important organs for NH_3 metabolism. How, then, is nitrogen homeostasis maintained elsewhere in the body, such as within the cardiovascular and respiratory systems? In the brain, we have an interesting phenomenon. The metabolism of neurotransmitter glutamate depends on that of gasotransmitter ammonia. Beyond their metabolic correlation do they affect each other's signaling downstream pathways? How does NH_3 affect brain functions as well as other non-neuronal cells and tissues? Another intriguing challenge is the interaction of ammonia with other gasotransmitters. H_2S and NH_3 can be generated under identical conditions in the same reverse-transsulfuration pathway [1,6]. Are their cellular and molecular effects antagonized or potentiated by each other?

A signaling role of ammonia gas in eukaryotes has emerged [30]. The expression of inducible nitric oxide synthase (iNOS) in cultured astrocytes was increased by ammonia at pathophysiologically relevant concentrations [31]. Increased expression of neuronal nitric oxide synthase (nNOS) and iNOS in the striatum of portocaval shunted rats, an animal model of chronic hyperammonemia, was also observed [32]. As glutamine is a precursor to the antioxidant glutathione, ammonia may affect the redox status of cells through the glutamine cycle. Ammonia-induced protein tyrosine nitration was found in cultured astrocytes as well as in the cerebral cortex from portocaval shunted rats [31]. The modified proteins, such as glutamine synthetase, were inactivated. A recent study on cultured rat astrocytes demonstrated that ammonia can induce another post-translational modification, O-GlcNAcylation [30]. Many 25–50-kDa proteins are modified by ammonia in this way, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Ammonia meets all six criteria for being a gasotransmitter (Box 1). As such, this 'pungent gas' should be classified as a gasotransmitter.

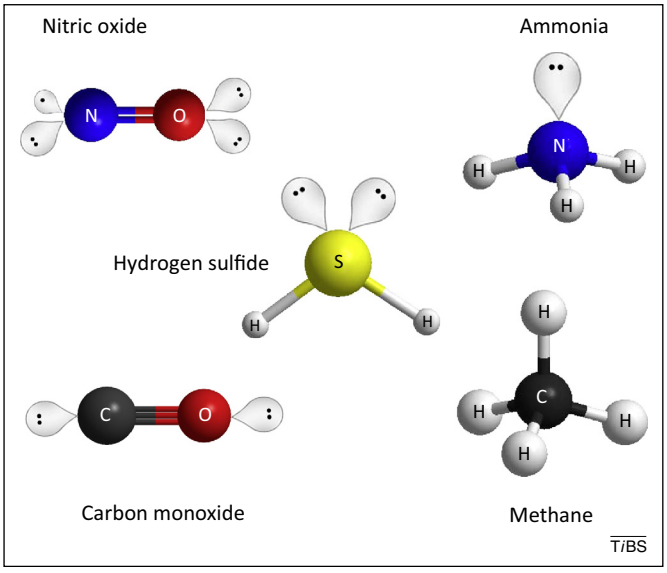

Figure 3. Molecular models of gasotransmitters. The 'balloons' attached to an atom indicate electron orbitals. The dots inside balloons are electrons, either being a lone pair or single in the case of nitric oxide (NO). The number of lone pairs is defined using the Lewis structure of molecules. The number of lone pair electrons plus the number of bonding electrons gives the total number of valence electrons around an atom. Lone pairs are also used in the formation of dative bonds as in the case of carbon monoxide (CO). They are located closer to the atom nucleus compared with the bonding electron pairs. The presence of lone pairs of electrons also impacts on the reactivity of molecules. Lone pairs of electrons usually have high charge density and thus negative polar character. Molecules with lone pairs of electrons can act as Lewis bases, nucleophiles, or ligands and this explains their high activity in organic reactions.

Methane (CH_4)

Similar to H_2S, CH_4 is a greenhouse gas. As the simplest hydrocarbon, CH_4 can be generated from organic material breakdown almost everywhere H_2S bubbles out. The anaerobic bacteria in our bodies produce significant amounts of methane via the methanogenesis pathway. The catalyzing enzyme of this pathway is methyl co-enzyme M reductase [33], which uses H_2/CO_2 and acetate as its main substrates. Formate, methanol, CO, and methylamines can also be converted to methane.

Non-microbial CH_4 release from methionine has been confirmed in fungi under aerobic conditions [34] and in mitochondria from cultured plant cells [35]. Most interestingly, methane can be produced in mammalian cells [36]. This notion is supported by the observation that antibiotic treatment of rats to purge intestinal methanogenic flora did not affect the whole body production of methane [36]. The carbon precursors for mammalian CH_4 production may be the electrophilic methyl groups bound to positively charged nitrogen moieties in methionine or choline [37]. The catabolism of methane in mammals is also known. Methane can be released into the blood circulation and then diffuses into the alveolar space or the gastrointestinal tract where it is excreted.

Methane has been shown to have biological and/or signaling functions *in vitro* and *in vivo* based on the observed effects of exogenously applied methane gas. The inhalation of 2.5% methane by dogs decreased oxidative and nitrosative stress with reduced ischemia/reperfusion damage [38]. This effect may be mediated by the immune system, as the same study demonstrated that exogenous methane inhibits leukocyte activation *in vitro* [38]. Alternatively, methane may directly affect oxidative stress level. Hypoxia [37] or the blockade of mitochondrial complex IV, which both increase oxidative stress, increases methane production from rat mitochondrial subfractions and from cultured bovine endothelial cells [39]. Oxygen-derived free radical production in these preparations was inhibited in proportion to methane production [39]. Intestinal methane gas infusion slowed intestinal transit in dogs, and the authors showed that the contractility of intestinal muscles and/or their contraction rhythm were determined by methane-induced decreases in the postprandial serotonin level [40]. In addition, high levels of methanogen-produced methane were found in Ob/Ob mice and in obese human subjects, and the extent of colonization of methanogens in the gastrointestinal tract of animals and humans was positively correlated to the development of obesity [41]. Unfortunately, the role of endogenous methane produced by our own cells (rather than by methanogens) in obesity has yet to be examined.

The chemical reactivity of methane is different from other gasotransmitters. NO, CO, H_2S, and NH_3 are inorganic molecules, whereas CH_4 is an organic molecule. Also, methane has a tetrahedral electron pair geometry without lone pairs of electrons (Figure 3). However, although it does not take part in nucleophilic reactions and is not very active in acid-base reactions, methane is engaged in many other reactions such as halogenation and hydrogen production and possesses the reactive capacity as a gasotransmitter.

Scientific interrogation to date suggests that methane (CH_4) is conceivably a gasotransmitter because it fully satisfies the first two criteria of gasotransmitters and partially meets criteria three and five (Box 1). However, this conviction has not been proven beyond a reasonable doubt. Specifically, the biochemical reactions leading to mammalian CH_4 production and the related catalyzing enzymes have not been identified (criterion three); the physiological levels of methane and its physiological functions have not been defined (criterion four); and the signaling functions and molecular targets of methane in mammalian cells remain questionable (criterion six).

Hydrogen gas (H_2)

A colorless and odorless diatomic gas, hydrogen is naturally produced by certain microbes. Microorganism-generated H_2 affects other microbes in the gastrointestinal tract and can serve as a biomarker for certain intestinal diseases. Hydrogen has low blood solubility. After being metabolized by various microbes in the gut, the remaining hydrogen gas enters the blood circulation and diffuses into the alveolar space to be exhaled later.

The conventional belief that H_2 is inert to human health was challenged by a 2007 study showing that molecular hydrogen selectively scavenged hydroxyl radicals in cultured cells [42]. Furthermore, inhaling H_2 gas decreased rat brain damage induced by acute focal ischemia and reperfusion [42]. Since then, the therapeutic utilization of hydrogen in various pathophysiological simulations has been reported. For instance, inhalation of H_2 protected various organs from ischemia/reperfusion injuries [43], suppressed inflammation, and improved

lipid and glucose metabolism in metabolic syndrome [44]. Drinking hydrogen-enriched water also prevented atherosclerosis development in ApoE knockout mice and reduced noise-induced hearing loss [45]. The therapeutic effects of H_2 have been generally ascribed to its antioxidant action because molecular hydrogen is a reducing agent. Interestingly, decreased CO production (due to the inhibition of HO-1) inhibits the anti-inflammatory effect of H_2 in lipopolysaccharide-stimulated macrophages [46], suggesting potential interactions of hydrogen with the known gasotransmitters.

The advances in therapeutic applications of hydrogen gas invite the inquiry on whether H_2 is a gasotransmitter. Unfortunately, this gas just does not measure up against the gasotransmitter criteria for now except for criteria one and two (Box 1). Specifically, there is no evidence that mammalian cells produce H_2 (criteria three and four); no physiological or biological functions can be assigned to endogenous as well as exogenous H_2 (criteria four and five); and no specific signaling roles or targeting molecules of H_2 can be identified in the body (criterion six).

Concluding remarks

The original article that coined the term 'gasotransmitter' was published in 2002 [1]. I hope that the current article is a worthy sequel that will further inspire interest and exploration into gasotransmitter research for researchers in all areas of life sciences. This sequel reaffirms the existence and importance of gasotransmitters to 'SAVE' the integrity and functions of many organs and systems in our bodies. It reminds us of some crucial challenges facing current gasotransmitter research. The outcomes of and mechanisms for the interactions among gasotransmitters on each other's production and functions should be further clarified. The conditions and mechanisms governing the effects of different gasotransmitters on the same molecule targets to produce the same effects in some cases, but the opposite effects in others, remain puzzling. It is also important to gain a better understanding of the mechanisms for the same functional outcomes of different gasotransmitters, which act on different molecular targets. Finally, this article expands this modern family of gasotransmitters from the original three (NO, CO, and H_2S) to include ammonia because this gas molecule meets all six criteria of being a gasotransmitter. Methane is a likely candidate of gasotransmitter considering its gas nature, high membrane permeability, endogenous production and catabolism in mammalian cells, and the biological and cellular effects produced by its exogenous donors. More extensive studies are needed, however, before we can fully qualify methane as a gasotransmitter. By contrast, hydrogen gas does not meet most of the criteria of gasotransmitters, although its therapeutic potential has been observed in recent years.

With the rapid advances in gasotransmitter research, I am optimistic that we will not need to wait another decade to justify a new sequel. Stay tuned.

Acknowledgments

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