CARBON MONOXIDE DETECTION AND BIOLOGICAL INVESTIGATIONS

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INTRODUCTION

The yellow-orange jaundice pigment, bilirubin (BR), has attracted the attention of physicians who have described the many syndromes associated with its accumulation in tissues and easily observed as jaundice. Because its accumulation is considered a sign of disease, BR, its synthesis, and elimination from the body have been studied extensively.

Investigation of the control of BR formation has directed the attention of scientists towards heme degradation and, ironically, a reconsideration of the roles of BR (1–3) and its biochemical siblings, carbon monoxide (CO) (4), biliverdin (5), and iron (6) in biological systems. These studies have shown that most of the heme entering the pathway is derived from the red blood cell (RBC) recycling process (7) (Figure 1). The remainder arises from the turnover of many non-hemoglobin hemoproteins, such as myoglobin, catalase, cytochromes, glutathione peroxidase, and nitric oxide synthase (NOS) that play essential roles in physiologic homeostasis (8). Although most of the aged RBCs are trapped and degraded by the spleen, it has been suggested that the liver may also play an important role in this process. Furthermore, the heme from hemoprotein turnover is most likely degraded locally within the cell.

The enzyme responsible for the degradation of heme is heme oxygenase (HO). In conjunction with cytochrome (P_{450}) reductase, it controls the rate-limiting step in the heme degradation pathway, binds with heme, and in turn binds to oxygen. In the presence of NADPH, the complex then oxidizes the tetrapyrrole ring structure at the α-carbon, to yield equimolar quantities of the green-colored linear tetrapyrrole, biliverdin, and CO. This reaction also results in the release of the central iron ion (Fe^{2+}) (9,10). The biliverdin is then immediately re-

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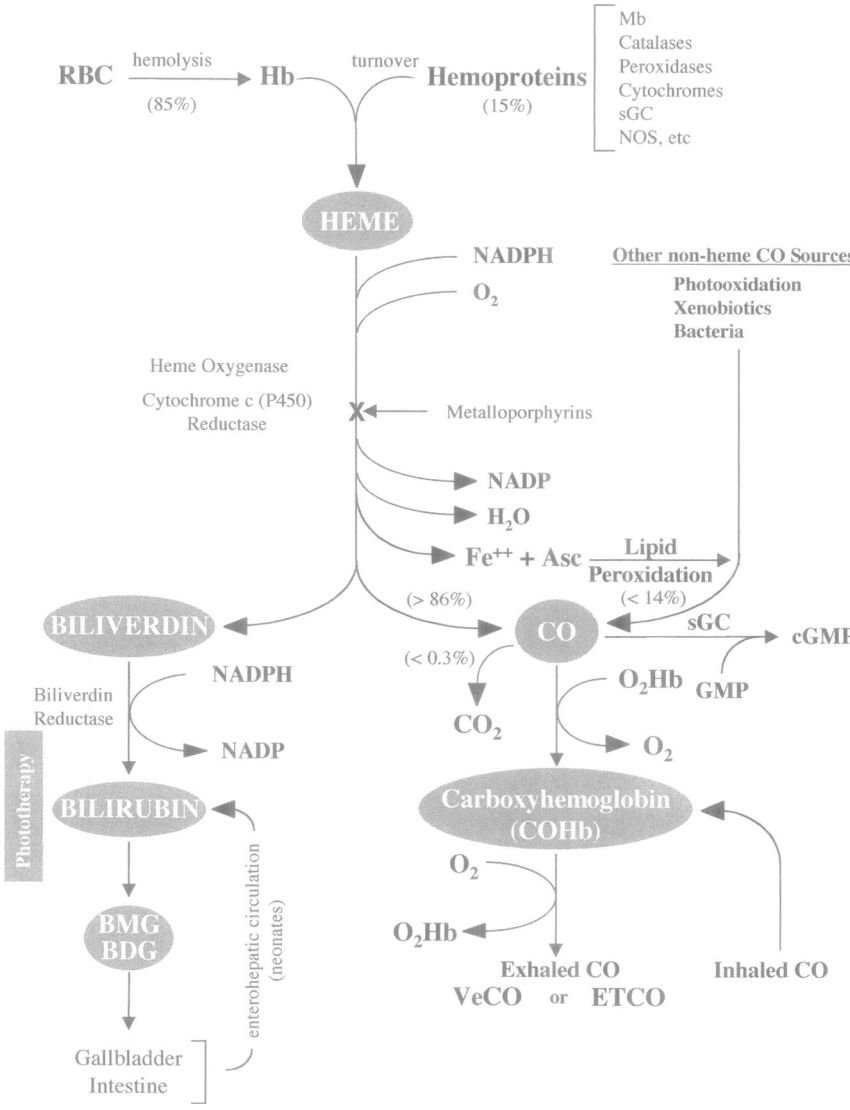


FIG. 1. Heme degradation pathway.

duced to BR by an excess of biliverdin reductase (11). In adult humans, BR is conjugated with one or two sugar molecules to form water-soluble mono- or diglucuronides, which facilitate its transport into the circulation and subsequent excretion from the body with the bile into the intestine (12).

The CO, on the other hand, binds to the hemoglobin of circulating

RBCs to form carboxyhemoglobin (COHb) for ultimate transport to the lungs, where COHb equilibrates with the inhaled oxygen and then CO is excreted from the body in the breath. Because CO is produced primarily from the degradation of heme in a one to one ratio and is excreted from the body primarily via the lungs, measurements of CO in the blood (i.e. COHb) and in the breath can serve as indicators of the rate of heme degradation as well as BR formation (7,13). Consequently, much of our information on the rates of heme degradation and BR formation in animal models and humans has been obtained through invasive as well as noninvasive measurements of total body CO production.

Until recently, the HO pathway was considered to be primarily a process for the conservation and recycling of valuable iron. The Fe^{2+} released as a result of heme degradation is sequestered by transferrin and is returned to iron stores as ferritin, the major iron storage protein (6). The mechanisms and regulation of iron reutilization in the bone marrow and other cells is still incompletely understood (14). What is known is that free Fe^{2+} and/or Fe^{3+} are powerful promoters of oxidative reactions (Fenton chemistry) that result in membrane damage and CO production (15–17).

Endogenous Sources of CO

A long time ago, Paracelsus (1493–1541) remarked that "All substances are poisonous. Only the dose differentiates a poison from a remedy." His observation very aptly describes the components of the heme degradation pathway. Carbon monoxide, like BR, has had a long-standing reputation as a biological villain. It is an odorless, tasteless, and invisible poison, lethal to humans through its interference with oxygen delivery and use. A telltale rosy hue of a victim's skin and mucous membranes belies the fatal truth that CO has surreptitiously replaced oxygen on the hemoglobin molecule, rendering its circulation, not only a futile, but also fatal, exercise. Recently, however, CO has been credited with beneficial biochemical and physiologic properties similar to those of its companion gas, nitric oxide (NO) which, when bound to soluble guanylyl cyclase (sGC), stimulates the production of cyclic GMP (18–21).

In humans, the production rate of CO per kg body weight is 2 to 3 times higher in newborns than in adults (22–24). Under pathologic conditions, however, such as hemolysis, increased ineffective erythropoiesis, or increased hemoprotein turnover, the rate of CO produc-

tion can increase many fold in the adult male from 18–160 μmol/hr (22).

The non-enzymatic production of CO is one of the more intriguing phenomena recently confirmed in biological systems devoid of heme. Originally reported in 1968 (25), lipid peroxidation was suggested as a source of CO again in 1976 and 1978 (17,26). A role for CO-derived from *in vivo* lipid peroxidation in tissues, like the brain, remains speculative, but worthy of serious consideration as potential CO-mediated processes are investigated (15). Photooxidation, mediated by natural (riboflavin and BR) or synthetic photosensitizers [some metalloporphyrins (MPs)], may be another potential source of CO, especially in more translucent subjects, such as premature infants receiving phototherapy (27,28). As animals have become dependent upon bacteria, for example intestinal colonization, CO produced by bacteria (29) may also turn out to be relevant to the understanding of not only intestinal physiology but also dysfunction.

Heme Oxygenase

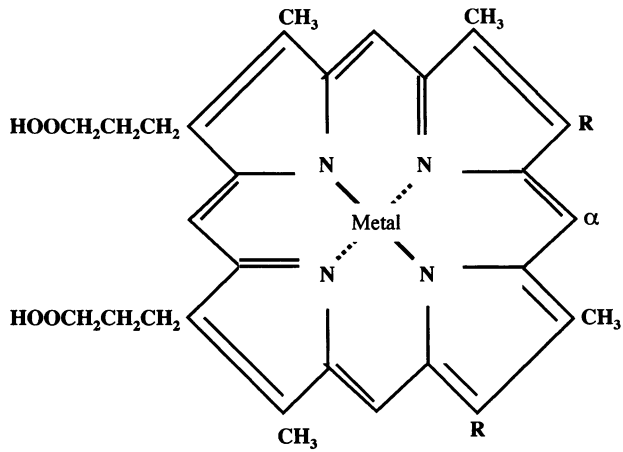
Although HO biology is a formidable topic in itself, CO detection can not only provide insight into the various aspects of the HO reaction, but also information about CO biology itself, a burgeoning field of investigation. Measurements of CO production in animals can serve as a means to expand investigation into the role of CO in the physiology of animals and possibly plants. More detailed and informative reviews on HO- and CO biology have been presented earlier (13,18,20,21,27,30–33).

CO production by lower animals, and possibly plants, is also linked to the enzymatic formation of biliverdin, a precursor of chromophores, that are attached to proteins functioning as photoreceptors involved in regulating important metabolic pathways. For example, the photosynthetic light-harvesting biliproteins of the red alga, *Cyanidium caldarium*, are derived from biliverdin (34). CO and biliverdin are formed in equimolar quantities by algal HO (35), which can be inhibited by the synthetic heme analogue, tin protoporphyrin (34). Phytochrome, which regulates photo-induced morphogenesis in higher plants, also contains a biliverdin-derived chromophore. In studies of vascular plants, we have found HO-like activity to vary between plant species, organs (root, stem, leaf, fruit, flower), and subcellular organelles, reminiscent of the mammalian circumstance (36). HO activity has also been observed in vertebrates, such as the frog and chicken, while biliverdin and BR have been identified in fish (37,38). In addition, biliverdin is incorporated into

the blue hemolymph protein, insecticyanin, used by the invertebrate tobacco hornworm in camouflage (39). Although the role of CO has not been thoroughly investigated in these few examples, the remarkable conservation of the heme degradation pathway and its regulation among diverse phylogenetic groups suggests an important role for this process in both animals and plants, and supports a legitimate inquiry into the physiologic roles that CO might play in biological systems.

HO is nearly ubiquitous throughout the body. Only anucleated, mature RBCs have been found to lack HO (40). At this time, three isozymes have been identified. HO-1 is the isoform inducible by a large number of physiological stresses, such as heavy metals, UV light (free radicals), heat, hyperoxia, infection, etc. (41). HO-2 is the constitutively expressed (housekeeping) isozyme, which appears to control the basal heme degradation process. HO-3 is the putative third isozyme with very little demonstrable activity and an as yet unknown function (42).

The HO isozymes are not distributed evenly across tissues. Not



Porphyrin Type Based on Ring Substituent

Metal	Deuteroporphyrin (R = -H)	Mesoporphyrin (R = $-CH_2-CH_3$)	Protoporphyrin (R = $-CH=CH_2$)	Bis Glycol Porphyrin (R = $-CH_2OH-CH_2OH$)
Iron (Fe^{2+})	FeDP	FeMP	**FePP (Hemin)**	FeBG
Zinc (Zn^{2+})	ZnDP	ZnMP	ZnPP	ZnBG
Tin (Sn^{4+})	SnDP	SnMP	SnPP	SnBG
Chromium (Cr^{2+})	CrDP	CrMP	CrPP	CrBG

FIG. 2. Basic metalloporphyrin (MP) structure with central metal and ring modifications representing the various MPs and their abbreviations.

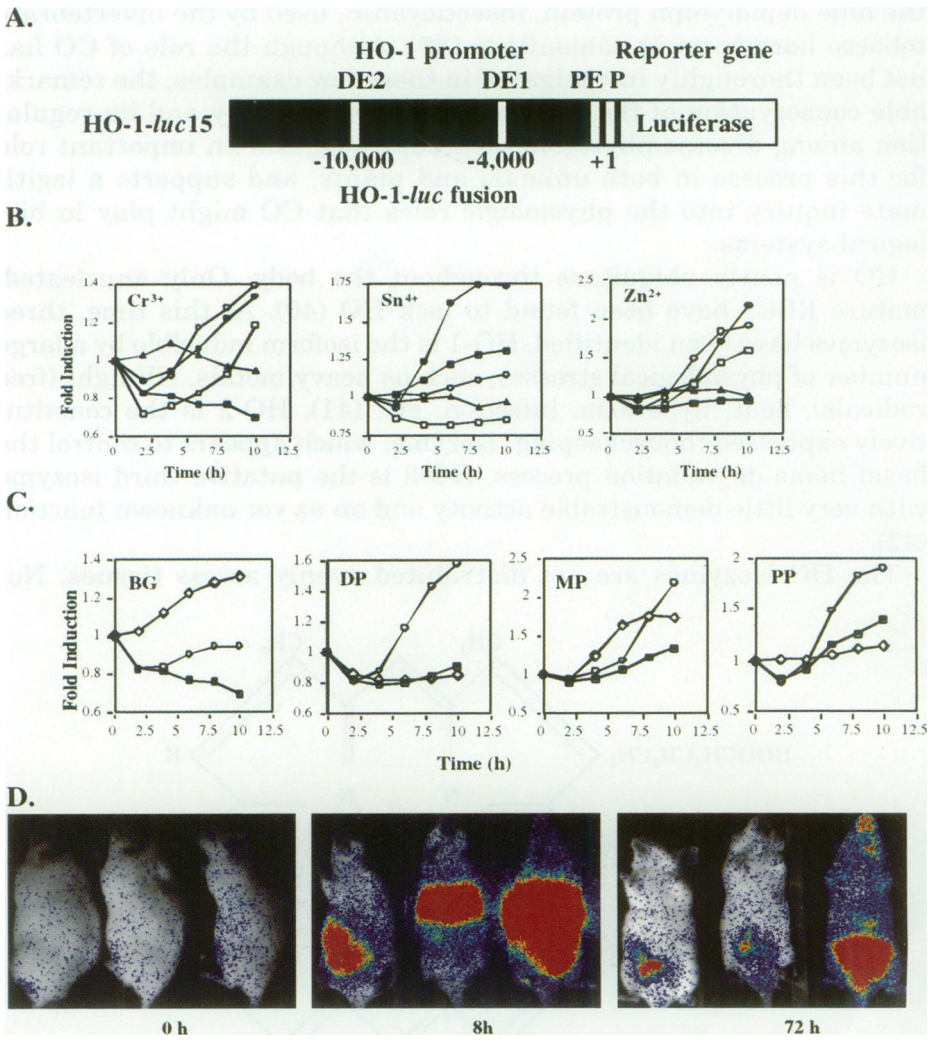


FIG. 3. A. HO-*luc* reporter construct. The HO-1-*luc*15 expression vector was constructed by cloning a 15 kb, 5′-upstream regulatory region of HO-1 gene from pMHO-cat15 (kindly provided by Dr. J. Alam et al (91), a 1.8 kb DNA fragment containing a modified firefly luciferase coding sequence (Promega, Madison WI), and 3′ poly-A signal sequence into pBluescriptKS(+) vector (**P**-promoter, **PE**-proximal enhancer, **DE**-distal enhancer); B. Effects of metalloporphyrins (MPs) on HO-1 transcription in a stable HO-1-*luc*15 murine cell line in culture. MPs with chromium (Cr^{3+}), tin (Sn^{4+}), or zinc (Zn^{2+}) and various ring substituents were tested and compared to chloride salts of each metal. The symbols indicate side groups for each set of MPs containing a specific metal (see Figure 2): ■ bis glycol porphyrin; □ deuteroporphyrin; ● mesoporphyrin; ○ protoporphyrin; and ▲ chloride salt. C. Effects of MPs on HO-1 transcription in a stable HO-1-*luc*15 murine cell line in culture. In these comparisons the porphyrin ring was unaltered but the metals were changed. The symbols indicate metals for each set of MP

surprisingly, the spleen contains predominantly HO-1, whereas, the HO activity of neuronal- and testicular tissues is almost exclusively due to HO-2. The liver, however, contains both HO-1 and HO-2 isozymes in nearly equal amounts. Tissue HO enzyme activity is the combined action of all isozymes and can be quantitated *in vitro* via measurements of BR (43). More convenient and less subject to error and matrix limitations are assays based on the quantitation of CO in tissue homogenates, cells, slices, and even intact animals (13,40).

HO isozymes are competitively inhibited *in vitro* and *in vivo* by micromolar concentrations of natural and synthetic derivatives of heme or metalloporphyrins (MPs) (Figure 2). The administration of these MPs allows clinicians the potential for preventive control of hyperbilirubinemia (44,45). Even though tin proto- (46) and meso-porphyrin (47) have undergone clinical trials, chromium mesoporphyrin and zinc bis glycol porphyrin warrant further consideration as therapeutic agents because they lack undesirable side effects (48). However, the ultimate "magic bullet" for this approach does not exist. Some of the MPs are long-acting (months). Others are photosensitizers (48), yet others increase transcription of the HO-1 mRNA gene, possibly producing more of the enzyme that they are supposed to inhibit (Figure 3). Others are not orally absorbable, requiring invasive intravenous or intraperitoneal injection. Even though different MPs inhibit HO-1 and HO-2 to varying degrees, the difference is not great enough for any MP studied to completely inhibit HO-1 without inhibiting the housekeeping HO-2 (32). Furthermore, MPs also bind to NOS and sGC, thereby affecting the production of NO and the processes it regulates (49). All these properties of MPs need to be investigated further.

The observed increase in HO-1 mRNA levels after treatment with inhibitors is another subject deserving closer scrutiny. It needs to be determined unequivocally if increases in HO-1 transcription lead to proportional increases in HO-1 protein and activity, which could complicate the efficacy and safety of MP administration. Nonetheless, the various MPs affect HO-1 transcription differently, depending on both metal and ring substituents (50) (Figure 3). Therefore, regulation of

families: □ = Cr^{3+}; ○ = Zn^{2+}; and ◇ = Sn^{4+}; D. Effects of 10 μM $CdCl_2$ on HO-1 transcription in HO-1-*luc* Tg mouse line. The second HO-1-*luc* founder line was bred to homozygosity and the level of induction of the HO-1 promoter was assessed over time following the injection of $CdCl_2$ by measuring the luminescent signal over the animals. Pretreatment signals are noted at the 0-time point and levels at 8 and 72 h are shown. This demonstrates that the spatiotemporal regulation of gene expression can be monitored in living animals (51).

HO-1 by MPs at the level of transcription should be considered when these compounds are evaluated for clinical use. The use of unique tools such as HO-1 promoter-luciferase constructs in transformed cell lines and transgenic animals in conjunction with *in vivo* CO-measuring technology may provide access to these levels of regulation in real time (51) (Figure 3).

That the activity of HO has been linked in nature to the production of CO, biliverdin (and BR), and iron suggests that understanding the interrelationships between heme and the products of the heme degradation pathway may yield some interesting insights into how life has adapted to Earth's oxidative challenge.

Physiologic Role of Carbon Monoxide

Since 1991, CO has been considered as possibly more than an inert waste product (Figure 1) (4). Instead, this small volatile diatomic molecule may be an important physiologically active gas, with a biochemistry as complex as that of NO. In fact, it is now suggested that CO may regulate the production of cGMP through the activation of sGC (18–21,52). In particular, the HO-CO-cGMP pathway in the central nervous system is of interest, and similar mechanisms have been proposed for regulation of vascular smooth muscle tone (53), myometrial contractility (54), carotid body sensory activity (55), and olfactory neuroreceptors (56). However, it is unlikely that the role of CO in physiology is singular and simplistic. It is more likely that the heme catabolic pathway participates in orchestrated responses to a variety of stimuli, mediated through regulation of HO-1, for example by oxidative stress (57). That intracellular heme levels can affect protein phosphorylation, protein synthesis, and cellular differentiation, and BR can inhibit protein phosphorylation, suggests a coordinated regulation of intracellular heme, CO, and BR levels related to cellular adaptation to changing environments (57). The effects of MPs on this regulation are important to understand, and may be more complicated because some MPs can inhibit other hemoproteins like NOS and sGC (52,58). Even presumed beneficial effects, such as the modulation of excessive BR production or inhibition of oxidation of membrane lipids, which influence intracellular and pericellular membrane integrity, require further study. A special emphasis, the interrelationships between CO- and NO-producing processes and their metabolites, should yield fundamental information about cellular homeostasis as well as disease.

Historical Perspective

Roughton and Root conclusively demonstrated in 1946 that human blood carries a small, but measurable amount of CO (59). Sjöstrand in 1949 and 1952 (60,61) and Coburn and others in 1964 and 1967 (30,62,63) demonstrated that its source was heme. They improved CO-measuring technology for physiologic and pathophysiologic (hemolytic) studies in humans. Since then, physicians and scientists have been stimulated to learn more about CO biology. While Tenhunen and colleagues devised biochemical methodologies for the study of HO (64), the gas chromatographic (GC) assay for CO developed by Collison and co-workers (65) laid the groundwork for a modification that provided scientists improved accuracy, sensitivity, and decreased sample volume and throughput time. Additional adaptations have been devised for detection of tissue CO (66), inviting researchers to explore the role of extravascular CO in regulation of tissue function, independent of intravascular and intracellular CO.

CO Technology and Applications

In our ongoing investigations, we employ *in vitro* and *in vitro* CO-measuring methodology to monitor perturbations that impact upon endogenous CO production. *In vitro* measurements of CO are applied primarily to the studies quantitating endogenous CO generation as a measure of HO enzyme activity (40). Determinations of basal and upregulated HO enzyme activity have been reported in many animal and plant species and tissues, such as newborn and adult rats (67,68), neonatal monkeys (69–71), and mice (72) at various developmental stages (73) and using various drug administration routes (68,74). Furthermore, (48,49,75) MP inhibitory effects on HO activity have also been widely studied *in vitro* and *in vivo*. In order to assess MP-induced photosensitization, *in vitro* (48,76,77) and *in vivo* (78) CO measurements can be used to quantitate the severity of potential photoreactivity of MP compounds. *In vitro* CO measurements can also be adapted to study non-heme CO-producing processes, such as lipid peroxidation, in order to assess the potential for oxidative tissue damage under certain conditions (15).

In addition, *in vivo* CO measurements, such as COHb, total body CO production (VeCO), and end-tidal breath CO corrected for inhaled CO (ETCOc), can also be used to monitor heme degradation, and thus BR formation, in human neonates and in animal models. Reports on hemolytic disease such as glucose-6-phosphate dehydrogenase deficiency (79–83), ABO-(84,85), Rh isoimmune diseases (84,86), sickle cell ane-

mia (87), and thalassemia (87,88) show that all hemolytic conditions lead to increased production and excretion of CO (and BR). In addition, evaluation studies of different populations of premature and newborn babies on the basis of sex, ethnicity (84,89), and health factors, such as diabetes in the mother (90), polycythemia (84), and sequestered blood (84), can also be performed using *in vivo* CO measurements.

Finally, it may be possible to uncover, under rigorously controlled experimental conditions, the contribution to endogenous CO by other processes, such as lipid peroxidation. Consequently, this finding could complicate the interpretation of measurements of CO resulting from heme degradation. However in this case non-CO based tests could possibly be used to differentiate the possible origins of CO.

SUMMARY

Even though the heme degradation pathway consists of only two reactions, it and its major enzyme (i.e. HO), nonetheless, impact other processes not only through the removal of excess heme, but also through the production of several metabolically active compounds. Thus CO and biliverdin along with reactive iron, Fe^2, are the primordial products of this ancient, highly conserved reaction. That every component of the heme catabolic pathway is directly or indirectly related to other reactions involving oxygen or light is, perhaps, no accident of nature. That a fundamentally destructive event can be linked with a multiplicity of synthetic events and various biological effects, depending on the timing and location of the HO activity, is testament to the economy and the ultimate beauty of nature. Furthermore, the interaction of the heme catabolic pathway with that of the NOS system may lead to even more exciting avenues of research. It may be shown that the integrity of the heme catabolic pathway, which is ever present and plays a role in every tissue, is central to the existence of most complex organisms.

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DISCUSSION

BLOOMER, Alabama: You have talked about the possible biological roles of carbon monoxide. There have been some studies to indicate that carbon monoxide alters vascular tone. Do you see any situations in which overproduction of carbon monoxide could exert a beneficial effect on some pathophysiologic process by altering vascular tone?

STEVENSON: That is an interesting and important question. I do not know the answer to it yet. We do know that vascular tissue in vitro can produce CO via the heme oxygenase reaction. In vivo it is less clear whether the vascular system is affected to a measurable extent during pathologic hemolysis, upon administration of heme, or through upregulation of the heme oxygenase gene. For that matter, it is not known conclusively whether endogenous CO production does affect neuronal transduction in the brain in vivo, which is another area of great interest. Your question does raise issues, of course, about environmental exposure to CO or conditions that might induce the production of CO in the body; and one of the most intriguing things to me is the potential for having non-enzymatic, non-heme source of CO —sometimes endogenous, perhaps exogenous to the body —generate amounts of the gas large enough to influence the brain or some other tissue and lead to fairly profound effects on biological processes.