# 1 The Evolution of Gasotransmitter Biology and Medicine

## *From Atmospheric Toxic Gases to Endogenous Gaseous Signaling Molecules*

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*CONTENTS*



## SUMMARY

Overproduction of many atmospheric gases, from natural resources and anthropogenic activities, impose a serious environmental concern with adverse health effects. Among pollutant gases are nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide ($H_2S$). Over several decades, studies from numerous laboratories have demonstrated that gases such as NO, CO, and $H_2S$ not only are generated in the human body but also play important physiological roles. These particular gases share many common features in their production and function but carry on their tasks in unique ways, which differ from classic signaling molecules, in the human body. Collectively, these endogenous molecules of gases or gaseous signaling molecules compose a family of "gasotransmitters." The regulation of ion channels by gasotransmitters, either directly via chemical modification of ion channel proteins or indirectly via second messengers, exerts significant influence on cellular functions. *S*-nitrosylation, carboxylation, and sulfuration may represent mechanisms of direct interaction of NO, CO, and $H_2S$ with ion channel proteins, respectively.

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This chapter summarizes the history and evolution of the concept of the gasotransmitter and outlines the criteria used to identify novel gasotransmitters. Gasotransmitter research is accelerating into the next phase. Many new gasotransmitter candidates are being investigated. Alterations in the metabolism and functions of gasotransmitters under different pathological conditions are being explored, which may shed light on the pathogenesis and management of many diseases. Thus, research on gasotransmitters is certainly as important to clinical practice and community health as it is to basic research, if not more so.

**Key Words:** Gasotransmitter; nitric oxide; carbon monoxide; hydrogen sulfide; signal transduction.

*"Air is a physical substance; it embraces us so intimately that it is hard to say where we leave off and air begins. Inside as well as outside we are minutely designed for the central activity of our existence—drawing the atmosphere into the centre of our being, deep into the moist, delicate membranous labyrinth within our chests, and putting it to use."—David Suzuki, The Sacred Balance*

## 1. INTRODUCTION

Humans tend to treat atmospheric gases, such as oxygen, carbon dioxide ($CO_2$), nitrogen, carbon monoxide (CO), and hydrogen sulfide ($H_2S$), like sunshine and water—nature's gifts to us. Accompanying the arrival of the Industrial Revolution, the Third Wave is a high tide of natural gas production from industrial sources. In the public eye, most natural gases are nothing but toxicants, wastes, and pollutants, with oxygen as possibly the only exception. By definition, environmental toxicants are "agents released into the general environment that can produce adverse health effects among large numbers of people" *(1)*. Gas pollutants as environmental toxicants can induce both acute and chronic health hazards at societal as well as individual levels. The health hazards of these toxic gases become magnified in our public life. When this is coupled with public concern about the production of natural gases, it then becomes a health issue impacting both environmental and occupational health.

Scientists have worked with two schools of thought searching for the biological production and the physiological function of natural gases, be it detrimental or beneficial. One ancient frontier is the study of the biological production of gases. Archaea and microbes produce great amounts of gas, not only for their own use, but also for the necessity of life in their environment. Interestingly enough, these studies consistently demonstrate the production of numerous natural gases by microorganisms. For example, many bacterial types, such as Proteus vulgars, produce CO *(2)*. The biological production and utilization of $H_2S$ have been best known for particular bacteria and archaea *(3)*. Human beings sit on top of the genomic life tree. Do we inherit or share any of these abilities from low forms of life to produce gases in our body? Plant life generates oxygen from light, a process of photosynthesis through the use of chlorophyll. Humans are not equipped in this way. However, our bodies do produce $CO_2$, ammonium, and other gases. The human body is often in this way treated as a pollutant when an analogy is drawn to the automobile or even a restaurant kitchen, which also generates useless gases, toxicants, or other types of harmful byproducts. The records of endogenous production of CO and $H_2S$ in human tissues can be traced back hundreds of years. The human body can generate a myriad of gases with unknown functions—*the truth is still out there.* This body of knowledge, unfortunately, has not been completely used to facilitate the understanding of human physiology.

Scientists working in the second frontier—the physiological function of biological gases, a natural extension of the first frontier—brought about this revolution. In this regard, nitric oxide (NO), a pioneer gas, is doubtless the molecule of a new era. Over the last several decades, studies from thousands of worldwide laboratories have demonstrated that gases such as NO, CO, and $H_2S$ are not only generated in humans but also have important physiological properties.

These gases share many common features in their production and function while carrying out their tasks in unique ways that differ from classic signaling molecules in the human body. Collectively, these endogenous molecules of gases, or gaseous signaling molecules, compose a family of "gasotransmitters," a nomenclature composed of "gas" and "transmitters."

This introductory chapter is devoted to discussing the conceptual transition of biological gases from toxic wastes and pollutants to important physiological gasotransmitters.

## 2. PRODUCTION AND HEALTH HAZARDS OF ATMOSPHERIC GASES

### *2.1. Nitric Oxide*

Natural causes—lightening, forest fires, and organic decay—lead to the generation of oxides and nitrogen (NOx). Soil microorganisms also produce NOx. NO and $N_2O$ are emitted from anaerobic soils by denitrifiers such as *Pseudomonas* spp. or *Alcaligenes* spp. and from aerobic soil by autotrophic nitrifiers such as *Nitrosomonas europaea (4)*. Motorized vehicles are the major mobile combustion source of NOx production. In 1994, one study showed that in a long, 7.5-km Norwegian road tunnel, with traffic flowing in both directions, the atmospheric $NO_2$ concentration exceeded the Norwegian air quality limits for road tunnels 17% of the time. When traffic was reduced through the tunnel, the mean $NO_2$ concentration was significantly lowered *(5)*. Stationary combustion sources of NOx include heat power plants and industrial factories *(6)*. Cigarette smoking generates a considerable amount of NO and $NO_2$ *(7)*. The biological treatment of nitrogen-rich wastewater with a high concentration of ammonium likewise yields NO and $NO_2$, although this might not contribute significantly to general environmental pollution with NOx *(8)*.

As the initial product of NOx from a reaction between nitrogen and atmospheric oxygen, NO quickly transforms to $NO_2$ either through simple oxidation involving molecular oxygen or through a photochemical reaction involving irradiation by sunlight. As a result, health hazards of atmospheric NO must be considered in conjunction with $NO_2$. Mercer et al. *(9)* found that after adult rats were exposed to 0.5–1 ppm of NO for 9 wk, the fenestration numbers in the alveolar septa of the lung increased more than 30-fold in the control rats, and 3-fold of $NO_2$ in the exposure group. The number of interstitial cells in the NO group was significantly reduced by 29%. Likewise, a significant reduction in the thickness of interstitial space was observed in the NO-treated rats, but not in the $NO_2$-treated rats, compared with the control rats *(9)*. Their study demonstrated that a low level of atmospheric NO exposure is more potent than $NO_2$ in producing interstitial lung damage. It is believed that most NO toxicity is mediated by the interaction of NO with superoxide producing peroxynitrite. This leads to oxidative damage to targeted cells and tissues. Epidemiological data often show controversial results on the adverse health effects of $NO_2$ *(6)*, partially because of the difficulty in determining the actual atmospheric $NO_2$ levels to which a specific portion of the population was exposed. Controlled animal and human studies provide evidence that high $NO_2$ levels weaken pulmonary

defense mechanisms and change human airway responsiveness. Lipid peroxidation *(10)* and protein oxidation *(11)* have been described as part of the cellular mechanisms of $NO_2$-induced health hazards. The most important and consistent conclusion is that exposure to high $NO_2$ concentrations may exemplify a specific health risk for a subpopulation of people with respiratory diseases, such as asthma and chronic obstructive pulmonary disease.

Over a 1-yr period, Giroux et al. *(12)* examined the correlation of acute myocardial infarction, atmospheric levels of NOx, temperature, and relative humidity. Among 282 patients with acute myocardial infarction, it was determined that the infarction area was reduced when the daily NO level in the atmosphere was higher than 13 $\mu g/m^3$ and the average daily temperature was lower than 13°C.

NO and $NO_2$ act as phytotoxic agents, damaging plant health as well. The growth of plants becomes poorer and productivity lower when exposed to high NOx levels *(13)*.

### 2.2. Carbon Monoxide

The toxicology profile of CO has been portrayed for hundreds of years. CO is among the most abundant air pollutants in North America. Because it is colorless, odorless, and noncorrosive, intoxication by CO is hard to detect, which earns CO the reputation of the "silent killer." A report in 1982 by the US Centers for Disease Control revealed that approx 4000 deaths and 10,000 cases of individuals requiring medical attention occur annually because of acute CO intoxication *(14)*.

All types of incomplete combustion of carbon-containing fuels yield CO. Natural processes such as metabolism and production of CO by plants and oceans release CO into the atmosphere. Oxidation of methane and nonmethane hydrocarbons by hydroxyl radicals and ozone, either natural or anthropogenic, is also a significant mode of CO production in the atmosphere. The most notable ways that humans contribute to the production of CO are the operation of internal combustion engines; the fueling of appliances with gas, oil, wood, or coal; and the disposal of solid waste. Cigarette smoking also produces a substantial amount of CO.

Whether an elevated environment of CO levels leads to human intoxication is influenced by the exposure and duration of pulmonary ventilation function, as well as the endogenous buffering capacity (i.e., the level of carbonmonoxy-hemoglobin A [HbCO A]), and the partial pressures of CO and oxygen. Acute ambient CO poisoning occurs as suddenly elevated CO concentration accelerates the binding of CO to normal adult hemoglobin (Hb) (Hb A), forming HbCO A. The formation of HbCO A impairs two functions of Hb. The oxygen storage function of Hb A is significantly reduced because the affinity of CO to Hb A is approx 250 times greater than that of oxygen *(15)*. The affinity of myoglobin to CO is approx 25-fold that of oxygen. The oxygen transportation function of Hb A is also reduced, because the release of oxygen from HbCO A to the recipient tissue becomes more difficult. CO binds to one of the four oxygen-binding sites of Hb A via the formation of a hydrogen bond between CO and the distal histidine residues of Hb A *(16)*. This binding, in turn, increases the affinity of oxygen to HbCO A. With tissue hypoxia being the major toxicological consequence of CO poisoning, the combination of CO with other heme-proteins, such as cytochrome P450, cytochrome-*C* oxidase, catalase, and myoglobin, may also in part account for the toxic effects of CO *(2,17)*. Because of their high demand for oxygen, the brain and heart are the most vulnerable organs, to the CO-induced acute hypoxia. Neurological and myocardial injuries associated with acute CO intoxication can be fatal unless medical treatment is provided immediately. The

normal background of the HbCO A level in a healthy nonsmoker is about 0.5–1% *(18)*. Early neurological symptoms such as headaches, dizziness, nausea, vomiting, disorientation, and visual confusion occur when the HbCO A level reaches 10–30%. Depending on the CO exposure level, duration, and treatments, the prognosis in patients with acute CO poisoning varies *(17)*.

Chronic environmental CO exposure may constitute one risk factor for cardiovascular diseases. A retrospective study of 5529 New York City bridge and tunnel officers unmasked the relationship between occupational exposure to CO and mortality from heart disease *(19)*. The CO exposure level of the tunnel officers was much higher than that of the bridge officers. There were 61 deaths from arteriosclerotic heart diseases in tunnel officers, which was higher than the expected 45 deaths based on the New York City population. Once the exposure was eliminated, the high risk of arteriosclerotic heart disease in the tunnel officers dissipated.

There has been a long-lasting debate on whether chronic CO inhalation as intrinsically linked to cigarette smoking acts either alone or with other environmental stressors to induce hypertension *(20,21)*. Increases, decreases, or no change in blood pressure after CO exposure has been reported. What should be remembered is that the adverse health effect of cigarette smoking is not a simple mirror image of CO inhalation. Immediately following cigarette smoking, an acute but transient increase in the smoker's blood pressure occurs, which has been largely ascribed to the nicotine in smoking. This hypertensive effect of nicotine is overcompensated by CO in the end. The blood pressure of these long-term smokers is decreased, or at the very least not increased, without other cardiovascular complications *(22)*. This notion was further supported in an animal study in which borderline hypertensive rats were exposed to chronic CO. This treatment actually led to hypotension, not hypertension, in these animals *(20,22)*. Chronic CO inhalation leads to many diseases, chiefly those linked to hemodynamic responses to CO and hypoxia-adaptive changes *(23)*. Cardiac hypertrophy exemplifies the cardiovascular complications of chronic CO exposure. Continuous exposure in adult male rats to 700 ppm of CO for 27 d *(24)* or 500 ppm CO for 30 d *(25)* induced volume-overload cardiac hypertrophy. Hypertrophy of both the left ventricle (22%) and right ventricle (37%) developed with hematocrit increased nearly 50%. Chronic CO exposure also alters normal development of the cardiovascular and other systems. In one experiment, 1-d-old rat pups were exposed to 500 ppm of CO for 30 d, and cardiac histology analysis was performed at 61 and 110 d of age *(26)*. One notable alteration was the significant increase in small arteries across all heart regions. The diameter of the large arteries in the entire heart region was also greater than that in the control rats. The architectural impact of coronary vessel changes following chronic neonatal CO exposure would be considerable on cardiovascular functions, especially those at different developmental stages and in adulthood.

### *2.3. Hydrogen Sulfide*

The presence of $H_2S$ in our environment is easily recognizable for its peculiar rotten-egg smell *(27,28)*. Atmospheric $H_2S$ has both natural and anthropogenic sources. Volcanic gases, marshes, swamps, sulfur springs, and decaying matter such as from mushrooms all release $H_2S$ into the environment. Emissions from oil and gas refineries, paper mills, and sewer networks also result in odor, health, and corrosion problems. Acute intoxication of $H_2S$ can be lethal *(29)* and is one of the leading causes of sudden death in the workplace *(30)*. At least 5563 cases of intoxication and 29 deaths resulting from $H_2S$ exposure occurred in the United States between 1983 and 1992 *(31)*. Loss of the central

respiratory drive is one of the major mechanisms for acute $H_2S$ death *(27,28,32,33)*. The interaction of $H_2S$ with many enzymes and macromolecules, including Hb, myoglobin, and cytochrome oxidase, exerts a profound effect on the vitality of cells *(34–36)*. Disorders of the central nervous, cardiovascular, respiratory, and gastrointestinal systems have been reported with acute $H_2S$ intoxication *(34,37)*.

The health hazard of chronic $H_2S$ exposure has also been observed *(36)*. Bates et al. *(38–40)* carried out a series of studies in the city of Rotorua, New Zealand, which is located over an active geothermal field. Approximately one-quarter of the population had been exposed regularly to high concentrations of $H_2S$ from 143 to 1000 ppb. During 1981–1990, a higher mortality risk for respiratory diseases and a higher morbidity risk for neuronal diseases (both peripheral and central nervous systems) were observed in the Rotorua population compared with the rest of the population of New Zealand *(38,39)*. Another improved survey based on 1993–1996 morbidity data linked adverse health outcomes of Rotorua to other regions within Rotorua with high, medium, or low $H_2S$ exposure levels *(40)*. This recent study again demonstrated an $H_2S$ exposure-response tendency for disorders of the nervous system and sense organs as well as circulatory and respiratory diseases. Furthermore, a retrospective epidemiological study examined 2853 married, adult, nonsmoking women in a petrochemical complex in Beijing, China *(41)*. During their first trimester of pregnancy, about 57% of the surveyed woman had been exposed to petrochemicals. The results showed a significantly increased risk of spontaneous abortion when exposed to $H_2S$ (odds ratio [OR]: 2.3; 95% confidence interval [CI] 1.2–4.4), benzene (OR: 2.5; 95% CI: 1.7–3.7), and gasoline (OR: 1.8; 95% CI: 1.1–2.9).

The average odor threshold for $H_2S$ is about 0.5 ppb *(42)*. A low level of $H_2S$ exposure does not appear to have had any adverse long-term health effect *(42)*. According to the Agency for Toxic Substances and Disease Registry, the acute minimal risk level for $H_2S$ currently is set at 70 ppb, i.e., 24-h daily exposure to 70 ppb of $H_2S$ over a period of 14 d or less *(42)*. An investigation was conducted in a Pennsylvania elementary school that complained of $H_2S$ odors putatively related to the nearby mushroom-composting operations *(43)*. During the spring of 1998, 1-h averages of atmospheric $H_2S$ levels were found to be consistently below 10 ppb at a control school, but between 11 and 59 ppb for 7 d for the outside air, and 5 d for the inside air at the exposed school. During the autumn of 1998, 1-h averages of atmospheric $H_2S$ levels were consistently below 10 ppb at the control school, but between 11 and 129 ppb for 9 d for the outside air, and 7 d for the inside air at the exposed school. The investigators stated: "No consistent association was found between exposure to low levels of hydrogen sulfide and any adverse health effects. It was concluded that the students attending the elementary school near the mushroom-composting operations were not exposed to any significant public health hazard" *(43)*.

More details about the chemical and physical properties and toxicology profile of $H_2S$ are discussed in Chapter 17.

## 3. PRODUCTION AND PHYSIOLOGICAL EFFECTS OF ENDOGENOUS GASES

Decades of environmental and occupational health studies describe NO, CO, and $H_2S$ as vicious toxicants that exert a detrimental influence only on human health. This conventional thinking has gradually lost ground. First is the evidence that NO is actually endogenously generated with profound biological and physiological effects. The endog-

enous production of CO, on the other hand, has been known for a long time. The re-evaluation and realization of the physiological importance of CO to the homeostatic control of the human body have been achieved only in the past 10 yr or so *(44)*. Like NO and CO, $H_2S$ at physiologically relevant levels affects structures and functions of the human body at the molecular, cellular, tissue, and system levels.

### *3.1. Nitric Oxide*

Application of nitrate-containing compounds, starting with nitroglycerin, for medicinal purposes can be traced back more than 150 yr. Less than two decades ago, the discovery that a simple gas, NO, was critical for endothelium-dependent vasorelaxation led to a revision of the doctrine about cell signal transduction *(45)*. The enzymatic synthesis of NO from L-arginine occurs in almost every type of cell, catalized by NO synthases. Many endogenous substances modulate the activities of NO synthases. The first discovered was a neurotransmitter, acetylcholine. Decomposition and biotransformation of NO in vivo have also been clearly demonstrated *(46)*. To capitalize on the discovery of endogenous NO, on October 12, 1998, Robert Furchgott, Louis Ignarro, and Ferid Murad were awarded a Nobel Prize in Medicine and Physiology for their discoveries concerning NO as a signaling molecule in the cardiovascular system. Today, the physiological importance of NO has been extended far beyond the cardiovascular system. NO has critical regulatory roles in physiological functions of many different types of cells, tissues, organs, and systems. Abnormal metabolism and/or functions of NO have also been described for pathogenic processes of many diseases. On the incomplete list of diseases involving NO are hypertension, diabetes, ischemia/reperfusion heart damage, cardiac attack, inflammation, stroke, erectile dysfunction, aging, menopause, hyperlipidemias, atherosclerosis, cancer, drug addiction, intestinal motility, memory and learning disorders, neuronal degenerating diseases, septic shock, sunburn, anorexia, tuberculosis, and obesity.

### *3.2. Carbon Monoxide*

In 1898, Saint-Martin and Nicloux gave the first indication of endogenous CO. In 1950, Sjöstrand provided experimental evidence for the endogenous production of CO *(47)*. The biological and physiological function of endogenous CO had been either unknown or ignored for the ensuing half-century. Although lipid peroxidation yields endogenous CO, breakdown of the α-methane bridge of heme is the major route for the endogenous production of CO. Three isoforms of microsomal heme oxygenases (HOs) are involved in the enzymatic CO production in vivo. For more details about endogenous CO production and regulation, refer to Chapter 10.

Endogenous CO plays an important role in long-term potentiation (LTP) as a retrograde messenger in the brain *(48,49)*. This role of CO is similar to that of NO but may be mediated by different mechanisms. One hypothesis is that NO induces LTP by stimulating NMDA receptors, whereas it induces CO by stimulating metabotropic glutamate receptors. The involvement of 5-HT(3) receptors in the induction of ganglionic LTP by CO has also been suggested.

CO released from the vascular wall modulates proliferation and apoptosis of smooth muscle cells as well as endothelial cells. Relaxation of various types of smooth muscles by CO has also been consistently shown. Endogenous cellular levels of CO vary under different pathophysiological conditions, contributing to different disorders. Readers are referred to two recently published books for more detailed descriptions of the different biological effects of CO under physiological and pathophysiological conditions *(50,51)*.

Regarding regulation of heme metabolism, the physiological importance of HO has long been recognized. In addition to the degradation of heme, HO catalyzes the production of CO as well as biliverdine and ferrous iron. However, CO had not been taken into account for its beneficial effects of HO until little more than a decade ago. The breakthrough discovery of NO opened the way to further research on membrane/receptor-independent signaling by gas molecules. In 1991, Marks and colleagues *(52)* hypothesized that CO might be another important endogenous gaseous molecule. This pioneering thinking stirred up the resurgence of CO as a physiological signaling molecule *(44)*.

As CO biology has bloomed in recent years, more and more enthusiasm has been injected into HO biology. Research on CO and HO is now closely interacted and coevolved. This HO/CO field is experiencing phenomenal growth, spurred on by scientists and health workers, from the laboratory bench to the hospital bedside and by trainees from graduate students to postdoctoral fellows.

### *3.3. Hydrogen Sulfide*

A significant amount of $H_2S$ is produced by mammalian cells, and this substance has been measured in both circulatory blood and in isolated tissues and cells *(53)*. Two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase [CBS] (EC 4.2.1.22) and cystathionine γ-lyase [CSE] (EC 4.4.1.1), are responsible for the majority of the endogenous production of $H_2S$ in mammalian tissues, which use L-cysteine as the main substrate *(53)*. Ammonium and pyruvate are two other end products, in addition to $H_2S$, of CBS- and/or CSE-catalyzed cysteine metabolism. $H_2S$ is also produced endogenously through the nonenzymatic reduction of elemental sulfur using reducing equivalents obtained from the oxidation of glucose *(53)*.

The elimination of $H_2S$ from the body takes place mainly in the kidney. Mechanisms for biotransformation and scavenging of $H_2S$ in vivo include oxidation in mitochondria, methylation in cytosol, and scavenging by methemoglobin or metallo- or disulfide-containing molecules such as oxidized glutathione. The appendix to this chapter gives detailed descriptions of the metabolism of $H_2S$ *(53)*.

Similar to the story of CO, in which HO captured all of the glories initially, $H_2S$ has lived for a long time in the shadow of $H_2S$-generating enzymes. These enzymes initially were characterized in the liver and kidney *(54,55)*. The physiological processes modulated by these enzymes were also elucidated in the liver and kidney, but the role played by $H_2S$ was not studied further. Even homocysteine, a precursor of $H_2S$ that is catabolized by the same $H_2S$-generating enzymes, received more attention from the perspective of atherosclerosis.

Recent studies have contributed significantly to our understanding of the physiological roles of $H_2S$ in the nervous and cardiovascular systems. At physiologically relevant concentrations, $H_2S$ reduced KCl-stimulated releases of the corticotropin-releasing hormone *(56)*. NaHS, a donor of $H_2S$, induced a concentration-dependent (27–200 μ*M*) hyperpolarization and reduced input resistance of CA1 neurons or dorsal raphe neurons *(34)*. This concentration range is physiologically relevant in the brain *(57)*. Changes in $K^+$ conductance were identified to be the main ionic basis for these effects of NaHS, and $K_{ATP}$ channels in neurons were speculated as the specific targets.

*N*-methyl-D-aspartate (NMDA) receptors are another target of $H_2S$. In the presence of a weak tetanic stimulation, NaHS at 10–130 μ*M* facilitated the induction of hippocampal long-term potentiation in rat hippocampal slices by enhancing the NMDA-induced inward current *(57)*. Activation of the cyclic adenosine monophosphate-dependent protein kinase pathway likely mediates the interaction of $H_2S$ and NMDA receptors *(58)*.

In the cardiovascular system, $H_2S$ has been demonstrated at physiologically relevant concentrations to relax vascular tissues by opening $K_{ATP}$ channels in vascular smooth muscle cells (VSMCs) *(59,60)*. In this case, NO serves as a trigger to increase $H_2S$ production and release *(59)*. Evidence has also been presented for the relaxant effects of NaHS on rabbit isolated ileum, rat vas deferens, and guinea pig isolated ileum at physiologically relevant concentrations *(61)*. Inhibition of the $H_2S$-generating enzyme CSE caused a slowly developing increase in the contraction of the guinea pig ileum as a result of field stimulation *(61)*.

## 4. GASOTRANSMITTERS IN EVOLUTION

**Table 1** lists organized activities for promoting research on and advancing our understanding of gasotransmitters. A 2-yr span saw the birth of a scientific society, a scientific journal, and the first scientific conference specifically devoted to NO (1996–1998). Since then, NO biology and chemistry have been the subject of many international meetings. Following the first world Internet meeting on cardiovascular effects of CO in 1998, two HO/CO conferences were held in 2000 and 2002 and another HO conference in 2003. **Table 2** lists selective monographs and books on the different types of gasotransmitters. Most of these books are on NO, and two are related to endogenous CO.

While this book was being edited, the *Antioxidants and Redox Signaling* journal published a special forum issue entitled "Gaseous Signal Transducers," discussing the biological roles of NO, CO, and $H_2S$. Another cheering development was the creation of the first strategic training program for gasotransmitter research in 2003, entitled "Gasotransmitter REsearch And Training" (GREAT). More than 15 researchers from four Canadian universities participated in this 6-yr program, supported by the Canadian Institutes of Health Research. The GREAT program will provide trail-breaking interdisciplinary and transdisciplinary training for local and international students, postdoctoral fellows, and researchers on sabbatical. The training program will be delivered through an array of courses; a trainee exchange program; laboratory, clinical, and community health research; and training-mentoring initiatives. A compulsory component of the GREAT program is a three-credit course, "Gasotransmitter Biology and Medicine." Another course offered through this program is "Career Development Essentials for Gasotransmitter Trainees."

Determination of endogenous levels of NO, CO, and $H_2S$; identification of the enzymes responsible for the production of these gases; and, most important, elucidation of the physiological functions of these gaseous molecules pave the way for the development of a general concept to envelop all these gases into one family. As can be seen from the aforementioned organized activities, one can only conclude that the era of gasotransmitters is coming and "the medium is the message" (Marshall McLuhan).

## 5. GASOTRANSMITTERS: DEFINITION OF THE CONCEPT

Vehicles for intercellular communication are either electrical signals via gap junction or chemical substances. The latter category is composed of hormones, autocoids, and transmitters. Hormones are released from endocrine cells into the bloodstream. The concentration of hormones is diluted to a relatively stable level when they reach distant multiple organs and cells. This endocrine mode of action is distinctive from the paracrine action of transmitters, in which transmitters, once released, usually act on adjacent postsynaptic cells. A definition of autocoids is not strictly precise. In general, autocoids (such as prostaglandins, adenosine, and platelet-activating factor) act on the same cells from which they are produced. Similar to the effects of hormones and transmitters,

Table 1
Chronicle of Organized Activities Related to Evolution of Gasotransmitter Biology and Medicine

| *Event* | *Location* | *Year* | *Reference* |
|---|---|---|---|
| Founding of Nitric Oxide Society | | 1996 | http://darwin.apnet.com/no/ |
| Founding of *Journal of Nitric Oxide: Biology and Chemistry* | | 1997 | |
| First official conference of Nitric Oxide Society: Biochemistry and Molecular Biology of Nitric Oxide[a] | Los Angeles, CA | 1998 | |
| Discovery of NO as signaling molecule in cardiovascular system and awarding of Nobel Prize in Medicine and Physiology to Robert Furchgott, Louis Ignarro, and Ferid Murad | | 1998 | www.nobel.se/medicine/laureates/1998/index.html |
| Invited Symposium of "Carbon Monoxide and Cardiovascular Function" | Internet World Congress '98, INABIS '98 | 1998 | www.mcmaster.ca/inabis98/toc.html |
| Sixth International Meeting on the Biology of Nitric Oxide | Stockholm, Sweden | 1999 | www.ki.se/org/nitric-oxide-99/ |
| The 1st International Conference on Heme Oxygenase (HO/CO) | New York, NY | 2000 | |
| The 2nd International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide | Prague, Czech Republic | 2002 | |
| The 2nd International Conference on Heme Oxygenase (HO/CO) and Cellular Stress Response | Catania, Italy | 2002 | |
| Initiation of 6-yr GREAT program | University of Saskatchewan; Queen's University; University of Calgary; University of Montreal, Canada | 2003 | |
| Conference on HO regulation, functions, and clinical applications | Uppsala, Sweden | 2003 | |

[a] This is the third in a series of conferences on biochemistry and molecular biology of NO.

cognate membrane receptors are still essential for the biological effect of autocoids. Some endocrine hormones, such as melatonin, can also act as autocoids *(62)*. In conventional signal transduction processes, the binding of neurotransmitters, certain endocrine hormones, or autocoids to receptors located on the plasma membrane is the essential triggering event. The ligand-receptor interaction generates intracellular second messengers that relay and direct the extracellular signals to different intracellular destinations, resulting in modulated cellular activity.

A neurotransmitter is a chemical substance that is released from a neuron either by exocytosis or directly from cytoplasm. It binds to specific receptors in the postsynaptic cell membrane and affects the function of postsynaptic cell(s). In some circumstances, neurotransmitters also act on "autoreceptors" located on presynaptic membranes to regulate the progress of synaptic transmission. Since the discovery of acetylcholine release from vagus terminals in frog hearts by Otto Loewi and Henry Dale while studying cholinergic and adrenergic systems in the early 1930s, the neurotransmitter concept has evolved and been constantly redefined.

Generally, a neurotransmitter is gauged against the following criteria:

1. It is synthesized in the neuron.
2. It is present in the presynaptic terminal and is released in amounts sufficient to exert a defined action on the postsynaptic neuron or effector organ.
3. When administered exogenously (as a drug) in reasonable concentrations, it mimics the action of the endogenously released transmitter exactly (for example, it activates the same ion channels or second messengers pathway in the post-synaptic cell).
4. A specific mechanism exists for removing it from its site of action (the synaptic cleft) *(63)*.

Acetylcholine, catecholamines, serotonin, histamine, glutamate, glycine, γ-aminobutyric acid, and adenosine triphosphate or its metabolites are among a handful of the identified low-molecular-weight neurotransmitters.

NO, CO, and $H_2S$ are distinctive from classic neurotransmitters and humoral factors while sharing common characteristics among themselves (**Table 3**). These endogenous gaseous transmitters have been defined as *gasotransmitters*, measured by the following criteria *(53)*:

1. They are small molecules of gas.
2. They are freely permeable to membranes. As such, their effects do not rely on the cognate membrane receptors. They can have endocrine, paracrine, and autocrine effects. In their endocrine mode of action, for example, gasotransmitters can enter the bloodstream, be carried to remote targets by scavengers and released there, and modulate functions of remote target cells.
3. They are endogenously and enzymatically generated and their production is regulated.
4. They have well-defined and specific functions at physiologically relevant concentrations. Thus, manipulating the endogenous levels of these gases evokes specific physiological changes.
5. Their functions can be mimicked by their exogenously applied counterparts.
6. Their cellular effects may or may not be mediated by second messengers but should have specific cellular and molecular targets.

The gasotransmitter family may consist of many as-yet-unknown endogenous gaseous molecules, such as $NH_3$ and acetaldehyde. It is also worth noting that the effects of gasotransmitters may not always be beneficial. Under certain circumstances or in specific cellular environments, some gasotransmitters may inhibit physiological cellular function.

Table 2
Selective Monographs and Books on Different Gasotransmitters[a]

| *Authors/editors* | *Title and publisher* | *Year* |
|---|---|---|
| Topics on NO | | |
| S. Moncada, M. A. Marletta, J. B. Hibbs Jr., E. A. Higgs | *The Biology of Nitric Oxide: Part 1—Physiological and Clinical Aspects.* California Princeton Fulfillment | 1992 |
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[a] Not including books and monographs on atmospheric gases or toxicology and environmental concerns.

Table 3
Comparison of the Action Modes of Neurotransmitters and Gasotransmitters

| | *Release* | *Re-uptake* | *Removal mechanism* | *Revert direction* | *Membrane receptors* |
|---|---|---|---|---|---|
| Neurotransmitters | Exocytotic vesicle | Yes | Enzyme dependent | Pre- to postsynaptic membrane (one direction) | Necessary |
| Gasotransmitters | Cytoplasm release | No | Nonenzymatic: oxidation, scavenging, methylation, etc. | Bidirectional | Not necessary |

In this scenario, these gasotransmitters under physiological conditions would be maintained at low levels, thereby ensuring homeostasis of specific organs or cells. Significant differences among gasotransmitters exist regarding their mechanisms of production and function. For example, the effects of NO and $H_2S$, but not CO, may involve the production of free radicals. The biological outcome of the activation of NO synthase can be easily explained by its end product of NO, but upregulation of HO may alter cellular functions via its end products of iron and biliverdin, other than CO, in some cases. Differences in chemical and physical properties, cellular production levels, signal transduction pathways involved, and so on for various gasotransmitters are discussed in detail in other chapters of this book.

The birth of the gasotransmitter concept—with NO, CO, and $H_2S$ its current stars—is just the first exploratory step on an unknown path. Much more work remains to be done. The physiological roles and importance of CO and $H_2S$ still need to be vigorously tested. More gasotransmitters may be discovered and identified in the future. The interaction among gasotransmitters should be investigated. Physiological levels, both circulatory and cellular, of gasotransmitters as well as molecular switches to turn on or off the production of gasotransmitters should be determined. Pathological actions of gasotransmitters should be assessed. Before all these concerns and challenges can be addressed, answered, and articulated, the jury is still out on the case of gasotransmitters *(64)*.

The concept of gasotransmitters initially was framed in a *FASEB Journal* article by Wang *(53)*, which is provided as an appendix at the end of this chapter.

## 6. GASOTRANSMITTERS AND ION CHANNELS

Gasotransmitters are freely permeable to biological membranes and very likely interact with ion channels in the plasma membrane and intracellular organelle membranes. The interaction of gasotransmitters with ion channels is the focus of discussion in this book for the following considerations: First, direct modification of ion channels by gasotransmitters, independent of conventional second messengers, has been demonstrated in many cases for NO, CO, and $H_2S$. Key discoveries in this regard are summarized in **Table 4**. This direct modulation of ion channels by gasotransmitters represents a novel class of signal transduction mechanism. Conventional dogma argues that membrane ion channels can only be modified by endogenous substances via membrane receptor–related second-messenger systems. Second, the structure and function of ion

Table 4
Key Discoveries on Direct Interaction of Gasotransmitters with Ion Channels

| *Authors* | *Discovery* | *Year* | *References* |
|---|---|---|---|
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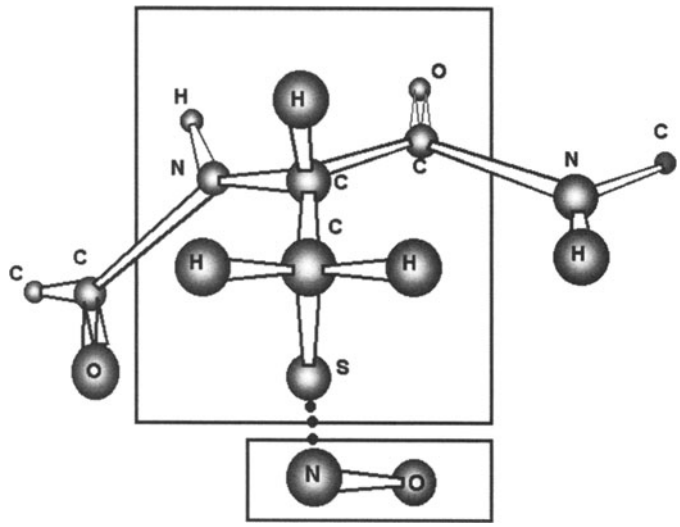


Fig. 1. Modification of ion channel proteins by NO through a *S*-nitrosylation mechanism.

channels on cell membranes affect general as well as many specific cellular functions. Third, by conducting specific ions, ion channels themselves serve as important signal transduction links. Fourth, the complexity of ion channel families is directly coupled to diverse biological functions. Finally, the modulation and mobilization of classic second messengers by gasotransmitters has been the topic of numerous peer-reviewed articles, monographs, and books. The direct interaction of gasotransmitters with membrane ion channels has not previously been systematically described and encapsulated.

Three specific modes of direct interaction of gasotransmitters with membrane ion channels are discussed in this book. NO covalently modifies free cysteine residues in proteins via *S*-nitrosylation *(65)*. The *S*-nitrosylation of ion channel proteins by NO would directly change the function of these channels (**Fig. 1**). This mechanism is specifically discussed in Part II of this book. Direct interaction of gasotransmitters with ion channel proteins also applies in the case of CO. Many reported effects of CO on $K^+$ channels are not regulated by known second messengers. Chemical modification of histidine residues of $K^+$ channel proteins by CO via the formation of hydrogen bond (**Fig. 2**), a process of carboxylation, has been indicated *(66–68)*. Chapters 12 and 13 of this book give detailed descriptions of the ion channel carboxylation. Direct modulation of $K_{ATP}$ channels by $H_2S$ is recently reported, which is not mediated by cyclic guanosine *S'*-monophosphate or other known second messengers *(59)*. A chemical interaction of $H_2S$ with sulfhydryl groups of ion channel proteins is, as such, hypothesized. The formation of adduct of $HS^-$ with free sulfhydryl group, a sulfuration mechanism, and the breakdown of disulfide bonds, a reducing mechanism, by $H_2S$ are alternative molecular mechanisms, which are further discussed in Chapter 21.

Notwithstanding the focus on the direct interaction of gasotransmitters on ion channel proteins, this book also gives a balanced view to include the effect of gasotransmitters on ion channels mediated by different second messengers.

## 7. PERSPECTIVES ON GASOTRANSMITTER RESEARCH

Gasotransmitters are recent discoveries emanating from both the laboratory and clinical research ends of the health research spectrum. There are already more than 97,000

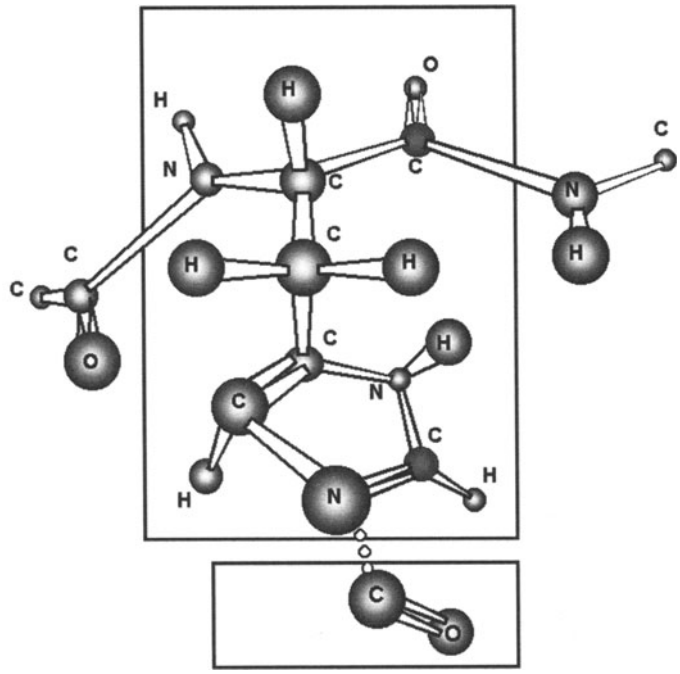


Fig. 2. Modification of ion channel proteins by CO through a carboxylation mechanism.

articles incorporating the terms NO, CO, or $H_2S$. Numerous laboratories worldwide are studying these gasotransmitters. It seems probable, if not certain, that new members of the gasotransmitter family will come to light in a few years. Chapter 22 discusses many other candidates for potential gasotransmitters.

### *7.1. Growth of Gasotransmitter Research*

By March 2004, Medline searchers found approx 60,000 articles incorporating the term *nitric oxide*, with some 17,000 using the term *carbon monoxide* and more than 21,000 using the term *hydrogen sulfide*. Not only is the information base in this area exploding, but some researchers expect the roster of proven gasotransmitters to grow dramatically in the future, citing such biomolecules as formaldehyde ($CH_2O$), ethylene ($CH_2CH_2$), and ammonia ($NH_3$) as potential new members in this class. As new gasotransmitter molecules appear on the scene, membership in the gasotransmitter family will be enlarged and updated by incorporating these substances. This new and challenging field of gasotransmitter medicine encompasses biomedical, clinical, health services, and population health studies.

### *7.2. Link Between Gasotransmitters and Human Diseases*

Numerous human diseases are linked to abnormal metabolism and functions of gasotransmitters. This knowledge will significantly affect the pathogenesis, diagnosis, therapeutics, and prevention strategies for gasotransmitter-related diseases. Therefore, gasotransmitter research is as important for clinical researchers and practitioners as it is for basic researchers—if not more so. **Table 5** summarizes Medline search results reflecting links between gasotransmitters and circulatory and respiratory diseases.

Table 5
Relevant Medline-Indexed Disease-Related Publications
(Dated to March 15, 2004)

| | *NO* | *CO* | *$H_2S$* |
|---|---|---|---|
| Stroke | 821 | 106 | 12 |
| Hypertension | 5065 | 214 | 73 |
| Transplantation | 1321 | 153 | 115 |
| Atherosclerosis | 1840 | 171 | 110 |
| Ischemia and reperfusion | 1919 | 66 | 8 |
| Heart failure | 1087 | 105 | 14 |
| Asthma | 825 | 221 | 29 |
| Chronic obstructive pulmonary disease | 98 | 222 | 5 |

As research progresses, more implications of the role gasotransmitter molecules play in human health are emerging. Elucidation of the roles of NO, CO, and $H_2S$ in the mechanisms of specific human diseases will enable future discovery, development, and clinical use of innovative therapeutic interventions *(69)*. For instance, Medline search reveals about 2000 publications on hyperhomocysteinemia *(70,71)*. This clinical problem is now known to be related to the metabolism of homocysteine, an endogenous precursor of $H_2S$. A better understanding of the metabolism of $H_2S$ will greatly illuminate clinical practice for many hyperhomocysteinemia-related cardiovascular diseases.

Another example is the application of inhaled gasotransmitters to treat human diseases, including the understanding of the technology to administer gasotransmitters, their action mechanisms, and their indication for the treatment of different pathologies. These gasotransmitters can be administered alone or in combination. Human clinical trials have been conducted to determine the role of inhaled NO in the treatment of severe acute respiratory distress syndrome in adults and in the treatment of pulmonary hypertension during surgery *(72)*. Inhaled NO decreases the pulmonary inflammation induced by the extracorporeal circulation in swine *(73)*. The inhaled NO also has major extrapulmonary effects particularly on renal function, preventing the detrimental renal effects of cyclooxygenase inhibitors.

The application of gene therapy to protect the heart from ischemia/reperfusion damage by the overexpression of HO has proven to be effective *(74)*. Intramyocardial delivery of the human HO-1 gene by the adenoassociated virus protected the heart from reperfusion injury. An upregulated HO/CO system inhibited cardiac anaphylaxis *(75)* and lowered blood pressure in young spontaneously hypertensive rats *(76)*. As the link between HO/CO and heart transplants becomes increasingly known *(77,78)*, it will be important for clinicians to learn about the analysis and regulation of HO/CO level to perform successful heart transplantation to protect the heart from ischemia. In this case, gasotransmitter research at the laboratory level will elucidate the mechanisms for HO/CO protection. Clinical research will bring a new paradigm into organ and tissue transplant technology. Community and population health research will provide specific populations with abnormal CO metabolism more knowledge regarding their cardiac health, stress management, and prevention of cardiovascular diseases.

### *7.3. Triad Frame of Transdisciplinary Gasotransmitter Research*

The clinical relevance of gasotransmitter research has been delineated. Linkages between gasotransmitter research and community health can be viewed from at least two

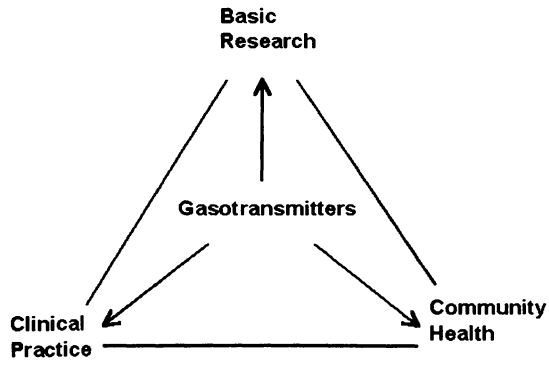


Fig. 3. Bridging and branching of gasotransmitter research.

angles (**Fig. 3**): (a) several community health issues are related to diseases caused by abnormal metabolism and functions of gasotransmitter molecules; and (b) the long-recognized health hazards of these gases, at higher ambient and pollution levels, pose environmental and community health questions, which may have relevance to or share characteristics with their endogenous levels. High concentrations of CO, NO, $NO_2$ *(5)*, and $H_2S$ *(34,79)* are especially hazardous for people working or living in specific environments and communities. Levels of NO and $NO_2$ are constantly increasing in the urban community atmosphere, particularly in areas close to automobile traffic and airports *(13)*. Exposure to high concentrations of these gases may present a specific health concern for the population's health *(5)*. Gasotransmitter research will arm community health researchers and workers with a better knowledge of and expertise in the metabolism of these gases at toxic levels in our bodies, their specific cellular targets, toxicological mechanisms, and specific detoxification maneuvers.

### *7.4. Future Directions*

More organized activities for promoting research on gasotransmitters are expected. Capacity building by recruiting new researchers into the field of gasotransmitters and training more highly qualified personnel is becoming a priority in research agendas throughout the globe.

## ACKNOWLEDGMENTS

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## APPENDIX

# Two's company, three's a crowd: can $H_2S$ be the third endogenous gaseous transmitter?

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ABSTRACT **Bearing the public image of a deadly "gas of rotten eggs," hydrogen sulfide ($H_2S$) can be generated in many types of mammalian cells. Functionally, $H_2S$ has been implicated in the induction of hippocampal long-term potentiation, brain development, and blood pressure regulation. By acting specifically on $K_{ATP}$ channels, $H_2S$ can hyperpolarize cell membranes, relax smooth muscle cells, or decrease neuronal excitability. The endogenous metabolism and physiological functions of $H_2S$ position this gas well in the novel family of endogenous gaseous transmitters, termed "gasotransmitters." It is hypothesized that $H_2S$ is the third endogenous signaling gasotransmitter, besides nitric oxide and carbon monoxide. This positioning of $H_2S$ will open an exciting field—$H_2S$ physiology—encompassing realization of the interaction of $H_2S$ and other gasotransmitters, sulfurating modification of proteins, and the functional role of $H_2S$ in multiple systems. It may shed light on the pathogenesis of many diseases related to the abnormal metabolism of $H_2S$.—Wang, R. Two's company, three's a crowd: can $H_2S$ be the third endogenous gaseous transmitter? *FASEB J.* 16, 1792–1798 (2002)**

*Key Words: carbon monoxide · cardiovascular system · gasotransmitter · neuron · nitric oxide*

THE CELLULAR SIGNALING process is usually initiated by the binding of neurotransmitters or humoral factors to receptors located on the plasma membrane. The ligand–receptor interaction generates intracellular second messengers that relay and direct the extracellular signals to different intracellular destinations, resulting in modulated cellular activity. The discovery of nitric oxide (NO) elucidates more than just the nature of the endothelium-derived relaxing factor (1). It presents a membrane receptor-independent signaling mechanism, emphasizing the necessity to modify the conventional doctrine about cellular signal transduction. The subsequent resurgence of carbon monoxide (CO) as another important endogenous signaling gas is embraced by researchers in almost every field of life sciences (2). To distinguish NO and CO from the classical neurotransmitters and humoral factors while acknowledging the common nature of these two gases, an effort has been made to classify these endogenous gaseous transmitters against several criteria (**Table 1**). I would recommend designating these gaseous transmitters as gasotransmitters. NO and CO are the first two identified gasotransmitters. In this hypothesis study, arguments are made to entitle hydrogen sulfide ($H_2S$) as the third gasotransmitter. Important implications of this identification are explained.

### Physical and chemical properties of $H_2S$

$H_2S$ is a colorless gas with a strong odor of rotten eggs. The detectable level of this gas by the human nose is at a concentration 400-fold lower than the toxic level. Oxidation of $H_2S$ yields elemental sulfur, sulfur oxide ($SO_2$), and sulfates such as sulfuric acid. $H_2S$ can be hydrolyzed to hydrosulfide and sulfide ions in the following sequential reactions: $H_2S \Leftrightarrow H^+ + HS^- \Leftrightarrow 2H^+ + S^{2-}$. Even in an aqueous solution, about one-third of $H_2S$ remains undissociated at pH 7.4. $H_2S$ is permeable to plasma membranes as its solubility in lipophilic solvents is ~ fivefold greater than in water.

### Endogenous generation and metabolism of $H_2S$

The biological production and utilization of $H_2S$ have been best known for certain bacteria and archae (3). A sobering fact is that mammalian cells also produce $H_2S$. The $H_2S$ concentration of rat serum is ~ 46 μM (4). Aside from circulating $H_2S$, a significant amount of $H_2S$ is produced in various tissues. For instance, the physiological concentration of $H_2S$ in brain tissue has been reported to be 50–160 μM (5, 6). Recent studies have shown that vascular tissues generate measurable amounts of $H_2S$ (4, 5).

Two pyridoxal-5′-phosphate-dependent enzymes—cystathionine β-synthase or CBS (EC 4.2.1.22) and cystathionine γ-lyase or CSE (EC 4.4.1.1)—are responsible for the majority of the endogenous production of $H_2S$ in mammalian tissues that use L-cysteine as the main substrate (7–9). In some tissues CBS and CSE are both needed for generation of $H_2S$, whereas in others one enzyme suffices (**Fig. 1**). Thus, it comes as no surprise that the expression of CBS and/or CSE is tissue specific. The expression of CBS (5, 10) and CSE (11–14) has been identified in many human and other mammalian cells, including those from liver, kidney,

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TABLE 1. *Classification of gasotransmitters (gaseous transmitters)*

(1) They are small molecules of gas, like nitric oxide (NO) and carbon monoxide (CO).
(2) They are freely permeable to membrane. As such, their effects will not rely on cognate membrane receptors.
(3) They are endogenously and enzymatically generated and their generation is regulated.
(4) They have well-defined specific functions at physiologically relevant concentrations. For instance, NO and CO both participate in vasorelaxation and synaptic transmission in the central nervous system.
(5) Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets. For instance, NO and CO activate $K_{Ca}$ channels in plasma membrane either directly or mediated by the cGMP pathway.

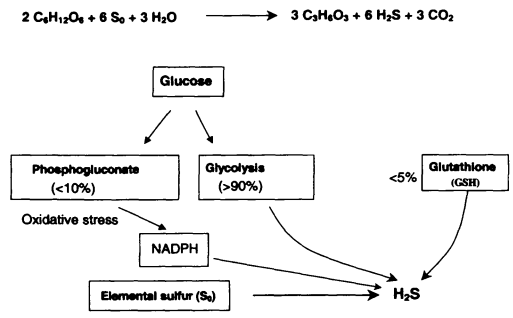


**Figure 2.** Endogenous nonenzymatic production of $H_2S$.

brain, skin fibroblasts, and blood lymphocytes. As the end product of CBS- and CSE-catalyzed cysteine metabolism, $H_2S$ exerts a negative feedback effect on the activity of these enzymes. Elevated $H_2S$ level inhibited CSE activity (15) and the rate of gluconeogenesis from cysteine (16). Another less important endogenous source of $H_2S$ is the nonenzymatic reduction of elemental sulfur to $H_2S$ using reducing equivalents obtained from the oxidation of glucose (17) (**Fig. 2**). All essential components of this nonenzymatic pathway are present in vivo, including the supply of reducible sulfur. The presence of millimolar concentration of sulfur in blood circulation has been reported in humans (18) or mice (19).

$H_2S$ in vivo is metabolized by oxidation in mitochondria or by methylation in cytosol (Fig. 1). $H_2S$ can be scavenged by methemoglobin (20) or metallo- or disulfide-containing molecules such as oxidized glutathione (21). $H_2S$ is excreted mainly by the kidney as free or conjugated sulfate (20). The interaction of hemoglobin and $H_2S$ calls for special attention. Hemoglobin may be the common "sink" for CO in forming scarlet carboxyhemoglobin (22), for NO in forming nitrosyl hemoglobin, and for $H_2S$ in forming green sulfhemoglobin (23). If this sink is filled with one gas, the binding of other gases would be affected and their individual availability to act on targeted cells would be altered. A

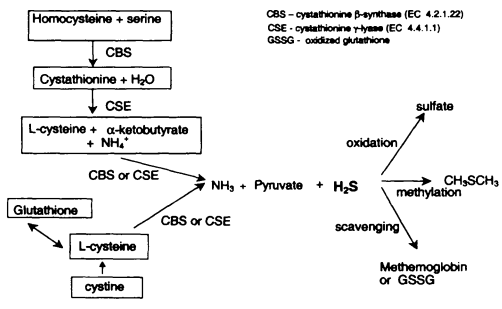


**Figure 1.** Endogenous enzymatic production and metabolism of $H_2S$.

case in point is the observation that after pretreatment of human erythrocytes with CO to saturate the hemoglobin sink, the accumulated amount of endogenous $H_2S$ was significantly enhanced (17).

## Physiological effects of $H_2S$ and the underlying mechanisms

The physiological functions of endogenous $H_2S$ may be multifaceted. In liver and kidney, activities of the $H_2S$-generating enzymes have been studied in great detail (8, 9, 24, 25). To be succinct, a discussion of this study focuses on the physiological role of $H_2S$ in nervous and cardiovascular systems.

## Physiological effects of $H_2S$ on the nervous system

The first and most important evidence for the physiological role of $H_2S$ was obtained in 1989 when endogenous sulfide levels in rat brain tissues (1.6 μg/g) (26) and in normal human postmortem brainstem (0.7 μg/g) were reported (26, 27). Endogenous sulfide level in mice brain (28) was similar to that of rats, but threefold lower than that of bovine cerebral cortex (29). The study by Awata et al. in 1995 (30) provided the enzymatic mechanisms for this endogenous $H_2S$ in rat brain, in which activities of CBS and CSE in six different brain regions were detected though the activity of CBS was > 30-fold greater than that of CSE. Brain activities of CBS and CSE gradually increased after birth and reached adult level at 2–4 wk. The transcriptional expression of CBS in rat brain (hippocampus, cerebellum, cerebral cortex, and brainstem) was later confirmed using Northern blot analysis but no CSE mRNA was detected (6). The reduced $H_2S$ production after the inhibition of CBS further pinpointed CBS to be the major endogenous enzyme for $H_2S$ production in brain (6).

The functional role of $H_2S$ at physiologically relevant concentrations in brain was gradually uncovered in early 1990s. Chronic exposure of neonatal rats to $H_2S$ altered the release of neurotransmitters in brain with increased serotonin and norepinephrine levels in rat

cerebellum and frontal cortex (31, 32). Application of NaHS, which generates $H_2S$ once in solution, to rat hypothalamic explants in vitro did not affect the basal secretion of corticotropin-releasing hormone (CRH), but consistently reduced KCl-stimulated CRH release from the explants (33). This effect of exogenous $H_2S$ was consistent with the observation that the intramuscular application of S-adenosyl-L-methionine, an endogenous precursor of $H_2S$, to conscious rats reduced the hypothermia-induced increase in serum level of corticosterone (33).

Voltage-dependent and TTX-sensitive $Na^+$ channels may be targeted by $H_2S$ in neurons. In cultured neuroblastoma cells, NaHS or taurine alone did not alter $Na^+$ channel currents. After pretreatment of these cells with NaHS, taurine dramatically inhibited $Na^+$ channels in a reversible fashion (34). This effect of NaHS was mimicked by disulfide-reducing agents dithiothreitol and β-mercaptoethanol. A reduction of disulfide bonds between $Na^+$ channel subunits by $H_2S$ was thus suggested. Since taurine is an inhibitory neurotransmitter and a short exposure to NaHS (<2 min) resulted in a twofold increase in taurine levels in brainstem (35), the interaction between NaHS and taurine suggests that certain neuronal effects of $H_2S$ could be mediated by the alteration in taurine levels. However, the physiological importance of this study is limited since the concentration used for NaHS (10 mM) was far outside the physiological range.

NaHS induced a concentration-dependent (27–200 μM) hyperpolarization and reduced input resistance of CA1 neurons or dorsal raphe neurons (36). This concentration range is physiologically relevant in the brain (6). Changes in $K^+$ conductance were identified to be the main ionic basis for these effects, since the presence of extracellular barium or intracellular cesium abolished the NaHS-induced membrane hyperpolarization. NaHS-induced neuronal hyperpolarization was blocked by a high concentration of TEA (50 mM) but not by a low concentration of TEA (10 mM) or 4-aminopyridine (1 mM). Thus, the involvement of either calcium-activated $K^+$ channels or voltage-dependent $K^+$ channels in NaHS effect was not supported. Activation of ATP-sensitive $K^+$ ($K_{ATP}$) channels by NaHS was proposed in these experiments as the consequence of ATP depletion due to the inhibition by sulfide of the oxidative phosphorylation (36). This hypothesis was not without ambiguity, since in the same experiments manipulation of intracellular ATP concentrations did not affect the NaHS-induced membrane hyperpolarization and no $K_{ATP}$ channel currents were directly examined. Electrophysiological measurement of $K^+$ channel currents in neurons with tight control of intracellular ATP levels in the presence of NaHS/$H_2S$ would help clarify the interaction of $H_2S$ and neuronal $K_{ATP}$ channels.

In addition to $K_{ATP}$ channels, NMDA receptors may be the target of $H_2S$. In the presence of a weak tetanic stimulation, NaHS at 10–130 μM facilitated the induction of hippocampal long-term potentiation (LTP) in rat hippocampal slices by enhancing the NMDA-induced inward current (6). Interaction of $H_2S$ and NMDA receptors was possibly mediated by the activation of a cAMP-dependent protein kinase pathway. NaHS (1–100 μM) increased cAMP production in primarily cultured rat cerebral and cerebellar neurons or in selected rat brain neuronal and glial cell lines (37). By enhancing the production of cAMP, NaHS increased the sensitivity to NMDA stimulation of NMDA receptors expressed in oocytes (37).

### Physiological effects of $H_2S$ on the cardiovascular system

It has been a conventional view that $H_2S$ interferes with cardiovascular function as a result of the secondary anoxia rather than a direct action of the gas on cardiac myocytes or vascular smooth muscle cells (SMCs) (36). However, this doctrine has started to become shaky in light of two aspects of development. The location of the $H_2S$-generating enzymes as well as the detection of endogenous levels of $H_2S$ in cardiovascular system provides the endogenous sources of $H_2S$. In-depth study of the whole animal and at tissue and cellular levels defines the functional role of $H_2S$ in the cardiovascular system.

Chen et al. (38) found no activity or expression of CBS in human atrium and ventricle tissues. The activity and/or expression of CBS were also lacking in human internal mammary arteries, saphenous veins, coronary arteries, or aortic arteries (38, 39). Thus, CBS does not appear to play a major role in generating $H_2S$ in cardiovascular tissues under physiological conditions. On the other hand, expression of CSE and the endogenous production of $H_2S$ have been shown in rat portal vein and thoracic aorta (5). In rat mesenteric artery and other vascular tissues, CSE is the only $H_2S$-generating enzyme that has been identified, cloned, and sequenced (4). mRNA of this enzyme was expressed solely in vascular SMCs as detected by RT-PCR and in situ hybridization (4). No transcript of CSE was found in the endothelium layers of intact vascular tissues or cultured endothelial cells (4). Expression levels of CSE mRNA varied in different types of vascular tissues, with an intensity rank of pulmonary artery > aorta > tail artery > mesenteric artery (4). Endogenous production of $H_2S$ depends on the types of vascular tissues. For instance, the homogenates of thoracic aortas yielded more $H_2S$ than that of portal vein of rats (5).

The physiological function of $H_2S$ in the cardiovascular system has been studied recently. An intravenous bolus injection of $H_2S$ transiently decreased blood pressure of rats by 12–30 mmHg, an effect mimicked by pinacidil (a $K_{ATP}$ channel opener) and antagonized by glibenclamide (a $K_{ATP}$ channel blocker) (4). At the tissue level, $H_2S$ at physiologically relevant concentrations ($IC_{50}$, 125 μM) induced in vitro relaxation of aorta and portal vein of rats (4, 5). Whether this vasorelaxant effect was due to a direct action of $H_2S$ on vascular SMCs has been questioned. Zhao et al. (4) showed that the $H_2S$-induced relaxation of rat aortic tissues was due mainly to a direct interaction of $H_2S$ and SMCs, based on the failure of denervation of vascular

tissues in vitro to alter $H_2S$ effects and on the observation that $H_2S$ still significantly relaxed vascular tissues after endothelium removal. Zhao et al. (4) showed that a small portion of the $H_2S$-induced vasorelaxation was attenuated by either removal of the endothelium or the application of L-NAME (an inhibitor of NO synthase) in the presence of the endothelium. This endothelium-dependent effect of $H_2S$ could be explained by the release of endothelium-derived vasorelaxant factors in response to $H_2S$ stimulation. The presence of an intact endothelium might serve as a buffer to retain $H_2S$ in the blood vessel wall so that its vasorelaxant effect can be potentiated and prolonged. Another interesting observation was that the coapplication of apamin and charybdotoxin, a protocol to block the effect of endothelium-derived hyperpolarizing factor (EDHF) (40), to the endothelium-intact rat aortic tissues reduced the vasorelaxant effect of $H_2S$. It seems that $H_2S$ might release EDHF from vascular endothelium. It should be borne in mind that endothelium dependency of the vascular effects of $H_2S$ has been controversial. One study concluded that the vasorelaxant effect of $H_2S$ was independent of endothelium, even though no experimental data were shown to support this conclusion (5).

Mechanisms for the direct effect of $H_2S$ on vascular SMCs have been explored. Unlike NO or CO, $H_2S$ relaxed vascular tissues independent of the activation of cGMP pathway. Whereas the vasorelaxation induced by NO was virtually abolished by ODQ, a specific inhibitor of soluble guanylyl cyclase, the $H_2S$-induced vasorelaxation was not inhibited by ODQ (4). In fact, ODQ even potentiated the vasorelaxant effect of $H_2S$. The synergistic actions of $H_2S$ and ODQ cannot be fully understood yet. Hypothetically, the interaction between ODQ and $H_2S$ may have generated vasorelaxant free radicals, which further relaxed vascular tissues.

The most recent significant advance in our understanding of the vascular effects of $H_2S$ was the identification of $K_{ATP}$ channels in vascular SMCs as the target protein of $H_2S$. When isolated rat aortic tissues were precontracted with 20 or 100 mM KCl, the maximum vascular relaxation induced by $H_2S$ was ~ 90% or 19%, respectively (4). This difference in relaxation potency of $H_2S$ represents the portion of relaxation possibly mediated by potassium conductance. Furthermore, $H_2S$-induced relaxation of the aortic tissues precontracted with phenylephrine was mimicked by a $K_{ATP}$ channel opener pinacidil but concentration-dependently inhibited by glibenclamide. Results from these tissue contractility studies were substantiated in isolated single SMCs. $K_{ATP}$ channel currents in rat aortic SMCs were significantly and reversibly increased by either $H_2S$ or pinacidil. A direct action of $H_2S$ on $K_{ATP}$ channel proteins, rather than the interfered ATP metabolism by $H_2S$, was proposed based on three lines of evidence. First, intracellular ATP concentration in these studies was clamped at a fixed level (e.g., 0.5 mM) by dialyzing cells with the pipette solution. Second, the effect of $H_2S$ on $K_{ATP}$ channels was quickly reversed on washing out $H_2S$ from the bath solution. Third, intentionally varying ATP concentrations inside the cell (from 0.2 to 3 mM) did not change the excitatory effect of $H_2S$ on $K_{ATP}$ channels. Together, these results demonstrate that $H_2S$ is an important endogenous vasoactive factor and is the first identified gaseous opener of $K_{ATP}$ channels in vascular SMCs.

### Physiological vs. toxicological effects of $H_2S$

The toxicity of $H_2S$ has been known for ~ 300 years. The major lethal consequence of $H_2S$ intoxication is the loss of central respiratory drive due to biochemical lesions of the respiratory centers of the brainstem (41). For a complete toxicological profile of $H_2S$, readers are redirected to two excellent reviews by Beauchamp et al. (20) and Reiffenstein et al. (36). Note first that the endogenously generated $H_2S$ under physiological conditions is hardly accumulated or toxic to cells due to the balanced cellular metabolism of the gas (Fig. 1). In the presence of > 30 μM $HS^-$, no apparent disturbance in oxidative phosphorylation could be observed likely due to the rapid oxidation of $H_2S$ in mitochondria (42, 43). Second, the line between toxicological and physiological effects of $H_2S$ is very thin. The reported toxic level of $H_2S$ is < twofold greater than its endogenous level in rat brain tissues (26). Intoxication of mice with NaSH only elevated the sulfide concentration from the endogenous level by 57%, 18%, and 64% in brain, liver, and kidney, respectively (28). It is thus reasoned that the dose-response relationship of $H_2S$ at the physiological concentration range must be very steep before the physiological effect of $H_2S$ sharply transforms into a highly toxic effect (4). Moreover, mammalian cells must possess a delicate regulatory mechanism to control the endogenous $H_2S$ level within the physiological range.

### Interaction of $H_2S$ with other gasotransmitters

Given that $H_2S$, NO, and CO can all be gasotransmitters, they are not redundant (**Table 2**). For example, $H_2S$, NO, and CO facilitate the induction of hippocampal LTP. This effect of $H_2S$ depends on the activation of NMDA receptors (6) whereas that of NO and CO does not. NO can act as a reactive oxygen species by impairing the reduced glutathione/oxidized glutathione balance and/or by inhibiting enzymes and ion channels through S-nitrosylation processes. $H_2S$ may also be involved in the reduction of thiols, whereas CO is not directly involved in redox reactions. Gasotransmitters may interact with each other. As discussed above, competition for the common hemoglobin sink by one gasotransmitter would potentiate or unmask the biological effect of other gasotransmitters.

Published data have shown that the endogenous production of $H_2S$ from rat aortic tissues is enhanced by NO donor treatment (4). The NO donor also enhances the expression level of CSE in cultured vascular SMCs. Similar to the release of NO by acetyl-

TABLE 2. *Metabolism and function of gasotransmitters*[a]

| | $H_2S$ | CO | NO |
|---|---|---|---|
| Main substrates | L-cysteine | Heme | L-arginine |
| Generating enzymes | CBS, CSE | Heme oxygenases | NO synthases |
| Inducer | NO | Free radicals | Acetylcholin, endotoxin |
| Scavenger | Hemoglobin | Hemoglobin | Hemoglobin |
| Inhibitor | D,L-propargylglycerine | Zinc-PPIX | L-NAME |
| Protein targets | $K_{ATP}$ channel, cAMP (?) | cGMP, $K_{Ca}$ channel | cGMP, $K_{Ca}$ channel |
| Amino acid targets | ? | Histidine | Cysteine |
| Half-life in solution | Minutes | Minutes | Seconds |
| Production tissue source | SMC, not in EC | EC < SMC | EC > SMC |

[a] Only examples, not a complete list, are given. SMC, smooth muscle cell; EC, endothelial cell; zinc-PPIX, zinc protoporphyrin-IX; L-NAME, $N^G$-nitro-L-arginine methyl ester.

choline, release of $H_2S$ by NO adds a line of essential evidence for the physiological role of $H_2S$.

Finally, the integrated vascular effect of $H_2S$ and NO may not be a simple algebraic summation of their individual actions. Hosoki et al. (5) observed that the vasorelaxant effect of sodium nitroprusside (SNP), a NO donor, was enhanced by incubating rat aortic tissues with 30 μM NaHS. On the contrary, pretreating aortic tissues in another study with 60 μM $H_2S$ inhibited the vasorelaxant effect of SNP. This discrepancy may be partially explained by the experimental conditions of these studies, including differences in tissue preparations and tension development before the application of $H_2S$. The putative interactions of NO and $H_2S$ are hypothetically presented in **Fig. 3**.

## CONCLUDING REMARKS AND PERSPECTIVES

In keeping with the criteria listed in Table 1, $H_2S$ might be classified as the third gasotransmitter besides NO and CO. This gas is endogenously generated and manifests significant effects at physiologically relevant concentrations. The effect of $H_2S$ on $K_{ATP}$ channels may represent an important endogenous mechanism in vascular SMCs, neurons, and other excitable cells to couple cellular metabolism to excitability. By demonstrating the role of NO as an inducer or as a molecular switch for endogenous $H_2S$ production, we can begin to understand how the interaction between $H_2S$ and NO provides an integrated regulation of vascular tone. These advances in $H_2S$ research may revolutionize many conventional doctrines. For example, hyperhomocystinemia is a disease with a deficient expression of CBS. The role of a low level of endogenous $H_2S$ in the pathogenesis of this disease has been largely overlooked or simply neglected (13), yet it may be an important cause of atherosclerosis and thrombotic complications associated with hyperhomocystinemia. We still have a long way to go before a complete understanding of cellular metabolism and functions of $H_2S$ is achieved. The following future studies of $H_2S$ physiology serve only as examples.

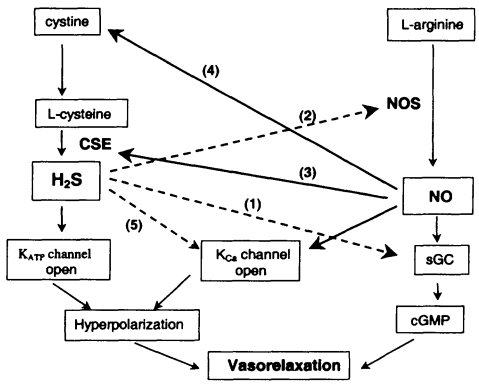


**Figure 3.** Hypothesized scheme of the interaction of $H_2S$ and NO in vascular tissues. The solid lines indicate the stimulatory inputs and the dashed lines, inhibitory inputs. (*1*) $H_2S$ may decrease the sensitivity of the cGMP pathway to NO (27). (*2*) $H_2S$ may reduce the expression level of NO synthase (NOS). (*3*) NO may increase the expression of CSE. (*4*) NO may increase the cellular uptake of cystine. (*5*) $H_2S$ may modify $K_{Ca}$ channels to decrease their sensitivity to NO.

*1)* Molecular mechanisms of the interaction of $H_2S$ and $K_{ATP}$ channels should be further investigated. As expression of different $K_{ATP}$ channel subunits is tissue-type specific, whether $H_2S$ stimulates $K_{ATP}$ channels in other tissues (e.g., lungs, kidney, pancreas) as it does in vascular SMCs and neurons may be a key to the differential effects of $H_2S$ on different tissues. Direct evidence, including single channel recording on heterologously expressed $K_{ATP}$ channels in the presence of $H_2S$, should be collected. $H_2S$ may interact with membrane and/or cytosol proteins to form reactive and unstable persulfides (44). These persulfides may take different forms, including protein-SSH, thiotaurine, thiocysteine, thiocystine, or mercaptopyruvate (45). The persulfide-related sulfuration and structural changes of the targeted proteins are recognized mechanisms for the biological effects of sulfide donors. This mechanism may underlie the interaction of $H_2S$ and $K_{ATP}$ channel proteins.

*2)* $H_2S$ may alter cellular redox status. $H_2S$ in an aqueous solution is a weak reducing agent. Vasorelaxation induced by $H_2S$ was not mimicked by the disulfide bond-reducing agents (5) but the $H_2S$-induced modulation of $Na^+$ channels in neurons was (34). This controversy

supports, rather than denounces, the importance of the reducing capability of $H_2S$. Quite likely, manifestation of the reducing effect of $H_2S$ depends on the tissue-specific targets and the tissue-specific redox environment. Does $H_2S$ have an oxidative potential? This is unsettled given the reported yield of free radicals from $H_2S$. In the presence of peroxidase and $H_2O_2$, $H_2S$ produced thiyl free radicals ($SH^{\bullet}$ and $S^{\bullet}$) (46). More vigorous studies are needed to investigate the physiological effects of $H_2S$ in the presence of different antioxidants, especially the scavengers for thiyl free radicals.

*3)* The endogenous inhibitors and stimulators for $H_2S$ production should be explored. Since CBS is a heme-containing protein (10) and heme-containing proteins are common targets of NO and CO, the activity of CBS might be under the influence of both CO and NO (47). CSE activity is increased by L-cysteine (48), but this substance is not stable and may have neurotoxicity. Steroid hormones are putative modulators of CBS functions; one such example is the testosterone-induced increase in the activity of CBS (49). The expression of CBS is also inducible. Although no CBS protein could be detected in freshly isolated human aortic tissues, primarily cultured human aortic SMCs within five passages exhibited clear CBS activity and protein expression (38). This may imply a regulatory role of endogenous $H_2S$ in the proliferation of vascular SMCs, which are normally quiescent.

*4)* Pharmacological or genomic manipulation of $H_2S$ production is an underdeveloped area with great potential. Enhancement of CBS activity by S-adenosyl-methionine (6, 9, 50) may find novel applications in dealing with some brain disorders. However, S-adenosyl-methionine may have other effects unrelated to the endogenous generation of $H_2S$ due to its methyl donor role. Specific activators of CSE, which is uniquely expressed in vascular tissues, are not available at present, but these agents can be important tools in the regulation of abnormal cardiovascular functions related to the altered endogenous $H_2S$ metabolism. Most if not all of the currently available inhibitors for different types of the $H_2S$-generating enzymes are not membrane permeable, which significantly impedes their applications under physiological conditions. A heterozygous deficiency of CBS mice has been established (51). The transgenic animal model with CSE deletion will be needed to establish the contribution of this enzyme to endogenous $H_2S$ levels in vascular tissues.

*5)* Investigations should begin to look into the pathological role of endogenous $H_2S$. Deficiency in CBS expression causes hyperhomocystinemia, which leads to premature peripheral and cerebral occlusive arterial disease (52). The pathogenic role of low levels of $H_2S$ in this disease has not been explored. Similarly, homocystinuria is an autosomal recessively inherited disorder (53) that may be closely related to the low endogenous production of $H_2S$. On the other hand, Down syndrome with elevated CBS expression, low plasma homocysteine, and significantly increased thiosulfate urinary excretion (54) may couple to abnormally high $H_2S$ levels. These observations have led to the hypothesis that the accumulation of $H_2S$ in the brain could cause the metabolic intoxication (55). Sudden infant death syndrome may be related to the abnormally higher taurine levels induced by $H_2S$ (34). The development of vascular diseases after heart transplantation is accompanied by increased total plasma homocysteine concentrations (56). In this case and other vasculopathy circumstances, a potentially lower endogenous level of $H_2S$ may be an important pathogenic factor.

Now that the role of $H_2S$ has been identified as sharing metabolic mechanisms and cellular effects similar to NO and CO, it is the time to call the family of gasotransmitters to 'please stand up.' It is expected that the gasotransmitter family will be expanded to include other yet undefined endogenous gaseous molecules. FJ

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