

Carbon Monoxide: Endogenous Production, Physiological Functions, and Pharmacological Applications

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| | |
|--|-----|
| Abstract | 587 |
| I. Introduction | 587 |
| II. Milestones for the biological and physiological studies of carbon monoxide | 588 |
| A. Endogenous production of carbon monoxide | 588 |
| B. Physiological functions of carbon monoxide | 589 |
| III. Heme-dependent and -independent endogenous production of carbon monoxide | 590 |
| A. Primary structure of different heme oxygenase isoforms | 590 |
| B. Tissue-specific distribution of heme oxygenases | 590 |
| C. Subcellular localization of heme oxygenases | 591 |
| D. Up-regulation of the expression and activity of heme oxygenases | 591 |
| E. Down-regulation of the expression and activity of heme oxygenases | 592 |
| F. Biological functions of heme oxygenases | 592 |
| 1. Production of carbon monoxide, biliverdin/bilirubin, and ferrous iron | 592 |
| 2. Heme metabolism | 593 |
| 3. Heme-containing protein and heme-binding protein | 594 |
| G. Nonheme sources of carbon monoxide production | 595 |
| IV. Catabolism of endogenous carbon monoxide | 596 |
| A. Expiration | 596 |
| B. Scavenging | 596 |
| C. Oxidation | 596 |
| V. Physiological roles of carbon monoxide | 596 |
| A. Carbon monoxide and circulatory system | 596 |
| 1. Cardiac function | 596 |
| 2. Vascular contractility | 597 |
| 3. Platelet aggregation and monocyte activation | 597 |
| B. Carbon monoxide and nervous system | 597 |
| 1. Hypothalamic-pituitary-adrenal axis | 598 |
| 2. Glia | 599 |
| 3. Circadian rhythm control | 599 |
| 4. Odor response adaptation | 599 |
| 5. Nociception and chemoreception | 599 |
| 6. Thermal regulation | 600 |
| 7. Learning, memory, and behavior | 600 |
| 8. Vision | 600 |
| 9. Hearing | 600 |
| 10. Peripheral autonomic nervous system | 601 |
| C. Carbon monoxide and respiratory system | 601 |
| D. Carbon monoxide and reproductive system | 601 |
| E. Carbon monoxide and gastrointestinal system | 602 |
| F. Carbon monoxide and liver | 603 |

| | | |
|-------|--|-----|
| G. | Carbon monoxide and kidney | 603 |
| H. | Carbon monoxide and pancreas | 604 |
| VI. | Pathophysiological implications of abnormal heme oxygenase/carbon monoxide system | 604 |
| A. | Neurodegenerations and brain disorders | 604 |
| B. | Hypertension | 605 |
| 1. | Hypertension induced by heme oxygenase inhibitors | 605 |
| 2. | Spontaneously hypertensive rats | 605 |
| 3. | Salt-induced hypertension | 606 |
| 4. | Angiotensin II-induced hypertension | 606 |
| 5. | One kidney-one clip renovascular hypertension | 606 |
| 6. | Pulmonary hypertension | 606 |
| 7. | Portal hypertension | 606 |
| C. | Carbon monoxide and inflammation | 606 |
| D. | Cardiac hypertrophy and heart failure | 607 |
| E. | Transplantation | 608 |
| 1. | Allograft survival | 608 |
| 2. | Xenograft survival | 608 |
| F. | Apoptosis and cellular proliferation | 608 |
| 1. | Vascular smooth muscle cells | 608 |
| 2. | Endothelial cells | 609 |
| 3. | Other types of cells | 609 |
| G. | Oxidative stress | 610 |
| VII. | Heme protein-dependent cellular and molecular mechanisms for carbon monoxide effects | 611 |
| VIII. | Interaction of carbon monoxide with different ion channels | 612 |
| A. | Carbon monoxide and K_{Ca} channels | 612 |
| B. | Heme, heme oxygenase, 20-hydroxyeicosatetraenoic acid, and K_{Ca} channels | 614 |
| C. | Carbon monoxide and K_{ATP} channels | 614 |
| D. | Carbon monoxide and calcium channels | 614 |
| E. | Carbon monoxide and other ion channels | 615 |
| 1. | Na^+ channels | 615 |
| 2. | Nonselective cationic channel | 615 |
| 3. | Cyclic nucleotide-gated ion channels | 615 |
| IX. | Interaction of heme oxygenase/carbon monoxide and nitric-oxide synthase/nitric oxide systems | 615 |
| A. | Influence of carbon monoxide on nitric-oxide synthase/nitric oxide system | 616 |
| 1. | Carbon monoxide potentiates the activity of nitric-oxide synthase/nitric oxide system | 616 |
| 2. | Carbon monoxide reduces the activity of the nitric-oxide synthase/nitric oxide system | 616 |
| 3. | Carbon monoxide reduces the expression of nitric-oxide synthase | 616 |
| B. | Influence of nitric oxide on heme oxygenase/carbon monoxide system | 617 |
| 1. | Nitric oxide potentiates the activity of heme oxygenase/carbon monoxide system | 617 |
| 2. | Nitric oxide reduces the activity of heme oxygenase/carbon monoxide system | 617 |
| X. | Therapeutic applications of carbon monoxide | 617 |
| A. | Up-regulating the expression of heme oxygenase | 617 |
| 1. | Genetic approaches | 617 |
| 2. | Nongenetic approaches | 618 |
| B. | The more the merrier? Down-regulating the expression or activity of heme oxygenase | 618 |
| 1. | Genetic approaches | 619 |
| 2. | Nongenetic approaches | 619 |
| C. | Inhalation of carbon monoxide | 619 |
| D. | Use of carbon monoxide-releasing compounds | 620 |
| E. | Use of prodrugs to generate carbon monoxide | 620 |
| XI. | Conclusions and perspectives | 620 |
| | Acknowledgments | 622 |
| | References | 622 |

Abstract—Over the last decade, studies have unraveled many aspects of endogenous production and physiological functions of carbon monoxide (CO). The majority of endogenous CO is produced in a reaction catalyzed by the enzyme heme oxygenase (HO). Inducible HO (HO-1) and constitutive HO (HO-2) are mostly recognized for their roles in the oxidation of heme and production of CO and biliverdin, whereas the biological function of the third HO isoform, HO-3, is still unclear. The tissue type-specific distribution of these HO isoforms is largely linked to the specific biological actions of CO on different systems. CO functions as a signaling molecule in the neuronal system, involving the regulation of neurotransmitters and neuropeptide release, learning and memory, and odor response adaptation and many other neuronal activities. The vasorelaxant property and cardiac protection effect of CO have been documented. A plethora of studies have

also shown the importance of the roles of CO in the immune, respiratory, reproductive, gastrointestinal, kidney, and liver systems. Our understanding of the cellular and molecular mechanisms that regulate the production and mediate the physiological actions of CO has greatly advanced. Many diseases, including neurodegenerations, hypertension, heart failure, and inflammation, have been linked to the abnormality in CO metabolism and function. Enhancement of endogenous CO production and direct delivery of exogenous CO have found their applications in many health research fields and clinical settings. Future studies will further clarify the gasotransmitter role of CO, provide insight into the pathogenic mechanisms of many CO abnormality-related diseases, and pave the way for innovative preventive and therapeutic strategies based on the physiologic effects of CO.

I. Introduction

Were scientists to remake the classic 1932 film *Dr. Jekyll and Mr. Hyde*, the starring role would go not to Frederic March but to an infamous gas molecule, namely carbon monoxide (CO¹). The “evil side” of CO has been known for hundreds of years, far longer than Mr. Hyde’s name. As a result, we are already accustomed to the idea that CO is nothing but a toxicant, waste, or pollutant. As an environmental toxicant, pathological levels of CO can induce both acute and chronic health hazards at societal and individual levels. On the other hand, CO has earned

the image of Dr Jekyll in recent years. Mounting evidence now speaks loudly and clearly for the vital importance of CO, which is generated within our body, in regulating many biological functions. The sources of CO, its local concentrations, and its interaction with a specific environment integrally determine which side of this double-natured gas will dominate.

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¹ Abbreviations: CO, carbon monoxide; AD, Alzheimer’s disease; Ang-II, angiotensin-II; ACTH, adrenocorticotropic hormone; icv, intracerebroventricular; AP-1, activator protein-1; AVP, arginine vasopressin; CRF, corticotropin releasing factor; CrMP, chromium mesoporphyrin; EC, endothelial cell(s); ET-1, endothelin-1; 20-HETE, 20-hydroxyeicosatetraenoic acid; HIF-1, hypoxia-inducible transcription factor-1; HPA, hypothalamic-pituitary-adrenal; HO, heme oxygenase; hHO, human heme oxygenase; IAS, internal anal sphincter; HEK, human embryonic kidney; LTP, long-term potentiation; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NANC, nonadrenergic noncholinergic; NF- κ B, nuclear factor- κ B; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inhibition of purified macrophage nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; NOSVF, ventricular fibrillation; PDGF, platelet-derived growth factor; GSH, glutathione; GSSG, glutathione disulfide; ODQ, [1,2,4]-oxadiazolo-[4,3- α] quinoxaline-1-one; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; POMP, pro-opiomelanocortin; PVN, paraventricular; RPE, retinal pigment epithelium; siRNA, small interfering RNA; sGC, soluble guanylyl cyclase; SD, Sprague-Dawley; SHR, spontaneously hypertensive rats; SnPP, tin protoporphyrin; TEA, tetraethylammonium; CORM-3, tricarbonylchloro-(glycinato)ruthenium(II); TNF- α , tumor necrosis factor- α ; SMC, smooth muscle cell(s); ORN, olfactory receptor neuron; CNG, cyclic nucleotide-gated; L-NAME, N^w-nitro-L-arginine methyl ester; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal polypeptide; WKY, Wistar-Kyoto rats; ZnPP, zinc protoporphyrin; DPBG, deuteroporphyrin 2,4-bis glycol; DMSO, dimethyl sulfoxide; DCM, dichloromethane; EFS, electrical field stimulation; FS, febrile seizure(s).

CO is the diatomic oxide of carbon. At temperatures above -190°C , CO is a colorless and odorless gas. The specific gravity of CO is 0.967 relative to air, and its density is 1.25 g/l at standard temperature and pressure. CO is a chemically stable molecule because of its formal triple bond. Chemical reduction of CO requires temperatures well above 100°C . The water solubility of CO is very low (354 ml/dl; 44.3 ppm by mass) at standard temperature and pressure (Allen, 1977). CO cannot react with water without substantial energy input. Even for molecular oxygen, the reaction rate of CO is slow and needs a high activation energy (213 kJ/mol). Theoretically speaking, CO can be involved in redox reactions (Allen, 1977). Free CO does not readily react with reducing agents, including hydrogen. The coordinated CO has greater reactivity than the free gas, and the reduction of CO can be greatly facilitated by transition metals (Shriver, 1981). Once formed, metal carbonyls are relatively stable until CO is displaced, e.g., by molecular O₂.

All types of incomplete combustion of carbon-containing fuels yield CO. Natural processes such as metabolism and production of CO by plants and oceans or wildfires 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 noticeable human activities which produce CO are internal combustion engines, appliances fueled with gas, oil, wood, or coal, and solid waste disposal. The use of smoking tobacco or inadequately vented stoves is examples of CO accumulation in a closed field. CO intoxication mainly results from expo-

sure to environmentally generated CO at high concentrations and/or prolonged exposure periods. Being one of the most abundant air pollutants in North America, CO readily accumulates in the atmosphere and in our bodies, yet its physiochemical properties are hard to detect. CO intoxication can be fatal, the reason for its being known as a "silent killer". A simple increase in ambient CO levels will not necessarily lead to human intoxication, which is also influenced by the functional status of pulmonary ventilation, the endogenous buffering capacity, i.e., level of carbonmonoxy-hemoglobin A (COHb), and the partial pressures of CO and oxygen. Elevated CO concentration in the bloodstream accelerates binding of CO to normal adult hemoglobin (HbA), forming COHb. The formation of COHb impairs two functions of HbA. The oxygen storage function of HbA is significantly reduced since the affinity of CO to HbA is about 210 to 250 times greater than that of oxygen. The oxygen transportation function of COHb is also reduced, as the release of oxygen from COHb to the recipient tissue becomes more difficult. Both decreased arterial O₂ content (poor O₂ binding to hemoglobin) and decreased tissue PO₂ (increased affinity of COHb for O₂) (Stewart, 1975) cause hypoxia. The brain and heart are the organs most vulnerable to CO-induced acute hypoxia, due to their high demand for oxygen. Neurological injuries are manifested as headaches, dizziness, weakness, nausea, vomiting, disorientation, visual confusion, collapse, and coma. Without immediate treatment, the neurological injuries can be fatal. Severe or chronic CO intoxication of a developing fetus may increase the risk of retarded development of the central nervous system with abnormalities in visual perception, manual dexterity, learning, driving performance, and attention level. Chronic exposure of adult humans or experimental animals to CO has also been shown to induce cardiovascular disorders, including arteriosclerotic heart diseases and cardiac hypertrophy (Wang, 2004). 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 (Piantadosi, 2002). However, it has to be pointed out that living cells can tolerate CO in the concentration range of 0.01% (100 ppm) for several hours (Otterbein and Choi, 2000). Exposure to 500 ppm CO continuously for up to 2 years without deleterious effects has been reported in rodents (Stupfel and Bouley, 1970; Otterbein and Choi, 2000).

CO is a paradigm in itself. Advances in several frontiers in the last decade have given the evil image of CO a make-over. Endogenous CO production has been illustrated in great details, from its enzymatic catalyzation process to its variations in different tissues under different conditions. The physiological importance of endogenous CO to the homeostatic control of the human body has been reevaluated and realized in neuronal and cardiovascular systems and almost every other system and every tissue type. The numbers of publications and

conferences dedicated to CO physiology and CO pharmacology have been increasing at a stunning speed with contributions from numerous research laboratories around the world. Although there is still reasonable skepticism on the physiological importance of CO, the current research advancements have led to a significant acknowledgment that CO, joining with other endogenous gases including nitric oxide (NO) and hydrogen sulfide (H₂S), is one member of a new class of physiologically important "gasotransmitters", a nomenclature composed of "gas" and "transmitters" (Wang, 2002).

II. Milestones for the Biological and Physiological Studies of Carbon Monoxide

A. Endogenous Production of Carbon Monoxide

As early as the 1850s, French physiologist Claude Bernard recognized the reversible binding of CO with hemoglobin as a potent chemical reaction that could cause asphyxia (Bernard, 1857). A later study in 1895 showed the antagonistic effect of high partial pressure of O₂ on CO binding to hemoglobin (Haldane, 1895). The first indication for endogenous CO production was made by Saint-Martin and Nicloux in 1898. Warburg reported in 1930 that CO could inhibit respiration in yeast in a light-sensitive manner, extending the original discovery that COHb could be dissociated by exposure to light of appropriate wavelengths (Haldane and Smith, 1896). These pioneer studies initiated a century of investigations for the biological actions of CO. In the early 1950s, Sjöstrand for the first time provided actual experimental evidence for the existence of CO in our body (Sjöstrand, 1950 and 1952). He observed that decomposition of hemoglobin *in vivo* produced CO. Endogenous production of CO can be regulated. Increased heme levels after erythrocyte destruction increased endogenous CO production, reflected by the elevated COHb level (Coburn et al., 1965 and 1966). When heme metabolism is abnormally increased, such as in hemolysis, CO production rate in our body can increase tremendously (Coburn et al., 1966). In the late 1960s, Tenhunen and colleagues ascribed the driving force for endogenous source of CO production to heme oxygenase (HO) (Tenhunen et al., 1968, 1969, 1970; Landaw et al., 1970). The inducible HO isoform, HO-1, was identified in 1974 in two different laboratories (Maines and Kappas, 1974; Yoshida et al., 1974). Maines' laboratory in 1986 identified the constitutive HO isoform, HO-2, from rat liver microsomes (Maines et al., 1986; Trakshel et al., 1986). About 10 years later, the same laboratory identified the third HO isoform, HO-3 (McCoubrey et al., 1997b) (Fig. 1).

The production rate of CO is 16.4 $\mu\text{mol/h}$ in the human body (Coburn, 1970) and the daily production of CO is substantial, reaching more than 12 ml (500 μmol) (Coburn et al., 1965). The average physiological concentrations of CO in tissues are rather low, in the nanomolar range if based on normal levels of COHb of 1 to 2%

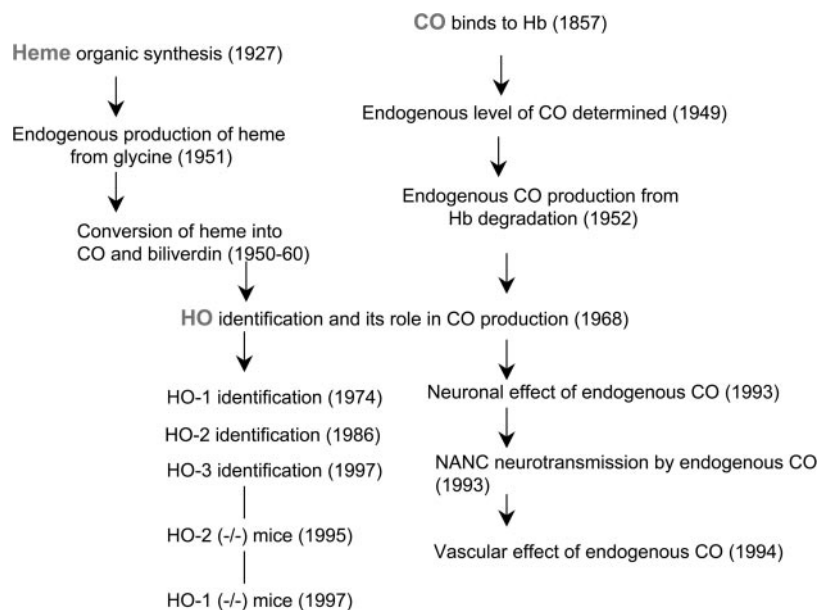


FIG. 1. Chronological development in discovery of the biological and physiological importance of endogenous CO.

(Piantadosi, 2002). However, the accuracy of this estimation is questionable, since CO generated in living cells would first be scavenged in the cytosol before being released into the bloodstream where COHb is formed. Previous studies have shown poor correlation of COHb levels with biological changes induced by CO (Stewart, 1975) and remnant effects of CO after COHb elimination (Halperin et al., 1959).

B. Physiological Functions of Carbon Monoxide

While investigation on endogenous production of CO and its regulation progressed continuously, especially in regards to the expression and biological implications of HO, the questions of how our body uses, or why it needs, endogenous CO had not been answered by conventional understanding of this gas as an endogenous waste or by-product of heme metabolism. It was not until little more than a decade ago that an appreciation of the biological and physiological functions of endogenous CO, and the role of CO in the beneficial effects of HO, was made. The break-through discovery of NO opened the way to further research on membrane/receptor-independent signaling gas molecules. In 1991, Marks and colleagues predicted that there was a metabolic reason and a physiological meaning for the production of CO in our bodies (Marks et al., 1991). This pioneering thinking stirred up the resurgence of CO as a physiological signaling molecule (Wang, 1998). Two years later, Snyder's research team provided the first comprehensive evidence for the role of CO as an endogenous neural messenger, based on the effect of HO inhibitors and the histological location of HO (Verma et al., 1993). Rattan and Chakder (1993), also using HO inhibitors, demonstrated that endogenous CO was involved in the relaxation of opossum internal anal sphincter (IAS) in re-

sponse to nonadrenergic noncholinergic (NANC) nerve stimulation.

The relaxation of pulmonary vasculature induced by exogenous CO under normoxic conditions was reported as early as the late 1970s (Sylvester and McGowan, 1978). However, cardiovascular researchers struggled for many years to find the evidence for the vasoactive effect of endogenous CO. By inhibiting HO activity with zinc protoporphyrin-IX (ZnPP), Suematsu et al. (1994) provided the evidence that ZnPP treatment reduced endogenous CO generation and increased vascular resistance in rat liver. Other laboratories at the same time argued that the vascular effect of ZnPP might not be related to inhibition of HO provoked (Ny et al., 1995; Zygmunt et al., 1994). Ensuing studies applying HO substrate and/or up-regulating HO-1 expression confirmed the vasorelaxant effect of endogenous CO (Wang 1996; Wang et al., 1997a).

The identification of the physiological role of endogenous CO was greatly facilitated by the use of gene knockout or gene overexpression techniques. The first HO-2 null mutant mouse was produced in 1995 (Poss et al., 1995). Poss and Tonegawa (1997) first generated mice deficient in HO-1 by targeted deletion of a 3.7-kb region including exons 3 and 4 and a portion of exon 5 of the mouse HO-1 gene. The direct relevance of the HO/CO pathway to human health was drawn by the reported first human case of HO-1 deficiency in Japan in 1999 (Yachie et al., 1999; Ohta et al., 2000). This HO-1 deficient patient died at age 6, showing growth retardation, anemia, thrombocytosis, hyperlipidemia, leukocytosis, elevated serum levels of ferritin and heme, and lower serum levels of bilirubin.

Clearly, the momentum in the study on CO biology in recent years has injected more and more enthusiasm

into study on HO biology. Research on CO and HO is now closely integrated and coevolving. This HO/CO field is experiencing phenomenal growth, spurred on by scientists and health workers, from the laboratory bench to the hospital bed side, and by trainees, from graduate students to postdoctoral fellows.

III. Heme-Dependent and -Independent Endogenous Production of Carbon Monoxide

Upon the action of hydrogen peroxide or ascorbic acid, heme methylene bridges can be broken and CO released (Bonnert and McDonagh, 1973; Brown et al., 1978; Guengerich, 1978). Cytochrome P450 can be inactivated by free NADPH oxidation, NADPH-dependent monooxygenase reactions and lipid peroxidation. This self-inactivation also leads to the breakage of the bond between heme and apoenzyme and heme degradation (Karuzina et al., 1999).

Although nonenzymatic heme metabolism as mentioned above occurs *in vivo*, the majority of CO in our body is produced by enzymatic heme metabolism. This metabolism is catalyzed by HO, mainly occurring in the reticuloendothelial system of the spleen and liver (Maines, 1988). There are three isoforms of HO. HO-1 is the inducible isoform. An increased cellular stress level is one common denominator for most of the stimuli to up-regulate *de novo* transcription of HO-1 (Applegate et al., 1991; Tyrrell, 1999). HO-2 is constitutively expressed in many mammalian cells. HO-3 is also a constitutive isoform of HO (McCoubrey et al., 1997b). One theory suggests that HO-3 may be derived from retrotransposition of the HO-2 gene since the HO-3 gene does not contain introns (Scapagnini et al., 2002b).

A. Primary Structure of Different Heme Oxygenase Isoforms

Four mammalian HO-1 genes have been cloned and sequenced, including rat (Muller et al., 1987), mouse (Alam et al., 1994), human (Yoshida et al., 1988), and chicken (Lu et al., 1998). Their protein homology is about 80%. Human HO-1 gene is located on chromosome 22q12. HO-2 has been cloned from rat, mouse, and human and they share more than 90% protein homology. Human HO-2 is mapped to chromosome 16q13.3 (Kutty et al., 1994a; Abraham et al., 1996).

The HO-1 gene consists of 5 exons and 4 introns, spanning ~14 kb. A promoter sequence is located approximately 28 base pairs upstream from the starting site of transcription. Different transcriptional enhancer elements reside in the flanking 59 region (Fogg et al., 1999). Consensus binding sites for oxidative stress-responsive transcription factors, including nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), AP-2, Sp1, upstream stimulatory factor, *c-myc/max* and interleukin-6 response elements have been reported in the promoter region of the human

HO-1 gene (Deramautd et al., 1999; Lavrovsky et al., 1994; Muraosa et al., 1996; Sato et al., 1990; Tyrrell et al., 1993), suggesting a potential role for these factors in modulating HO-1 induction. Positive regulatory regions of the human HO-1 promoter contain consensus binding sites for AP-1, STATx, c-Rel, hepatocyte nuclear factor-1 and factor-4, GATA-X, and cadmium response element (Takahashi et al., 1999b and 2002; Takeda et al., 1994). Negative regulatory regions of the human HO-1 promoter contain consensus binding sites for negative regulatory elements (NRE) boxes (Takahashi et al., 1999b) and a polymorphic GT repeat region (Yamada et al., 2000). It should be pointed out that some of these regulatory regions may function in one type of mammalian cells, but not necessarily in others (Takahashi et al., 1999b). The HO-1 gene from different species may have quite different responses to the given inducers, depending on variances in the promoter regions of the HO-1 gene. For example, in response to interferon- γ or hypoxia, mouse HO-1 gene is activated but repression of human HO-1 gene occurs (Sikorski et al., 2004). Accordingly, hypoxia up-regulates the expression of HO-1 in rat, bovine, mouse, and monkey cells but is a repressor of HO-1 expression in human cells (Kitamuro et al., 2003, Lee et al., 1997). In another case, heat shock induced the expression of HO-1 in rodent cells (Shibahara et al., 1987), but HO-1 expression in human cells was not altered by heat shock stimulation (Okinaga et al., 1996; Sato et al., 1990; Shibahara et al., 2002). Finally, an internal enhancer region was identified in human HO-1 promoter (Hill-Kapturczak et al., 2003). This region is only responsive to heme and cadmium.

The HO-2 gene consists of 5 exons and 4 introns. As the products of different genes, HO-1 (32 kD) and HO-2 (36 kD) share roughly 40% amino acid homology (Maines, 1997). HO-1 and HO-2 share a common 22 amino acid domain (differing in just one residue), named "HO signature". This signature sequence may be responsible for the heme degradation capacity (Maines, 1997). HO-1 and HO-2 proteins are immunologically distinct. HO-3 (33 kD) shares about 90% amino acid homology with HO-2. There are no introns in HO-3 gene (Scapagnini et al., 2002a).

Both HO-2 and HO-3, but not HO-1, are endowed with 2 Cys-Pro residues as the core of the heme-responsive motif, a domain critical for heme binding but not for its catalysis (Hon et al., 2000; McCoubrey et al., 1997a). HO-3 is a poor heme catalyst and its role may be limited to heme binding and/or sensing, considering the presence of 2 heme-responsive motifs in its amino acid sequence.

B. Tissue-Specific Distribution of Heme Oxygenases

The spleen has the most abundant expression of HO-1. In fact, under physiological conditions, the spleen may be the only organ in which HO-1 overpowers HO-2. In many other mammalian tissues, HO-1 is ubiquitously induced. HO-2 is predominantly expressed in the brain and testes and also constitutively expressed in other tissues, including endothelium, distal nephron seg-

ments, liver, myenteric plexus of the gut, and in other tissues at low levels (Ewing and Maines, 1992). The profile of HO isoform expression can change under different conditions. Upon stimulation, HO-1 expression in the testes overpowers the expression of HO-2. The same profile shift holds true for the lung, brain, and other tissues. Although early studies did not find HO-1 proteins in the brain (Ewing and Maines, 1992), recent studies have detected the HO-1 mRNA in different regions of the brain, especially in the hippocampus and the cerebellum (Scapagnini et al., 2002a). The expression of HO-1 protein in rat hippocampus at different ages was also reported (Huang et al., 2004). HO-3 has only been found in rat tissues, including brain, liver, kidney, and spleen (McCoubrey et al., 1997b). Using RT-PCR method, Scapagnini et al. (2002a) found high levels of HO-3 transcript in rat cerebellum and the hippocampus. HO-3 transcript was also detected in primarily cultured rat type I astrocytes, but not cortical neurons. In situ hybridization using an HO-3 specific riboprobe showed the expression of HO-3 mRNA in hippocampus, cerebellum and cortex. The expression of functional HO-3 protein, even in rat tissues, is in doubt since no report is available to convincingly demonstrate the detection of HO-3 proteins in any tissues. Hayashia et al. (2004) recently re-examined the expression of HO-3 gene in rat tissues. They concluded that the reported HO-3 genes might be processed pseudogenes derived from HO-2 transcripts. Genomic PCR, RT-PCR, and Western blot studies all lead these authors to the same conclusion that HO-3-related protein(s) is unlikely expressed in rat tissues.

C. Subcellular Localization of Heme Oxygenases

HO-1 is traditionally viewed as a microsomal protein, primarily localized in endoplasmic reticulum (Maines, 1988). This protein can be translocated to the nucleus of differentiated astroglial cells (Li Volti et al., 2004), where it may participate in the regulation of heme metabolism. Since nuclear heme can activate a high molecular weight complex by altering the affinity of HapI to Hsp90, the translocation of HO-1 to the nucleus would be a factor in the regulation of different transcriptional factors (Li Volti et al., 2004). Subcellular localization of HO-1 has also been detected in cytoplasm, nuclear matrix, mitochondria, and peroxisomes of parenchymal and nonparenchymal liver cell populations (Immenschuh et al., 2003). By monitoring the formation of bilirubin as an indicator of HO activity, Srivastava and Pandey (1996) detected significant HO activity in mitochondria from liver, spleen, kidney and brain, but not cerebral mitochondria. Since these activities can only be detected after the animals were treated with cobalt and hemin, HO-1 localization in mitochondria under these conditions was suggested. HO-2 proteins are anchored to the endoplasmic reticulum by a hydrophobic sequence of amino acids at the carboxyl terminus of the protein

(Wagener et al., 2003). The possibility has also been raised that HO-2 may be anchored to plasma membranes directly or indirectly through big-conductance K_{Ca} channels (Williams et al., 2004).

D. Up-Regulation of the Expression and Activity of Heme Oxygenases

HO-1 is also known as the stress protein HSP32 (Keyse and Tyrrell, 1989). A great array of endogenous and exogenous stimuli can induce the expression of HO-1. Among the known HO-1 inducers are heme and heme derivatives, heat shock, heavy metals, NO and NO donors, oxidized lipids, hyperoxia, lipopolysaccharides, phorbol ester, sodium arsenite (Sardana et al., 1981), radiation, ultraviolet, hydrogen peroxide, hypoxia, endotoxin, growth factors [platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β)], various electrophiles, okadaic acid, methylglyoxal (Wu, 2005), curcumin (Mottetlini et al., 2000), oxidative stress (Nath et al., 2001), cytokines (interleukin-1, interleukin-6, interleukin-10, TNF- α , interferon- γ), shear stress (Wagner et al., 1997), intensive light, angiotensin II (Ang-II), glucose deprivation, and other injuries. HO-1 expression is up-regulated by exogenous CO (Ndisang and Wang, 2003a and 2003b; Carraway et al., 2002). Depending on the cell types and the nature of the stimuli, HO-1 induction may be mediated by different signaling pathways. These pathways may include cAMP-dependent mechanisms (Durante et al., 1997a; Immenschuh et al., 1998), protein kinase C (PKC), Ca^{2+} -calmodulin-dependent protein kinase and the phosphoinositol pathway (Terry et al., 1999). Mitogen-activated protein kinases (ERK and P38) and tyrosine phosphorylation are also involved in HO-1 induction in some tissues (Elbirt et al., 1998; Shan et al., 1999; Alam et al., 2000; Chen and Maines, 2000). A role for PI3K activation was demonstrated for the effect of hepatocyte growth factor (Tacchini et al., 2001).

Being a constitutive isoform, HO-2 can also react to certain stimuli by changing its expression level or activity. A consensus sequence of the glucocorticoid response element is located in the promoter region of the HO-2 gene (Liu N et al., 2000). Correspondingly, the expression of HO-2 is up-regulated by adrenal glucocorticoids (Weber et al., 1994) and opiates (Li and Clark, 2000a). Induction of HO-2 by cortisteron has been shown in neonatal rat brain (Raju et al., 1997). In addition, endothelial cells treated with the NO synthase (NOS) inhibitor L-NAME and HO inhibitor zinc mesoporphyrin exhibited a significant up-regulation of HO-2 mRNA. Estrogen up-regulates HO-2 in endothelial cells (Tschugguel et al., 2001). Several mechanisms are responsible for the regulation of HO-2 activity. 1) HO-2 activity can be rapidly and transiently increased by calcium-calmodulin binding to HO-2 during neuronal activity (Boehning and Snyder, 2004). Boehning et al. (2004) demonstrated, with the aid of a yeast two-hybrid screen assay, the calcium-dependent high-affinity binding of

calmodulin to HO-2. Consequently, the catalytic activity of HO-2 was increased significantly in stimulated neurons presumably within milliseconds. 2) PKC and phorbol esters phosphorylate HO-2 and increase the HO-2 activity (Dore et al., 1999). 3) Selective phosphorylation of HO-2 by CK2 (formerly known as casein kinase 2) has been shown, which markedly augments the catalytic activity of HO-2 (Boehning et al., 2003). CK2 is activated by PKC. This activation process is much slower than the calcium-calmodulin-dependent activation of HO-2 (Boehning et al., 2004). 4) Glutamate, via activation of the metabotropic glutamate receptor (mGluR), stimulates HO-2 activity in neurons (Boehning et al., 2004). Nathanson et al. (1995) found that glutamate augmented endogenous CO production, which was abolished after inhibition of HO-2 and PKC. HO-2 is also activated by glutamate in cerebral vascular endothelium (Parfenova et al., 2001) and smooth muscle cells (Leffler et al., 2003), leading to increased CO production. This effect of glutamate, independent of cytosolic calcium, was abolished by inhibiting protein tyrosine kinase but potentiated by inhibiting protein tyrosine phosphatases (Leffler et al., 2003).

E. Down-Regulation of the Expression and Activity of Heme Oxygenases

HO-1 expression can be reduced by interferon- γ (Takahashi et al., 1999a) or hypoxia (Nakayama et al., 2000) in human glioblastoma, human umbilical vein endothelial cells, coronary artery endothelial cells, astrocytes, and many other human cell lines (Kitamuro et al., 2003). Bach1, a member of basic leucine-zipper factors, is a heme-regulated transcriptional repressor for the HO-1 gene (Kitamuro et al., 2003; Sun et al., 2002; Kitamuro et al., 2003). The reduced expression of HO-1 may help to preserve intracellular heme as an important substrate of certain heme proteins, and reduce energy expenditure for heme catabolism. It seems that the repression of HO-1 by hypoxia or heat shock is unique for many types of human cells, and the same hypoxia stimuli increase the expression of HO-1 in rodent cells (Shibahara et al., 2003). However, hypoxia does increase the expression of HO-1 in certain types of human cells, such as human retinal pigment epithelium (RPE) cells (Udono-Fujimori et al., 2004). Down-regulation of HO-2 expression has not been reported.

Whereas little is known about an endogenous HO activity inhibitor, an array of pharmacological blockers of total HO activity (HO-1 and HO-2) have been used as an invaluable tool in dissecting out the physiological role of endogenous CO. The most widely studied and used HO blockers are metalloporphyrins, including chromium mesoporphyrin (CrMP), manganese protoporphyrin, manganese mesoporphyrin, zinc protoporphyrin (ZnPP), tin mesoporphyrin, and tin protoporphyrin (SnPP) (Vreman et al., 1993). The majority of these metalloporphyrins are light sensitive. Therefore, by al-

tering lighting conditions one can either optimize the specificity of action and preserve the integrity of most metalloporphyrins or purposely inactivate these agents as internal controls. Depending on the species of metal cations that is linked to the porphyrin ring as well as the substitutions in the ring, the potency of metalloporphyrins varies (Vreman et al., 1993). By carefully choosing the concentrations, these metalloporphyrins can specifically inhibit HO without interacting with other cellular components. This is specifically relevant to the reported inhibition of NOS and soluble guanylyl cyclase (sGC) activities by metalloporphyrins (Luo and Vincent, 1994; Grundemar and Ny, 1997). For example, SnPP is 10 times more potent in inhibiting HO-2 activity than NOS or sGC activities when used at 7.5 μ M (Zakhary et al., 1996). Inhibition of muscle relaxation and suppression of cAMP and cGMP by the metalloporphyrins have also been reported (Ny et al., 1995), possibly due to the drug's interaction with membrane receptors or their downstream signal transduction pathways. The permeability of the blood-brain barrier to different metalloporphyrins varies. SnPP can easily pass the blood-brain barrier, whereas ZnPP cannot (Li and Clark, 2000b). This property will be important in determining the administration routes for applying metalloporphyrin in vivo for different purposes.

F. Biological Functions of Heme Oxygenases

1. Production of Carbon Monoxide, Biliverdin/Bilirubin, and Ferrous Iron. HO forms a complex with NADPH-dependent flavoprotein reductase (cytochrome P450 reductase) and biliverdin reductase (a cytosolic enzyme) on the endoplasmic reticulum (Maines, 1988). In the presence of functional HO, the porphyrin ring of heme (ferroprotoporphyrin IX) is broken and oxidized at the α -methene bridge, producing equimolar amounts of CO, ferrous iron, and biliverdin (Tenhunen et al., 1968; Maines, 1988; Ortiz de Montellano, 1998). The heme-CO metabolism pathway, as illustrated in Fig. 2, also requires the participation of NADPH and O₂. HO has an apparent K_m for O₂ of 12 mM in liver. Therefore, the presence of O₂ is required for HO activity, but even during severe hypoxia HO continues to produce CO. Cytochrome P450 reductase transfers electrons to the HO-heme complex. The process of endogenous CO production displays a wide color spectrum. Black heme breaks down to green biliverdin and colorless CO. Biliverdin complexes with iron until its final release. Yellowish bilirubin is generated from biliverdin, catalyzed by biliverdin reductase (Tenhunen et al., 1968).

Biliverdin and bilirubin are believed to be the most powerful endogenous antioxidants, efficiently scavenging peroxy radicals and inhibiting lipid peroxidation (Baranano et al., 2002; Stocker et al., 1987). Bilirubin protects cells from a 10,000-fold excess of hydrogen peroxide (Baranano et al., 2002; Dore et al., 1999; Llesuy and Tomaro, 1994; Stocker et al., 1987). Over production

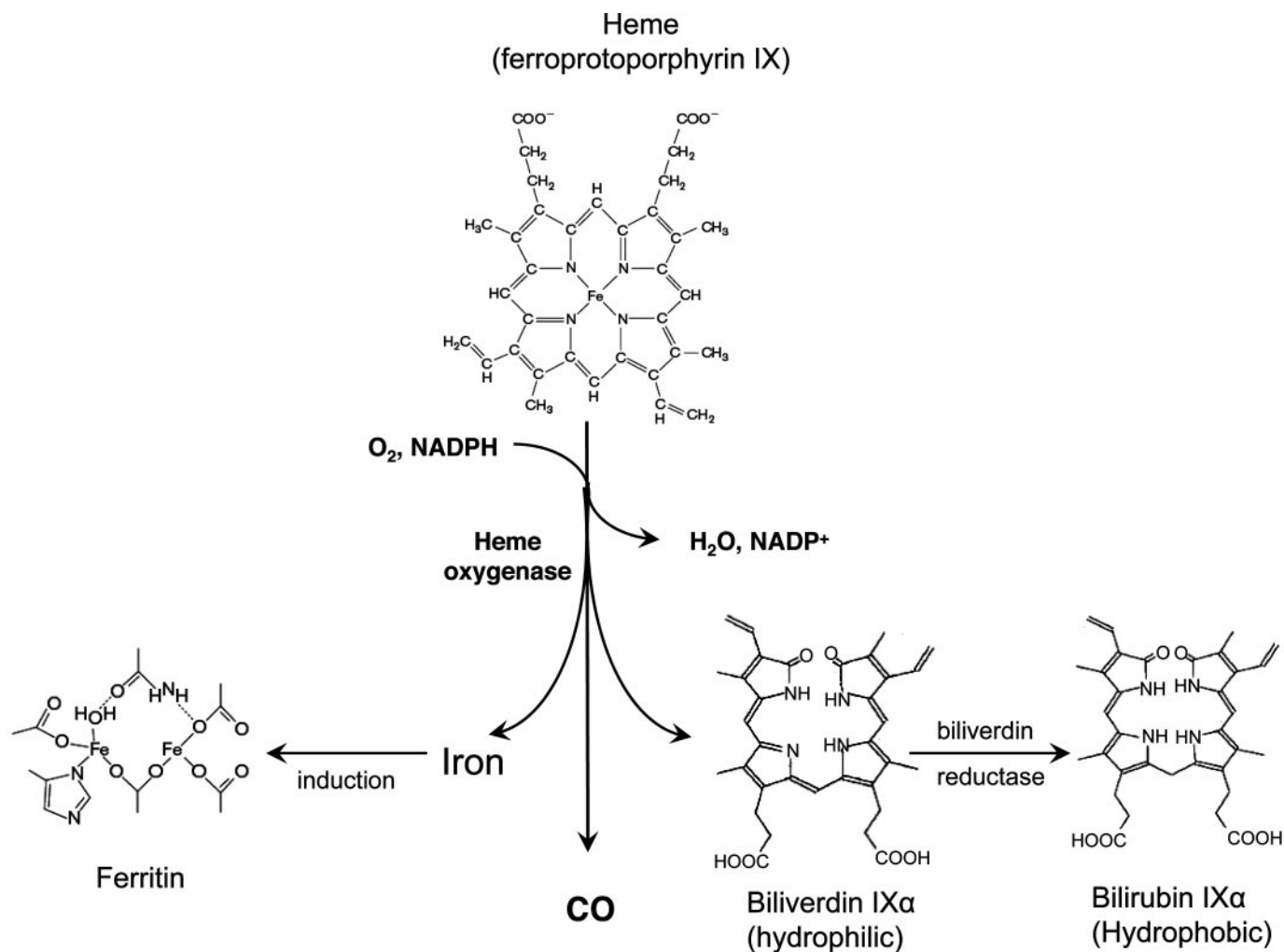


FIG. 2. HO-catalyzed heme metabolism pathway.

of bilirubin leads to jaundice, a clinical syndrome of hyperbilirubinemia. Beyond its antioxidant capability, biliverdin is also an endogenous inhibitor of sGC (Koglin and Behrends, 2002). At the micromolar range, biliverdin, but not bilirubin, inhibits the basal and NO-stimulated activity of recombinant $\alpha(1)/\beta(1)$ isoform of sGC without affecting the affinity of sGC for GTP.

Heme catabolism-generated ferrous iron, being an oxidant, stimulates the synthesis of ferritin (Eisenstein et al., 1991) through its regulatory protein binding and activation of iron response elements (Hanson et al., 1999; Pantopoulos et al., 1997). Ferritin, an intracellular iron repository, allows safe sequestration of unbound iron liberated from heme degradation. In this way, ferritin possesses additional antioxidant capabilities (Balla et al., 1992; 1995). It has been estimated that each apo-ferritin molecule (450 kD) can sequester about 4500 iron atoms (Harrison and Arosio, 1996). Modulation of intracellular iron stores and increased efflux of free iron has recently been suggested as a mechanism for the cytoprotective effects of HO-1 (Ferris et al., 1999). In this activity HO-1 cooperates with a recently identified Fe-ATP pump (Baranano et al., 2000). Once HO-1 was

knocked out, iron accumulated in the cells. The HO-1 knockout mice exhibit hepatosplenomegaly, lymphadenopathy and leukocytosis. Massive iron overload in the liver and kidneys in these mice leads to death at a young age (Poss and Tonegawa, 1997a; 1997b). Iron accumulation in the absence of HO-1 makes these animals much more susceptible to oxidative stress damage. Transfection of cells derived from HO-1 knockout animals with HO-1 cDNA restored the normal cellular iron levels in these cells (Ferris et al., 1999). Iron accumulation was also documented in the liver and kidney of the human HO-deficiency case (Yachie et al., 1999). Finally, a feedback mechanism between iron and HO-1 exists. Iron has been shown to regulate transcriptional expression of HO-1 and iNOS (Abraham et al., 1988; Baranano et al., 2002).

2. Heme Metabolism. Regulating cellular heme level is another important function of HO. After completing their life cycle of 120 days, red blood cells release hemoglobin into circulation. Haptoglobin captures free hemoglobin and transports it to the reticuloendothelial system in the spleen, liver, and bone marrow. The rapid transformation of hemoglobin to methemoglobin also oc-

curs, leading to the release of the incorporated heme. The free heme will then be carried by hemopexin or albumin to the reticuloendothelial system. In reticuloendothelial system, HO functions as the rate-limiting enzyme in further heme degradation (Abraham et al., 1988). HO activity in different types of cells is also largely responsible for the degradation of heme derived from denatured heme proteins other than hemoglobin.

Heme consists of ferrous iron complexed with the four porphyrin groups (Beri and Chandra, 1993). The organic synthesis of heme was realized as early as 1927 in Hans Fischer's laboratory (Watson, 1965). The full elucidation of all enzymes involved in heme synthesis in mammalian cells was not available until about 25 years later (Shemin and Wittenberg, 1951). Now we know that heme is synthesized in all human nucleated cells using glycine and succinyl CoA as the precursors. Involving 8 different enzymes, heme synthesis starts in the mitochondria, continues in the cytosol, and is completed in the mitochondria. The iron is eventually inserted by ferrochelatase. Heme can also be derived from the degradation of hemoproteins. This recycling process would be energy efficient without the need to start over again from glycine and succinyl-CoA.

The turnover of heme is rapid. For instance, cultured cerebellar granule cells consume 17% of the total heme pool to produce about 1 to 5 $\mu\text{mol CO}$ in 5 h (Ingi et al., 1996b). The physiological level of free heme in normal cells is below 1 μM (Sassa, 2004). At this concentration range, free heme down-regulates δ -aminolevulinic synthase and reduces the expression of Bach1. The latter will lift inhibition of HO-1 gene expression (Sassa, 2004).

Extracellular heme is transported into cell via a heme transporter (Worthington et al., 2001). Due to its lipophilic nature, heme readily moves around among different organelles (Ingi et al., 1996b) and it interacts with many cellular membranes and organelles, including lipid bilayers, mitochondria, cytoskeleton, nuclei, and several intracellular enzymes (Nath et al., 1998; Ryter and Tyrrell, 2000). Free heme at high concentrations catalyzes oxidative reactions to generate reactive oxygen species (Jeney et al., 2002), mainly due to the catalytic effect of heme. This would explain increased expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin on endothelial cells in the presence of high levels of heme (Wagener et al., 2003). The pro-oxidant effect of free heme is not due to the release of iron from heme molecule (Vincent et al., 1988) since heme induced more peroxidation of rat liver microsomal lipid in the presence of H_2O_2 than iron and that iron release is very low under the conditions employed. Increased oxidative stress affects a variety of substrates, including lipids, thiols, proteins, carbohydrates, and nucleic acids (Ryter and Tyrrell, 2000). Normal cellular functions would be disturbed and pathological cellular injuries exacerbated (Dennery et al., 1998; Ryter and Tyrrell, 2000). An ab-

normally high heme level is correlated with many diseases, such as nephrotoxin-induced renal injury (Agarwal et al., 1995). The elevated circulatory heme level results from either excessive filtration of heme proteins as would occur in rhabdomyolysis (Nath et al., 1992) or from the destabilization of intracellular heme proteins (e.g., cytochromes) in ischemia-reperfusion and nephrotoxin-induced renal injury (Balla et al., 1993; Agarwal et al., 1995; Shimizu et al., 2000). When free heme exceeds the physiological range, its cytotoxic role dominates its constitutive role in heme protein formation. Heme-responsive genes remain repressed when heme at low concentrations binds to Bach1 with MARE sequences (Ogawa et al., 2001). However, at higher concentrations heme inactivates the binding of Bach1, allowing access of transcription factors such as Nrf2 to interact with the MARE sequences (Ogawa et al., 2001). This in turn activates the heme-responsive gene. In this regard, intact HO activity is crucial for the removal of the prooxidant heme (Balla et al., 1991; Jeney et al., 2002). Cell lines derived from the HO-1 deficient human were strongly sensitive to the injury induced by hemin (Jeney et al., 2002).

3. Heme-Containing Protein and Heme-Binding Protein. Most mammalian cells contain a "free" heme pool, i.e., nonprotein bound heme, providing heme for the synthesis of heme-containing proteins and for CO production (Ponka, 1999). In this article, we refer to heme-containing proteins as heme proteins. As the prosthetic moiety for heme proteins, the availability of heme influences the metabolism of hemoglobin, myoglobin, cytochromes, prostaglandin endoperoxide synthase, NOS, catalase, peroxidases, respiratory burst oxidase, guanylyl cyclase, tryptophan dioxygenase, pyrrolases, and many others (Table 1). These heme proteins play important roles in regulating cellular functions, from oxygen delivery and mitochondrial respiration to signal transduction (Platt and Nath, 1998). Among heme proteins also are cyclooxygenase isoforms that need the heme prosthetic group for their catalytic activity (Smith and Marnett, 1991).

The function of HO is also linked to another class of protein, i.e., heme-binding proteins. These proteins, without heme in their molecular structure, have vivid affinities for it. As such, they regulate the availability of heme for the catalytic activity of HO and CO production as well (Table 2). Examples include HBP23 (Iwahara et al., 1995), and the glutathione *S*-transferases. Hemopexin, which has the highest affinity for circulatory heme of all heme-binding proteins (Muller-Eberhard, 1988), completely inhibited heme-catalyzed lipid peroxidation at concentrations slightly higher than that of heme, suggesting a unique role for this acute phase protein in antioxidant defense mechanisms. The protein itself was not oxidized, presumably because the putative bis-histidyl heme-hemopexin complex cannot interact with H_2O_2 . Rat and human

TABLE 1
Selective list of the potentially targeted heme proteins by CO

| Name of Protein | Effects of CO or HO Up-Regulation | Reference |
|--------------------------|-----------------------------------|--|
| Catalase | Inhibition | Hu and Kincaid (1992) |
| Cyclooxygenase | Inhibition | Alcaraz et al. (2003) |
| | Activation | Lee et al. (2001) |
| Cytochrome c | Binding | Bhuyan and Kumar (2002) |
| Cytochrome P450 | Inhibition | Estabrook et al. (1980) |
| Cytochrome c oxidase | Inhibition | Wohlrab and Ogunmola (1971) |
| Guanylyl cyclase | Activation | Stone and Marletta (1994) |
| Hemoglobin | Inhibition | Forster (1970) |
| Neuroglobin | Inhibition | Burmester et al. (2000) |
| Myoglobin | Inhibition | Coburn and Mayers (1971) |
| Cytoglobin | Inhibition | Geuens et al. (2003); Sawai et al. (2003) |
| NADPH oxidase | Inhibition | Taille et al. (2004) |
| NO synthases | Inhibition | Willis et al. (1995); Thorup et al. (1999) |
| Peroxidases | Binding | Carlsson et al. (2005) |
| Prostaglandin H synthase | Binding | Lou et al. (2000) |
| Tryptophan dioxygenase | Inhibition | Piantadosi (2002) |
| NPAS2 | Inhibition | Dioum et al. (2002) |

TABLE 2
Selective list of heme-binding proteins

| Name of Protein | References |
|----------------------------------|--------------------------|
| Albumin | Monzani et al. (2001) |
| Hemopexin | Baker et al. (2003) |
| Haptoglobin | Wang et al. (2001b) |
| HO-1 | Maines (1997) |
| HO-2 | Wagener et al. (2003) |
| HO-3 | Wagener et al. (2003) |
| Liver fatty acid-binding protein | Vincent et al. (1988) |
| Glutathione S-transferase | Immenschuh et al. (1995) |
| Heme-binding protein 23 | Iwahara et al. (1995) |
| P22 heme-binding protein | Immenschuh et al. (1995) |
| Hap 1 | Hon et al. (1999) |
| BK _{Ca} channel (?) | Tang et al. (2003) |

albumin and rat glutathione *S*-transferases, proteins with moderate affinities for heme, decreased heme-catalyzed lipid peroxidation in a dose-dependent manner but were subject to oxidation. Bovine albumin and rat liver fatty acid-binding protein have lower affinities for heme. These proteins enhanced, instead of inhibiting, lipid peroxidation. In short, heme-binding proteins may enhance, decrease, or completely inhibit heme-catalyzed oxidations and in doing so the proteins themselves may be oxidized depending upon their relative affinities for heme, the nature of the amino acids in the vicinity of the bound catalyst, and the availability of a free coordination site on the iron.

In summary, the levels and activities of heme, HO, and CO are interrelated closely (Fig. 3). HO-2 may function as a physiological regulator of cellular functions via controlling the size of the free heme pool and providing physiologically important heme metabolites. The elevated heme level itself presents a pro-oxidant threat. The cell copes with this threat by up-regulating HO-1 expression. The latter degrades heme to increase the production of CO. The role of HO-3 in heme degradation is very limited. It may function as a heme sensing or heme binding protein (McCoubrey et al., 1997b). Thus, an appropriate heme level is reached and cellular homeostasis maintained.

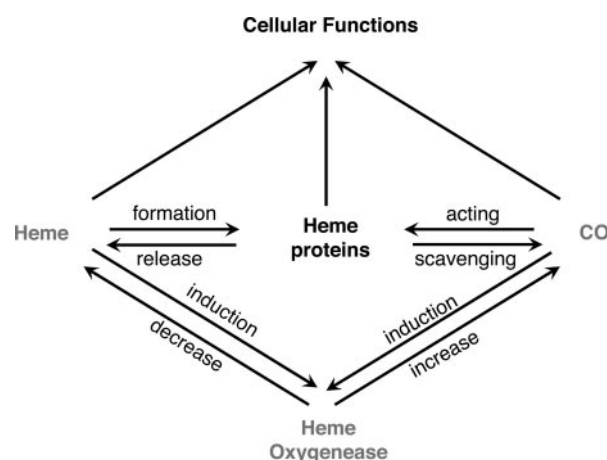


FIG. 3. Interplay among heme, HO, and CO on cellular functions.

G. Nonheme Sources of Carbon Monoxide Production

Heme-independent sources for endogenous CO production have been reported (Rodgers et al., 1994). In comparison with HO-catalyzed reactions, heme-independent CO production is minor and its physiological importance has not been properly evaluated. The CO thus formed diffuses into the blood, is carried via hemoglobin, and excreted in the lungs.

Phenobarbital and diphenylhydantoin enhanced CO production in humans (Coburn, 1970a) and so did progesterone (Delivoria-Papadopoulos et al., 1974). CO production during auto- and enzymatic oxidation of phenols was also reported (Miyahara and Takahashi, 1971). Photo-oxidation of organic compounds is another source of endogenous CO production (Rodgers et al., 1994). Iron-ascorbate-catalyzed lipid peroxidation of microsomal lipids and phospholipids results in endogenous CO production, a process usually linked to oxidative stress (Nishibayashi et al., 1968; Vreman et al., 1998; Wolff and Bidlak, 1976). Reduction of cytochrome *b*₅ is accompanied by emergence of the absorption peak at 450 nm (Archakov et al., 1975). This likely reflects the yield of endogenous CO formation during lipid peroxidation. Ar-

chakov et al. (2002) further investigated the CO production from rabbit liver microsomes during iron-dependent lipid peroxidation, induced by phenobarbital (a specific inductor of cytochrome P450 2B4). This CO production was NADPH-dependent and required Fe^{2+} . Whereas inhibition of HO activity with Zn-PP did not alter the CO production, addition of cytochrome P450 inhibitors SKF 525A and metyrapone reduced CO formation rate. Furthermore, there was no heme destruction in microsome preparation used in this study. Desferal and α -tocopherol potently inhibit lipid peroxidation via acting on the protein bound Fe^{2+} in microsomes. After microsomes were treated with these two antioxidants, CO production decreased, accompanied by a decline in the formation rate of malonyldialdehyde (MDA), an end product of lipid peroxidation. These data convincingly demonstrated lipid peroxidation-induced, but not heme-dependent, CO production.

Endogenous production of CO from lipid peroxidation is ubiquitous to many types of cells, including brain, kidney, lung, spleen and blood (Vreman et al., 1998). Although no CO production from lipid peroxidation has been detectable in rat liver and heart (Vreman et al., 1998), CO thus generated was observed in rabbit (Archakov et al., 2002) and mouse (Usami et al., 1995) hepatic microsomes.

IV. Catabolism of Endogenous Carbon Monoxide

A. Expiration

The majority of CO from systemic production is exhaled through the lung. CO rapidly diffuses across the alveolar–capillary membrane, a process influenced by alveolar gas volume, ventilation, and the concentration of hemoglobin in the pulmonary capillaries (Coburn and Forman, 1987). The local partial pressures of both CO and O_2 determine the cellular concentrations of CO since these two gases compete for the same iron or copper binding sites. Exhaled CO from humans has been recently used as a novel noninvasive indication of heme metabolism and the related endogenous levels of CO and bilirubin. The end-tidal breath CO, corrected for ambient CO, has been clinically used for understanding the mechanisms of jaundice in healthy term infants in a variety of conditions (Stevenson et al., 1994; Okuyama et al., 2001). Seshadri et al. (2003) monitored exhaled CO in patients with advanced ischemic and nonischemic cardiomyopathy. It was found that exhaled CO was lower in patients with cardiomyopathy at rest and immediately after exercise than healthy people. A lower CO production in these patients was thus indicated.

B. Scavenging

Under normal conditions, most of our body CO is endogenously generated from HO-catalyzed heme metabolism. Also contributing to our body CO store are exogenously inhaled ambient CO gas or xenobiotics

which is metabolized to CO by cytochrome P450 in the liver (Kubic and Anders, 1978). CO is bound to hemoglobin in the red blood cells as COHb (80%). Other intracellular heme proteins are responsible for the residual CO loading (Coburn, 1970b). The CO body stores are exchangeable. It was suggested that during hypoxia more CO would move from blood to tissue where CO binds to heme proteins (Coburn and Mayers, 1971). However, this redistribution of CO has not been demonstrated under physiological conditions.

C. Oxidation

Oxidation of CO in mammalian tissues under physiological conditions has not been shown. It has been believed, however, for a long time that a small metabolic endpoint of CO in living tissues is the oxidation to CO_2 (Fenn and Cobb, 1932). The catalyzation of this process by reduced cytochrome *c* oxidase in mitochondria was reported in 1965 (Tzagoloff and Wharton, 1965) and later confirmed by many other laboratories (Young and Caughey, 1986; Young et al., 1979; Vijayasathy et al., 1999). It seems that whether the interaction of CO with cytochrome *c* oxidase leads to ferrous carbonyl formation or the formation of CO_2 depends on the CO/O_2 ratios. The latter determined molecular configuration of the oxidase (Young and Caughey, 1990). The oxidation of CO in vivo is much slower than the rate of endogenous CO production. The rate of CO oxidation, however, increases in proportion to tissue CO store (Luomanmaki and Coburn, 1969). In microbes living on CO, CO is oxidized to CO_2 in the presence of CO dehydrogenase (Ragsdale, 2004). The oxidation or oxygenation of CO to CO_2 is also well appreciated in chemistry, e.g., the metal-catalyzed water gas shift reaction generates H_2 and CO_2 from CO and H_2O , but this CO oxidation also requires temperatures beyond physiological tolerance of most organisms. CO in the atmosphere can react with hydroxyl radicals to yield HO_2 (hydroperoxyl radical) and CO_2 (Allen and Root, 1957).

V. Physiological Roles of Carbon Monoxide

A. Carbon Monoxide and Circulatory System

1. Cardiac Function. The cardiac distribution of HO-1 has been largely detected in heart vascular wall, not in cardiomyocytes. The expression level of HO-1 proteins in normal myocardium often falls below the detectable level by Western blot (Nishikawa et al., 2004; Lakkisto et al., 2002). However, HO-1 protein expression is significantly up-regulated by hemin or other pathological stimuli, such as myocardial infarction (Lakkisto et al., 2002). Hypoxia-induced up-regulation of HO-1 in heart also increased CO production. The latter improves cardiac blood supply by relaxing vascular tone in heart, constituting a cardiac defense mechanism (Grilli et al., 2003). CO perfusion reduced the contractility of isolated rat papillary muscle (Liu et al., 2001).

Clark et al. (2003), using a CO carrier, demonstrated that CO protected myocardial cells and isolated rat hearts against ischemia-reperfusion (I/R) injury as well as cardiac allograft rejection in mice. Exogenous CO also limited ischemia-reperfusion injury in vivo in a mouse model of myocardial infarction (Guo et al., 2004). Hearts from HO-1 knockout mice have greater susceptibility to I/R injury (Yoshida et al., 2001). Cardiac-specific overexpression of HO-1 leads to attenuated myocardial injury after I/R in transgenic mice (Yet et al., 2001).

Two HO-2 homologous transcripts (1.9 and 1.3 Kb) and HO-2 proteins are constitutively expressed in the atrium, ventricles and descending aorta (Ewing et al., 1994). HO-2 is also expressed in intracardiac neurons (Hassall and Hoyle, 1997). Therefore, cardiac CO production can be catalyzed by HO-2 from different types of cells, cardiomyocytes, vascular smooth muscle cells, and neurons.

2. Vascular Contractility. The phenomenon of CO-induced vasorelaxation was originally unmasked in 1984 when McGrath and Smith showed the relaxation of rat coronary artery in response to exogenous CO. It was quickly demonstrated in the following years that the vasorelaxant effect of exogenous CO was not mediated by endothelium (Coceani et al., 1988; Graser et al., 1990; Vedernikov et al., 1989). The vasorelaxant effect of endogenous CO was indicated as inhibition of HO activity with metalloporphyrins either increased the perfusion pressure of isolated rat liver (Suematsu et al., 1994) or decreased the diameter of pressurized gracilis muscle arterioles (Kozma et al., 1999). Pretreatment of rat tail artery tissue with hemin to promote HO activity suppressed phenylephrine-induced vasoconstriction in a time- and concentration-dependent manner (Wang et al., 1997b). Moreover, hemin-dependent vasorelaxation was abolished after the tail artery tissues were coincubated with either oxyhemoglobin to scavenge CO or ZnPP to inhibit HO, suggesting that hemin increased endogenous production of CO (Wang et al., 1997b). Mounting evidence now demonstrates that the CO-induced vascular relaxation is ubiquitous (Wang, 1998). The long list of CO-relaxed vascular tissues includes rat tail artery (Wang, 1996; Wang et al., 1997a), lamb ductus arteriosus (Coceani et al., 1988), rat and rabbit thoracic aorta (Lin and McGrath, 1988; Furchgott and Jothianandan, 1991), canine carotid, coronary and femoral arteries (Vedernikov et al., 1989), rat coronary artery (McGrath and Smith, 1984), guinea pig coronary artery (Gagov et al., 2003), porcine coronary artery and vein (Graser et al., 1990), rat hepatic vein (Pannen and Bauer, 1998), piglet mesenteric artery (Villamor et al., 2000) and pial arterioles (Leffler et al., 1999), and many other types of blood vessels (for review see Ndisang et al., 2004).

Exogenous CO had no effect on the contractile status of canine or rabbit basilar and middle cerebral arteries (Brian et al., 1994). Extramural arteries of

pig bladder (Werkstrom et al., 1997) and hepatic arteries from rats (Pannen and Bauer, 1998) were also resistant to CO modulation. Whether the lack of vasoactive effect of CO on these blood vessel preparations is due to species/tissue type differences or to the variances in the experimental conditions has not been made clear. Another unique vascular reaction to CO was observed in rat gracilis muscle arterioles. In this preparation both exogenous and endogenous CO induced vasoconstriction (Johnson and Johnson, 2003; Kozma et al., 1999).

The vascular effects of CO on pulmonary circulation are variable. In response to hypoxia, systemic blood vessels dilate, but pulmonary vessels constrict. Ventilation with CO prevents hypoxic pulmonary vasoconstriction in dogs (Miller and Hales, 1979). In contrast, low concentrations of CO do not alter the hypoxia induced pulmonary vasoconstriction in isolated rat lungs (Cantrell and Tucker, 1995). Penney et al. (1992) showed that after exposure of neonatal rats to CO the development of hypoxic pulmonary hypertension was delayed.

3. Platelet Aggregation and Monocyte Activation.

Earlier studies showed that exposure of minipigs to 160 and 185 ppm CO significantly increased platelet aggregation. Adhesions of shape-changed platelets on the arterial endothelium were revealed under scanning electron-microscopy after elevating CO concentration to 420 ppm (Marshall and Hess, 1981). However, later studies mostly showed inhibition of platelet aggregation by CO. Platelet release of ADP and serotonin was inhibited by CO (Mansouri and Perry, 1984). The inhibitory effect of CO on platelet aggregation was mainly mediated by activation of the cGMP pathway. It seems that in platelets, CO had no effect on cytochrome P450 proteins, cyclooxygenase or 12-lipoxygenase, arachidonate, intracellular calcium levels, or phospholipase C (Brune and Ullrich, 1987).

Monocytes function at the front line of self-defense. As either innate or adaptive antigen-specific responses, monocytes ingest opsonized pathogens, digest and process foreign antigens, and release inflammatory effector cytokines. Yachie et al. (2003) reported that freshly isolated monocytes express HO-1 under basal conditions and with in vitro stimulation. Previous reports also showed that CO enhanced the production of proinflammatory cytokines and interleukin-10 from monocytes (Otterbein et al., 2000). Together, it is reasonable to suggest that HO-1 up-regulation increases CO production in monocytes, and the latter functions as an anti-inflammatory mediator.

B. Carbon Monoxide and Nervous System

More often than not, the literature highlights the involvement of different HO isoforms in the production of CO. How much difference does it make whether CO is produced by HO-1 or HO-2? It is true that CO is CO, no matter where it comes from or which enzyme is involved

in its production. It is also true that a large difference can be perceived because the availability under different conditions, the tissue distribution patterns, the enzymatic kinetics and affinities to heme of HO-1 and HO-2 are different. The central and peripheral nervous systems serve as good examples in this regard. HO-2 is widely and predominantly expressed in the brain and plays a major role in neuronal production of CO. For example, HO-2 is present in mitral cells in the olfactory bulb, pyramidal cells in the cortex and hippocampus (Verma et al., 1993; Ewing and Maines, 1997), granule cells in the dentate gyrus, many neurons in the thalamus, and hypothalamus, cerebellum and caudal brainstem (Vincent et al., 1994). HO-2 expression has the potential to protect these neurons by reducing lipid peroxidation via the catabolism of free heme. On the other hand, at different developmental stages or in discrete brain regions HO-1 expression and its role in neuronal CO production cannot be ignored (Bergeron et al., 1998; Nimura et al., 1996). Weak immunoreactivity of HO-1, for example, has been detected in some single cortical satellite cells in the whole cortex and in the pyramidal layer of the hippocampus. In the stratum oriens of CA3 and in the PoDG, much stronger immunoreactivity of HO-1 was found (Bidmon et al., 2001). HO-1 protein has been localized to selective neuronal and non-neuronal cell populations in forebrain, diencephalon, cerebellum, and brainstem regions (Ewing et al., 1992). The importance of HO-1 expression in brain becomes even more noticeable under certain pathophysiological conditions.

1. *Hypothalamic-Pituitary-Adrenal Axis.* Speculation that CO might influence neurotransmission like NO was made based on the neuronal location of HO and its colocation with NOS in the central nervous system (Verma et al., 1993). The hypothalamic-pituitary-adrenal (HPA) axis constitutes an important stress response effector pathway for preservation of an organism's internal homeostasis. In response to different stressors, neurons in the paraventricular (PVN) and supraoptic nuclei of the hypothalamus, the brain area that controls and initiates the HPA response, synthesize corticotropin releasing factor (CRF) and arginine vasopressin (AVP) and then release these peptides into the hypothalamo-hypophyseal portal vessels of the median eminence. At the anterior pituitary, CRF in conjunction with AVP stimulates the synthesis of pro-opiomelanocortin (POMC) and release of the POMC-derived adrenocorticotrophic hormone (ACTH). ACTH is transported via the systemic circulation to the adrenal gland, where it acts on the adrenal cortex to stimulate the synthesis and release of glucocorticoids. The important role of CO in the modulation of HPA response has been described previously (Mancuso et al., 1997b; Snyder et al., 1998). HO-2 expression (Vincent et al., 1994; Weber et al., 1994) was found in hypothalamic nuclei known to control the release of CRF/AVP. Mancuso et al. (1997a) reported that endogenous CO inhibited the release of AVP from rat

hypothalamus. CO also inhibited the hypothalamic secretion of oxytocin (Kostoglou-Athanassiou et al., 1996). The secretion of gonadotropin releasing hormone seems to be stimulated by CO in vitro (Lamar et al., 1996). However, this observation cannot be confirmed by in vivo study during the estradiol-induced luteinizing hormone surge (Kohsaka et al., 1999).

Using cultured primary rat hypothalamic cells, Parkes et al. (1994) showed that treatment of cells with 100% gaseous CO or with hematin (100 μM), a heme analog, significantly increased basal secretion of CRF. ZnPP (0.3–100 μM), a selective inhibitor of CO formation, decreased CRF secretion in a dose-dependent manner. Using acute rat hypothalamic explants, Pozzoli et al. (1994) reported inhibition of KCl-stimulated CRF release by hemin with a maximal effect at 1 μM , whereas hemin had no effect on basal CRF secretion. ZnPP had no effect on basal or stimulated CRF release up to a maximal dose of 10 μM . When hemin and ZnPP were given together, hemin-induced inhibition of CRF release was completely antagonized by the enzyme inhibitor. Tissue preparation aside, the concentration of CO and CO precursor seems to be a determining factor in these different observations. At low and more physiological concentrations, CO seems to inhibit CRF release, whereas at high and probably toxic levels, CO stimulates CRF release.

Systemic or intracerebroventricular (icv) injections of HO inhibitor attenuated ACTH response to different stressors (Turnbull et al., 1998; Kim and Rivier, 2000). For instance, prior icv injection of SnPP (20–25 μg) in Sprague-Dawley (SD) rats (180–220 g) attenuated electroshock-induced ACTH release and concurrently decreased HO activity in the hypothalamus, but not in the anterior pituitary (Kim and Rivier, 2000). However, the proposed role of hypothalamus-generated CO in ACTH release is not without doubt. The basal ACTH release level was not affected by SnPP, indicating that the basal CO level would not be sufficient to release ACTH. Within 1 to 2 h of electroshock stimulation, the plasma ACTH level was significantly increased. Considering the time course of ACTH release upon stress stimulation, increased activity of existing HO proteins, rather than HO expression up-regulation, likely occurred. However, this notion was inconclusive since hypothalamus HO activity was not changed by electroshocks (Kim and Rivier, 2000), indicating that increased ACTH release may not be due to increased HO activity, nor to CO. Furthermore, since the whole hypothalamus was used in the HO activity assay, the specific change in HO activity in the PVN nucleus may be masked. Given all these considerations, the mechanism for the interaction of icv-injected SnPP with ACTH release is still unknown and whether or not CO acts on the hypothalamus remains to be determined.

In the isolated rat hypothalamus, endotoxin decreased AVP secretion. However, with inhibition of HO or in the presence of CO scavenger endotoxin stimulated AVP

secretion (Kostoglou-Athanassiou et al., 1998). Whereas the mechanisms for these observations are open to debate, a neuroendocrine role of CO is supported. Endotoxin may stimulate endogenous pathways that lead to the generation of CO, which modulates the release of AVP.

HO activity has been assessed in rat adrenal gland. The expression of different isoforms of HO and the regulation of HO activity in adrenal gland were recently investigated (Pomeraniec et al., 2004). Both HO-1 and HO-2 proteins were expressed in zona fasciculata adrenal cells as well as Y1 cells. The latter were derived from murine adrenal cortex and behaved like normal steroidogenic cells. These cells produce steroid in response to ACTH in a cAMP-dependent pathway (Schimmer 1979). Only HO-1 expression was up-regulated by ACTH in a time- and concentration-dependent fashion in zona fasciculata adrenal and Y1 cells. Accumulation of HO-1 proteins under these conditions correlated with an increase in HO activity as increased bilirubin production. Bilirubin increase reflects an equimolar increase in CO production, although such an increased endogenous CO production was not directly measured. After application of HO-inhibitor SnPP to these cells, HO activity was abolished both in the absence or presence of ACTH and steroid production was significantly increased. Since adrenal steroidogenesis in rat adrenal cells is not regulated by cGMP and endothelial NOS activity (the only isoform detected in Y1 cells) was not inhibited by SnPP at the given concentration, a nonspecific effect of SnPP in this case can be excluded (Pomeraniec et al., 2004). These indirect lines of evidence suggest an inhibitory role of HO/CO for steroid production.

2. *Glia.* High levels of HO-1 mRNA have been reported in rat astrocytes (Scapagnini et al., 2002a). HO-1 proteins, however, were hardly detectable in astrocytes within one week of primary culture. The faint immunoreactivity of HO-1 proteins was primarily localized in the cytosol and the perinuclear region. As the primary culture was prolonged to 14 and 21 days, an abundant expression of HO-1 proteins in astrocytes was detected in the nucleus and nucleoli (Li Volti et al., 2004). It seems that the dynamic pattern of HO-1 expression in astroglial cells may give this protein an important role in brain development and neurodegenerative diseases (Li Volti et al., 2004). For example, the expression of HO-1 in astrocytes was very weak in mouse hippocampus. A significant up-regulation of HO-1 expression was detected 2 weeks after traumatic brain injury in microglia/macrophages and reactive astrocytes in areas adjacent to the site of cortical impact (Chang et al., 2003). In human brains following traumatic injury, accumulation of HO-1 plus microglia/macrophages at the hemorrhagic lesion were detected as early as 6 h post-trauma and were still pronounced after 6 months (Takeda et al., 2000). Similar up-regulation of HO-1 expression in glia was reported in adult rat brain after experimental sub-

arachoid hemorrhage (Matz et al., 1996). After injections of lysed blood, whole blood, or oxyhemoglobin into the cisterna magna, increased HO-1 immunoreactivity was observed in all of the cortex, hippocampus, striatum, thalamus, forebrain white matter, and in the cerebellar cortex. HO-1-positive cells were predominately microglia and to a lesser degree, astrocytes (Beschoner et al., 2000). It seems that, following cell lysis or hemorrhage, heme induces the transcription of HO-1 in microglia.

3. *Circadian Rhythm Control.* Circadian clocks can quickly respond to environmental variations, including light, oxygen, redox potential, and physical activity (Rutter et al., 2002). It has been reported that CO participates in the regulation of circadian rhythms (Artinian et al., 2001). The major circadian clock in mammals is the suprachiasmatic nucleus (SCN), which can be altered by the muscarinic receptor-mediated activation of sGC and cGMP synthesis. Hemin-stimulated HO activity mimicked cholinergic effects on circadian timing. Inhibition of HO activity, or scavenging endogenous CO, blocked cholinergic clock resetting as well as cholinergic stimulation of cGMP synthesis. The modulatory effect of CO on circadian clocks can also be regulated by its interaction with transcription factors, such as NPAS2 (neuronal PAS domain protein) (Dioum et al., 2002). While NO fails to bind to NPAS2 at physiologically relevant concentrations, CO binds to the heme-containing, but not heme-depleted, NPAS2 with a dissociation constant of about 1 μ M. As such, CO inhibits the DNA binding activity of NPAS2. Since CO production is synchronized with the circadian rhythm of heme metabolism (Dioum et al., 2002), the interaction of CO with NPAS2 functions as one of the underlying molecular mechanisms of circadian rhythm control.

4. *Odor Response Adaptation.* Endogenous production of CO in cultured rodent olfactory neurons was demonstrated as the generation of 14 C (Ingi and Ronnett, 1995). This CO production was associated with increased cGMP production, which was reversed by inhibition of HO activity (Ingi and Ronnett, 1995). Decreased CO production was observed in the presence of TGF- β , an olfactory neurogenic factor. CO regulates long-term adaptation to odorant stimulation in olfactory neurons via its effect on cGMP levels, which represents a long-term slow action of CO (Zufall and Leinders-Zufall, 1997; Leinders-Zufall et al., 1995). In the presence of ZnPP, basal cGMP levels in primarily cultured olfactory neurons were reduced and the generation of cGMP in response to odorants was abolished as well (Verma et al., 1993). The similar odorant adaptive effect of CO has also been observed in olfactory interneurons in a terrestrial mollusk (Gelperin et al., 2000).

5. *Nociception and Chemoreception.* The role of the HO-2/CO system in nociception regulation has been shown in models of neuropathic and incisional pain. When exposed to these chronic pain stimuli, HO-2 $^{-/-}$ mice exhibited significantly less hyperalgesia and allo-

dynia than the wild type mice (Li and Clark, 2003). Systemic application of SnPP, a blood-brain barrier-permeable HO inhibitor, also significantly reversed pain development in these models (Li and Clark, 2000b). On the other hand, systemic injection of the blood-brain barrier-impermeable HO inhibitor ZnPP had little analgesic activity in these models. Together, these results indicate that an increase in spinal cord HO activity at least partially underlies the allodynia and hyperalgesia seen in rat models of neuropathic and incisional pain.

CO is also a signaling molecule in carotid body oxygen chemoreception. HO-2 has been located in glomus cells. The production of CO in carotid body critically relies on the availability of molecular oxygen. Once generated, CO seems to inhibit carotid body sensory activity. During hypoxia, the biosynthesis of CO would be decreased and the sensory discharge would be increased (Prabhakar, 1999).

6. Thermal Regulation. After rat brains were hyperthermally stimulated, HO-1 expression at the mRNA level increased 20-fold within 1 h, and HO-1 protein expression increased 4-fold within 6 h. This increased HO-1 protein expression mainly and intensively occurred in glia throughout the brain, ependyma lining the ventricles of the brain, paraventricular nucleus, Purkinje cell layer of the cerebellum, and cochlear nucleus of brainstem (Ewing et al., 1992). Whether this fast and significant up-regulation of the HO-1/CO system during hyperthermia can offer neuroprotection is not clear yet.

7. Learning, Memory, and Behavior. Long-term potentiation (LTP) is one of the cellular mechanisms underlying learning and memory. Mostly occurring at postsynaptic sites, LTP is also manifested as changes in presynaptic neurotransmitter release. Functional studies suggest that CO may act as a retrograde messenger, which links post- and presynaptic sites in LTP. This notion is derived from the observation that metabotropic receptor activation in the brain regulates the conductance of specific ion channels via a cGMP-dependent mechanism that is blocked by HO inhibitors (Glaum and Miller, 1993). Application of HO inhibitors prevented induction of LTP (Stevens and Wang, 1993). Direct application of CO, synchronized with weak tetanic stimulation, to hippocampal slices increased the evoked synaptic potentials. A long-term activity-dependent presynaptic enhancement during LTP was also induced by CO (Zhuo et al., 1993). It should be noted that HO-2-generated CO is not the sole retrograde messenger in LTP since LTP remained unaltered in HO-2 knockout mice (Poss et al., 1995).

Behavioral importance of endogenous CO has been examined in HO-2 knockout mice. These mice manifested normal olfactory ability, motor coordination, motor strength, and visual activity (Burnett et al., 1998). Knocking out HO-2 in mice also did not change LTP in hippocampal slices from these animals (Poss et al., 1995). On the other hand, an open field model of “anxi-

ety” measurement, which tests an animal’s reluctance to move around in an area, shows that HO-2 knockout mice have increased movement. These mice also display less “fear of falling” when suspended from a wire.

8. Vision. Cao et al. (2000) immunocytochemically localized HO-2 to retina. In turtles, HO-2-like immunoreactivity (-LI) was found in all photoreceptors, some amacrine cells, and in numerous bipolar and ganglion cells. HO-2-LI colocalized with sGC activity in many cells. In rats, HO-LI was found only in the inner retina, in ganglia and amacrine cells. Stimulation with CO alone primarily increased cGMP-LI in turtle bipolar cells in the visual streak. Coapplication of CO and NO dramatically increased cGMP-LI throughout the retina, more so than the changes seen with NO or CO alone. These data suggest that CO is an endogenous modulator of the sGC/cGMP signaling pathway in many retinal neurons, and can dramatically amplify NO-stimulated increases in cGMP. Local sGC function at different retinal layers is differentially modulated by CO stimulation as the NO availability of these layers is different. Based on the changes in immunoreactivities of the mAb3221 (an anti-sGC monoclonal antibody) induced by application of HO inhibitor ZnPP and NOS inhibitor L-NAME, Kajimura et al. (2003) demonstrated that endogenous CO levels increased basal sGC activity in external limiting membrane and optic fiber layers. It is the housekeeping level of endogenous NO, not CO, which activates sGC at the inner nuclear layer and inner plexiform layer. Furthermore, 24 h green light exposure significantly up-regulated the expression of HO-1 in Muller’s glia cells and in RPE. Correspondingly, sGC activity was increased in the external limiting membrane, but reduced in the inner nuclear layer and inner plexiform layer.

The RPE forms a single cell layer, separating retinal photoreceptors from the vascular-rich choroids. RPE is critical for the visual function and survival of the photoreceptors. These cells are usually exposed to great oxidative stress from light exposure and during active phagocytosis of shed outer segments. The expression of HO-1 in RPE (Schwartzman et al., 1987; Hunt et al., 1996) provides an antioxidative protective and survival mechanism for RPE.

CO inhibited a K^+ channel current in rabbit corneal epithelial cells (Rich et al., 1994). The expression of HO-1 in these cells has been demonstrated (Neil et al., 1995). Furthermore, endogenous generated CO in retina may be involved in dark adaptation and light sensitivity (von Restorff and Heibisch, 1988).

9. Hearing. Local production of CO in the human middle ear has been indicated as the immunoactivity of HO-1 and HO-2 was identified in human middle ear epithelium (Andersson et al., 2002). The physiology of gas exchange across middle ear mucosa results in the difference in the gas mixture of the middle ear from that of the atmosphere. Changes in gas composition and pres-

sure would alter the physiological conditions in the middle ear cavity. Whether or not locally produced CO regulates the cellular response of the middle ear and affects hearing function has not been studied.

10. Peripheral Autonomic Nervous System. CO is one of the two signaling molecules for inhibitory NANC neurotransmission, NO being the other. Rattan and Chakder (1993) demonstrated that CO caused a concentration-dependent and tetrodotoxin-resistant fall in the resting tension of the isolated opossum IAS. This relaxant effect of CO is due to the increase in tissue cGMP levels. IAS relaxation induced by NANC stimulation or vasoactive intestinal polypeptide (VIP) was suppressed by ZnPP, as was the increase in cGMP in response to NANC nerve stimulation. Furthermore, IAS exhibited significant HO activity, which was increased by NANC nerve stimulation and VIP. Since ZnPP did not alter the IAS responses to NO, CO, or isoproterenol, it seemed that NANC stimulation released CO via activation of HO, and the latter enhanced cGMP production in IAS smooth muscles. A relaxation was consequently induced. Another piece of proof of concept for this scheme was that CO relaxed the isolated smooth muscle cells. This pioneer study indicated the signaling role of CO in NANC neurotransmission. Subsequent studies using HO-2 knockout mice more directly addressed the endogenous CO involvement in NANC neurotransmission. NANC neurotransmission as well as cGMP levels in the intestine were reduced by 50% in HO-2 knockout mice (Zakhary et al., 1997; Xue et al., 2000). This reduction was rescued by adding exogenous CO. Furthermore, the resting membrane potential in jejunal smooth muscle cells from HO-2 knockout mice was depolarized and the inhibitory NANC junctional potential was decreased (Xue et al., 2000).

HO-2 knockout mice are deficient in their NANC neurotransmission in the urogenital system (Burnett et al., 1998). This leads to the notion that the neuronal mediation of ejaculation also involves CO.

C. Carbon Monoxide and Respiratory System

CO inhalation leads to tissue hypoxia by binding to hemoglobin and reducing the oxygen carrying capacity of blood. Paradoxically, inhaling CO also reduces the pulmonary ischemia damage. Inhalation of CO (0.1%) inhibited the hypoxic induction of plasminogen activator inhibitor in HO-1 knockout mice, resulting in higher activity of tissue plasminogen activator and decreased fibrin deposition and lung inflammation (Fujita et al., 2001). The synthesis of surfactant by cultured human type II pneumocytes was inhibited by exogenously added CO, as indicated by decreased D-¹⁴C]glucose incorporation into phosphatidylcholine (Arias-Diaz et al., 1997). The inhibitory effect of endogenous CO on surfactant synthesis was demonstrated as hemin increased CO and cGMP production and decreased phosphatidylcholine synthesis. Inhibition of HO activity with ZnPP also an-

tagonized the excitatory effect of TNF- α on cGMP and phosphatidylcholine synthesis (Arias-Diaz et al., 1997).

Overexpression of HO-1 in human lung epithelial cells decreased cell growth and protected cells against hyperoxia-induced cell death (Lee et al., 1996). Gene transfer using a recombinant adenovirus containing the rat HO-1 cDNA in rat lung after intratracheal administration also reduced hyperoxia-induced lung injury in vivo (Otterbein et al., 1999).

One essential event in the development of pulmonary fibrosis is the apoptotic death of respiratory epithelial cells that results in collagen deposition. As CO effectively modulates cellular apoptosis in different systems, the involvement of CO in pulmonary fibrosis becomes a real possibility. In C57BL/6 mice, adenovirus-mediated HO-1 overexpression by intratracheal administration suppressed bleomycin-induced pulmonary fibrosis. HO-1 overexpression also enhanced interferon- γ production in lung and reduced respiratory epithelial damage (Tsuburai et al., 2002). Therapeutic effect of HO-1 overexpression did not manifest itself, however, in preventing pulmonary fibrosis induced by agonistic anti-Fas antibody inhalation in C57BL/6 or ICR mice, a strain known to develop pulmonary fibrosis via the Fas-Fas ligand (FasL) pathway. These observations suggest that using HO-1 overexpression strategies to treat idiopathic pulmonary fibrosis, or fibrotic disorders of other target organs, by attenuating apoptotic cell death would be effective in clinical situations independent Fas-FasL pathway. The protective effect of HO-1 overexpression has been ascribed to CO-induced elevation of cGMP or activation of p38 MAPK (Tsuburai et al., 2002).

D. Carbon Monoxide and Reproductive System

HO activity has been detected in human umbilical cord (Vreman et al., 1999), human placenta (McLaughlin et al., 2000; Lyall et al., 2000), placental bed (Lyall et al., 2000), and guinea pig placenta (Odrreich et al., 1998). HO activity affects trophoblast invasion and spiral artery transformation in general and HO-derived CO may dilate the fetoplacental circulation in particular (Lyall et al., 2000; Barber et al., 2001; Navarra et al., 2001). Uterus has both HO-1 and HO-2 expression (Acevedo and Ahmed, 1998). The expression of HO-1 and HO-2 in human and rat testis (Trakshel and Maines, 1988) is cell-type specific. Sertoli cells mainly express HO-1 within seminiferous tubules whereas HO-2 is mainly expressed in germ cells of the testis from rat (Ewing and Maines, 1995) and humans (Middendorff et al., 2000). HO activity can be increased by chorionic gonadotropin in testis (Kutty and Maines, 1989) or by stress (Maines and Ewing, 1996). For the modulation of spermatogenesis in response to stress, Leydig cell-derived HO-1 activity, more specifically ZnPP-inhibitable CO production, is involved by inducing apoptosis of permeiotic germ cells (Ozawa et al., 2002). Similar up-regulation of

HO-1 induction by hyperthermal stress was observed in Sertoli cells (Maines and Ewing, 1996).

In the ovary, Cd^{2+} stimulated HO activity (Maines and Kutty, 1983). HO-1 and HO-2 were expressed in theca cells, granulosa cells of follicles, and luteal cells of mature rat ovaries. HO-2 was also expressed in the ovarian stroma (Alexanderanu and Lawson, 2003). Hemin injection in vivo increased androstenedione and estradiol but not progesterone production. However, inhibition of HO activity with CrMP reduced progesterone and androstenedione but not estradiol secretion. A possible stimulatory role of endogenous CO in the production of ovarian steroids is suggested (Alexanderanu and Lawson, 2003).

Endogenous CO also affects normal course of the estrous cycle, parturition or lactation. No significant change in the estrous cycle or in milk production during lactation was observed with daily injection of hemin to normal cycling rats for more than 10 days (Alexandeanu and Lawson, 2003). However, the estrous cycle was significantly affected by CrMP treatment. Daily CrMP injection at 2 and 4 $\mu\text{mol/kg}$ for 11 days significantly reduced the occurrence of estrus phase of the estrous cycle. Even after the discontinuation of CrMP, this suppressive effect continued for an additional two weeks' observation (Alexandeanu and Lawson, 2003). The seemingly decreased estrogen secretion from the ovaries with CrMP treatment indicates that endogenous levels of CO may promote ovarian estradiol production. Lactational performances were decreased by CrMP injection (4 $\mu\text{mol/kg}$) due to reduced milk production, not to the suckling intensity of the pups (Alexandeanu and Lawson, 2003). These studies suggest that the basal level of endogenous HO-derived CO is important in supporting female reproductive processes and lactation. In fact, blocking HO activity, and presumably CO production, by CrMP administration blocked parturition (Alexandeanu and Lawson, 2003).

The correlation of the HO/CO system with pregnancy has been controversial. Some reports indicate that HO/CO system may be up-regulated during pregnancy, contributing to uterine quiescence during pregnancy. The lines of evidence include higher expression of HO induced by progesterone in pregnant myometrium than that of nonpregnant myometrium (Acevedo and Ahmed, 1998), and increased CO production and HO expression in rat uterus and placenta toward the end of pregnancy (Kreiser et al., 2000). In addition, both the spontaneous and oxytocin-induced contractions of myometrial strips were inhibited by hemin injection (Acevedo and Ahmed, 1998; Longo et al., 1999). Contradictory reports showed that HO-1 and HO-2 were not up-regulated during pregnancy in myometrium at all (Barber et al., 1999). Therefore, role of the HO/CO system in maintaining uterine quiescence during pregnancy is still not settled.

The uterine effects of CO have been reported as this gasotransmitter inhibited myometrial contractility in

humans (Acevedo and Ahmed, 1998). This observation was in line with the report that the end tidal CO level was lower in pregnant woman with premature uterine contractions (Seidman et al., 2000).

Whereas NO is well known for its role in penile erection, CO also participates in erectile regulation. The expression of HO-2 has been documented in rat corpus cavernosum (Ushiyama et al., 2004). Although few HO-1-immunoreactive nerve fibers were observed along strands of smooth muscle in human corpus cavernosum and spongiosum, HO-1 and HO-2 immunoactivity were clearly identifiable in the endothelium lining penile arteries and the sinusoidal walls of the corpus cavernosum and spongiosum (Hedlund et al., 2000). CO alone decreased the isometric tension of rat corpus cavernosum tissues. In the presence of HO inhibitors, the relaxation of these tissues in response to electrical field stimulation (EFS) was suppressed. These observations suggest that neurogenic relaxation by EFS is mediated by the HO/CO system in the corpus cavernosum (Ushiyama et al., 2004). It is worth mentioning however, that the EFS-induced relaxation of isolated strips of rabbit corpus cavernosum was not affected by zinc deuteroporphyrin treatment at different concentrations in the absence or presence of guanethidine and atropine (Kim et al., 1994). More rigorous studies need to be performed to investigate the direct effect of CO on and the regulation of HO expression and activity in these tissues before one can conclude that the role of CO in erectile regulation is different in rabbits than in other species.

In addition to involvement in penile erection control, CO also plays an important role in regulating ejaculation (Burnett et al., 1998). HO-2 has been located in the majority of ganglia in the myenteric plexus and other autonomic ganglia including the petrosal, superior cervical and nodose ganglia. After deletion of the HO-2 gene in mice, myenteric plexus neurotransmission became dysfunctional. Furthermore, HO-2 knockout lead to a markedly diminished reflex activity of the bulbospongiosus muscle of mice, which mediates ejaculation and ejaculatory behavior (Burnett et al., 1998). In contrast, another study showed that prenatal exposure to CO (150 ppm) lead to an increased mount/intromission latency, decreased mount/intromission frequency, and a significant decrease in ejaculation frequency of 80-day male rats (Cagiano et al., 1998). Prenatal exposure to exogenous CO and endogenous production of CO in adult animals may explain these seemingly inconsistent observations.

E. Carbon Monoxide and Gastrointestinal System

The effects of CO on the gastrointestinal (GI) system can be largely ascribed to its role in mediating inhibitory NANC neurotransmission, which has been discussed in the preceding sections of this article. In addition to neuronal expression of HO-2 in the submucosal and myenteric plexus of the GI tract (Ny et al., 1996; Zakhary et

al., 1996), HO-2 immunoreactivity was also discovered in non-neuronal cells, including the smooth muscle and arterial endothelium (Zakhary et al., 1996). HO activities have been detected in IAS and the pylorus of the stomach of mice (Watkins et al., 2004). Exogenously applied CO evoked a concentration-dependent relaxation of the feline lower esophageal sphincter of cats, accompanied by an increase in cyclic GMP (Ny et al., 1996). Although ZnPP caused a right shift of the concentration-response curves for the relaxant responses to VIP, inhibition of HO with ZnPP and SnPP did not affect the NANC-induced by EFS. This observation would suggest that endogenous CO may not be a NANC transmitter in this preparation. In contrast, NANC relaxation of IAS from HO-2 null mice was profoundly reduced in comparison with that of WT mice (Watkins et al., 2004; Zakhary et al., 1997). Moreover, VIP-induced relaxation of IAS was significantly reduced by SnPP, a HO inhibitor. The relaxant effect of VIP was significantly reduced in IAS from HO-2 null mice. These observations suggested that VIP stimulates HO-2, likely via activation of CK2. Thus generated CO is the major mediator for the relaxation of the intestine induced by VIP (Watkins et al., 2004). Considering that IAS has relatively slow relaxation and contraction rates, the stable nature of CO and constitutive expression of HO-2 make HO-2 generated CO a more suitable NANC transmitter than NO.

In contrast to the neuronal source of HO-2, HO-1 immunoreactivity was confined to the smooth muscle, but not neurons, of the GI tract. When HO-1 proteins are up-regulated in the intestinal tract in response to oxidative stress or inflammation (Naito et al., 2004), the GI tract is protected from these damages. The role of HO-1 generated CO in HO-2 null mice remains to be determined.

F. Carbon Monoxide and Liver

Detoxification of biologically active molecule xenobiotics occurs mainly in the liver. Hepatic biliary excretion through specific transporters expressed at the hepatocellular membrane provides further antioxidant protection that ameliorates acute inflammatory processes. Endogenous CO at submicromolar level was detected in the effluent of isolated perfused rat liver (Suematsu et al., 1994). Constitutive expression of HO-2 has been confirmed in hepatocytes, Kupffer cells, endothelial cells and Ito cells. HO-1 is constitutively expressed in Kupffer cells (the liver-specific tissue-fixed macrophages) and its inducible expression occurs in both nonparenchymal and parenchymal liver cells (Bauer and Bauer, 2003). Hepatic HO activity is induced in vivo by glucagon, insulin, epinephrine (Bakken et al., 1972) and Ang-II (Immenschuh et al., 1998). Up-regulation of HO-1 expression reduced ischemia-reperfusion injury in the liver of Zucker fatty rats (Amersi et al., 1999), supporting a functional role of HO/CO in the liver.

Perfusing isolated rat liver in vitro with HO inhibitor (ZnPP) increased the perfusion pressure under the constant flow conditions. Addition of CO (2 μ M) to the perfusate reversed ZnPP-induced increase in perfusion pressure (Suematsu et al., 1994). Unfortunately, whether CO alone could alter the basal perfusion pressure was not tested in this study. In another recent study from the same research team, it was found that inclusion of CO (4 μ M) in the perfusate did not alter the vascular resistance of the isolated rat liver (Norimizu et al., 2003). At cellular level, CO activates sGC in hepatic stellate cells (Suematsu et al., 1995), which are sinusoidal pericytes controlling sinusoidal tone and blood flow distribution. While the hepatic arterial inflow of the liver is subject to control by NOS/NO (Pannen and Bauer, 1998), the effect of HO-derived CO on portal circulation may help provide sufficient blood supply under varied disease conditions as well as physiological circumstances (Goda et al., 1998; Suematsu et al., 1995). CO regulates bile canalicular contractility in cultured rat hepatocytes (Shinoda et al., 1998). Norimizu et al. (2003) reported that in perfused rat livers, transportal administration of CO at 4 μ M induced choleresis and increased baseline bile output by stimulating biliary excretion of bilirubin-IXa and glutathione. When the CO concentration increased beyond 10 μ M, bile output was no longer affected by CO. This is possibly linked to increased paracellular junctional permeability by excess CO. CO at 4 μ M also increased the fluxes of HCO_3^- and K^+ . Coperfusion of livers with CO and tetraethylammonium (TEA) abolished the CO effects on the fluxes of HCO_3^- and K^+ . Since TEA also blocked the CO-induced choleresis, the stimulation of K channels in hepatocytes and/or the biliary system by CO was suggested, at least in part, to underlie the hepatic effect of the gas.

G. Carbon Monoxide and Kidney

Both HO-1 and HO-2 are expressed in renal vascular and tubular structures and glomeruli (Hill-Kapturczak et al., 2002; Botros et al., 2002). Accordingly, renal production of CO has been detected in rat urine and renal microdialysate (Rodriguez et al., 2003). Glucagon and Ang-II induced HO-1 expression in kidney cells (Haugen et al., 2000). In isolated and perfused rat kidney, Ang-II (1 μ M) increased perfusion pressure from 97 to 150 mm Hg. The concentration of CO in the venous effluent after 2 h Ang-II was increased from 27 to 102 nM. At the same time, HO-1 expression increased, whereas HO-2 expression remained unchanged (Li P et al., 2004).

The protective effect of up-regulated HO-1 expression and activity against renal injury has been extensively studied. HO-1 is induced in rat models of acute renal injury including glycerol-induced renal failure (Nath et al., 1992), nephrotoxic serum nephritis (Vogt et al., 1996), cisplatin nephrotoxicity (Agarwal et al., 1995; Shiraishi et al., 2000), ischemia/reperfusion-induced renal failure (Maines et al., 1993; Shimizu et al., 2000),

and rhabdomyolysis (Nath et al., 2000). Overexpression of HO-1 is cytoprotective in cisplatin-induced renal epithelial cell injury. HO-1 null mice treated with cisplatin develop more severe renal failure with increased apoptosis and necrosis, compared with cisplatin-treated wild-type or heterozygote mice (Shiraishi et al., 2000). The protective effects of HO-1 in the glycerol model of acute renal injury have been confirmed in HO-1 knockout mice that demonstrate significantly worse renal function and tubular injury with 100% mortality in the HO-1 null mice compared with WT mice (Nath et al., 2000).

H. Carbon Monoxide and Pancreas

HO-2 immunoreactivity was detected in ganglionic cells located in the periphery of the islets (Lundquist et al., 2003) and in all four types of islet endocrine cells (Alm et al., 1999; Henningson et al., 1997 and 1999). In glucose-stimulated isolated islets, the HO substrate hemin stimulated, whereas the HO inhibitor ZnPP suppressed, the release of insulin in parallel with the rate of CO production (Henningson et al., 1999; 1997). Hence HO-2-derived CO was proposed to be a novel, stimulatory regulator of glucose-induced insulin release. On the other hand, HO-1 expression cannot be detected in freshly isolated islets from rats (Ye and Laychock, 1998) or from normal lean mice (Lundquist et al., 2003). Up-regulation of HO-1 occurred either in cultured islets or after the pancreatic islets were exposed to different stimuli (Henningson et al., 2001; Ye and Laychock, 1998).

Synchronization of calcium oscillation within and among the islets in the pancreas by CO was recently reported (Lundquist et al., 2003) in adult obese-hyperglycemic mice (*ob/ob*) and lean ($-/-$) C57BL/6J mice. In the islets of *ob/ob* mice, HO-2 expression was about 3 times higher than in the lean control mice, and HO-1 became detectable. Production of CO from islets of *ob/ob* mice was 6 times higher than in the lean controls. This higher CO level was accompanied by a 16 times higher frequency of $[Ca^{2+}]_i$ transients in *ob/ob* β -cells. Exogenous CO application at 1 to 100 μ M resulted in increased $[Ca^{2+}]_i$ transients. ZnPP treatment of *ob/ob* mice significantly reduced glucose-stimulated insulin release from islets. The firing and synchronization of calcium oscillations were promoted by hemin and inhibited by ZnPP or CrMP. Thus, not only does this suggest a potentially causative role of pancreatic-produced CO in the hyperinsulinemia of *ob/ob* mice, it also suggests that the underlying mechanism for the CO effect is that CO may coordinate the β -cell rhythmicity by propagating Ca^{2+} signals among the β cells.

In other models of hyperglycemia, such as partial pancreatectomy with normal plasma insulin levels, HO-1 expression in islets was also up-regulated (Laybutt et al., 2002). This up-regulation of HO-1 was reversed by 2-week treatment with phlorizin to normalize hyperglycemia. Therefore, hyperglycemia rather than

hyperinsulinemia is responsible for this increased HO/CO activity. As hypertrophy is an important compensatory response to insulin resistance, increased HO/CO activity would contribute to the survival of hypertrophied β -cells.

CO, exogenously applied or endogenously generated, protected cultured murine insulinoma cells β TC3 from apoptosis induced by TNF- α or serum starvation. Exposure to CO for 24 h also protected isolated murine (C57/BL6) islets of Langerhans from TNF- α plus CHX-mediated apoptosis. These antiapoptotic effects of CO are mediated by activation of the sGC/cGMP pathway. Since the failure of islet transplantation is at least in part due to the apoptosis of β cells, preventing apoptosis might improve islet survival/function after transplantation. This goal can be achieved by CO treatment of islets. Gunther et al. (2002) further showed that purified murine islets exposed to CO for 2 h exhibited much better function, in terms of normalization of blood glucose levels, after transplantation to a diabetic syngeneic recipient than did other islets that were not exposed to CO.

VI. Pathophysiological Implications of Abnormal Heme Oxygenase/Carbon Monoxide System

A. Neurodegenerations and Brain Disorders

The functions of the HO/CO system in the brain has been linked to many devastating neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis. Increased HO-1 expression has been found in AD brains in association with neurofibrillary tangles (Premkumar et al., 1995; Takahashi M et al., 2000), senile plaques, and glial fibrillary acidic protein-positive astrocytes (Schipper et al., 2000). AD neocortex and cerebral vessels also have increased HO-1 expression (Schipper et al., 2000). This AD-related HO-1 up-regulation may be induced by increased free heme associated with neurodegeneration and represents a compensatory reaction to convert the highly damaging heme into the antioxidants biliverdin and bilirubin (Schipper et al., 1998). The time sequence of HO-1 expression and AD progression is not clear yet.

The protective effect of HO-1 up-regulation has been shown in experimental autoimmune encephalomyelitis (Liu et al., 2001), an animal model for human multiple sclerosis. Hemin reduced the severity of encephalomyelitis, but tin mesoporphyrin exacerbated encephalomyelitis (Liu et al., 2001).

The involvement of the HO/CO system in the pathogenesis of seizure disorders has been examined. Prolonged and recurrent febrile seizures (FS) represents a health hazard, causing hippocampal damage that leads to temporal lobe epilepsy. Immunohistochemical studies detected the expression of HO-1 protein in rat hippocampal neurons, which was significantly up-regulated after induction of FS (Yang and Qin, 2004). A 96% increase in plasma CO level was also observed in FS rats in com-

parison with that of control rats. The functional consequence of this up-regulated HO/CO system regarding the pathologic progress of FS is not clear. Inhibition of HO activity with ZnPP lowered endogenous CO levels in FS rats. As would have been expected, this treatment worsened hippocampus neuronal damage induced by recurrent FS. Transmission electron microscopy examination of hippocampal neurons showed that the rough-surfaced endoplasmic reticulum was highly dilated after ZnPP application (Yang and Qin, 2004).

HO-1 protein expression was up-regulated in the hemisectioned spinal cord. This up-regulation was limited to degenerating fiber tracts after spinal cord injury, and mainly in microglia and macrophages. Whether increased HO-1 expression offers a protective or detrimental effect on the injured neuronal tissues is not clear (Mautes et al., 2000). In addition, substantia nigral dopaminergic neurons of Parkinson's disease patients exhibited up-regulated HO-1 in response to oxidative stress (Yoo et al., 2003).

B. Hypertension

Abnormal metabolism and functions of CO have been linked to the pathogenesis and maintenance of hypertension due to elevated peripheral resistance, unbalanced cellular apoptosis and proliferation in the vascular wall, increased oxidative stress, and deranged interaction of CO and NO, etc. (Ndisang et al., 2004). Depending on the models of hypertension, the pathogenic contributions from reduced expression and activity of HO-1, lowered CO production, and dysfunctional signaling pathways mediated by CO will be different.

1. Hypertension Induced by Heme Oxygenase Inhibitors. HO-1 deficiency does not per se lead to hypertension development in mice (Wiesel et al., 2001). Treatment of normotensive SD rats (Johnson et al., 1995) with inhibitors of HO (metalloporphyrins) increased systemic arterial pressure with an upsurge in peripheral resistance. Reduction in HO-catalyzed CO production, rather than biliverdin and iron, was believed to underlie the induced hypertension (Johnson et al., 1995). Biliverdin and iron per se do not induce vasorelaxation (Johnson et al., 1996). Ndisang and Wang (2002) showed that daily injection of CrMP (4 μ mol/kg i.p.) for 4 days elevated the already high blood pressure in 8-week spontaneously hypertensive rats (SHR). Interestingly, this treatment did not change the blood pressure of 8-week normotensive Wistar-Kyoto rats (WKY). Different blood pressure responses of SHR, SD, and WKY rats to HO inhibition may reflect the strain-specific contribution of the HO/CO system to blood pressure regulation.

2. Spontaneously Hypertensive Rats. SHR are the closest genetic animal model for human essential hypertension. The role of the HO/CO system in the hypertension development in SHR and its treatment has been extensively studied. Since this subject has been discussed in great detail and length in another recent re-

view article (Ndisang et al., 2004), only several key issues will be addressed here.

1) Abnormality of the HO/CO system in SHR is manifested differentially at different animal ages or stages of hypertension development. HO-1 expression in vascular tissues is lower in SHR at an prehypertensive stage (<4 weeks) than in age-matched WKY. The activities of the down-stream targets of CO, including the sGC/cGMP pathway are also accordingly reduced in SHR. When SHR enter an evolving hypertension stage (around 8 weeks old), vascular HO-1 expression levels remains lower than in the age-matched WKY. When SHR reach a stage of established hypertension (at ages older than 12 weeks) HO-1 expression levels are actually much higher than at younger ages, becoming comparable with that of age-matched WKY (Ndisang and Wang, 2002). These results suggest that the suppression of the HO/CO system in vascular tissues precedes the development of hypertension, possibly constituting a pathogenic factor for hypertension in SHR. Increased blood pressure and other pathological changes in adult SHR, in turn, would provide stress stimulation to up-regulate HO-1 as a compensative reaction. Changes in HO-2 expression in SHR exhibit tissue-type specificity. While the expression level of HO-2 in other vascular tissues in young SHR (8 weeks) remains unchanged, a decreased expression of HO-2 protein and decreased HO activity were observed in the aorta and pulmonary arteries. Furthermore, the basal HO-1 and HO-2 levels in the pulmonary artery and aorta of adult SHR (20 weeks) are generally greater than those of age-matched WKY rats, an observation not found in mesenteric and tail artery tissues.

2) An altered HO/CO system in SHR affects many different signaling pathways. The most significant changes are the reduced expression and function of the sGC/cGMP pathway (Kojda et al., 1998; Papapetropoulos et al., 1995). It has been shown that sGC expression levels and cGMP contents were significantly lower in tail and mesenteric arteries from 8-week or 4-week SHR than in age-matched WKY rats (Ndisang et al., 2004). BK_{Ca} channels in vascular smooth muscle cells are another major target of CO. BK_{Ca} α subunit expression and BK_{Ca} channel currents were both increased in aortic SMC from SHR. BK_{Ca} channels exhibit a greater probability of being open at resting membrane potential in aortic SMC from SHR (Ndisang et al., 2004). These increases in BK_{Ca} channel expression and function are believed to compensate reduced endogenous CO production and increased blood pressure in SHR.

3) Up-regulation of HO-1 or directly applying CO can reverse the blood pressure development in SHR. Sacerdoti et al. (1989) and Escalante et al. (1991) have demonstrated that either acute or chronic administration of an inducer of HO-1 (stannous chloride) to SHR led to a normalization of blood pressure. Similar results with hemin were also recently reported (Ndisang and Wang,

2002). Administration of CO (i.p.) decreased the arterial blood pressure in SHR (Johnson and Johnson, 2002).

3. *Salt-Induced Hypertension.* After feeding Dahl salt-sensitive rats with high-salt (8% NaCl) diet for 4 weeks, mean arterial blood pressure, carboxyhemoglobin level, HO-1 protein expression in abdominal aortas, and CO production were all elevated (Johnson et al., 2003). This model proposed that instead of lowering blood pressure, CO even worsened the hypertension. CO was proposed to reduce the vasorelaxant response of gracilis muscle arterioles to acetylcholine and L-NAME. Acute treatment of the vascular tissues with an inhibitor of HO, chromium mesoporphyrin, partially normalized the vascular responses (Johnson et al., 2003). This phenomenon may be unique to Dahl salt-sensitive rats since the CO-induced vasoconstriction of gracilis muscle arterioles has not been reported in normotensive SD rats (Kozma et al., 1999) or in other hypertensive animal models such as the SHR. Is the elevated level of endogenous CO a causative factor for the salt-induced hypertension in Dahl salt-sensitive rats? This possibility remains to be investigated. One critical experiment would be to determine whether inhibition of HO activity in vivo in high salt-fed Dahl salt-sensitive rats can normalize NO production and blood pressure in these animals.

4. *Angiotensin II-Induced Hypertension.* Chronic administration of Ang-II induces hypertension in rats. The blood pressure development in this animal model is accompanied by increased HO-1 expression in cardiac (Ishizaka et al., 2000), aortic (Ishizaka and Griendling, 1997; Ishizaka et al., 1997), and renal (Aizawa et al., 2000) tissues. Once again, these changes in HO-1 expression have been described as a compensatory protective reaction to counteract the complications of hypertension. Taking the kidney as an example, the intraperitoneal administration of ZnPP or hemin to Ang-II-infused rats either decreased or increased the glomerular filtration rate, respectively (Aizawa et al., 2000).

5. *One Kidney-One Clip Renovascular Hypertension.*

The kidney plays an important role in the chronic regulation of blood pressure. A unilateral nephrectomy and a partial occlusion of the renal artery of the remaining kidney (1K1C) in animals result in fluid retention by the single stenotic kidney. This eventually leads to the development of volume-dependent renovascular hypertension. Wild mice receiving 1K1C operation developed hypertension with a systolic blood pressure of 140 mm Hg, and cardiac hypertrophy. The blood pressure of sham-operated wild mice was only 108 mm Hg (Wiesel et al., 2001). The same 1K1C procedure produces much more severe hypertension in HO-1 knockout mice. Extensive renal ischemic injury was unmasked in 1K1C HO-1 null mice. These mice also exhibit an at least 2-fold higher death rate than the wild type after surgery (Wiesel et al., 2001). This indicates a protective role for HO-1 in renovascular hypertension and cardiac hypertrophy.

6. *Pulmonary Hypertension.* The antihypertensive effect of the HO/CO system is not only applicable to systemic arterial hypertension. Chronic exposure of rats to an intermittent normobaric hypoxic environment induced pulmonary hypertension. In this model, the expression of HO-1 mRNA in rat lung tissues and COHb levels were significantly increased in comparison to normal control rats, which could represent a compensatory reaction to hypertension. Treatment of these pulmonary hypertension rats with hemin to further increase HO-1 expression and CO production reduced the elevated right ventricular systolic pressure and partially normalized the thickened intra-acinar pulmonary arteries (Zhen et al., 2003). On the prevention side, treatment of rats with HO inducers inhibited the development of structural remodeling of pulmonary arteries and hypoxic pulmonary hypertension (Christodoulides et al., 1995; Christou et al., 2000). Similar protection against the development of pulmonary hypertension by overexpression of HO-1 has also been reported in transgenic mice (Fagan et al., 1999).

HO-1 and eNOS mRNA expressions in the lungs of infants who died of severe congenital diaphragmatic hernia and persistent pulmonary hypertension were significantly decreased in comparison with the normal lungs from age-matched newborns who died of sudden infant death syndrome as control. More specifically, the immunoreactivities of HO-1 and eNOS proteins in the endothelium and arterial wall in the congenital diaphragmatic hernia samples were markedly reduced. The reduced endogenous vascular production of CO and NO, due to the lower expression levels of HO-1 and eNOS in the congenital diaphragmatic hernia lung, may stimulate vascular smooth muscle cell proliferation and increase the pulmonary vascular tone, constituting one of the causative factors for persistent pulmonary hypertension (Solari et al., 2003).

7. *Portal Hypertension.* Dilatation of the mesenteric vasculature and consequently increased portal inflow in cirrhosis contributes to the generation of portal hypertension (Gatta et al., 1999). This also occurs in prehepatic portal hypertension (Vorobioff et al., 1984; Gatta et al., 1999). In rats with prehepatic portal hypertension induced by ligation of the portal vein, HO-1 activity significantly increased and inhibition of HO activity reduced mesenteric circulation (Fernandez et al., 2001). In rats with cirrhotic portal hypertension induced by CCl₄, HO-2 expression, not that of HO-1, was increased (Sacerdoti et al., 2004). Inhibition of HO activity with SnMP in cirrhotic rats normalized mesenteric perfusion pressure and normalized the response of the mesenteric vasculature to vasoconstrictor agents (Sacerdoti et al., 2004).

C. Carbon Monoxide and Inflammation

A physiologically relevant concentration of CO in body fluid is in the range of 100 to 500 ppm. At this concen-

tration range, CO inhibited the production of proinflammatory cytokines, such as TNF- α , MIF and interleukin-1, from macrophages (Otterbein et al., 2000) and the secretion of interleukin-2 from the activated T cells (Pae et al., 2004). CO stimulated the synthesis of the anti-inflammatory cytokine interleukin-10 (Otterbein et al., 2000). The anti-inflammatory effect of CO is mediated by p38 kinase, but independent of the cGMP pathway (Otterbein et al., 2000). Interleukin-10, in turn, induces HO-1 expression and the latter produces more CO (Lee and Chau, 2002). In this way, CO self-amplified its anti-inflammatory effect.

The anti-inflammatory effect of endogenous CO is closely related to the expression of HO-1 under different conditions. HO-1 null mice exhibit enlarged spleen and lymph nodes, high peripheral white blood cell counts, and high splenic and lymph node CD4⁺:CD8⁺ T cell ratios with numerous activated CD4⁺ T cells, all indicative of a chronic inflammatory status (Poss and Tonegawa, 1997a and 1997b). An inflammatory syndrome that developed in a HO-1 deficient human was one of the reasons of death (Kawashima et al., 2002). One important mechanism underlying the HO/CO-mediated anti-inflammatory effect is the down-regulated expression of adhesion molecules. These adhesion molecules are mainly from three families, i.e., selectin, integrin, and the immunoglobulin superfamily (Wagener et al., 2003). For example, induction of HO-1 down-regulated the H₂O₂-evoked P-selectin expression, leading to decreased leukocyte binding (Hayashi et al., 1999). When HO-1 expression was first up-regulated by local stress conditioning, expression of ICAM-1 induced by subsequent injury was significantly decreased (Rucker et al., 2001). These HO-yield effects, however, have been mainly ascribed to increased enzymatic production of bilirubin. The role of CO in altered adhesion molecules is not clear. The anti-inflammatory role of endogenous CO has been documented much more solidly with activated CD4⁺ T cells. Whereas bilirubin or free iron does not offer any antiproliferative effect on cultured CD4⁺ T cells, CO blocked early entry of T cells in the cell cycle and reduced secretion of interleukin-2 from these cells activated by anti-CD3 plus anti-CD28 antibodies (Pae et al., 2004).

CO elicits a cytoprotective and anti-inflammatory response by selective activation of MAPK (Otterbein et al., 2000). Other pathways involve the modulation of proinflammatory cytokine GM-CSF via the NF- κ B pathway. Sarady et al. (2002) reported that exposure of macrophages to a low concentration of CO (250 ppm) as well as overexpression of HO-1 in these cells inhibited lipopolysaccharide-induced production of granulocyte macrophage colony-stimulating factor (GM-CSF). This effect of CO was mediated by inhibition of the transcription factor NF- κ B. Consequently, the phosphorylation and degradation of the regulatory subunit I κ B- α was prevented. In activated CD4⁺ T cells, CO inhibited ERK phosphor-

ylation, suppressing the cell proliferation (Pae et al., 2004).

The anti-inflammatory activity of CO may underlie the defensive effect of HO against atherogenesis and atherosclerosis (Ishikawa and Maruyama, 2001; Ishikawa et al., 2001a and 2001b). HO-1 overexpression has been shown to prevent the development of atherosclerosis (Shih et al., 2000). The products of HO activity play different roles in this process. Biliverdin scavenges oxygen free radicals, attenuating the adhesiveness of leukocytes to the vessel wall and chemotaxis (Duckers et al., 2001; Hayashi et al., 1999; Ishikawa, 2003). The lipid peroxidation in plasma and artery wall can also be reduced by biliverdin. HO-derived CO, on the other hand, suppressed blood vessel constriction (Suematsu et al., 1995). Increased HO-1 activity facilitates iron extrusion from the cells of the blood vessel wall (Juan et al., 2001). For a more detailed perspective on the role of CO in inflammation, readers are referred to a recent excellent review by Wagener et al. (2003).

D. Cardiac Hypertrophy and Heart Failure

To maintain cardiac output and tissue perfusion in many cardiovascular pathologies including various cardiomyopathies, myocardial infarction, valvular heart disease, and hypertension, the heart adaptively and chronically increases its external or internal work load. This eventually leads to cardiac hypertrophy with increased surface area of cardiomyocytes, sarcomere assembly, and preproatrial natriuretic peptide mRNA expression. HO-2 is constitutively expressed in canine myocardium. During myocardial ischemia, HO-2 is activated and CO produced. CO increased the cGMP level, likely inducing the relaxation of the coronary artery (Nishikawa et al., 2004). The mechanism by which ischemia activates HO-2 is not clear yet and further studies are merited.

Oxygen availability is a necessity for HO activity (Appleton et al., 2002). Lower PO₂ during ischemia would compromise total HO activity unless additional HO-1 can be induced. Adenoviral overexpression of HO-1, but not β -galactosidase, has been shown to significantly inhibit endothelin-1 (ET-1)-induced cardiac myocyte hypertrophy. Since the antihypertrophic effects of HO-1 were mimicked by biliverdin (10 μ M) and the CO-releasing molecule [Ru(CO)₃Cl₂]₂ (10 μ M), biliverdin and CO may be responsible for the antihypertrophic effect of HO-1 (Tongers et al., 2004). Furthermore, biliverdin and CO suppressed extracellular signal-regulated kinases (ERK1/ERK2) and p38 MAPK activation by ET-1 stimulation. The antihypertrophic effect of CO is independent of cGMP and cGMP-dependent protein kinase type I (Tongers et al., 2004). Yet et al. (1999) reported the occurrence of severe right ventricular enlargement after chronic hypoxia in HO-1 null mice compared with wild-type mice. In 24 patients with advanced ischemic and nonischemic cardiomyopathy and in 13

control subjects without known cardiac disease, exhaled CO level was monitored. At rest and 1 and 5 min after exercise testing, exhaled CO was lower in patients with cardiomyopathy than in the healthy controls at rest (1.66 versus 1.8 ppm) and after exercise; however, exercise did not affect exhaled CO in healthy humans (Seshadri et al., 2003). These studies indicate that a lower endogenous CO level may be correlated to the pathogenesis of cardiomyopathy in patients.

CO suppresses proinflammatory cytokines (Otterbein et al., 2000) and ameliorates postischemic myocardial dysfunction (Clark et al., 2000). The development of reperfusion-induced ventricular fibrillation (VF) is related to the expression level of HO-1 and CO production. After exposure to ischemia/reperfusion damage, the isolated hearts from HO-1 homozygous knockout mice developed VF. The same damage to the hearts from wild-type mice leads to significant increase in endogenous CO production and HO-1 up-regulation, which protected the hearts from the reperfusion-induced VF (Bak et al., 2003).

E. Transplantation

HO activity is indispensable for inhibition of transplantation rejection. Use of CO precursors, such as methylene chloride, has been shown to prolong the graft survival.

1. Allograft Survival. An allograft is a transplant of organ or tissue between allogeneic individuals of the same species. Also named homograft, allograft is significantly prolonged by an up-regulated HO-1 activity with reduced intragraft apoptosis, devoid of arteriosclerosis (Hancock et al., 1998), less ischemia/reperfusion damage (Katori et al., 2002), and suppressed vascular injury (Soares et al., 1998). Up-regulation of HO-1 by cobalt-protoporphyrin treatment of the donor before organ harvesting increases the survival chance of the kidney graft. The levels of TNF- α mRNA were decreased and those of interferon- γ and bcl-x, increased by HO-1 up-regulation (Tullius et al., 2002).

2. Xenograft Survival. A xenograft is a transplant of organ or tissue taken from a donor of one species and grafted into a recipient of another species (or genus or

family). An early study showed that HO activity aided the survival of the cardiac transplants in rodents (Sato et al., 2001). CO mediated this HO-1 facilitated mouse-to-rat cardiac transplantation (Sato et al., 2001). Application of HO inhibitor abolished this protective effect of HO (Soares et al., 1998). HO-1 gene transfer also improved liver allograft survival. For this HO-mediated protective effect, CO is also the key signaling molecule (Ke et al., 2002).

F. Apoptosis and Cellular Proliferation

The effects of CO on cell apoptosis are summarized in Table 3.

1. Vascular Smooth Muscle Cells. Gene transfer of HO-1 (Duckers et al., 2001; Liu et al., 2002b; Peyton et al., 2002) or delivery of CO (Morita et al., 1997) inhibited serum-stimulated SMC proliferation. In line with this, it has been reported that inhibition of HO-1 activity potentiated SMC growth (Togane et al., 2000; Peyton et al., 2002). SMC growth increased when cells were treated with hemoglobin, a CO scavenger (Peyton et al., 2002). In one study, CO reportedly inhibited proliferation of cultured hypoxic vascular smooth muscle cells (Morita et al., 1997). However, the 5% concentration of CO used in this study may be higher than the physiological level.

One interesting study showed that HO-1 has a proapoptotic effect on vascular SMC, mediated by the proapoptotic protein p53. This effect was mimicked by exogenous biliverdin and bilirubin, but not by CO or iron (Liu et al., 2002b). Contrary to the reported proapoptotic effect of HO-1 activity, exogenous CO inhibited the cytokine-mediated apoptosis of cultured vascular smooth muscle cells (Liu et al., 2002a). This effect was partially dependent on activation of sGC. Inhibition of mitochondrial cytochrome *c* release and the suppression of p53 expression were also associated with the antiapoptotic effect of CO. Evidence was also presented that the up-regulated HO-1 activity has an antiapoptotic effect, like exogenous CO. The antiapoptotic effect of CO against the endoplasmic reticulum stress-induced apoptosis of vascular SMC was recently shown by the same laboratory and linked to a decrease in the expression of the proapoptotic protein, GADD153 (Liu et al., 2005). There

TABLE 3
The effects of HO/CO system on cellular apoptosis

| Cell Type | Treatment | Apoptosis Outcome | References |
|--------------------------|---------------------|-------------------|--|
| Smooth muscle cells | HO-1 overexpression | Stimulation | Liu et al. (2002b); Peyton et al. (2002) |
| Smooth muscle cells | CO | Inhibition | Liu et al. (2002a) |
| Endothelial cells | HO-1 overexpression | Inhibition | Brouard et al. (2000, 2002) |
| Endothelial cells | HO-1 overexpression | Inhibition | Soares et al. (1998) |
| Endothelial cells | CO | Stimulation | Thom et al. (2000) |
| Epithelial cells | HO-1 overexpression | Inhibition | Inguaggiato et al. (2001) |
| Fibroblasts | HO-1 overexpression | Inhibition | Petrache et al. (2000) |
| Pancreatic β cells | CO | Inhibition | Gunther et al. (2002) |
| Jurkat cells | CO | Stimulation | Song et al. (2004) |
| Jurkat cells | CO | Inhibition | Song et al. (2004) |
| Hepatocyte | CO | Inhibition | Zuckerbraun et al. (2003) |
| Thymocyte | CO | Stimulation | Turcanu et al. (1998a) |

may be many explanations for these two seemingly contradictory conclusions, the proapoptotic and antiapoptotic roles of HO-1 in vascular SMCs, but a comprehensive understanding has yet to be reached.

Activation of ERK1/2 has been proposed as one of the mechanisms for inhibition of cell proliferation by CO. ERK1/2 is a family of cell-cycle specific transcription factors that regulate the expression of many genes involved in cell proliferation, and govern the transition of cells from the G₁ to S phase. Activation of ERK1/2 by CO is dependent on the cGMP pathway (Durante, 2002). Conditioning sGC with YC-1 decreased SMC growth (Durante, 2002), and inhibition of sGC with methylene blue or ODQ prevented the effect of CO on SMC cell cycle progression (Morita et al., 1997).

The physiological importance of CO-suppressed cell proliferation can be multifaceted. For example, after vascular injury or therapeutic balloon angioplasty, HO-1 is induced, and the produced CO helps limit narrowing of the vessels. The questions remaining to be answered are also multifold. Does CO also inhibit the regeneration and reformation of blood vessels? What is the switch or threshold level for CO that decides when and in which direction the antiproliferation effect of CO will kick in? In this hypothetical switch role, CO levels might be low at the early developmental stage when the growth of SMC is critical, so that the inhibitory effect of CO might be blocked. Many growth factors such as PDGF-BB (Durante et al., 1999) and TGF- β (Kutty et al., 1994b; Hill-Kapturczak et al., 2000) induce cell proliferation. These factors also induce HO-1 expression and increase CO production. HO-1 up-regulation and CO production may serve as a negative feedback mechanism to restrain the growth factor-induced cell proliferation within a physiological range.

2. Endothelial Cells. HO-1 overexpression protected endothelial cells from TNF- α -mediated apoptosis. Brouard et al. (2000) found that CO inhibited apoptosis in endothelial cells by stimulating the p38MAPK signaling pathway, not cGMP, and the subsequent activation of NF- κ B (Brouard et al., 2002). Other studies demonstrated that CO (or HO-1 transfection) enhanced proliferation of EC (Deramaudt et al., 1999; Li Volti et al., 2002; Malaguarnera et al., 2002). Inhibitory effect of CO on EC proliferation has also been reported. Exposure of bovine pulmonary artery EC to 100 ppm CO for more than 1 h caused cell death, which could be prevented by a caspase-1 inhibitor. Preconditioning of these EC with 10 ppm CO enabled EC to resist the detrimental effects of 100 ppm CO exposure (Thom et al., 2000). Abraham et al. (2003) showed that transfection of human vascular EC with a retroviral vector encoded with human HO-1 gene significantly increased the expression of the proliferation genes, such as cyclin A1, D3 and E, and vascular endothelial growth factor (VEGF), but down-regulated the expression of CDK6, CDK4, BAK, DAD 1, and caspases 2 and 6. The down-regulated expression of

caspase family genes is in line with decreased apoptosis of endothelial cells by the HO/CO system.

Hypoxia-inducible transcription factor-1 (HIF-1) activates hypoxia responsive genes, including NOS, VEGF, erythropoietin, glycolytic enzymes, and HO-1 (Lee et al., 1997; Semenza et al., 1997; Palmer et al., 1998). The relationship of CO and HIF-1 is intriguing. Exogenous CO at 5% decreased the binding of HIF-1 to hypoxia responsive genes (Liu et al., 1998). The amount of HIF-1 α protein seemed not to be influenced by 5% CO exposure (Liu et al., 1998), whereas it was decreased by 80% CO exposure (Huang et al., 1999). These concentrations of CO (5–80%) are not physiologically relevant, and how this relates to the physiological situation is unknown.

Exogenous CO has been shown to inhibit hypoxia-induced expression of erythropoietin (Huang et al., 1999), VEGF (Goldberg and Schneider, 1994), ET-1, and PDGF genes (Morita and Kourembanas, 1995). Similarly, endogenous CO inhibited hypoxic induction of PDGF-B and ET-1 in endothelium (Morita and Kourembanas, 1995). The reduced expression of these mitogens in endothelial cells inhibited the proliferation of the cocultured vascular SMC (Morita et al., 1997). How CO inhibits the expression of these mitogens is unknown. Inhibition of cytochrome P450 could be one mechanism (Wang, 1998). CO is an inhibitor of cytochrome P450, and the level of cytochrome P450 is controlled by the availability of cellular heme. Increased CO production, due to increased heme breakage, would be linked to decreased cytochrome P450 levels (Wang, 1998).

Although inconsistent results have been reported on the effects of CO on the apoptotic status of SMC and EC, the majority of these studies still support the general consensus that CO stimulates EC proliferation but inhibits SMC proliferation through different mechanisms. CO is not alone in having dual effects on cell proliferation. NO also stimulates EC proliferation, but inhibits the growth of SMC (Tzeng et al., 1997; Kibbe et al., 2000). One theory proclaims that CO or NO may up-regulate the expression of VEGF. This specific mitogen acts on EC, but not SMC, to promote EC proliferation (Dulak and Józkwicz, 2002). A VEGF-independent effect of CO may prevail in SMC to inhibit SMC proliferation. The challenge for this theory is to find the threshold when the VEGF-dependent stimulatory CO effect can overpower the VEGF-independent inhibitory CO effect on cell growth. Since CO has opposite effects on SMC and EC, it would also be interesting to know the relative rate of proliferation of these two types of cells, which would dictate the outcome of vascular restenosis.

3. Other Types of Cells. The antiapoptotic effect of CO has also been shown in fibroblasts, and this effect is mediated by activation of the sGC, not MAPK, pathway (Petrache et al., 2000). An accentuated death rate in fibroblasts, induced by serum deprivation or other fatal stimuli, was encountered after targeted HO-1 gene de-

letion and reversed by HO-1 overexpression (Ferris et al., 1999). The protective effect of HO-1 activity was interpreted as the consequence of HO-1-induced expression of an iron pump in fibroblasts (Ferris et al., 1999). The consequence is reduced intracellular iron content, less oxidative stress through the Fenton reaction, and less apoptosis. Since activation of the sGC/cGMP pathway does not protect the HO-1^{-/-} fibroblast cells from apoptotic damage, it was assumed that the HO protection was not offered by CO. One should be very cautious in making this assumption, however, considering the fact that numerous effects of CO are cGMP-independent.

CO protects hepatocytes (Zuckerbraun et al., 2003) from TNF- α induced apoptosis. Its activation of the cGMP pathway as well as the MAPK pathway underscores the antiapoptotic effect of CO on pancreatic β cells (Gunther et al., 2002). Inguaggiato et al. (2001) showed that genetically engineered LLC-PK1 renal epithelial cells with stable overexpression of HO-1 exhibited growth arrest and cellular hypertrophy. The TNF- α /cycloheximide or staurosporine or serum deprivation-induced apoptosis in these cells was significantly reduced. The underlying mechanisms could be HO-1-induced expression of the cyclin-dependent kinase inhibitors p21CIP1, WAF1, and SDI1. Application of hemin to wild-type cells similarly increased expression of p21 and decreased the induced apoptosis. This observation contrasts somewhat with the findings in SMCs, where HO-1 stimulated p21 but inhibited cell growth (Duckers et al., 2001).

It has been suggested that CO may activate p38 MAPK to inhibit mitochondrial pathway-dependent apoptosis but prevent phosphorylation of the ERK MAPK pathway to augment death receptor pathway-dependent apoptosis (Song et al., 2004). Fas/CD95-mediated apoptosis plays a pivotal role in regulating immune functions. Activation of this cascade may represent a potential therapeutic approach to induce tolerance against a transplanted organ, autoimmune disorders, or cancer. Fas/CD95-induced apoptosis of Jurkat cells was augmented by CO exposure. In part, this was related to CO-induced up-regulation of the proapoptotic protein FADD as well as activation of caspases 8, 9, and 3. This proapoptotic effect of CO was not mediated by free radical production but was nullified after the overexpression of FADD siRNA. Inhibition of Fas/CD95-induced activation of the prosurvival ERK MAPK could be the key event. CO also decreased the expression of the antiapoptotic protein *Bcl-2*. Interestingly, when the apoptosis of Jurkat cells was induced by TNF- α and etoposide, CO became an antiapoptotic agent. It seems that, not only the cell type, but also the specific apoptosis inducers as well as ligand-dependent and -independent pathways, may determine whether CO offers an antiapoptosis protection or proapoptosis stimulation. Turcanu et al. (1998a) showed that CO exposure for 6 h induced apoptosis of thymocytes. This proapoptotic ef-

fect of CO was reduced by Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an antioxidant vitamin E analog, but not by sGC/cGMP pathway blockers.

The antiapoptotic effect of CO on tumor cells has been shown. The importance of endogenous CO in this regard can be perceived by the observation that HO inhibitors offered an antitumor effect, constituting a promising novel anticancer chemotherapy. Inhibition of HO with ZnPP and its water-soluble analog significantly suppressed the growth of rat hepatoma AH136B and mouse sarcoma S-180 by increasing caspase-3 activity and inducing a significant apoptosis (Doi et al., 1999; Fang et al., 2003, 2004). A similar antiapoptotic effect of HO-1 was reported in human colon cancer SW480 cells, which exhibited apoptotic changes after receiving polyethylene glycol-ZnPP treatment (Fang et al., 2004). After transfected with small interfering RNA (siRNA) to silence HO-1, these cancer cells become apoptotic (Fang et al., 2004).

G. Oxidative Stress

Unlike the high reactivity of NO, which by itself is a free radical, CO does not contain free electrons. This stable chemical nature dictates that CO would not by itself give out oxygen reactive species. However, CO may be involved in oxidative stress indirectly, especially under pharmacological and toxicological conditions. Toxic and presumed subtoxic CO exposures are associated with significant oxidative (Thom, 1990; Zhang and Piantadosi, 1992; Thom et al., 1999b) and nitrosative stress (Thom et al., 1997). After poisoning rats with CO (>1000 ppm), plasma levels of oxidized proteins and lipid peroxidation were significantly increased (Thom, 1990; Thom et al., 1997). This CO-induced increase in oxidative stress was not related to hypoxic stress from formation of carboxyhemoglobin or changes in circulating platelets or neutrophils. However, CO-dependent lipid peroxidation was prevented or reduced by inhibition of xanthine oxidase or superoxide dismutase and iron chelators (Thom, 1990). It has also been shown that intracellular H₂O₂ production in the brain was increased by high concentrations of CO, accompanied by increases in hydroxyl radical production and decreases in the reduced to oxidized glutathione (GSH/GSSG) ratio in mitochondria (Lautier et al., 1992; Piantadosi et al., 1995). These observations on CO-induced oxidative stress may be useful from a perspective of emergency evaluation of CO-poisoning victims. Whether endogenous CO under phy-siological conditions contributes to the modulation of oxidative stress has been unknown.

NO-derived reactive species has been proposed to underlie CO-mediated oxidative stress (Thom et al., 1997). Nitrotyrosine formation in cerebral endothelial cells after prolonged CO exposure has been reported (Koehler and Traystman, 2002). It is possible that CO-induced nitrosative stress inhibits mitochondrial cytochromes,

causing the formation of reactive oxygen species (Agarwal et al., 1995; Koehler and Traystman, 2002). CO can bind to certain heme proteins, alter their role in oxidative stress control, and, as such, indirectly change the cellular redox status. The pro-oxidant effects of CO are exemplified by the elevated oxidative stress by CO in vascular endothelium (Thom et al., 1999a, 2000) and other cell types (Piantadosi, 2002). Inhibition of catalase activity (Zhang and Piantadosi, 1992) or up-regulation of the expression of manganese superoxide dismutase (Frankel et al., 2000) also underlie the CO-induced hydrogen peroxide production. CO alters mitochondrial redox states and energy provision in the brain (Brown and Piantadosi, 1992). NADPH oxidases, a group of heme proteins, are significantly involved in free radical production in mitochondria. CO can inhibit NADPH oxidase to yield oxygen free radicals.

VII. Heme Protein-Dependent Cellular and Molecular Mechanisms for Carbon Monoxide Effects

The major molecular targets of CO are heme proteins, including hemoglobin, myoglobin, prostaglandin endoperoxide synthase, NOS, catalase, peroxidases, respiratory burst oxidase, pyrrolases, cytochrome *c* oxidase, cytochrome P450, sGC, tryptophan dioxygenase, and many others (Table 1). The interaction of CO with iron is coordinated at the center of all heme proteins. CO binds iron at its reduced state [Fe(II)], resulting in characteristic shifts in the optical spectrum of the heme proteins. Several specific reactions underlie the CO effect on heme proteins. The binding of CO to HbA not only reduces the subsequent binding of O₂, it also hinders the release of O₂ from HbA in the needed tissues (Roughton and Darling, 1944). CO interacts with cytochrome to disturb oxidation-reduction (redox) reactions, requiring the transfer of electrons through the heme moiety, usually, to molecular O₂. Generally speaking, the binding of CO to heme proteins inhibits their functions, except for sGC,

which is activated (Fig. 4). CO also binds to proteins that contain other transition metals at the active site, e.g., copper, and by doing so interferes with their functions. Examples include heme-copper cytochrome *c* oxidase (Heitbrink et al., 2002), the half-apo derivative of peptidylglycine monooxygenase (Jaron and Blackburn, 2001), and cytochrome bo quinol oxidase (Ciccognani et al., 1992). Although CO's interaction with heme proteins underlies most of its physiological effects, it also targets the signaling pathways that are not categorized as heme proteins, such as different ion channels. CO activates sGC in many different cell types (Karlsson et al., 1985; Brune et al., 1990; Furchgott and Jothianandan, 1991; Verma et al., 1993; Morita et al., 1997; Ingi et al., 1996a; Xue L et al., 2000). This signaling cascade is largely responsible for the CO-induced activation of neurotransmission and vasodilation. By activating sGC, CO also inhibits platelet aggregation (Brune and Ullrich, 1987). CO can increase the activity of purified bovine lung sGC severalfold (Stone and Marletta, 1994; Kharitonov et al., 1995), although its effect is 30 to 100 times less than that of NO (Stone and Marletta, 1994). The relative low potency of CO in activating sGC does not seem to defer the physiological importance of CO since certain putative endogenous substances may greatly boost the stimulatory effect of CO on sGC. A benzyloid derivative, YC-1, has been shown to amplify the effect of CO on sGC to an extent similar to that of NO (Friebe et al., 1996; Friebe and Koesling, 1998). The intriguing questions are when we can find an endogenous counterpart of YC-1 and what it would be. Direct evidence for in situ CO binding to signaling molecules, including sGC, has also not been reported.

Another pathway for the interaction of CO and sGC is via activation of NOS. CO may activate NOS, and the subsequently synthesized NO stimulates sGC. In other words, CO may be a modulator of NO signaling. Supporting evidence for this hypothesis includes the studies of the enteric nervous system and enteric smooth muscle

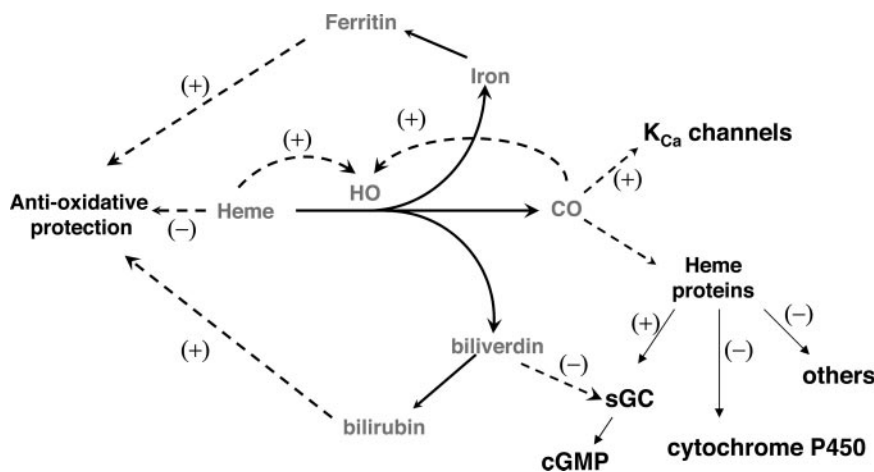


FIG. 4. Interrelations and interactions of HO/CO system. Solid lines represent metabolic routes or subgroups. Dashed lines represent main functional consequences. (+), stimulation; (-), inhibition. Exceptions exist for the depicted functional consequences.

in HO-2 knockout mice and HO-2 and nNOS double knockout mice. In these preparations, the CO-mediated inhibitory NANC neurotransmission seems to rely on the synthesis of NO. In the presence of nNOS, the phenotypic change occurring in NANC neurotransmission in HO-1 knockout mice can be rescued by providing CO. CO failed to do so once nNOS was also knocked out (Ingi et al., 1996a; Xue L et al., 2000). Validating this hypothesis, i.e., that CO activates sGC via modulating NO production, requires proof that novel mechanisms exist to quickly regulate CO synthesis.

The direct downstream target of cGMP is cGMP-dependent protein kinase (PKG). Activation of PKG phosphorylates several important regulators of $[Ca^{2+}]_i$, inhibiting Ca^{2+} gates and activating Ca^{2+} pumps on the endoplasmic reticulum and activating K_{Ca} channels on the plasma membrane (Wang, 1998). cGMP also regulates several classes of phosphodiesterases and is itself rapidly degraded by phosphodiesterases. The physiological outcome of the decrease in $[Ca^{2+}]_i$ varies among different cell types. In vascular smooth muscle, a drop in $[Ca^{2+}]_i$ leads to relaxation, and a rise in $[Ca^{2+}]_i$ leads to vasoconstriction.

Cytochromes P450 are a group of mixed-function oxidases or oxygenases. These heme proteins are characterized by their 450-nm absorption peak of the CO-dithionite-reduced difference spectrum. The hydroxylation reactions catalyzed by cytochrome P450 involve the uptake of a pair of electrons from NADPH with reduction of one atom of O_2 to H_2O and incorporation of the other into the substrate (White and Coon, 1980). Widely distributed in mammalian tissues, cytochromes P450 participate in steroid and xenobiotic metabolism (Estabrook et al., 1980). Mediation of biological effects of CO by cytochrome P450 was suggested long ago. The sensitivity of cytochrome P450 to CO increases during rapid electron transport (Estabrook et al., 1980). There is no doubt that CO can bind to and inhibit cytochrome P450, but the CO level has to be abnormally increased beyond physiological levels. This would explain the CO-induced changes in biological functions and drug metabolism (Montgomery and Rubin, 1973) when COHb reaches toxic levels.

It had been proposed that CO may reduce the cytochrome P450-dependent synthesis of endogenous vasoconstrictor substances (Coceani et al., 1988; Wang, 1998). Moreover, increased CO production due to increased HO-catalyzed heme metabolism would reduce the availability of cellular heme, which subsequently controls the synthesis of cytochrome P450 (Levere et al., 1990). Observations on CO-induced relaxation of the lamb ductus arteriosus supported a cytochrome P450-dependent mechanism because the relaxing effect of CO was maximally reversed by light illumination at 450 nm (Coceani et al., 1984). On the other hand, Utz and Ullrich (1991) found that CO-induced relaxation of ileal smooth muscles was maximally photoreversed at 422

nm, indicating formation of a reduced heme-CO complex. For example, the maximum absorption of a guanylyl cyclase-CO complex is at 422 nm (Gerzer et al., 1981). Inhibition of cytochrome P450 in isolated rat tail artery tissues with 4-phenylimidazole, an antagonist of cytochrome P450, caused vasorelaxation. However, CO-induced vasorelaxation in the same vascular tissues was not altered by 4-phenylimidazole, suggesting that inhibition of cytochrome P450 was additive, not permissive, to the vasorelaxant effect of CO on rat tail artery tissues (Wang, 1998). The discrepancy in the roles played by cytochrome P450 in CO-induced relaxation of the lamb ductus arteriosus and tail arteries from adult rats may be partially explained by different vascular preparations from different species at different development stages.

VIII. Interaction of Carbon Monoxide with Different Ion Channels

The interaction of CO and ion channels constitutes an important mechanism for the biological effect of CO. Most noticeable among the CO-targeted ion channels are K^+ channels. This superfamily is composed of voltage-dependent K_v , ATP-sensitive K_{ATP} , and calcium-activated K_{Ca} channels. The stimulation of big-conductance K_{Ca} channels (BK_{Ca}) by both endogenous and exogenous CO has been documented in many studies from many different laboratories. Calcium-spark activated transient K_{Ca} channels are also activated by CO. The interaction of CO and K^+ channels may be the dominant force in driving the CO-induced vasorelaxation in specific types of blood vessels, especially peripheral resistance and cerebral arterioles. In other types of blood vessels, CO effects on K^+ channels may become less important compared with activation of the cGMP pathway by CO.

A. Carbon Monoxide and K_{Ca} Channels

The first evidence for a direct interaction of CO and BK_{Ca} channels was presented in a series of papers published in 1997 (Wang and Wu, 1997; Wang et al., 1997a,b). These studies showed that CO relaxed isolated rat tail artery tissues in vitro. Partially blocked by inhibition of cGMP, the CO-induced vasorelaxation was also partially blocked by pharmacological inhibition of BK_{Ca} channels. Whole-cell and single-channel recordings on isolated single vascular SMC from rat tail arteries demonstrated that CO directly opened BK_{Ca} channels (Fig. 4). This effect was not mediated by the sGC/cGMP pathway, and CO did not directly affect intracellular calcium homeostasis (Wang, 1998). Rather, an interaction of CO with histidine residue of BK_{Ca} channel proteins was necessary for CO to increase opening probability of this channel (Wang and Wu, 2003). Recently, the same group reported that, whereas NO stimulated BK_{Ca} channels by targeting the cysteine residues of the $BK_{Ca,\beta}$ subunits, the stimulatory effects of CO on BK_{Ca} channels depend on specific interactions of the gaso-

transmitter with the BK_{Ca}, α subunit (Wu et al., 2002). The saturated amplification of BK_{Ca} channels by a specific agonist for the β subunit of BK_{Ca} channels, dehydrosoyasaponin-I, can be further increased by CO, not NO. CO still stimulated the native BK_{Ca} channels in vascular SMC that were pretreated with anti-K_{Ca}, β subunit antisense oligodeoxynucleotides, but the stimulatory effect of NO on BK_{Ca} channels was abolished by this treatment (Wu et al., 2002). Finally, CO, but not NO, amplified BK_{Ca} current densities in HEK-293 cells that were transfected with cloned BK_{Ca}, α subunit cDNA. These observations indicate that the BK_{Ca}, β subunit is not indispensable for the effect of CO, whereas it is for NO. In newborn porcine cerebral arteriole SMC, CO at submicromolar concentrations significantly increased whole-cell and inside-out single BK_{Ca} channel activity. This excitatory effect of CO on BK_{Ca} channel was not mediated by the cGMP pathway since inhibition of sGC did not alter the effect of CO on whole-cell current and a functional cGMP pathway had been removed from inside-out membrane preparations (Xi et al., 2004). The stimulation of heterologously expressed BK_{Ca} channels in HEK-293 cells by CO, administered via a CO-releasing compound, was recently confirmed using the inside-out single channel recording technique (Williams et al., 2004). Another recent study also confirmed stimulatory effect of CO on the heterologously expressed cSlo- α subunit of BK_{Ca} channels in HEK-293 cells (Xi et al., 2004).

The interaction of CO with BK_{Ca} channels has also been shown in other types of vascular smooth muscle cells. TEA and iberiotoxin, two blockers of BK_{Ca} channels, abolished the vasodilation of the pial arterioles of newborn pigs induced by CO or by heme-L-lysinate, a HO substrate, indicating a mediatory role of BK_{Ca} channels in the CO effect (Leffler et al., 1999). Heme-L-lysinate or exogenous CO also induced concentration-dependent relaxation of isolated pressurized mesenteric arterioles from chronically hypoxic rats (Naik and Walker, 2003). This relaxation was blocked by iberiotoxin, but not by ODQ or ryanodine, indicating a BK_{Ca}-sensitive, sGC/cGMP-independent mechanism for CO action. The ryanodine insensitivity suggested that the CO effect did not depend on Ca²⁺ sparks emanating from ryanodine-sensitive stores. At the isolated single cell level, exogenously applied or endogenously generated CO acutely stimulated both BK_{Ca} channels and Ca²⁺ spark-induced transient BK_{Ca} currents in porcine cerebral arteriole SMC (Jaggar et al., 2002). Inhibition of HO activity reduced BK_{Ca} currents in SMC isolated from rat gracilis muscle arteriole, which correlated with reduced endogenous CO production (Zhang et al., 2001). In vascular SMC from rat renal interlobar artery, CrMP decreased the open probability of a TEA-sensitive 105 pS K channel (Kaide et al., 2001) in a sGC-independent fashion. CO reversed the inhibitory effect of CrMP on K channels. In some cell preparations, the interaction of CO and K⁺ channels seems to involve a sGC/cGMP-dependent mechanism. CO donor or HO substrates also

increased the open probability of BK_{Ca} channels in inside-out patches from rat carotid body glomus cells (Williams et al., 2004). In smooth muscle cells from coronary arteries, CO and its precursor heme activated the iberiotoxin-sensitive K_{Ca} channel and hyperpolarized membrane. Instead of a direct interaction of CO with the channel, PKG-dependent activation of the Na⁺/Ca²⁺ exchanger by CO was proposed. This activation may increase the submembrane Ca²⁺ concentration in the vicinity of BK_{Ca} channels. A consequential opening of BK_{Ca} channels would then occur (Gagov et al., 2003).

It should be noticed that an activated cGMP pathway may be permissive, not prerequisite, for activation of BK_{Ca} channels by CO. Barkoudah et al. (2004) recently showed that application of a CO-releasing compound dilated arteriolar branches of the middle cerebral artery from piglets by activating BK_{Ca} channels in vascular smooth muscle cells. However, removal of endothelium or blocking the sGC-cGMP pathway abolished CO-induced vasorelaxation. Release of NO from endothelium or activation of sGC-cGMP pathway in vascular SMC allowed CO to cause vascular dilation. The mechanisms for this permissive effect of cGMP on CO's effect on BK_{Ca} channels are not clear yet.

CO also causes chronic regulation of K⁺ channel function. In pulmonary artery SMC, the resting membrane potential is controlled by BK_{Ca} channels and voltage-activated Kv channels (Bae et al., 1999). A 3-week exposure of pulmonary artery SMC to CO at 530 ppm hyperpolarized membrane potential and enhanced the iberiotoxin-sensitive whole-cell outward current without changing the membrane capacitance (Dubuis et al., 2002). The sensitivity of the whole-cell outward current to iberiotoxin, a selective blocker of BK_{Ca} channels, suggested that CO induced expression and/or function (or both) of BK_{Ca} channels in these cells. This hypothesis was further tested recently with inside-out patch clamp recordings of single BK_{Ca} channels in rat pulmonary artery SMC (Dubuis et al., 2005). The most significant change induced by chronic CO inhalation was a large increase in the sensitivity of BK_{Ca} channels to Ca²⁺, which allows BK_{Ca} channels play a more prominent role in controlling resting membrane potential of pulmonary artery SMC.

Neuronal K_{Ca} channels may also be modulated by CO. In rat chemoreceptor cells, the hypoxic inhibition of BK_{Ca} channels was reversed by CO at the whole-cell and the single-channel levels (Riesco-Fagundo et al., 2001). The competitive inhibition by CO of O₂ binding to membrane hemoproteins that act as an O₂ sensor to modulate channel activity has been suggested. Unfortunately, no result was provided pertinent to the direct effect of CO on K_{Ca} channels in the absence of hypoxia and a direct interaction of CO with K_{Ca} channels, without the complication of an unknown O₂ sensor in the membrane, is still plausible.

Norimizu et al. (2003) reported that TEA blocked CO-induced choleresis, suggesting that the stimulation of K^+ channels by CO underlay the hepatic effect of the gas. In rabbit corneal epithelial cells, exogenous CO (1%) increased both the whole-cell and cell-attached single-channel K^+ currents and hyperpolarized membrane potential (Rich et al., 1994). Once cell membrane patch was excised, CO failed to alter single K^+ channels in the patch. Together with CO-induced increase in intracellular cGMP concentration (Rich et al., 1994), the cytoplasmic milieu-dependent effect of CO on K^+ channels seems to suggest a cGMP-dependent mechanism in this cell preparation. However, a permissive role of cGMP for effect of CO on K^+ channels (Barkoudah et al., 2004) cannot be ruled out yet.

B. Heme, Heme Oxygenase, 20-Hydroxyeicosatetraenoic Acid, and K_{Ca} Channels

Being the inducer of HO and the precursor of CO, heme may also directly affect the function of BK_{Ca} channels. One such interaction may be caused by binding of heme to the heme-binding sequence motif on BK_{Ca} channel proteins. A recent study by Tang et al. (2003) showed that heme regulated the function of cloned human Slo1 channels and wild-type BK_{Ca} channels in rat brain. Structural evidence based on changes in ultraviolet-visible and electron paramagnetic resonance revealed heme binding to a synthesized 23-residue peptide (hSlo-HBP23) encompassing the putative heme-binding CKACH segment located at residues 612 to 616 of the channel protein. More specifically, the heme binding sites in the CKACH segment were localized to the histidine and cysteine residues by site-directed mutagenesis. Electrophysiological evidence showed that heme inhibited the heterologously expressed BK_{Ca} channels as well as single rat hippocampal native BK_{Ca} channels at 45 to 125 nM, but only when applied to the intracellular side of the membrane patch. Without changing the unit conductance, heme reduced the single channel opening frequency.

Williams et al. (2004) showed that HO-2 itself participated in the regulation of BK_{Ca} channels. In the native HEK-293 cells, HO-2 protein was constitutively expressed, and no HO-1 protein was detectable. $BK_{Ca,\alpha}$ subunit heterologously expressed in HEK-293 cells was coimmunoprecipitated with HO-2 proteins. The physical linkage of HO-2 to $BK_{Ca,\alpha}$ was thus indicated, which may underscore the modulation of BK_{Ca} channel activity by HO-2. Knockdown of HO-2 expression using siRNA reduced BK_{Ca} channel activity. CO rescued this loss of function. The addition of heme (1 nM) alone to the inside-out patch did not have any effect on the open probability of BK_{Ca} channels, which somewhat contradicts the inhibitory effect of heme on the same type of BK_{Ca} channels reported by Tang et al. (2003).

The metabolism and functions of the HO/CO system are closely linked to that of 20-hydroxyeicosatetraenoic

acid (20-HETE). The selective induction of cortical and outer medullary HO-1 was associated with a decrease in 20-HETE concentration and blood pressure in 7-week SHR after a single injection of cobalt protoporphyrin at a dose of 5 mg/100 g body weight (Abraham et al., 2002). Treatment with $SnCl_2$ increased the expression of HO-1 protein in the inner medullary collecting ducts and medullary thick ascending limb and decreased cortical and inner medullary formation of 20-HETE. On the other hand, the vasoconstrictive effect of 20-HETE has been linked to membrane depolarization and inhibition of BK_{Ca} channels (Zhang et al., 2001). In this regard, inhibition of 20-HETE formation by CO and NO would alter the BK_{Ca} channel function and vasoactive status. Currently, the mechanism by which CO decreases 20-HETE formation is not clear. At any rate, membrane hyperpolarization and BK_{Ca} channel activation after the CO-induced suppression of 20-HETE formation have been observed (Sun et al., 1998; Roman, 2002).

C. Carbon Monoxide and K_{ATP} Channels

Foresti et al. (2004) showed that CO released from tricarbonylchloro-(glycinato)ruthenium(II) (CORM-3) induced a concentration-dependent relaxation of phenylephrine-precontracted aorta tissues. This vasorelaxant effect of CO was significantly reduced after incubating the tissues for 15 to 30 min with a K_{ATP} channel blocker glibenclamide (10 μ M). No direct electrophysiology recording has yet been performed to test the interaction of CO and K_{ATP} channels.

D. Carbon Monoxide and Calcium Channels

Extracellular application of ZnPP in AtT-20 pituitary cells irreversibly inhibited the total Ca^{2+} currents conducted by L-, N-, and possibly P-type voltage-gated Ca^{2+} channels (Linden et al., 1993). However, the effect of ZnPP largely resulted from the generation of superoxide anion, instead of HO inhibition. A quite intriguing observation from this study was that two other HO-2 inhibitors, SnPP and Zn-deuteroporphyrin-bis-glycol, increased Ca^{2+} channel current. Even more controversial is the modulation of Ca^{2+} channel function by CO in chemosensitive cells of carotid body. Intracellular Ca^{2+} concentrations as well as Ca^{2+} channel currents in glomus cells in carotid body were elevated after ZnPP incubation, and application of exogenous CO abolished the effect of ZnPP. These results led Overholt et al. (1996) to conclude that endogenous CO suppressed intracellular Ca^{2+} level by inhibiting Ca^{2+} channels in carotid body (Parkes et al., 1994). Using the same glomus cells from carotid body, Mokashi et al. (1998) found that CO significantly increased intracellular Ca^{2+} concentration about 7-fold. The effect of CO on voltage-dependent calcium channels in guinea pig ileum smooth muscle cells was directly studied using the whole-cell patch clamp technique. CO (100 μ M) had little effect on the peak Ba^{2+} currents (I_{Ba}) when voltage was stepped from -60

to +50 mV (holding potential, -60 mV) (Kwon et al., 2001). It was thus concluded that the relaxant effect of CO on this tissue was not due to inhibition of L-type Ca^{2+} channels. One recent study, on the other hand, demonstrated that CO activated L-type Ca^{2+} channels in an NO-dependent fashion (Lim et al., 2005). Bath application of 0.2% CO or NO donor increased the maximal peak current (I_{Ba}) in transfected HEK cells. At the same concentration, CO increased NO production in transfected HEK cells. Exogenous CO also stimulated L-type Ca^{2+} channels in freshly dissociated human intestinal smooth muscle cells. This NO-dependent activation of L-type Ca^{2+} channels by CO may use the same mechanism as NO-dependent activation of sGC by CO (Ingi et al., 1996a; Xue L et al., 2000).

E. Carbon Monoxide and Other Ion Channels

Different types of ion channels conduct different functions in the control of membrane excitability or ionic regulation of cellular milieu. Beyond the effects of CO on K^{+} channels and Ca^{2+} channels, CO may also affect Na^{+} channels and other types of ion channels in different tissues.

1. *Na⁺ Channels.* The expression of a Na^{+} channel in rat peripheral nervous system was regulated by chronic CO exposure (75 and 150 ppm) during gestation (Carratu et al., 1993). It was found that the inactivation kinetics of transient Na^{+} current recorded from sciatic nerve fibers from 40-day rats were significantly slowed. The steady-state inactivation of Na^{+} channel also shifted to the right. Consequently, more Na^{+} channels in neurons from CO-treated rats were available for opening at a given membrane potential. These Na^{+} channel property changes, however, could not be observed in neurons from 270-day-old CO-exposed rats. Furthermore, a left shift of the current-voltage relationship of Na^{+} channels was induced in neurons from both 40- and 270-day-old rats with the reversal potential decreased from about +120 to +100 mV. This study suggests the importance of CO on the development of the neuronal system at specific developmental stages by regulating the behavior of Na^{+} channels in neurons. CO might directly modify Na^{+} channel macromolecules to keep the channel in immature status. Alternatively, membrane lipid distribution and density may be altered by CO exposure (Moxness et al., 1996), affecting the conformation and function of Na^{+} channel proteins embedded within. The protein change and the environmental lipid change could affect the voltage sensor and ion-selective filter of Na^{+} channels, influencing voltage-dependent activation and inactivation as well as ion selectivity.

2. *Nonselective Cationic Channel.* A nonselective cation channel was observed in the rat locus coeruleus neuron that was insensitive to tetrodotoxin and not gated by voltage. The reversal potential for this conductance was around -27 mV. Although no direct electrophysiology study was conducted to examine the effect of

CO on this nonselective cationic channel, an extrapolation was made based on the observation that the locus coeruleus neuron firing rate increased by 80% in 23 of 29 cells by the bath-applied CO within 3 to 10 min (Pineda et al., 1996). Similar effects were obtained with selective activators of cGMP-dependent protein kinase and the direct application of NO. The physiological implication of this study is not clear because a saturated CO solution with a CO concentration of around 1 mM was used.

3. *Cyclic Nucleotide-Gated Ion Channels.* Stimulation of olfactory receptor neurons (ORNs), mediated by the G-protein coupled cAMP pathway, activates cyclic nucleotide-gated (CNG) channels to generate cationic inward current and membrane depolarization. Leinders-Zufall et al. (1995) found that in isolated ORNs of the tiger salamander, exogenously applied CO consistently evoked prominent and reversible inward whole-cell CNG current with an EC_{50} of 2.9 μM . In line with the stimulatory effect of CO on CNG channels, CO (3.1 μM) depolarized the membrane of ORNs by about 22 mV. In the absence of GTP in the pipette solution or after activation of CNG channels with cGMP, CO lost its stimulatory effect on CNG channels. The blockade of sGC activation with LY85383 (10 μM) abolished the CO effect. Thus, CO plays an important role in olfactory signaling by generating cGMP, which stimulates CNG channels. This CO-cGMP pathway and odor-cAMP pathway converge on the same effector, CNG channels, to coordinate the activity of ORNs.

IX. Interaction of Heme Oxygenase/Carbon Monoxide and Nitric-Oxide Synthase/Nitric Oxide Systems

CO, like NO, binds to iron in the heme moiety of heme proteins. The two gasotransmitters share many common downstream signaling pathways and have the same regulatory functions. On the other hand, there are some functional differences between CO and NO. For example, NO mainly mediates glutamate effects at NMDA receptors, whereas CO is primarily responsible for glutamate action at metabotropic receptors. As another example, CO becomes a stimulatory modulator of sGC when the tissue level of NO is low. Conversely, CO inhibits sGC activity with a high-tissue NO level (Kajimura et al., 2003). The potential interplay between CO and NO also occurs at different levels, being synergistic or antagonistic, which provides an integrated mechanism for the finetuning of cellular functions.

HO resembles NOS in that the electrons for CO synthesis are donated by cytochrome P450 reductase, which is 60% homologous at the amino acid level to the carboxyl terminal half of NOS (Foresti and Motterlini, 1999).

A. Influence of Carbon Monoxide on Nitric-Oxide Synthase/Nitric Oxide System

1. *Carbon Monoxide Potentiates the Activity of Nitric-Oxide Synthase/Nitric Oxide System.* Maulik et al. (1996) reported that NO production protected isolated rat hearts from ischemia/reperfusion damage. Protoporphyrin, which reduced endogenous CO levels, seemed to reduce the NO protection. This suggests that CO has a permissive role in NO production by the heart. However, reduced cardiac protection due to suppressed bilirubin CO production after inhibition of HO should also be considered. Incubating turtle retinas in vitro with CO significantly increased cGMP production in bipolar and amacrine cells (Cao and Eldred, 2003). This stimulatory effect was completely blocked once NOS was pharmacologically inhibited with *S*-methyl-thiocitrulline and L-NAME. It was therefore hypothesized that the effect of CO on cGMP production might be ascribed, at least in part, to the displacement and release of NO from its intracellular storage pool(s). Is there an intracellular "NO pool"? It has been reported that the photorelaxation of rat vascular smooth muscle can be caused by the release of NO from a depletable store (Kubaszewski et al., 1993; Venturini et al., 1993). The existence of a saturable cellular sink for NO, at least in certain types of cells, has also been suggested (Griffiths and Garthwaite, 2001). More direct evidence for such an NO pool is derived from a recent NO imaging study that showed that NMDA-stimulated NO production in the retina was largely confined to the cytoplasm of some neurons (Blute et al., 2000). These aforementioned studies argue that NO produced inside the cells may not diffuse as easily as originally thought. It is likely that a cellular NO pool is composed of heme, hemoproteins, or thiols. For example, an NO-binding protein has been reported in neurons, namely neuroglobin (Burmester et al., 2000). Hemoglobin can bind NO in a light-sensitive fashion (Vladimirov et al., 2000). HO-2 can also bind NO at the two heme-binding sites (Ding et al., 1999). *S*-nitrosoglutathione, generated from the interaction of GSH with peroxynitrite, releases NO in a time-dependent manner from vascular tissues (Balazy et al., 1998) or in a light-sensitive manner from cultured leukemia cells (Sexton et al., 1994). In fact, even GSH alone can be a light-sensitive intracellular NO pool (Megson et al., 2000).

CO can cause a release of NO from its intracellular pool (Thorup et al., 1999; Marshall et al., 2000). In renal resistance arteries, 100 nM CO resulted in the release of NO. Consecutive applications of brief pulses of CO attenuated the amplitudes of the consecutive releases of NO (Thorup et al., 1999). In response to low-concentration CO stimulation, vascular endothelial cells and blood platelets release NO and generate peroxynitrite (Thom et al., 1997). Intracellular redistribution of NO is one hypothesis for this CO-mediated NO release. Different equilibrium constants for metallic binding of the two

gasotransmitters are the basis of this hypothesis. As is well known, NO is the most reactive physiological gas, comparable to O₂ in its effective size and polarity. NO binds to Fe(II)-heme protein as soon as it enters the binding pocket (Scott et al., 2001). NO has a much greater overall association constant with Fe(II)-heme protein than that of CO. The affinity of NO for Fe(II) of hemoglobin, for example, is 1500 times greater than that of CO (Keilin, 1966). On the other hand, CO has a significantly longer dissociation constant than that of NO or O₂ (Gibson et al., 1986). This property facilitates CO-induced NO release from Fe(II)-heme proteins (Moore and Gibson, 1976). The presence of NO sinks, such as reduced thiols, will facilitate the displacement of NO from Fe(II) by CO. It should be noticed that these rationales are based on chemical reactions in aqueous solutions in vitro. Whether or not the CO-induced NO release/displacement in living cells can be fast enough and efficient enough to have a physiological meaning has not been fully addressed. Furthermore, since CO does not bind to Fe(III)-heme proteins, direct CO-NO interactions will not occur in met-heme proteins.

2. *Carbon Monoxide Reduces the Activity of the Nitric-Oxide Synthase/Nitric Oxide System.* CO can directly bind to and inactivate NOS, decreasing the enzyme activity in some preparations (Ding et al., 1999). This may explain the arteriolar NO dysfunction induced by the overproduction of CO, leading to the development of salt-induced hypertension (Johnson et al., 2003). Endogenous CO has been suggested to control constitutive NOS activity (Turcanu et al., 1998b). Inhibition of purified macrophage NOS (iNOS), purified cerebellar nNOS, and endothelial NOS (eNOS) by CO has been reported (Willis et al., 1995; Thorup et al., 1999). There is no report to date that CO can directly activate NOS.

An indirect means by which CO reduces the activity of the NOS/NO system is the competition of CO with NO for binding to its targets, such as sGC. For example, cerebellar granule cells cultured for 1 week did not express significant NOS activity, and CO stimulated the production of cGMP. A prolonged culture of cerebellar granule cells induced NOS expression, and CO inhibited NO-mediated increases in cGMP (Ingi et al., 1996a). While acting on the downstream target of NO, CO had no significant effect on NOS activity in these granule cells (Ingi et al., 1996a).

3. *Carbon Monoxide Reduces the Expression of Nitric-Oxide Synthase.* The expression of iNOS was induced by cytokines in the human intestinal epithelial cell line DLD-1. Cavicchi et al. (2000) preincubated DLD-1 cells with heme (1–50 μM). This treatment inhibited cytokine-induced iNOS activity in a concentration-dependent manner, which was suppressed by SnPP. The cytokine-induced iNOS protein as well as iNOS mRNA expression were also abolished by heme treatment. It was thus concluded that HO-derived CO acted at the transcriptional level to inhibit iNOS expression. A re-

cent study showed that CO decreased cytokine- or hypoxia/endotoxin-induced iNOS/NO production in cultured IEC-6 cells, a rat enterocyte cell line (Zuckerbraun et al., 2005).

B. Influence of Nitric Oxide on Heme Oxygenase/Carbon Monoxide System

1. *Nitric Oxide Potentiates the Activity of Heme Oxygenase/Carbon Monoxide System.* NO has a beneficial hemodynamic effect and a cytotoxic effect, depending on the site and rate of NO production and the chemical fate of the NO produced (Yu et al., 1994). The cytotoxicity of NO is mediated by the generation of peroxynitrite, nitrosylation of thiols, and impairment of iron-sulfur clusters of proteins (Beckman et al., 1990). The detrimental effects of nitric reactive species, including NO and peroxynitrite, can be partially compensated by the induced expression of HO-1 (Maines, 1997). The latter offers strong antioxidant protection. Furthermore, increased CO production has the potential to inactivate NOS (Ding et al., 1999), reducing the production of nitric reactive species. The endpoints of this feedback loop would be reduced NO transformation to reduce oxidative stress and increased CO production to perform NO-equivalent signaling functions, such as stimulation of sGC and activation of K channels. Whenever iron and other redox-active reduced transition metals are available, the interaction of CO and NO becomes more significant since the generation of oxygen-derived free radicals will influence this interaction.

NO and NO donors have been reported to avidly induce HO-1 expression and CO production in different types of cells, including endothelial, smooth muscle, renal tubular, and mesangial cells, and hepatocytes (Durante et al., 1997b; Datta and Lianos, 1999; Bouton and Demple, 2000; Liang et al., 2000; Alcaraz et al., 2001). This interaction takes place over a relatively long period.

The activated transactivator AP-1 is one of the underlying mechanisms for NO-induced HO-1 expression, initiating de novo transcription of HO-1 gene (Durante et al., 1997b; Hartsfield et al., 1997). To date, whether or not NO directly acts on the promoter region of HO-1 gene is unclear. Is it possible that the reactive nitrogen species peroxynitrite, formed by NO interaction with superoxide anion, is responsible for the NO effect? The observations on bovine vascular endothelial cells and renal tubular epithelial cells support this hypothesis (Foresti et al., 1999; Liang et al., 2000). It has been shown that NO-mediated HO-1 induction is not mediated by the cGMP pathway (Motterlini et al., 1996; Liang et al., 2000). Increased HO-1 mRNA stability is another mechanism by which NO up-regulates HO-1 expression (Hartsfield et al., 1997; Bouton and Demple, 2000).

NO may stimulate and/or complement the activity of the HO/CO system. Topical application of CO or the HO substrate heme-L-lysinate produced dilation of pial ar-

terioles of piglets. However, this effect of CO was blocked by *N*^ω-nitro-L-arginine, an inhibitor of NO production. Sodium nitroprusside, an NO donor, recovered decreased vasodilation induced by CO in the *N*^ω-nitro-L-arginine-treated piglet. It was thus speculated that NO is a permissive factor for the vascular effect of CO. NO and CO may share the same target, such as K_{Ca} channels in vascular SMC. Preconditioning of K_{Ca} channels by NO may blunt the responses of these channels to CO (Wu et al., 2002). Some physiological effects of CO, such as cerebral vasodilation in adult rats, may even be directly mediated by NO (Meilin et al., 1996). Furthermore, NO has been reported to increase HO-2 activity and CO production in a cGMP-dependent manner in cerebral microvascular tissues (Leffler et al., 2005).

2. *Nitric Oxide Reduces the Activity of Heme Oxygenase/Carbon Monoxide System.* NO down-regulates the activity of constitutively expressed HO-2 (Ding et al., 1999) while up-regulating HO-1 protein expression (Durante et al., 1997b; Foresti et al., 2003). This inhibitory effect of NO may be more important in tissues that only express HO-2. Several vascular tissues, including cerebral blood vessels of newborn pigs (Leffler et al., 1999), rat gracilis muscle arterioles (Kozma et al., 1999), and rat renal interlobar arteries (Kaide et al., 2001), use HO-2 as the sole CO-generating enzyme. The potential down-regulation of HO-2 in these vascular tissues by NO would constantly reduce the production of CO, resulting in increased contractility and sensitivity to vasoconstrictors (Kaide et al., 2001). Under certain circumstances, such as severe hypoxia, NO production might be reduced due to a limited availability of O₂ as a substrate for NOS (Terraz et al., 1999). As a compensatory response of the vasculature during hypoxia, CO production from HO-2 would be increased to compensate for the shortage of NO.

X. Therapeutic Applications of Carbon Monoxide

A. Up-Regulating the Expression of Heme Oxygenase

By deciphering the underlying molecular mechanism that controls endogenous level of CO, it will be possible to finetune CO production and minimize the potential detrimental accumulation of iron, under different pathological conditions, to take advantage of using CO as an effective therapeutic agent.

1. *Genetic Approaches.* HO gene transfer is a powerful approach in preventing and/or treating certain HO/CO deficient diseases (Deramandt et al., 1998; Panahian et al., 1999). Many different vectors have been used to deliver HO-1 gene with different degrees of success. Sabaawy et al. (2001) overexpressed human HO-1 in 5-day-old SHR by a single intracardial injection of a retroviral vector containing human HO-1 cDNA (LSN-hHO-1). This gene transfer maneuver attenuates the development of hypertension and promotes somatic growth of these rats. Direct injection of human HO-1

(hHO-1) gene, using recombinant adeno-associated virus as a vector, into the myocardium of adult rats has also been attempted (Melo et al., 2002). This hHO-1 transgene procedure conferred a significant reduction in left ventricular myocardial infarction (75%) induced by ischemia/reperfusion myocardial injury. Coincidentally, myocardial lipid peroxidation and the abundance of proapoptotic Bax and proinflammatory interleukin-1 β were decreased, and antiapoptotic Bcl-2 protein levels increased. This study represents a preventive strategy for patients with a history of cardiac attack episodes or at risk for developing coronary ischemic events. An attempt has also been made to overexpress HO proteins using a TAT-HO fusion protein (Ribeiro et al., 2003). HO-1 was fused to an 11-aa cell-penetrating peptide from the human immunodeficiency virus TAT protein. The constructed fusion protein successfully delivered HO-1 proteins into pancreatic β -cells. The transduction of HO-1 fusion protein alone did not change the physiological status of these cells. Interestingly, these treated cells were protected from TNF- α -mediated cytotoxicity.

The safety of vectors is the primary consideration for HO gene transfer. Whether using viral vectors (adenovirus, retrovirus, adeno-associated virus, or lentivirus) or nonviral vectors, they should preferably be nonpathogenic and weakly immunogenic and not provoke robust immune and inflammatory reactions by the host. Depending on the ability to integrate into the host genome, different vectors provided variable expression durations of the transgene and the encoded therapeutic proteins. Site- and organ-specific delivery of HO-1 gene would offer a more specific cytoprotective effect. This can be achieved by using tissue type-specific promoters (Yang et al., 1999). HO-1 gene has been successfully delivered to renal structures in SHR using Na⁺-K⁺-Cl⁻ cotransporter (NKCC2 promoter). This maneuver normalized blood pressure and provided renal protection against oxidative injury (Abraham, 2003). Human HO-1 gene transfer into human endothelial cells in vitro has been shown to attenuate Ang-II-TNF- α - and heme-mediated DNA damage (Abraham, 2003).

2. Nongenetic Approaches. Many pharmaceutical drugs or compounds have been used to up-regulate expression of HO-1. SnCl₂ injection into 7-week SHR for 4 days lowered blood pressure (Sacerdoti et al., 1989). This synthetic substance with unique selectivity for renal HO, however, may induce nephrotoxicity. Cobalt chloride (CoCl₂) was another potent inducer of HO and exhibited antihypertensive effect on 7-week SHR (Sacerdoti et al., 1989). Special attention needs to be paid to excessive toxicity and the high index of animal mortality induced by CoCl₂. HO-1 protein levels before and after application of SnCl₂ or CoCl₂ have not been directly determined.

Mostly used HO-1 inducers are heme derivatives, especially hemin (Mosley et al., 1998). Hemin injection has been used to up-regulate HO-1 expression in different

hypertension animal models. As mentioned earlier, Ndisang and Wang (2002) showed that short-period administration of hemin (15 mg/kg/day i.p. for 4 days) to young SHR significantly up-regulated HO-1 expression and lowered blood pressure of these hypertensive animals. Similar observations with other HO inducers have been reported previously (Sacerdoti et al., 1989; Levere et al., 1990). Heme-L-lysinate and heme-L-arginate are heme derivatives that provide free-monomeric heme substrate for HO to increase CO production and to induce HO-1 expression. Application of heme-L-lysinate (45 μ mol/kg i.p.) or heme-L-arginate (45 μ mol/kg i.p.) to SHR, phenylephrine-induced, and deoxycorticosterone acetate-salt hypertensive rats has been shown to lower blood pressure within 1 h of the bolus injection (Johnson et al., 1996). The time course (<1 h) of blood pressure-lowering effects of heme-L-lysinate and heme-L-arginate in this study excludes putative up-regulation of HO-1 expression. Rather, increased production of CO, after increased supply of precursors, catalyzed by both HO-1 and HO-2, is a likely explanation for effects of the heme derivatives used in this study. In another study, heme-L-arginate was given to 7-week SHR (15 mg/kg body weight i.p.) for 4 days, and this maneuver lowered blood pressure by 22 mm Hg (Levere et al., 1990).

Since many HO-1 inducers are pro-oxidant, administration route and duration as well as dosage of these inducers should be carefully chosen. The half-life of in vivo metabolism of HO-1 inducers will also affect the effectiveness and potential side effects of HO-1 inducers. Furthermore, direct determination of up-regulated HO-1 protein expression induced by these inducers is critical for understanding molecular mechanism for their cellular actions.

Some immunosuppressive peptides were potent inhibitors of HO-1 activity in vitro. RDP1258 is one of them. Its HO-1 inhibitory effect was shown in vitro in a dose-dependent fashion (Cuturi et al., 1999). RDP1258, when administered to rats, significantly increased splenic HO-1 activity but did not significantly alter the expression of HO-1 at the mRNA level (Magee et al., 1999). These peptides suppress immunorejection, partially through their binding to HO-1 (Magee et al., 1999). Since HO-1 expression was not up-regulated by this peptide and since the binding of these peptides to HO-1 is their main interaction with HO-1 protein (Magee et al., 1999), the reason why this class of peptides inhibits HO-1 activity in vitro, but stimulates HO-1 activity in vivo, is puzzling.

B. The More the Merrier? Down-Regulating the Expression or Activity of Heme Oxygenase

HO-1 may have a dual role in tissue pathology and is not "therapeutic" in all instances (Platt and Nath, 1998; Suttner and Dennery, 1999). Too much HO-1 may, in fact, be a perpetrator of tissue injury—mostly due to iron intoxicity. Increased accumulation of bilirubin is associ-

ated with kernicterus in neonates (Dennerly et al., 2001). Too much CO may be equally bad. CO stimulates mitochondrial generation of free radicals and can poison heme proteins (Zhang and Piantadosi, 1992). This would be especially applicable to patients with impaired bilirubin disposition such as newborns and those with Crigler-Najjar type I syndrome (Kappas et al., 1993). Whether or not these situations are accompanied by higher CO levels, and thus the associated potential health hazards, has not been thoroughly studied. It is conceivable in this case that therapeutical approaches are also needed to down-regulate the expression and/or activity of HO under certain conditions.

1. Genetic Approaches. Down-regulating HO-1 expression may have the advantage of saving the energy consumed by heme catabolism. Local accumulation of CO, biliverdin, and iron, due to increased HO-1 activity, can also be prevented. To achieve a prolonged down-regulation of HO-1, Quan et al. (2001) delivered HO-1 gene in antisense orientation with a retroviral vector (LXSN) to cultured human endothelial cells. This represents the first trial to selectively block the expression of endogenous human HO-1 protein. A 45% decrease of endogenous HO-1 protein was achieved with a 167% increase in un-metabolized exogenous heme in these transfected cells. Importantly, CO production in transfected human endothelial cells after heme treatment was only 50% of that of nontransfected cells. HO-2 protein levels did not change after this virus transfection. The importance of this pioneering study goes far beyond the specific inhibition of HO-1. It also opens the door for regulating cellular heme levels by specifically inhibiting HO-2, which is constitutively expressed. Overexpression of HO-2 may have important pathophysiological implications. A focal trauma to the rat spinal cord resulted in profound up-regulation of local HO-2 expression 5 h after injury. This up-regulation of HO-2 is putatively injurious to spinal cord, leading to edema formation and nerve cell damage (Sharma and Westman, 2003).

Physiological stimuli can also repress the expression of HO-1. It has been reported that hypoxia, interferon- γ , or desferrioxamine (an iron chelator) decreased HO-1 mRNA expression in several human cells, including human glioblastoma cells, human lung cancer cells, and human umbilical vein endothelial cells. Bach1, a heme-regulated transcriptional repressor, is a basic leucine zipper protein, forming a heterodimer with a small Maf protein. Since induction of Bach1 mRNA by these stimuli (after 2 h) preceded the repression of HO-1 (after 12 h), it was postulated that Bach1 may function as a hypoxia-sensitive repressor for HO-1 gene. The repressive effect of hypoxia on HO-1 expression is specific for human cells because the same stimuli up-regulated HO-1 in all tested nonhuman cells (Kitamuro et al., 2003).

2. Nongenetic Approaches. As discussed previously, many relatively specific blockers are available for block-

ing HO activities. Metalloporphyrins are the most potent and frequently used to date (Vreman et al., 1993). Acute application of metalloporphyrins can evoke fast responses. One administration of ZnDPBG (45 $\mu\text{mol/kg}$ i.p.) increased total peripheral resistance and mean blood pressure in SD rats within 5 min. The altered hemodynamic changes lasted for about 1 h (Johnson et al., 1995). ZnPP (45 $\mu\text{mol/kg}$ i.p.) also increased arterial pressure within 2 min of injection, whereas nonmetallic DPBG (45 $\mu\text{mol/kg}$ i.p.) had no effect on blood pressure (Johnson et al., 1995). These findings suggest that ZnDPBG and ZnPP increased arterial pressure by specifically inhibiting HO activity, whereas nonmetallic DPBG is not an effective HO blocker. Metalloporphyrins have also been used chronically to inhibit HO activity for a prolonged period. Daily injection of ZnPP (45 $\mu\text{mol/kg}$ i.p.) or CrMP (4 $\mu\text{mol/kg}$ i.p.) for 4 days resulted in a striking increase in blood pressure in prehypertensive (4-week-old) and young (8-week-old) SHR (Ndisang et al., 2003). The advantages and disadvantages of using these metalloporphyrins to specifically inhibit HO activity have also been described previously (section III.E.) and will not be repeated here.

C. Inhalation of Carbon Monoxide

Inhalation of CO or NO has been viewed as a novel therapeutic approach for certain diseases. The potential therapeutic doses of inhaled NO are probably lower (65 ppm), due to the high reactivity of NO to generate more damaging peroxynitrite radical, than that of CO (500–1000 ppm) (Thiemermann, 2001). Fujimoto et al. (2004) applied inhaled CO to rats to examine whether cardiac ischemia-reperfusion injury in vivo could be ameliorated. Rats inhaled CO (250, 500, or 1000 ppm) for 24 h in a chamber, and myocardial ischemia/reperfusion damage was induced by occluding the left anterior descending coronary artery for 30 min. Although no protective effects were observed with low levels of inhaled CO, 1000 ppm CO significantly reduced the ratio of infarct areas to risk areas and suppressed the migration of macrophages and monocytes into infarct areas and the expression of TNF- α in the heart. Exposure to 1000 ppm CO activated p38MAPK, protein kinase B α , eNOS, and cGMP in the myocardium. There were no visible side effects with this CO inhalation therapy, as no alterations in the arterial blood pressure, pulse rate, and body temperature were detected in the treated animals.

Reports on tolerance to CO exposure have been contradictory. On the one hand, no deleterious effects were found for chronic exposure of the rodents to 500 ppm continuously for up to 2 years (Stupfel and Bouley, 1970; Otterbein and Choi, 2000). It has also been claimed that the exposure level of CO at 100 ppm can be harmless if the exposure duration is limited to a few hours (Otterbein and Choi, 2000). On the other hand, exposing rats for 20 h/day to CO (200 ppm) for 14 consecutive days induced a 21% increase in right ventricular hypertrophy

and a 7% increase in left ventricular hypertrophy (Loennechen et al., 2002). Similar health hazards of CO inhalation have also been reported (Penney and Formolo, 1993; Loennechen et al., 1999). Reasons for these opposite observations on the health consequence of CO inhalation are not clear.

D. Use of Carbon Monoxide-Releasing Compounds

Based on the capability of certain transitional metal carbonyls to release CO in biological fluids, a series of CO-releasing compounds were designed by Motterlini's research team. The first generation of CORM, such as tricarbonyldichlororuthenium(II) dimer (CORM-2), are soluble only in organic solvents, such as DMSO, which calls into question the safety of these compounds when used in vivo. Furthermore, the release of CO from these compounds usually requires physical (e.g., irradiating light) or chemical (e.g., strong ligands, such as DMSO) stimuli (Motterlini et al., 2002a,b). By coordinating glycine onto the metal center, the second generation of CORM, water-soluble and less toxic CO-releasing compounds, was formulated, e.g., CORM-3. This compound liberates CO in vitro, ex vivo, and in vivo (Clark et al., 2003; Foresti et al., 2004). CORM-3 has several advantages over the first generation of CORMs. 1) Water solubility. Approximately 1 mol of CO per mole of CORM-3 is liberated within 10 min after dissolving CORM-3 in water (Clark et al., 2003). 2) It is easy to control the concentration and release rate of CO. Both physiological pH and strong ligands present in the biological environment would suffice to dissociate CO from the metal center (Foresti et al., 2004). 3) No more CO will be released after keeping CORM-3 in Krebs-Henseleit buffer overnight at room temperature, thus providing a negative control to assess the biological function of CO released from the active CORM-3. It has been shown that CORM-3 administration significantly lowered mean blood pressure in vivo and relaxed precontracted aortic rings in vitro. The effective dosage of CORM-3 at a low micromolar range is relatively safe and physiologically relevant to endogenous CO. The cardioprotective functions of CORM (Clark et al., 2003; Guo et al., 2004) have also been reported. These studies shed light on the potential use of CORM for both basic biomedical research and future clinical applications to deal with different pathological situations.

E. Use of Prodrugs to Generate Carbon Monoxide

Dichloromethane (DCM), also named methylene chloride, is used as a degreaser, solvent, and extraction medium and is an important constituent of paint removers in industry. This compound has also been known for long time for its oxidation by primarily the 2E1 and 2B1 cytochrome P450 isoforms, yielding CO and carbon dioxide in vivo (Stewart et al., 1972; Ratney et al., 1974;

Milosz and Settle, 1975; Andersen et al., 1994). It is a high-affinity ligand for certain heme proteins with low capacity for oxidation in vivo. The effectiveness of biotransformation of DCM to CO in vivo has been documented. Six hours after administration of a single oral dose of DCM (6.2 $\mu\text{mol/kg}$) to rats, the mean maximum COHb level of 9.3% was detected. This level was significantly enhanced by prior administration of benzene, toluene, *o*- and *m*-xylene, and *p*-xylene (Pankow et al., 1991) or long-term ethanol treatment (Wirkner et al., 1997). Rats receiving injected DCM (3 $\mu\text{mol/kg}$ i.p.) (Kim and Kim, 1996) or inhaled DCM (100–500 ppm) (Wirkner et al., 1997) also exhibited a COHb level around 10 to 12%, which is the metabolism-delimited saturation level. The quantity of CO produced per mole of inhaled DCM in rats was about 0.7 to 0.8 mol (Andersen et al., 1994). The half-life of DCM in vivo is about 3 h, and CO released from DCM has a half-life of about 13 h (Wirkner et al., 1997; Sass et al., 2004).

Earlier studies showed the potential of this CO prodrug as an antisickling agent, which prevented the aggregation of hemoglobin S (Milosz and Settle, 1975). Sass et al. (2004) recently examined the protective effect of CO on the immune-mediated mouse liver injury, using DCM as a CO prodrug. An oral dose of 6.2 $\mu\text{mol/kg}$ DCM in corn oil was given 6 h before induction of liver damage. This treatment protected mice from liver damage based on the measurement of plasma enzyme activities of alanine aminotransferase and caspase-3 activation in liver tissues. The same liver protection effect was achieved by exposing mice to CO gas at 250 ppm for 24 h in a CO chamber.

Application of DCM in a recent transplantation study has generated interesting results. In a model of chronic allogeneic aorta rejection in rats, oral application of DCM immediately after transplantation (500 mg/kg/day for 15–30 days) significantly reduced the intimal thickness of the vessel wall, a comparable outcome with the use of AdHO-1 transgene approach (Chauveau et al., 2002). The number of leukocytes and expression of adhesion molecules, costimulatory molecules, and cytokines were also significantly reduced by DCM treatment. In fact, DCM produced a CO-mediated inhibition of vascular SMC accumulation in the intima more efficiently than AdHO-1 treatment. Application of DCM also prolonged liver allograft survival to more than 47 days with diminished apoptosis and preservation of hepatic architecture and function (Ke et al., 2002).

XI. Conclusions and Perspectives

Intra- and intercellular communications are conducted by mechanical, electrical, or chemical signals. Chemical signaling molecules include hormones, autooids, and transmitters. Whereas hormones act on distant targets via circulation in an endocrine mode, transmitters usually perform paracrine and/or autocrine

regulation for adjacent cells or the cells where the transmitters are produced. Autocoids (such as prostaglandins, adenosine, and platelet-activating factor) act on the same cells from which they are produced, albeit without a concrete and precise definition. Some endocrine hormones, such as melatonin, also function as autocoids (Tan et al., 2003). Cognate membrane receptors are the prerequisite for the biological effect of hormones, autocoids, and classic transmitters. The ligand-receptor interaction is the triggering event in generating intracellular second messengers and steering cellular activities.

Advances in cellular and molecular signaling studies have led to the classification of transmitters into two major categories, i.e., neurotransmitters (Kandel et al., 2000) and gasotransmitters (Wang, 2002). The first category includes a handful of identified low molecular weight endogenous substances, such as acetylcholine, catecholamines, serotonin, histamine, glutamate, glycine, GABA, and ATP or its metabolites. Categorizing CO together with NO and H₂S as one member of the second category, gasotransmitters, is based on the following criteria (Wang, 2002). 1) CO is a small molecule of gas. 2) CO is freely permeable to membranes. As such, its effects do not rely on the cognate membrane receptors. CO has paracrine and autocrine effects. It may also exert an endocrinal impact when carried by heme proteins and released at the remote targeting sites. 3) CO is endogenously and enzymatically generated, and its production is regulated. 4) The biological effects of endogenous CO can be mimicked by exogenously applied CO. 5) CO has well defined and specific functions at physiologically relevant concentrations. Thus, manipulating endogenous levels of CO gas evokes specific physiological changes. 6) The cellular effects of CO may or may not be mediated by second messengers. Activation of sGC is one example of the mediation of CO effects by known second messengers. Direct modulation of membrane BK_{Ca} channels represents a second messenger-independent mode of CO action. Table 4 summarizes the major differences between neurotransmitters and gasotransmitters.

The spectrum of ideas and perceptions on the physiological roles of CO ranges from suspicion to assurance. It may be argued that CO is a stable biological product, and, as such, it may not be physiologically important since its removal is not dynamic. The first counterargument for this perception is that a stable biological sub-

stance is also important for homeostatic control of given physiological functions. With the knowledge of endogenous production and physiological functions of CO, either in circulation or in specific tissues, CO can function as a tonic gasotransmitter that provides a constitutive force even without a "switch" to turn the production of CO on and off. The second counterargument comes from the fact that HO-1 expression can be up-regulated in a relatively fast fashion. Even the expression or activity of HO-2 can be regulated under specific conditions. Therefore, the endogenous level of CO can vary in response to different physiological or pathophysiological stimuli. It should be pointed out that precise time course for up-regulation of HO-1 leading to acute production of CO and target response has not been determined. Therefore, how fast and how precise CO can provide an instant regulation of certain cell functions, as NO does in many cases, are unknown. Among other concerns surrounding the physiological role of CO is the weak activation of sGC by CO (Stone and Marletta, 1994; Kharitonov et al., 1995). As discussed in previous sections, the existence of unidentified endogenous substances may greatly increase the stimulatory effect of CO on sGC. CO may also stimulate the production of NO, which acts on sGC. Finally, CO also exerts its physiological actions on cellular targets other than sGC.

The last several decades' research on CO metabolism and function has set the stage for the exploration of the physiological role of endogenous CO. Positioned still at the beginning of this exploration, we face many challenges. The physiological roles of CO and its acute as well as chronic regulation have not yet been fully addressed. The altered metabolism and functions of CO under different pathological conditions should be further articulated. The transference of our knowledge on the physiology/biology of CO to pharmacological and therapeutic applications should be promoted. Cautions in this regard are also necessary. For example, depending on interspecies differences in the promoter regions, HO-1 genes from different species may have quite different responses to the given inducers. In response to interferon- γ or hypoxia, mouse HO-1 gene is activated, but repression of human HO-1 gene occurs (Sikorski et al., 2004). Accordingly, hypoxia up-regulates the expression of HO-1 in rat, bovine, mouse, and monkey cells but is a repressor of HO-1 expression in human cells (Lee et al., 1997; Kitamuro et al., 2003). Keeping this optimism and

TABLE 4
Conceptual comparison of neurotransmitters with gasotransmitters

| | Source Cells | Release | Reuptake | Removal Mechanism | Synaptic Transmission | Receptors |
|-------------------|-----------------------------------|--|----------|---|----------------------------------|--------------------------------------|
| Neurotransmitters | Neuron | Exocytotic vesicle | Yes | Enzyme-dependent | Pre- to postsynaptic membrane | Membrane receptors a necessity |
| Gasotransmitters | All cell types, including neurons | Released as produced, (usually) not stored | No | Nonenzymatic: diffusion, oxidation, scavenging, methylation, etc. | Forward and retrograde diffusion | No classic membrane receptors needed |

these cautions in mind, we predict a mesmerizing and accelerating progress in this field.

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