CHAPTER 1

Overview of Gasotransmitters and the Related Signaling Network

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A system cannot be or remain a system without well-oiled and coordinated actions of all of its components or building parts which interact with each other to receive, deliver, integrate, and differentiate information *via* vast and intertwined networks. Using 'information' as signals and 'information webs' as signaling networks, all systems, as large as the world and as small as an intracellular organelle, function in the same fashion, from the distant past to the immediate present, and this will not change in the indefinite future.

Setting up flares to send out smoke signals in Ancient China alerted of an enemy invasion. A second beacon tower, upon seeing from a distance the smoke from the first beacon tower would light up a fire and send out its own smoke signal. This consequential smoke relay transmitted the signal of imminent danger over hundreds of kilometers within hours. Eventually, the visual signals would be transformed into army movement and a battle. In ancient Greek legends, a vocal signal was delivered over 35–40 km in over 3–4 h before the messenger collapsed. This messenger who ran from Marathon to Athens to report the victory of the Battle of Marathon is one of the most famous 'signal' carriers in human history. In the 21st century, our

Metallobiology Series No. 12
Gasotransmitters
Edited by Rui Wang

Published by the Royal Society of Chemistry, www.rsc.org

societies are bombarded by a large amount of signals and we have become accustomed to the velocity, versatility, and complexity of state-of-the-art signaling networks thanks to the internet, Twitter, Facebook, Instagram, and WeChat. The role of signals and signal networks in our modern society has become more important than ever.

You know by now what I am going to state next, and you are right. Gasotransmitters and their networks are the Twitter, Facebook, Instagram, and WeChat of life, human bodies, systems and organs, mammalian cells and intracellular organelles, bacteria and virus, or plants. The elucidation and description of the molecular and structural features of gasotransmitters, their production pathways, signaling mechanisms, cellular and molecular targets, and functional impact in prokaryotic and eukaryotic cells are the focus of this book. This chapter aims to provide an overview on the conceptualization of gasotransmitters as well as their interactions.

1.1 Conceptualization and Evaluation Systems for Gasotransmitters

Conceptual advances, scientific discoveries, and newly developed techniques impact our understanding of fundamental molecular and cellular events in biology and medicine. I proposed the establishment of the 'gasotransmitter' concept and framework firstly in 2002 to characterize and clarify a class of endogenous gas molecules, including nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S), that function as signaling molecules to direct various molecular and cellular events.[1] Over the last 15 years, this concept has continuously evolved and been refined[2] with recent updates.[3] The birth of the gasotransmitter framework deepened our understanding of cellular signaling processes, leading to the discovery of new pathogenic mechanisms and therapeutic strategies for related diseases. New gasotransmitters have been identified or suggested, such as NH_3.

Similar to the bacterial origin of H_2S in mammalian systems, methanogenesis in mammals is traditionally solely attributed to anaerobic microbial activity in the gastrointestinal tract. Studies in recent years have suggested that non-microbial methane formation may be a biologically relevant process in plants and animals.[4] Evidence has also been provided that endogenously generated methane may play a number of roles in the regulation of various and selective physiological functions. Interestingly, methanogenesis in mammals appears to be upregulated by hypoxia, suggesting the importance of this gas molecule for mammalian cells in dealing with the transition and adaptation to aerobic and anaerobic environments. Insufficient evidence, on the other hand, exists to support a full qualification of methane as a gasotransmitter against the six gasotransmitter criteria. However, the involvement of endogenous methane in redox regulation and mitochondrial function invites more intensive and in-depth research into methane-related physiological and biological events.

From the research capacity building point of view, the gasotransmitter concept has become a proliferative catalyst. Many programs or organizations on gasotransmitters have been created. The European Network on Gasotransmitters was created in 2011. The gasotransmitter concept has been included in the curriculum of many universities worldwide. A Google search of 'gasotransmitter' yields ~31 300 results (Mar. 28, 2018). An incomplete data search found four books with the word 'gasotransmitter' in their titles (Signal Transduction and the gasotransmitters: NO, CO, and H_2S in Biology and Medicine, 2004; Gasotransmitter – Physiology and Pathophysiology, 2012; Gasotransmitters: novel regulators of ion channels and transporters, 2012; Gasotransmitters in Plants – The Rise of a New Paradigm in Cell Signaling, 2016).

The following set of criteria defines the character and roles of gasotransmitters.

1. Gasotransmitters are small molecules of gas. In sharp contrast to numerous endogenous substances, gasotransmitters exist in gaseous form or are dissolved in circulation, interstitial fluid, lymph, or intracellular fluid. This criterion is inclusive, rather than exclusive, to account for derivatives of primary gasotransmitters. Gasotransmitters must have a light molecular weight, but their derivatives can present a light or heavy molecular weight and may no longer be in gas state. Regardless, these derivatives are still part of the gasotransmitter family. The derivatives of NO, such as nitrite (NO^{2-}), nitrate (NO^{3-}), nitrous oxide (N_2O), and nitroxyl (HNO), are examples of this inclusive concept of gasotransmitters. Together with persulfide, polysulfides are noticeable derivatives of H_2S, playing important gasotransmitter functions for H_2S or as H_2S.[5] These H_2S derivatives also help buffer fluctuations in the H_2S levels. Compared to endogenous H_2S levels, the endogenous levels of polysulfides in cells and tissues are much higher. This is somehow related to the fact that polysulfide store and/or release H_2S when needed.[5]

   Polysulfides can be formed in different ways. Enzymatically, 3-mercaptopyruvate sulfur transfurase (MST) decomposes mercaptopyruvate into pyruvate and sulfur. While pyruvate is rapidly released, sulfur remains bound to MST and accumulates as non-diffusible polysulfides.[6] After reaction of these polysulfides with thiols or sulfide, diffusible polysulfides may be consequently generated. The biological significance of this pathway is not clear as it relies on the cell type-specific expression of MST, and the diffusion and membrane permeability processes of the produced polysulfide are not straightforward. L-Cysteine competitively inhibits this pathway but mercaptoethanol activates it. The biogenesis of polysulfides can also stem from sulfide oxidation. One example of this path is the methemoglobin-dependent H_2S oxidation, leading to the generation of thiosulfate and hydropolysulfides.[7] Moreover, the interaction of H_2S with NO or nitrosothiols through HSNO or after decomposition of $SSNO^-$ leads to the formation of polysulfides.[8] It should

be noted that our understanding of the biosynthetic pathways and functional impact, as well as the underlying molecular and chemical mechanisms of polysulfides is still very limited.

Polysulfides can be reduced to sulfide in the presence of strong nucleophiles or enzymatically with the aid of the thioredoxin system or other enzymes. The endogenous conditions governing the bidirectional reactions between polysulfides and H_2S remain still unclear. The elucidation of the conditions and further insight into the kinetics of these reactions will help solve the puzzle of whether the stronger cellular signaling effect of polysulfide compared to that of H_2S at the same molar concentration is simply due to the fact that each polysulfide molecule contains multiple H_2S molecules and to the fast kinetics of the polysulfide reduction to H_2S. On the other hand, the polymerization of polysulfides affords cyclo-octasulfur (S_8), and homolytic cleavage of polysulfides leads to the formation of sulfur radicals.

Sulfur dioxide (SO_2) is another derivative of H_2S and its biological effects have been reported.[9] NADPH oxidase, glutathione-dependent thiosulfate reductase, and thiosulfate sulfur transferase catalyze the oxidation of H_2S to SO_2.[10–12] In aqueous solution, sulfites may be formed by reaction of sulfide with O_2 with formation of $SO_2^{\bullet-}$ and $S_2O_4^{2-}$.[13] Sulfur oxidation or sulfate reduction has been shown to generate SO_2 in certain prokaryotes. Calcium-stimulated production of SO_2 in porcine coronary arteries has been reported.[14] Currently, the *in vivo* SO_2 level is estimated using the proxy sulfite level. The sulfite level in rat plasma has been reported to range 10–15 μM.[9]

2. Gasotransmitters are freely permeable to cellular membranes. As such, their intracellular and intercellular movements do not *exclusively* rely on cognate membrane receptors or other transportation machineries.

   Lipid bilayers are the structural skeleton of plasmalemma and the membrane of intracellular organelles. Other constituents of cellular membranes include phospholipids, cholesterol, glycolipids, and proteins. Depending on the type of cells and organs where the lipid bilayer differs, membrane permeability to given substances is not always homogenous. Classical neurotransmitters cannot diffuse, freely or facilitated, through cell membranes. In order for neurotransmitters, secreted from pre-synaptic cells *via* exocytosis, to reach their targeted cells, they have to bind membrane receptors located on post-synaptic membranes to activate these receptors and the related downstream signaling events. The easiness of some gas molecules, such as CO_2 and NH_3, to diffuse through cell membranes may also be challenged in certain types of cells, such as those in renal thick ascending limbs or gastric glands.

   All identified gasotransmitters to date are polar molecules (H_2S, NO, CO, and NH_3) and able to permeate lipid-bilayer membranes. On the other hand, the ionic form or derivatives of these gasotransmitters may travel across lipid bilayers *via* facilitated machineries, free diffusion, or both. In this context, H_2S is freely permeable to membranes but

HS^- may require facilitated diffusion mechanisms, and protein-bound polysulfides would not be able to permeable membranes.[15,16] NO can diffuse freely through membranes, but NO^{2-} and NO^{3-} would not be able to cross said membranes in their ionic form unless facilitated transportation machinery is provided. The same contrast would apply to NH_3 and NH_4^+. Interestingly, no non-polar gas molecules, such as oxygen (O_2), carbon dioxide (CO_2), hydrogen (H_2), and nitrogen (N_2), are gasotransmitters. Methane (CH_4) is a non-polar gas and whether it is a gasotransmitter is under investigation. All these non-polar gases are hydrophobic gases. It has been suggested that, when hydrophobic gases penetrate the polar phospholipid head groups of lipid bilayers, significant energy consumption is expected and these gases may get trapped in the lipid phase of the cell membrane and unable to enter the aqueous phase of the cytoplasm or extracellular space.[17] As such, both the quantity and speed of free diffusion for these gases to pass through the lipid bilayer may not be sufficient to exert their paracrine or autocrine cellular effects. This reasoning leads to the idea that, in addition to free diffusion, facilitated diffusion may be needed for these hydrophobic gases, for which 'gas channels' may be the solution. How fast velocity is needed for a gas to penetrate a lipid bilayer and how much gas is needed to trigger a cellular reaction are both relative concepts. Gasotransmitters and non-gasotransmitter gases have different lifespans in gas and aqueous phases before they are transformed, ionized, decomposed, or excreted. The lifespan of NO in aqueous phase is in the range of seconds, while that of H_2S and CO ranges from minutes to hours. Therefore, NO needs to quickly cross the membrane before it is transformed, while this event is not that urgent for CO and H_2S. We also do not know the kinetics of fast free diffusion and facilitated diffusion of different gasotransmitters. Nevertheless, these ambiguities do not justify the need for gas channels to fulfill the gasotransmitter functionality.

The primary suspects regarding the molecular entities for gas channel proteins include aquaporins (AQPs), urea transporters (UTs), Amt/Mep proteins, and Rhesus (Rh) proteins.

Among the 13 identified mammalian AQPs, AQP-4 and AQP-1 have been proposed to serve as gas channels. AQP-1 is highly expressed in epithelial cells, endothelial cells, vascular smooth muscle cells, and red blood cells. AQP-1 has been suggested to be a gas channel for CO_2, based on the rate of acidification caused by the CO_2 influx across the cell membrane. AQP-4, which is highly expressed at the perivascular glia end-feet in the mammalian brain, has been reported as an NH_3 channel.[18] UTs have also been suggested to act as NH_3 channels.[19]

Amt/Mep proteins are also proposed gas channels for NH_3. Rh proteins are homologues of Amt/Mep proteins. Rh proteins have been suggested to function as CO_2 gas channels[20,21] or NH_3 gas channels[22] in red blood cells.

The arguments for the existence and function of 'gas channels' include the altered surface pH or intracellular pH values after the application of blockers of the corresponding channel proteins.[22] For example, AQPs can be blocked by 4,4′-diisothiocyanato-stilbene-2,2′-disulfonate (DIDS, a known inhibitor of ion exchangers) or DMSO. Decreased gas excretion after knocking out the expression of the proposed gas channel protein may also suggest the functional relevance of these channels to gas movement.

The arguments against the existence or importance of 'gas channels' are also multifarious. Knock-out of the expression of AQP-1[20,22] or Rh protein[22] did not change the CO_2 permeability of the mouse erythrocyte membrane. In many other studies, knocking out the expression of proposed 'gas channel' proteins only partially decreased, but *not eliminated,* the trans-membrane movement of the concerned gas. The partially decreased gas movement could be interpreted as a consequence of the altered molecular composition of the lipid bilayer in the genetically engineered preparations, which would then change the rate of gas diffusion. In any case, these studies showed that 'gas channels', if existing, are not the sole path of gases across cell membranes.[23]

Most studies on gas channels have concerned the conduits for CO_2 and NH_3 gases. To date, there is no direct measurement of gas molecule movement through these channels *per se*. This leads to another major concern on what the proposed 'gas channel' actually conducts. Gas molecules, such as NH_3 and CO_2, may be ionized as NH_4^+ and HCO_3^- on one side of the membrane or inside the lipid bilayer, and then cross the cell membrane through ionic channels, ion exchangers, ion co-transporters, or ion pumps. Once reaching the other side of the membrane, they could be de-ionized and return to the gaseous state. Under this assumption, the proposed 'gas channel' is not in fact a real gas channel but rather ionic transporting machinery.

There is no report on the existence of specific 'gas channels' for CO or H_2S. Some sporadic studies have suggested the existence of NO gas channels. Again, AQP-1 and AQP-4 were suspected to have this role. For example, the NO influx in Chinese hamster ovary cells, as measured by fluorescence microscopy to monitor the intracellular NO level, increased after AQP-1 was heterologously over-expressed in these cells. This was used as an indication that the AQP-1 protein channels NO through the cell membrane.[17] On the other hand, the same authors found free diffusion of NO across the lipid bilayer even in the absence of any protein, albeit much slower.[24] To summarize, current knowledge still validates that the membrane permeability of gasotransmitters does not require the presence of 'gas channels'.

Gasotransmitters do not have, neither need, cognate membrane receptors. Some studies use the terminology 'receptor' to describe the target proteins of gasotransmitters. This description is inaccurate and/or misleading. Receptors, in its scientific and stringent meaning, are

proteins located on plasma membranes or membranes of intracellular organelles. The receptor and its ligand, which cannot freely cross the lipid bilayer, are structurally cognate so that the ligand binds the corresponding receptor. The consequential configurational change of the receptor activates and triggers a cascade of molecular and cellular events *via* action of secondary messengers. One kind of ligand would usually bind specifically to one kind of receptor. For example, insulin or insulin-like proteins bind to the insulin receptor. From these considerations, it is clear that no receptors exist for gasotransmitters. NO binds the soluble guanylate cyclase (sGC) protein but sGC is not a receptor for NO since NO will bind to or act on numerous other proteins and sGC can interact with other gasotransmitters or endogenous substances. H_2S can induce the *S*-sulfhydration of numerous proteins. Are all these proteins receptors for H_2S? Absolutely not.

3. Gasotransmitters are endogenously generated in mammalian cells with specific substrates and enzymes. More than the products of metabolism, the production of gasotransmitters is regulated to fulfill signaling messenger functions.

   The message in this criterion is manifold. Gasotransmitters have to be endogenously generated, rather than inhaled from the environment. O_2 or H_2 cannot be produced by mammalian cells and, albeit important to life survival, are not gasotransmitters. Some potential gasotransmitters, such as methane, are produced in plants and prokaryotes with defined catalytic enzymes and endogenous substrates.[3] Likely, future studies may produce proof for the endogenous production of these candidates in eukaryotes once the specific enzymes and substrates are identified.

   Endogenous production alone would not suffice to establish a gasotransmitter role for a given gas molecule. Let's take CO_2 as a case in point. Oxidative phosphorylation in mammalian cells consumes oxygen (O_2) to produce energy (ATP), as well as CO_2 and water. The carbon in the CO_2 molecule originates from glucose ($C_6H_{12}O_6$) during the process. The endogenously generated CO_2 has a partial pressure of about 40 mmHg. There is no denial of the impact of CO_2 on the homeostatic control of numerous cellular and system functions, *e.g.*, pH regulation and chemoreflexes. However, CO_2 is a metabolism product. This gas is initially dissolved in a body fluid, carried to the lungs by circulation, and eventually breathed out of the body. The effects of CO_2 are exerted as a consequence of the oxygen/CO_2 metabolism, rather than as a result of the signaling role of CO_2 *per se*.

   Finally, the endogenous production of gasotransmitters has to be *regulated*, which is essential for the on–off switch of the related gasotransmitter signaling machine. The fastest regulatory mechanism is the activation of constitutive enzymes for given gasotransmitters to meet the minute-by-minute need of a specific cellular or molecular functional event. Acetylcholine stimulation of the muscarinic cholinergic receptor

in endothelial cells leads to increased intracellular calcium levels. Consequently, the formed calcium–calmodulin complex stimulates eNOS (endothelial NO synthase) to generate NO, or stimulates cystathionine γ-lyase (CSE) to generate H_2S, depending on the blood vessel type and functional needs. Now, the NO or H_2S signaling is turned on. Dissociation of acetylcholine from its receptor, decline of the increased intracellular calcium level, and deactivation of eNOS or CSE would then turn off the NO or H_2S signaling machine. In this way, a blood vessel is not always relaxed and a neuron is not always firing. Only in this way, a signal can be a signal to trigger the required on–off switching. A slower regulatory mechanism for endogenous production of gasotransmitters is the induction of gasotransmitter-producing enzymes. Inducible NO synthase (iNOS) and heme-oxygenase 1 (HO-1) can be induced by specific exogenous and/or endogenous stimuli, such as radiation, sheer stress, or hypoxia, to produce NO and CO, respectively. CSE and cystathionine β-synthase (CBS) have also been reported to be induced in some tissues where they are normally absent. An even slower mechanism for the regulation of gasotransmitter production is the genetic alteration of the expression and mutation of selective gasotransmitter-producing enzymes.

4. Gasotransmitters have well-defined specific functions at physiologically relevant concentrations. Why does the concentration matter? More often than not, the function of one endogenous substance is tested in an experimental setting with different concentrations of this substance. The observed effects provide information on the toxicological profile of the substance (bad effects), its potential therapeutic utilization (good effects), or its intrinsic role in maintaining our body running (physiological effects) if one knows the physiological concentration of this substance *in vivo*. This prerequisite of knowing the physiological concentration is complex and very challenging. Depending on the types of cell, organ, and system, the physiological concentrations of an endogenous substance may vary. Its levels in blood, urine, or breath may differ. Gender, age, and race may all contribute to the fluctuation of a given endogenous substance. This challenge is especially real for gasotransmitters due to their high volatility, brisk reactivity, and speedy turnover rate.[3] Gasotransmitters may quickly transform into secondary structures *in vivo*, such as the conversion of H_2S and polysulfides. Therefore, we need to make sure what we are really measuring under specific measurement conditions in order to determine the physiological concentration of the originating gasotransmitters or all of their derived species. Finally, can we ever be able to detect precisely the physiological concentration of a gasotransmitter? At this stage of the technology, the answer is NO. The moment you interfere with the homeostatic environment of a biological system, the physiological measurement ceases to be precise. Any measurement on isolated tissues and organs, body fluids, cultured cells, or the living body by introducing foreign probes or instruments will only afford results with

all kinds of interferences. The physiological concentration is only a RELATIVE term.

Given that the physiological concentration of a given gasotransmitter is a range rather than a precise value, let's try to define this range. The physiological concentration of total NO species has been reported to be around 20 μM in plasma and 30 μM in red blood cells (Suppl. Fig. 1 in Ref. 8]. The physiological level of H_2S is in the range of low micromolar to high nanomolar concentrations.[25]

When determining the cellular and molecular functions of gasotransmitters, researchers in biomedical and biological fields should all learn from our pharmacology colleagues, who usually systematically examine the full-scale of dose-dependent effects. Glancing through numerous published papers on gasotransmitters, one can easily find that the 'physiological effects' of a given gasotransmitter were tested at only one or two concentrations. The justification or fallacious argument for this choice is that these one or two concentrations are in the range of the reported 'physiological concentration' of the substance or that other researchers did the same before as reported in the literature. Here, important considerations on the physiological concentration, the differentiated sensitivity and tolerance to the tested substance in a given organ or cell type, and the second-order (and higher) kinetics of interaction between gasotransmitters and with other non-gasotransmitter substances are not considered.

5. The functions of gasotransmitters can be mimicked by their exogenously applied counterparts. A practical way to validate the physiological function of gasotransmitters is to test their effects by directly applying exogenously supplied gasotransmitters or their precursors *in vivo* or *in vitro*. In this way, the chemical nature of the applied compounds is certain, the elicited effects are specific, and cause–effect relationships can be defined.
6. Gasotransmitters are involved in signal transduction and have specific cellular and molecular targets.[3] Many macromolecules, such as actin and myosin or cholesterol, have important functions but they do not have a signal transduction function. They are either the structural components of the cell or the end targets of signal transduction cascades. On the other hand, gasotransmitters are not building blocks in a cell or at least their signaling functions are clearly distinct from the construction needs of other macromolecules. The major roles of gasotransmitters are to amplify, tune, and deliver a variety of signals to their targets, being micromolecules (*e.g.*, RNA and DNA) or macromolecules (*e.g.*, proteins, lipids, and carbohydrates). The value of gasotransmitters is manifested by the expression and/or functions of their targets *via* the signals they carry. The specificity of gasotransmitter signals ensures that the molecular and cellular functions are regulated purposely and orderly to avoid chaos, also enabling the specific chemical interactions of gasotransmitters with their targets, as

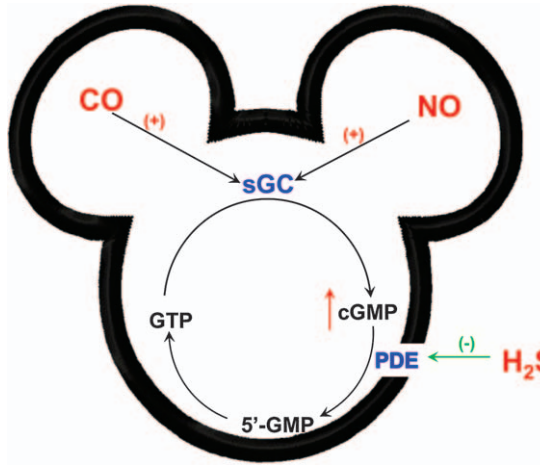

Figure 1.1 Interactions between gasotransmitters to increase the intracellular cGMP level.

well as the unique conditions and micro-environment where such interactions occur.

Many of the biological targets of gasotransmitters are known. NO activates the heme-containing enzyme sGC *via* coordination to the iron-heme, leading to increased levels of the second messenger cyclic guanosine monophosphate (cGMP). CO activates the same sGC with much weaker affinity. H_2S has no effect on sGC but it inhibits phosphodiesterase (PDE) to inhibit the degradation of cGMP (Figure 1.1). The mechanisms for the interaction of gasotransmitters with their targets are also known. For the aforementioned examples, NO and CO activate sGC *via* coordination to the iron-heme structure. H_2S induces the *S*-sulfhydration of specific cysteine residues of the K_{ATP} channel protein complex. On the other hand, the same biological targets can be stimulated or inhibited by other gasotransmitters and endogenous substances. The specificity of gasotransmitter action is a relative concept. It is neither necessary nor rational for any gasotransmitter to have only one or a few specific targets, unlike neurotransmitters which usually act only on their cognate receptors. NO-mediated signaling cascades include, but are not limited to, the simple activation of sGC. NO also causes global changes of cellular signaling network, such as thiol modification or oxidant formation (*via* interaction with other molecules).

1.2 Gasotransmitters – Why Does the Terminology Matter?

The concept and terminology of gasotransmitters have gained significantly recognition and acceptance by the majority of researchers in this promising

and young area. As its popularity grows and its impact becomes more obvious, alternative names for gasotransmitters have been suggested. This is not because of confusions or ambiguities caused by the gasotransmitter concept or anything alike. Rather, it shows that every researcher in the field cares for and desires to advance the field. Some of these suggested alternative names include 'small low-molecular weight molecules' and 'small signaling molecules'. Some wonder why not just call them 'gaseous messengers'.

To date, no one has challenged the importance of the six defining criteria for gasotransmitters, which are more important than the nomenclature *per se*. As long as you know what a neurotransmitter is, it does not matter whether it is called a neurotransmitter, neuronal messenger, synaptic messenger, or neuronal signaling molecule! Without the defining criteria, the alternative terminologies for gasotransmitter would fail to appropriately characterize this class of signaling molecules. For example, the proposed terminology of 'small-molecule signaling species' refers to small non-ionic molecules, which are freely diffusible, previously known mostly as toxic species that diffuse to relevant targets and should elicit a biological response. Some of these fingerprints have been included in the gasotransmitter framework and, as such, simply walking away from the nomenclature of gasotransmitter does not add any additional value but creates confusion. Some other critical fingerprints of gasotransmitters are not included and, as such, this alternative does not have the same rich and deep meaning as gasotransmitter. For example, the gasotransmitter family requires its members to have a molecular gaseous nature and to be endogenously synthesized. Dioxygen O_2 and reactive oxygen or nitrogen species are not gasotransmitters but are included within the framework of 'small-molecule signaling species'. Oxygen cannot be synthesized in our body. Period. This endogenous production concept also precludes the utilization of 'gaseous messenger' to replace 'gasotransmitter'. On the other hand, one may use 'gaseous messenger' as a general term to include all gas molecules that may have a signaling function in our body and to treat 'gasotransmitters' as a specific group within 'gaseous messengers', which have to fulfill the six criteria for gasotransmitters.

1.3 The Gasotransmitter Signaling Network in Eukaryotes

The production, sensing, and utilization of gasotransmitters are intrinsic and intertwined biological processes. Do all these processes involve or are regulated by the same proteins? How does one gasotransmitter-producing protein impact other gasotransmitter-producing proteins? How do gasotransmitter sensor proteins differentiate between different gasotransmitters? These questions have not been adequately addressed and answers are needed for the advancement and application of gasotransmitters in medicine and other life science disciplines.

1.3.1 Interaction of Gasotransmitters with Their Producers

CSE, CBS, and MST are responsible for the enzymatic production of H_2S. CSE and CBS are pyridoxal phosphate-dependent enzymes. Enzymatic production of NO is catalyzed by eNOS, iNOS, and neuronal NO synthase (nNOS). Deaminases, ureases, and CSE/CBS are involved in endogenous NH_3 production. Endogenous CO can be produced during the oxidation of heme catalyzed by the heme oxygenase (HO). HO presents two isoforms, the inducible HO-1 and constitutive HO-2.

1.3.1.1 H_2S Stimulates the eNOS Activity and NO Production

A decrease in the circulating nitrite and RXNO levels was observed in mice lacking CSE.[26] This result was ascribed to the lack of stimulatory effects of H_2S on the eNOS activity. NaHS treatment of mice resulted in increased eNOS expression.[27] We previously investigated the mechanisms for H_2S-induced activation of eNOS, which was heterologously expressed in HEK-293 cells.[28] It was found that NaHS increased the *S*-sulfhydration of eNOS, but decreased its *S*-nitrosylation induced by NO. The residue Cys443 of eNOS was pinpointed as a site for both *S*-sulfhydration and *S*-nitrosylation. We also examined the effect of H_2S on eNOS dimerization. Dimerized eNOS proteins were predominant in wide-type mice, but the absence of CSE in CSE-KO mice reversed this pattern with monomeric eNOS proteins being predominant. Similarly, *S*-nitrosylated eNOS proteins and heterologously expressed C443G-eNOS proteins exist mostly as monomers. These observations led to the conclusion that *S*-sulfhydration of eNOS increased the eNOS phosphorylation, decreased its *S*-nitrosylation, promoted the eNOS dimerization, and increased the NO production.

1.3.1.2 H_2S Increases the HO-1 Expression and CO Production

Administration of H_2S to rats with hypoxic pulmonary hypertension increased the plasma CO concentration and HO-1 expression in pulmonary arteries.[29] The same effect of H_2S on the HO-1 expression and CO production has also been observed in macrophages.[30] In contrast, NaHS treatment decreased the HO-1 expression in cultured aortic smooth muscle cells and the carboxyhemoglobin (COHb) levels in culture media.[31] NaHS treatment in Wistar rats also decreased the COHb levels in blood.[32] The reasons behind the conflicting observations in the aforementioned reports are not clear.

1.3.1.3 CO Regulates the Activity of CSE and CBS, as well as the H_2S Production

The mRNA and protein expression levels of hepatic CSE and serum levels of H_2S significantly decreased in cirrhotic rat livers compared to normal rat livers. After endogenous CO production in cirrhotic rats being inhibited with

HO inhibitors (cobalt protoporphyrin (CoPP) and zinc protoporphyrin IX (ZnPP)), the rats exhibited significantly lower levels of total bilirubin and arterial COHb, both proxies of elevated endogenous CO. Coincidently, hepatic CSE expression and the production of H_2S were significantly increased after CoPP or ZnPP treatment. It was concluded that endogenous CO downregulates the hepatic CSE expression and H_2S production in cirrhotic rat livers, and that the protective effects of the H_2S/CSE pathway against the progression of liver fibrosis are recruited when endogenous CO levels drop.[33] This study confirmed an earlier report describing the same effect of CO on the CSE/H_2S system in cultured aortic smooth muscle cells (SMCs).[31] The changes in plasma CO and HO-1 expression levels of guinea pig nasal mucosa were reversely correlated with H_2S levels during allergic rhinitis. Direct application of CO to the animals decreased the CSE expression and H_2S levels.[34]

A recent study showed that treatment of male Wistar rats with CORM-2, a CO donor, decreased the expression of CBS and H_2S production in gastric mucosa.[32] The effect of CO on the CBS activity has also been reported. The prosthetic heme in CBS offers a binding site for CO and the reduction of heme enables the binding of CO to CBS.[35] CO binding then inhibits the CBS activity and H_2S production.[36,37]

1.3.1.4 CO Upregulates the NOS Expression and Increases the NO Production

CORM-2 treatment has been found to upregulate the iNOS expression in rat gastric mucosa.[32]

1.3.1.5 NO Inhibits the CBS Activity but Increases the CSE Expression

NO has been shown to bind recombinant human CBS in ferrous state with an apparent $K_d \leq 0.23$ μM. This binding occurs rapidly and the dissociation is slow.[38] NO at physiological concentrations inhibits CBS, particularly in the presence of its allosteric activator *S*-adenosyl-l-methionine (AdoMet).[35,37] Interestingly, increased expression of CSE has been reported after treatment of mice with sodium nitroprusside (SNP).[27] Whether this NO-induced upregulation of CSE leads to altered endogenous H_2S levels is still unknown.

1.3.1.6 NO Induces HO-1 Expression and Activity

The role of NO as an inducer of HO-1 has been suggested and almost taken for granted. NO and its donors stimulate the *de novo* synthesis of HO-1 RNA and protein as a potential protection measure against nitrosative stress. This notion has been derived largely from experiments with NO donors. Depending on the type of NO donors, the actual impact of NO on HO-1 induction may vary. For example, neither nitroxyl (HNO/NO-) nor

NO-releasing agents could induce HO-1 expression without the addition of hemin during pre-incubation before the cell treatment.[39,40] While HO-1 induction induced by *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was mostly due to NO released thereof, it may not be the same case for SNP. SNP was reported to induce HO-1 expression in RAW 264.7 cells, but experimental evidence was submitted to demonstrate that the induction of HO-1 was mostly caused by the free iron released from SNP in aqueous solution.[41] Application of exogenous iron mimicked the effects of SNP with increased intracellular cyclic adenosine monophosphate (cAMP) and phosphorylated protein kinase A (PKA), leading to the upregulation of HO-1, which is antagonized by specific PKA inhibitors or an antagonist of cAMP. Deferoxamine (DFO) decreased the SNP-induced HO-1 expression but had no effect on the production of NO from SNP.[41] Another recent study reported that SNP increases the HO-1 activity in sunflower seedling cotyledons. The binding of NO, released from SNP, to the heme group of HO-1 was suggested as the underlying mechanism.[42] This study did not consider the potential role of the free iron released from SNP, neither compared the effect of SNP with other NO donors.

1.3.1.7 NH_3 Production Mediated by Other Gasotransmitters

Deamination of nucleotides and nucleosides, as well as nuclear acids, generates NH_3.[3] In this regard, NO has been reported at low micromolar concentrations to increase the DNA deamination products in human TK6 lymphoblastoid cells and decrease the cell viability.[43] SNP in aqueous solution was found to activate adenosine deaminase 2 purified from human blood plasma.[44] This effect of SNP involved certain amino acid residues, rather than SH-groups of cysteine. The reverse-trans-sulfuration pathway not only generates H_2S but also NH_3. As such, all aforementioned regulatory mechanisms for CSE/CBS have the potential to affect the NH_3 production. This area has not been actively explored yet.

1.3.2 Interaction of Gasotransmitters with Their Users/Targets

1.3.2.1 Iron-heme Proteins as Gasotransmitter Users/Targets

Many iron-heme proteins, including Hb, cytochrome c oxidase, cytochrome p450, sGC, HO-1, and CBS are the primary targets of gasotransmitters such as NO and CO. These gasotransmitter targets are able to coordinate metals and are sensitive to changes in oxygen levels.

1.3.2.2 Soluble Guanylate Cyclase

sGC catalyzes the production of cGMP from guanosine triphosphate (GTP). sGC is a heme protein. NO binds the ferrous (Fe^{2+}) heme with high affinity. Subsequently, the catalytic activity of sGC is increased and more cGMP is

produced. CO also acts on sGC but with lower affinity than NO, which explains the weaker vasorelaxant effect of CO compared to NO.[45] Under physiological conditions where NO levels are maintained, the stimulatory effect of CO on sGC activation would not been manifested. McLaughlin *et al.* demonstrated that, in the presence of YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole], the affinity of sGC toward CO increases to a similar level to that of NO.[46] Moreover, YC-1 potentiated the CO-induced relaxation of rat aortic strips by approximately 10-fold. This potentiating effect was mediated by the activation of sGC, since selective inhibition of sGC by ODQ (1*H*-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) abolishes the effect of YC-1 and CO.[46]

YC-1 is a synthesized compound and a similar endogenous substance has not been identified yet. It is clear that H_2S *per se* has no direct effect on sGC activation. Can H_2S fulfil the role of an endogenous enhancer for sGC activation? One hypothesis is that H_2S may regulate the sGC redox state and affect its responsiveness to NO and/or CO. Using cultured rat aortic smooth muscle cells, Zhou *et al.* observed that H_2S augmented the sGC response to the NO donor DEA/NO, which was related to the redox state of sGC.[47] When the oxidized sGC was activated by a heme-independent activator BAY58-2667, H_2S reduced the sGC activation. Over-expression of CSE in these cells also suppressed the stimulatory effect of BAY58-2667 on sGC activation. The authors further showed that H_2S converted the ferric status of purified recombinant human sGC into its ferrous state, resulting in the potentiation of the NO effect but suppression of the BAY58-2667 effect on the sGC activity.[47] Thus, the H_2S-induced reduction of the sGC heme iron is the key mechanism for the H_2S-facilitated sGC activation. The facilitating effect of H_2S on CO-stimulated sGC has not been reported.

1.3.2.3 Big-conductance Calcium-activated K Channel (BK_{Ca})

The BK_{Ca} is another interesting target for gasotransmitters. NO, CO, and H_2S regulate the activity of this channel, but target different units of the channel complex. NO may form covalent bonds with the C18 and C26 residues located on the extracellular loop of the BK_{Ca} beta subunit, whereas CO may interact *via* hydrogen bonding with the H6 and H37 located on the intracellular loop of the BK_{Ca} alpha subunit (Figure 1.2).[48]

The inhibition of BK_{Ca} by H_2S has been reported. NaSH inhibited heterologously expressed BK_{Ca} in a concentration-dependent manner by reducing the open probability of the single channels and shifting the half activation voltage by more than +16 mV. While the same BK_{Ca} alpha subunit is activated by CO, H_2S-induced inhibition is mediated by different mechanisms from those of CO. KCN completely suppresses the CO-evoked channel activation but without any effects on the H_2S-induced channel inhibition.[49] The same inhibitory effects of H_2S on the whole-cell BK_{Ca} currents have been observed in human-induced pluripotent stem cell (hiPSC)-derived mesenchymal stromal cells[50] and in rat colonic smooth muscle cells.[51] The effects of endogenous CO and H_2S on BK_{Ca} in rat carotid

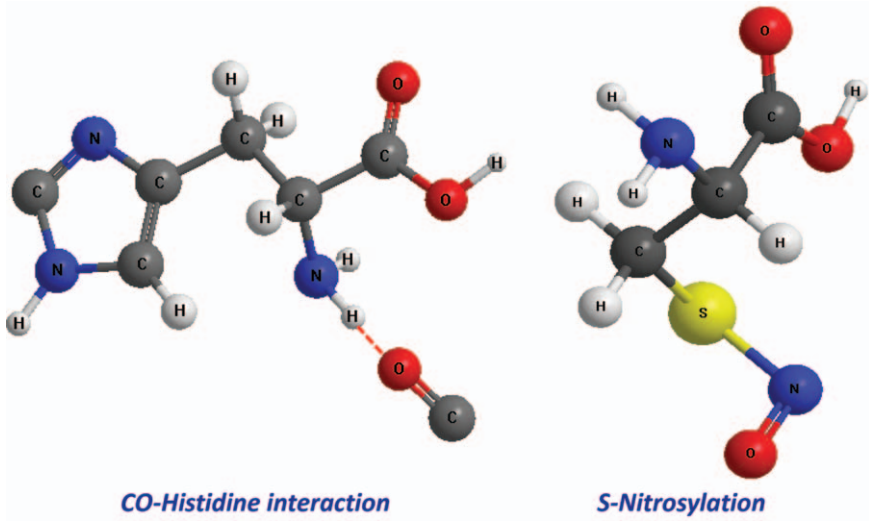

Figure 1.2 Interaction of CO and NO with different amino acids. CO interacts with the histidine residue by hydrogen bonding, while NO forms a covalent bond with cysteine.

body have also been suggested. BK_{Ca} is colocalized with HO-2. In the same cells, the expression of CBS and CSE produces endogenous H_2S. The existence of endogenous CO- and H_2S-generating enzymes creates the conditions for stimulation and inhibition of BK_{Ca} by endogenous CO and H_2S, respectively. During hypoxia, CO production from HO-2 in the cytosol is decreased. Hypoxia also reduces the mitochondrial oxidation of H_2S, which results in elevation of the cellular H_2S concentration. Thus, oxygen levels are linked to endogenous CO and H_2S levels, as well as the channel activity of BK_{Ca} in rat carotid body.[52]

1.3.2.4 K_{ATP} Channel

The K_{ATP} channel is the first identified molecular target for H_2S signaling action. Pharmacological blockade of K_{ATP} channels antagonized the H_2S-induced decrease in blood pressure in rats and relaxation of isolated rat aortic tissues *in vitro*. In isolated vascular smooth muscle cells, H_2S directly increased the whole-cell K_{ATP} channel currents and hyperpolarized the membrane.[53] Different molecular targets of H_2S on different subunits of the K_{ATP} channel complex have been reported. Mustafa *et al.* showed that H_2S covalently modified the Kir6.1 subunit of the K_{ATP} channel complex. Mutation of C43 in the Kir6.1 subunit prevented the H_2S-elicited hyperpolarization of vascular smooth muscle cells but did not alter the hyperpolarizing effect of cromakalim (a classical K_{ATP} channel opener) on the membrane potential.[54] It should be pointed out that the direct measurement of K_{ATP} currents was not conducted in this study, and that CSE-generated

endogenous H_2S caused the *S*-sulfhydration of the heterologously expressed Kir 6.1 in HEK293 cells.[54] To directly decipher the molecular interactions of H_2S with the K_{ATP} channel complex, Jiang *et al.* heterologously expressed different K_{ATP} channel subunits, Kir6.1 and SUR1, in HEK-293 cells and then recorded the whole-cell currents.[55] H_2S had no effect on the expressed Kir6.1 channel, but it stimulated the co-expressed Kir6.1/SUR1 K_{ATP} channels. Chemically modification of extracellularly located, but not intracellularly located, sulfhydryl groups inhibited the stimulatory effects of H_2S on Kir6.1/SUR1 currents. Point-mutation of cysteine residues (C6S and C26S) in the extracellular N-terminal of SUR1 subunits abolished the effects of H_2S on the Kir6.1/SUR1 currents.[55]

Many reports indicate that the effect of NO on K_{ATP} channels is mediated by cGMP/PKG or cAMP/PKA second messengers. SNP was found to have no effect on whole-cell K_{ATP} channel currents in vascular smooth muscle cells from rat mesenteric arteries.[56] Another study reported that NO donors potentiated the K_{ATP} channel currents induced by K_{ATP} channel openers in guinea-pig ventricular cells using whole-cell or cell-attached single channel recording patch-clamp configurations. In inside-out or outside-out patches, the potentiating effect of NO on the K_{ATP} channel currents was no longer observed.[57] In rat pancreatic beta cells, NO donors decreased or increased the K_{ATP} channel currents at low (0.5 μM) or high (10 μM) concentrations, respectively, in cell-attached and perforated whole-cell modes.[58] The inhibitory but not stimulatory effect of NO donors was mediated by the sGC/PKG pathway. However, neither the inhibitory nor the stimulatory effects of NO donors on K_{ATP} channels could be recorded in inside-out membrane patches.[58] The sGC/PKG pathway would have been kept intact in the cell-attached and whole-cell configurations of patch-clamp recording. Once in inside-out mode, all second messengers would be virtually eliminated and the channel proteins could not be regulated by cytosol second messengers. As such, the aforementioned observations suggest that NO does not have direct molecule-to-molecule interactions with K_{ATP} channel proteins.

The direct effects of NO, not mediated by other endogenous substances, on K_{ATP} channels have only been sporadically reported. For example, the outward-rectifying K_{ATP} channels of the inner mitochondrial membrane (mtK_{ATP}) in a human cell line (Jurkat cells) were reported to be *blocked* by NO, based on single channel patch-clamp measurements.[59] The direct stimulatory effect of NO on K_{ATP} channels has also been reported. In rat sensory DRG neurons, cell-attached and cell-free recordings revealed that NO donors activated single K_{ATP} channels by decreasing the channels' sensitivity to $[ATP]_i$. This stimulatory effect of NO could not be antagonized by specific inhibitors of sGC and PKG. These results indicate that NO directly acts on K_{ATP} channels, not mediated by the sGC/cGMP/PKG signaling pathway. DTT (a thiol-reducing agent) reversed and NEM (a thiol-alkylating agent) prevented the NO-induced activation of K_{ATP} channels, pointing to cysteine residues as the targets of NO.[60] Recombinant wild-type SUR1/Kir6.2 channels, heterologously expressed in COS7 cells, were activated by NO

donors. However, the NO donors had no effect on heterologously expressed truncated isoform Kir6.2 subunits without SUR1 subunits. Additional mutational studies showed that NO only interacted with residues in the NBD1 of the SUR1 subunit. This study suggests that NO may cause the *S*-nitrosylation of cysteine residues in the SUR1 subunit, leading to the activation of the K_{ATP} channel complex.[60]

A common theme for the regulation of K_{ATP} channels by gasotransmitters is the modification of cysteine residues by H_2S and NO. CO has no chemical potential to directly react with cysteine residues. Does it modulate the K_{ATP} channel activity? To date, there is no direct electronic evidence to show CO-induced changes of K_{ATP} channel currents. Foresti *et al.* showed that blockade of the K_{ATP} channel by glybenclamide partially suppressed the vasorelaxant effect of tricarbonylchloro-(glycinato)ruthenium(II) (CORM-3, a CO donor) in aortic tissues.[61] A similar pharmacological approach to demonstrate the involvement of K_{ATP} channels in CO effects was adopted in a later neural behavior study. Pereira *et al.* showed that intraplantar administration of hemin (as the HO substrate) or Tin protoporphyrin IX dichloride (an HO inhibitor) to rat right hindpaw inhibited or potentiated, respectively, carrageenan-induced hypernociception, as measured by electronic von Frey and Randall Selitto tests. Administration of glybenclamide abolished the hemin effect, indicating that the K_{ATP} channel is the molecular target of hemin/HO products in this setting.[62] In this study, the researchers did not directly administer CO into rat hindpaw; instead, biliverdin and iron(II) sulfate were injected in the same paw together with carrageenan. No attenuation of the carrageenan-induced hypernociception, however, was observed. Thus, it was reasoned that CO may be responsible for hemin/HO-induced activation of K_{ATP} channels in the peripheral nerve system.[62] Taking these limited studies together, it is premature to conclude whether CO has a direct effect on K_{ATP} channels. On the other hand, CO has been shown to impact the metabolism of NO and NO-related signaling pathways, including sGC/cGMP.[63] It would not be a surprise to see that CO may indirectly affect the K_{ATP} channel activity through the interaction with other gasotransmitters.

1.3.2.5 Thiol Proteins

S-sulfhydration is the reaction of sulfide with cysteine thiols in proteins to yield sulfurated thiol (−SSH) derivatives. Many cysteine-containing thiol proteins are common targets for gasotransmitters, especially NO and H_2S. The majority of the literature to date shows that H_2S-induced *S*-sulfhydration of cysteine residues of targeted proteins leads to increased protein activity, whereas NO-induced *S*-nitrosylation of selected cysteine residues results in decreased protein activity. However, this behavior has not been validated ubiquitously. It has been estimated that a small portion of NO-targeted proteins are *S*-nitrosylated (1–2%) and about 10–25% of H_2S target proteins are *S*-sulfhydrated.[64]

The SUR1 subunit of K_{ATP} channels can be modified by NO through *S*-nitrosylation or by H_2S through *S*-sulfhydration. Are the same cysteine residues of the SUR1 protein modified by NO and H_2S? The current literature does not provide an answer to this question.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a thiol protein and its cysteine residues are modified by both NO and H_2S. Being a classic glycolytic enzyme, GAPDH possesses multiple functions, such as the mediation of cell death. Endogenously generated NO has been shown to induce *S*-nitrosylation of Cys150, a catalytic cysteine of GAPDH. *S*-nitrosylation of GAPDH eventually leads to increased cytotoxicity and cell death.[65,66] GAPDH is also modified by H_2S through *S*-sulfhydration. GAPDH is sulfhydrated at Cys150, as confirmed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) on the full-length GAPDH protein immunoprecipitated from mouse liver lysate, as well as on purified full-length wild-type human GAPDH protein treated with NaHS. DTT treatment of the samples abolished the GAPDH *S*-sulfhydration.[67] *S*-sulfhydration enhanced the GAPDH activity with a maximum increase of 700% and half-maximal activation occurring at 15 μM NaHS. A recent study has shown that both H_2S and polysulfides induce the *S*-sulfhydration of GAPDH.[68] H_2S sulfhydrates Cys247 and polysulfides sulfhydrate the Cys156 and Cys247 residues of the reduced GAPDH. Polysulfides, but not H_2S, were found to inhibit the GAPDH activity by about 60%. The inconsistencies on the site(s) as well as the functional consequences of the *S*-sulfhydation of GAPDH in these reports have not been clarified.

NO directly modifies cysteine residues of the Klech-like ECH-associated protein 1 (Keap1) *via S*-nitrosylation after treatment of rat pheochromocytoma (PC12) cells[69] and human colon cancer HCT 116 cells.[70] Consequently, the NF-E2-related factor 2 (Nrf2) dissociates from Keap1, rapidly translocating from the cytosol to the nucleus, which increases its binding to DNA. Keap 1 is similarly *S*-sulfhydrated by H_2S at Cys151 in mouse embryonic fibroblasts.[71] A recent study confirmed the *S*-sulfhydration of Keap 1 at Cys151, but not at Cys273, in mouse vascular endothelial cells.[72] NaHS-induced Keap 1 *S*-sulfhydration has also been observed in gastric epithelial cells.[73]

1.3.3 Interaction of Gasotransmitters with Their Sensors

The concept of 'sensor' has to be considered in the context of the feedback regulatory system, which is composed of two key elements, the sensor and the regulator. A sensor detects the changed strength of the output signal and then compares this change with the intrinsically established reference strength. The difference in these strength levels is then forwarded to the regulator, which adjusts the system function to make sure that the strength of the input signal is altered opposite to the change in the strength of the output signal. The purpose of this system-wide sensing and feedback-controlling loop is to maintain the strength of the output signal constant, counteracting the fluctuations in the strength of the same input signal. The

operation of the feedback regulatory is aimed at achieving the status of homeostasis.

Oxygen sensing is the best example of this feedback regulatory system (Figure 1.3). When circulatory oxygen level is lower than the physiological level (the reference level), oxygen-sensor proteins firstly sense this change and then inform the regulatory molecules. The latter push the system to work harder, especially the cardiovascular and respiratory function, to increase the oxygen intake and transportation so that the oxygen supply to the system is renormalized. What is the oxygen sensor in this system? In a previous review article,[25] I specially stated that ''A molecule that is produced endogenously and can instantly interact with oxygen to yield the corresponding structural and functional changes that consequently trigger a series of cellular and body reactions is qualified as an oxygen sensor''. In our body, oxygen-sensing tissues include the carotid bodies in vasculature. Oxygen-sensing cells include glomus cells (chemoceptors of the carotid bodies), neuroepithelial cells in the gills, neuroepithelial bodies in airways, chromaffin cells in the adrenal medulla, or other ganglia of the sympathetic nervous system. This ambiguity on the identity of oxygen-sensing molecules has remained. Hypoxia-inducible factors (HIFs) are claimed to be among oxygen sensor proteins upregulated in response to decreased oxygen levels in cellular milieu. The function of HIFs is not changed by oxygen *per se* but their upregulated expression affects many other proteins that counteract the corresponding change in oxygen levels. Changes in HIF levels follow changes in oxygen levels. The utmost functional consequence of upregulated HIF expression is the increase of the oxygen supply systematically, regionally, and cellularly. In other words, O_2-sensors monitor blood and tissue oxygen tension levels and provide an informed reaction in order to renormalize the oxygenation of other types of cells, being located either adjacent or remote. In fact, we still do not know whether oxygen-sensing molecules are present in other cells beyond the specified cells or tissues, how many oxygen-sensing molecule(s) there are, and how these

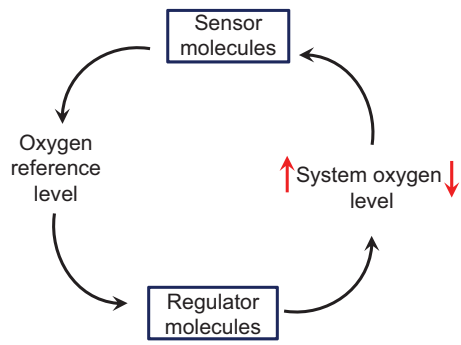

Figure 1.3 System-wide oxygen sensing and signaling loop.

sensors sense oxygen level changes. Some studies suggest that some membrane ion channels react with oxygen resulting in altered channel activity, but we do not have an idea on how oxygen interacts with channel proteins.

While *gas users* use gasotransmitters to alter their own functions, *gas sensors* should detect changes in the levels of specific gaseous molecules and correspondingly inform other molecules and cells about this change. Gas sensor proteins are involved in many biological regulatory and detoxification systems. Some proteins can function both as gas sensors and users.

Oxygen is NOT a gasotransmitter. Are there gasotransmitter sensors? To be more specifically, is there an H_2S or NO sensor protein in our body, for example? Are gasotransmitter-sensing machineries localized in specific tissues or cells, such as the oxygen-sensing tissues and cells in our body?

Hemoglobin in the blood binds oxygen and transports it to the organs. However, hemoglobin is not an oxygen sensor since it does not have the capacity to detect fluctuations in the oxygen levels and then activate other proteins to offset the hypoxia or hyperoxia. In other words, the gas-binding capacity of a protein does not crown it as a sensor protein for that gas. sGC is a gasotransmitter user, not a gasotransmitter sensor, since its interaction with NO directly changes its own function to produce more cGMP, rather than informing other molecules that the NO level has changed. The changes in sGC function do not necessarily reflect a change in NO levels since many other endogenous and exogenous substances can alter said function. Furthermore, the changes in sGC function would not necessarily lead to the normalization of NO levels.

From the above discussion, one may question the existence of real sensor proteins for the known gasotransmitters. Let me reinforce this notion by asking the alternative question – Is there any protein able to sense CO level changes and then trigger a series of reactions to renormalize the altered CO level? The same question applies to NO, H_2S, and other gasotransmitters. You may be surprised to find out that some candidate sensor proteins for gasotransmitters have been suggested, especially for heme-based CO sensor proteins. These CO sensor proteins sense changes in endogenous CO levels and correspondingly regulate the expression of other proteins to adjust the CO metabolism so that the endogenous CO level returns to normal values.

One of these CO sensor proteins is the neuronal PAS domain protein 2 (NPAS2). As a mammalian CO-dependent transcription factor, NPAS2 consists of a basic helix–loop–helix domain and two heme-containing Per-ARNT-Sim motives, *i.e.*, PAS domains (PAS-A and PAS-B). NPAS2 forms a heterodimer with BMAL1 to bind the E-box in the promoter of clock genes, such as Per1, Per2, Cry1, Cry2, and Rev-erb α, to regulate the circadian rhythm. Both PAS domains of NPAS2 bind heme as a prosthetic group, and the heme status controls the DNA binding *in vitro*. CO inhibits the DNA binding activity of heme-loaded holo-NPAS2, but not that of heme-free apo-NPAS2. The abundance of NPAS2-BMAL1 heterodimers decreases and the

number of inactive BMAL1 homodimers increases.[74] By analyzing the resonance Raman spectra of the wild-type and point-mutated NPAS2 proteins, Uchida *et al.* found that Cys170 is an axial ligand of the ferric heme. Upon heme reduction, cysteine becomes the ligand, replacing histidine.[75] A nu(Fe–CO) *versus* nu(C–O) correlation was found and a neutral His was reasoned to be the ligand *trans* to CO. This study indicated that the hydrogen bonding of His171 with the surrounding amino acids is disrupted once CO binds NPAS2. The consequential conformational changes in the His171-Cys170 moiety alters the function of NPAS2, triggering a feedback mechanism to regain homeostatic control of the endogenous CO level. This is accomplished by a reciprocal regulation of the heme biosynthesis and circadian clock.[76] The increased endogenous CO level is sensed by NPAS2, leading to the dissociation of NPAS2 from BMAL1 and decreased expression of the clock gene Per 2. Mammalian Per 2 stimulates the activity of the BMAL1–NPAS2 heterodimer. Decreased Per 2 expression in turn inhibits the NPAS2–BMAL1 activity. The expression of 5-aminolevulinate synthase 1 (Alas1) has been shown to be significantly higher in wide-type mice than in NPAS2-mutated mice, indicating that NPAS2 stimulates the transcriptional expression of Alas1.[76] Decreased NPAS2 activity leads to downregulation of Alas1. Alas1 is the first enzyme in the heme biosynthetic pathway and its downregulation would lead to decreased heme synthesis. Heme is the substrate of HO for the synthesis of CO. Thus, the endogenous CO level decreases at reduced heme levels. This feedback loop starts from the increased endogenous CO level, sensed by NPAS2 and regulated by the Per 2–Alas1–heme axis, and closes with the decreased and re-normalized endogenous CO level.

Some other heme-based 'sensor' proteins have been proposed, such as FixL, sGC, globin-coupled sensor (GCS), EcDos, AxPDEA1, HemAT-Hs, HemAT-Bs, and CooA.[77] All these proteins offer a heme group as the binding/targeting site for gas molecules. They can also couple a regulatory heme-binding domain to a neighboring transmitter, such as histidine protein kinases, cyclic nucleotide phosphodiesterases, chemotaxis methyl-carrier protein receptors, and transcription factors of the basic helix–loop–helix and helix–turn–helix classes. Again, the readers need to keep in mind the scientifically rigid definition of SENSOR in the context of signaling loops for feedback homeostatic control. It would be premature or incorrect to consider a protein as the sensor protein for a given gasotransmitter if the structural and functional changes of that protein do not lead to the re-normalization of the changed level of said gasotransmitter. For the sake of clarification, the existence of an intra-molecular sensing domain for gasotransmitters should not be confused with and misrepresented by the cellular/cellular identity of gasotransmitter sensors. These intra-molecular domains should be best and more accurately classified as binding domains rather than 'sensing' domains.[78]

Since the proposed CO sensor proteins are heme-proteins, it is natural to extrapolate that these same proteins may also sense changes in O_2 and NO

levels. For example, heme-NO-binding (HNOB) proteins or heme-NO/oxygen binding (HNOXB) proteins do bind NO and their activity is changed after NO/oxygen binding. What is not clear at this point is whether HNOB/HNOXB proteins also react with CO or whether NPAS2 can also sense changes in NO/oxygen levels. It is extremely interesting to consider the existence of H_2S sensor protein(s) since heme-proteins should also be capable to bind H_2S. They may be out there waiting to be discovered. Let's imagine this for a moment. An H_2S sensor protein in mammalian cells would be a heme protein, a metal protein, and/or a thiol protein based on the interaction of H_2S with heme, metals (iron, zinc, copper, *etc.*), and thiol groups. Upon sensing an increase in the endogenous H_2S level, for example, the H_2S sensing proteins would have their activity altered to inform the controlling proteins in the system of said increase. One or several things would happen thereafter. The function and/or expression of H_2S-generating enzymes would decrease, the substrate supply for H_2S-generating enzymes would decrease, and the catabolism of H_2S (oxygen-dependent or independent) would be accelerated. Finally, the increased endogenous H_2S level would return to a normal level.

1.3.4 Interactions Between Gasotransmitters

Gasotransmitters, including NO, CO, H_2S, and NH_3, have generally lone pairs of electrons in their structure to serve as Lewis bases, nucleophiles, or ligands.[3] Thus, the properties of high charge density, negative polar character, and high activity in organic reactions enable gasotransmitters to interact with other biomolecules and with themselves. To date, the chemical interactions between NO and H_2S have caught most of the attention of gasotransmitter researchers.

In the presence of oxygen, NO and H_2S readily yield oxidized nitrogen species and oxidized sulfur compounds. These species are capable of further interacting with each other *via* direct chemical reactions. NO interacts with thiols to form nitrosothiols (RSNO). As the simplest thiol molecule, H_2S is subject to *S*-nitrosylation by NO to produce thionitrous acid (HSNO) and/or nitroxyl (HNO). HSNO is the smallest RSNO with a very short half-life at physiological pH and ambient temperature.[79] HNO is the one-electron reduced and protonated sibling of NO. Sulfide at low concentrations quenches NO-mediated vascular responses *via* the formation of an uncharacterized RSNO, assumed to be HSNO.[80,81] A test-tube experiment by directly mixing NaHS with an NO donor or NO gas generated RSNO within seconds.[82] Incubating rat liver homogenates with NaHS or with precursors of endogenous H_2S (L-cysteine and pyridoxal phosphate) also led to increased NO_2 formation, an indication of the formation of RSNO. Interestingly, the newly yielded RSNO could not stimulate the cGMP production as NO or H_2S did alone.[82] Using a different approach, Yong *et al.* found that the direct interaction between an NO donor (SNP) and H_2S donor (NaHS) abolished the effects of SNP alone on calcium levels

and the contractility of ventricular myocytes. The formation of HNO out of this NO–H_2S interaction was suspected as the HNO donor (Angeli's salt) mimicked the effects of an SNP–NaHS mixture.[83] No direct chemical evidence has been obtained to confirm the precise identity of this novel molecule as HNO.

Not all studies support the importance of the direct chemical reaction between H_2S and NO. Injection of NaHS in Wistar rats significantly lowered the blood pressure without changes in the circulating levels of total NO species. However, the total nitroso (RXNO) species in plasma and NO-heme levels in red blood cells increased upon NaHS infusion. It appears that H_2S did not scavenge or affect the NO bioavailability, contrary to what the researchers claimed.[8] Should H_2S scavenge NO, the blood pressure would be expected to elevate, not decrease. This study also hinted that it is H_2S and/or its derivatives that lower the blood pressure through their direct effects on the cardiovascular system, rather than through NO scavenging. On the other hand, this study indeed showed that inhibition of NOS prolonged the cardiovascular effects of H_2S, which could be argued as evidence for the decreased formation of HSNO. Cortese-Krott *et al.* reported that the chemical reaction of H_2S and NO may yield three primary sulfur–nitrogen species: nitrosopersulfides (ONSS–), polysulfides (RSSN– and HS_n–), and dinitrososulfites (SULFI/NO).[8] Of these, ONSS– was the primary species capable of releasing NO, as verified by chemiluminescence detection. In comparison with HSNO, ONSS– is more stable and resistant to nucleophilic attack by other thiols and cyanide anions, whereas HSNO undergoes rapid isomerization, hemolysis, and polymerization.[8] ONSS– may function as a NO carrier and release species. However, whether the chemical reaction of H_2S and NO actually generates ONSS– under physiological conditions has not been firmly established. The rapid and spontaneous secondary reactions between sulfur–nitrogen species make the interpretation of H_2S and NO reactions *in vivo* more difficult. For example, polysulfides can enhance the formation of the ONSS– product.[8] Other sulfane sulfur molecules, including persulfides (RSSH), may also mediate the cellular and molecular effects of H_2S.[5] The reactions of these sulfane sulfur molecules with NO, as well as the functional consequence of these putative reactions, remain unknown.

Acknowledgements

This work has been supported by a Discovery grant from Natural Science and Engineering Research Council of Canada (NSERC) and an operating grant from Canadian Institutes of Health Research (CIHR).

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