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Pharmacological and Clinical Aspects of Heme Oxygenase

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Abstract——**This review is intended to stimulate interest in the effect of increased expression of heme oxygenase-1 (HO-1) protein and increased levels of HO activity on normal and pathological states. The HO system includes the heme catabolic pathway, comprising HO and biliverdin reductase, and the products of heme degradation, carbon monoxide (CO), iron, and biliverdin/bilirubin. The role of the HO system in diabetes, inflammation, heart disease, hypertension, neurological disorders, transplantation, endotoxemia and other pathologies is a burgeoning area of research. This review focuses on the clinical potential of increased levels of HO-1 protein and HO activity to ameliorate tissue injury. The use of pharmacological and genetic probes to manipulate HO, leading to new insights into the complex relationship of the HO system with biological and pathological phenomena under investigation, is reviewed. This information is critical in both drug development and the implementation of clinical approaches to moderate and to alleviate the numerous chronic disorders in humans affected by perturbations in the HO system.**

I. Introduction

Heme oxygenase (HO[1]) is the rate-limiting enzyme in the catabolism of heme, a process that leads to formation of equimolar amounts of the bile pigment biliverdin, free iron, and carbon monoxide (CO). Biliverdin formed in this reaction is rapidly converted to bilirubin. The enzymatic nature of the heme catabolic process and the essential features of its biochemistry were initially described by the Schmid group (Tenhunen et al., 1968, 1970). Similarities of this process to the cytochrome P450-dependent mixed function oxidase system, which had been earlier described by Estabrook and colleagues (Estabrook et al., 1963; Cooper et al., 1965), subsequently led to the proposed role of cytochrome P450 as the terminal oxidase in the heme catabolic system (Tenhunen et al., 1972). However, the disparity in organ distribution and tissue concentrations of cytochrome P450 and the HO system, among other considerations,

[1]Abbreviations: HO, heme oxygenase; CO, carbon monoxide; HO-1, heme oxygenase isozyme 1 (inducible form); HO-2, heme oxygenase isozyme 2 (constitutive form); ALA, δ-aminolevulinic acid; O_2^-; AP-1, activator protein-1; PKC, protein kinase C; $SnCl_2$, stannous chloride; HETE, hydroxyeicosatetraenoic acid; COX, cyclooxygenase; SnMP, tin mesoporphyrin IX dichloride; CoPP, cobalt protoporphyrin IX dichloride; NOD, nonobese diabetic; $CoCl_2$, cobaltous chloride; eNOS, endothelial nitric-oxide synthase; AKT, protein kinase (activator); p, phosphorylated; NO, nitric oxide; NOS, nitric-oxide synthase; ZnPP, zinc protoporphyrin IX dichloride; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole; SHR, spontaneously hypertensive rats; ZnDP, zinc 2,4-bis glycol deuteroporphyrin; NTS, nucleus of the tractus solitarius; BK_{Ca} channel, large-conductance calcium-activated potassium channel; CORM, carbon monoxide releasing molecule; sGC, soluble guanylate cyclase; GSH, glutathione; si, small interfering; ROS, reactive oxygen species; $ONOO^-$, peroxynitrite; NF-κB, nuclear factor-κB; CNS, central nervous system; LPS, lipopolysaccharide; LDL, low-density lipoprotein; HSP, heat shock protein; PGA, prostaglandin A; STAT, signal transducer and activator of transcription; IL, interleukin; SnPP, tin protoporphyrin IX dichloride; EC-SOD, extracellular superoxide dismutase; TNF, tumor necrosis factor; PPAR, peroxisome proliferator-activated receptor; VSMC, vascular smooth muscle cell; L or D-4F, amino acid apolipoprotein A1-4F; iNOS, inducible nitric-oxide synthase; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; cdk2, cyclin-dependent kinase 2; ERK, extracellular signal-regulated kinase; AG1067, unnamed derivative of probucol; ANP, atrial natriuretic peptide; AT_1, angiotensin II type 1; CCl_4, carbon tetrachloride; Nrf2, nuclear factor E2-related factor-2; TLR, toll-like receptor; TMMC, tris(methoxymethoxy) chalcone; JNK, Jun N-terminal kinase; HCV, hepatitis C virus; MnSOD, manganese containing superoxide dismutase; SIRS, systemic inflammatory response syndrome; T_3, 3,5,3′-L-triiodothyronine; Δ^{12}-PGJ_2, deoxy-$\Delta^{9,12}$-13,14-dihydro-prostaglandin D_2; AZT, 3′-azido-3′-deoxythymidine; BFU-E, burst-forming units–erythroid; EPO, erythropoietin; RSK, ribosomal S6 kinase; Bad, Bcl-xL/Bcl-2 associated death promoter; Apaf-1, apoptotic protease activating factor-1; HIV, human immunodeficiency virus; APP, amyloid precursor protein.

left the putative role of cytochrome P450 in heme catabolism an unsettled matter.

The issue was conclusively settled when it was shown that HO could be strongly induced in the liver by the inorganic element cobalt, independently of cytochrome P450, and that a complete separation of HO from cytochrome P450 could be achieved (Maines and Kappas, 1974, 1975a). Simultaneously the same conclusion was reached with respect to HO in the spleen (Yoshida et al., 1974). The identification of HO as an enzyme distinct from cytochrome P450 and one that was inducible by chemicals other than its natural substrate, heme, set the stage for the remarkable increase in HO research that followed in the succeeding years. In the reticuloendothelial system, HO functions as the rate-limiting enzyme in further heme degradation (Abraham et al., 1983). HO activity is present in different cell types, including hematopoietic stem cells, in bone marrow, and in all organs studied (Abraham et al., 1988b, 1989b; Abraham, 1991). Many of these studies focused on the diversity of chemicals that could down- or up-regulate HO activity, the mechanism of the HO induction process, the multiple biological responses that follow upon perturbations of HO expression in various cell types, and the possible clinical benefits that might accrue from controlling the activity of this enzyme in humans (Kappas and Drummond, 1986; Willis et al., 1996; Mingone et al., 2006).

The therapeutic potential inherent in devising a way to up- or down-regulate HO activity is of clinical interest. For example, down-regulating HO activity (Drummond and Kappas, 1981; Yoshinaga et al., 1982a) was first shown to be therapeutically important when a safe and effective method for transiently blocking bilirubin production in newborns was developed (Kappas et al., 1988, 1995, 2001a,b; Valaes et al., 1998; Martinez et al., 1999; Kappas, 2004). This method resolved the problem of progressive, unpredictable, and often undiagnosed jaundice in newborns—especially those infants born in deprived socioeconomic settings who had a particular risk of brain damage resulting from uncontrolled hyperbilirubinemia. The development of HO inhibitors having a pharmacological profile that permitted their use in infants to address a serious medical problem provided the first demonstration of the potential clinical uses to which agents that can control HO activity might be put. It can be anticipated that HO inducers will also have important applications in various diseases, such as hypertension, diabetes, and cardiovascular disease. HO-1-derived CO and biliverdin/bilirubin can be of potential use for clinical application. The delivery of increased levels of both of these products as a result of increased HO activity will permit their faster and sustained delivery and, as such, may have great significance in the clinic.

The myriad metabolic systems that have subsequently been shown to be responsive to the up-regulation of HO activity (Abraham et al., 1996, 2003b; Mingone et al., 2006) make it clear that enhancing HO activity by either pharmacological or genetic means offers potential for the development of new therapeutic modalities, which could moderate the course of various pathological processes in humans. This review highlights some of these possibilities and focuses on HO as an especially interesting target enzyme for future pharmaceutical development.

II. Heme Degradative Pathway

A. Characterization of Heme Oxygenase

Heme oxygenase, the rate-limiting enzyme in heme catabolism, catalyzes the stereospecific degradation of heme to biliverdin, with the concurrent release of iron and CO (Fig. 1). In mammals, biliverdin is then converted to bilirubin by the cytosolic enzyme biliverdin reductase; bilirubin is subsequently conjugated by UDP-glucuronyl transferase and then excreted into the bile. In addition to its role in regulating cellular levels of heme, HO is responsible for the recycling of iron from senescent red blood cells and extrahematopoietic cells, such as liver cells. Some 80 to 85% of the bilirubin formed in vivo is derived from hemoglobin released from aging or damaged erythrocytes (Schacter, 1988). This amount accounts for the high basal activity of HO within those tissues rich in reticuloendothelial cells, such as the spleen and bone marrow. Many cell types in culture, such as those of hepatic, renal, testicular, brain, intestinal, and ocular origin, catalyze heme degradation to biliverdin (Abraham et al., 1988a).

B. Heme Oxygenase Isozymes

Heme oxygenase, as described above, was first purified to homogeneity from rat liver and pig and bovine spleen and was shown to have a molecular mass of 32,000 Da (Maines et al., 1977; Yoshida and Kikuchi, 1978; Yoshinaga et al., 1982a). Isolation and characterization of human HO-1 was also reported (Abraham et al., 1987a). Human HO-1 activity can be increased in vitro by either NADH or NADPH as reducing agents (Abraham et al., 1987a), and its sensitivity to synthetic metalloporphyrins has been well studied (Mitrione et al., 1988; Chernick et al., 1989). Additional porphyrins and HO inhibitors are described by Kral et al. (2006).

It is now apparent that two isoenzymes of HO exist; the original enzyme was designated HO-1 and the second isoenzyme was designated HO-2. HO-2 has a molecular mass of 34,000 Da, and cDNA clones have been isolated for both rat and human HO-2 (Maines et al., 1986; Cruse and Maines, 1988; Trakshel and Maines, 1989). It appears that these isoenzymes are the products of two distinct genes, but share approximately 40% amino acid sequence homology (Müller et al., 1987; McCoubrey et al., 1992; McCoubrey and Maines, 1994). HO-1 is the product of only one transcript, but HO-2 is encoded by two transcripts from one gene (McCoubrey

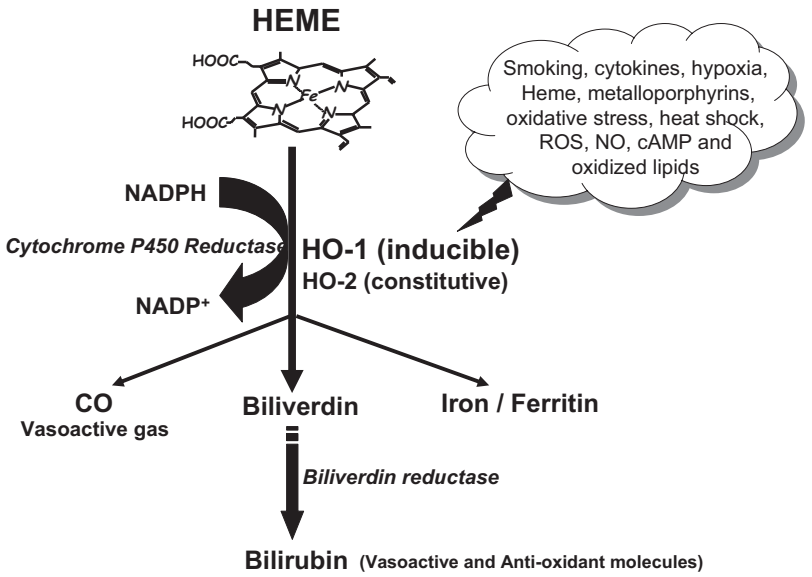

FIG. 1. Schematic representation of the heme degradative pathway. HO-1/HO-2 degrades heme, which is oxidatively cleaved at the methylene bridge to produce equimolar amounts of CO, biliverdin, and iron. Biliverdin is converted to bilirubin in a stereospecific manner by the cytosolic enzyme, biliverdin reductase. Both CO and bilirubin are bioactive molecules, and the iron generated by HO-1 and HO-2 is immediately sequestered by associated increases in ferritin. HO-2 is a constitutive enzyme, whereas HO-1 is inducible by heavy metals, cytokines, UV light, oxidative stress, inflammatory cytokines, and many drugs.

and Maines, 1994). These different transcripts arise from a difference in polyadenylation. Cloned cDNA encoding for human HO-2 was reported, confirming the presence of the two isoenzymes by examining their different regulation (Shibahara et al., 1993). It is apparent that HO-2 is constitutively expressed, whereas HO-1 is inducible by a large number of structurally unrelated pharmacological and other agents as well as by a variety of circumstances, such as heat shock and other forms of cellular stress.

In the early 1980s, Kappas' group originally investigated HO inhibitors in the control of hyperbilirubinemia in an animal model of jaundice (Drummond and Kappas, 1982a, 1986; Kappas, 2002, 2004). Subsequently, several HO inhibitors have been designed and constructed, including zinc 2,4-bis glycol, by Abraham's group (Abraham et al., 1988b; Martasek et al., 1988; Mitrione et al., 1988; Chernick et al., 1989) and showed the sensitivity of human tissues to these inhibitors (Martasek et al., 1988; Mitrione et al., 1988; Chernick et al., 1989). Both adult and fetal human HO-1 are sensitive to numerous synthetic metalloporphyrins, including zinc 2,4-bis glycol deuteroporphyrin (Mitrione et al., 1988; Chernick et al., 1989). Zinc-protoporphyrin was developed to treat cancer (Fang et al., 2004b). Recently, imidazole-dioxolane compounds have been shown to act as inhibitors of HO activity (Vlahakis et al., 2006; Sugishima et al., 2007) and shown to be effective as inhibitors of specific HO isoenzymes as well as HO activity (Kinobe et al., 2007).

HO-1 activity is increased in whole animal tissues after treatment with its natural substrate heme, as well as various metals, xenobiotics, endocrine factors, and synthetic metalloporphyrins. Many cells in culture, including hemopoietic, hepatic, epithelial, endothelial, and retinal pigmented epithelial cells, respond to these agents in a similar manner, i.e., by a marked increase in HO-1 activity (Matsuura et al., 1985; Stout and Becker, 1986; Yoshida et al., 1988; Lutton et al., 1991; Alam and Zhining, 1992; Kutty et al., 1994). Furthermore, HO-1 is a heat-shock protein (Shibahara et al., 1987; Mitani et al., 1989) and also a stress protein induced by several agents that cause oxidative damage (Keyse and Tyrrell, 1989; Nascimento et al., 1993). HO-1 is considered to be a key player in the development of tolerance in response to nitrates (McCoubrey and Maines, 1994). It is therefore possible that the induction of HO-1 may be an essential event for some types of acute reactions and for cellular protection after injury. This hypothesis implies that the induction of HO-1 enables the removal of the potentially toxic prooxidant molecule heme, a lipid-soluble transmissible form of iron, as well as the generation of bilirubin and biliverdin, metabolites with antioxidant properties (Stocker et al., 1987). HO-1 induction, coupled with ferritin synthesis, is a rapid, protective in vivo antioxidant response in rhabdomyolysis-induced kidney injury in the rat (Nath et al., 1992). This condition is characterized by an increased release of myoglobin and hemoglobin into the extracellular renal space, which initiates tissue toxicity with subsequent inflammation and renal failure. Along these lines, some have suggested that heme derived from cytochrome P450 may initiate tissue toxicity (Paller and Jacob, 1994).

Induction of HO-1 in skin fibroblasts is of value in protecting against UV light-induced oxidative stress (Vile and Tyrrell, 1993). These authors conclude that the

effect of such radiation, which also promotes an increase in ferritin levels, is mediated via the HO-1 release of iron and CO from endogenous heme sources. They propose that the increase in ferritin, after UV light exposure and the associated HO-1 induction, would decrease intracellular iron such that iron-catalyzed free radical reactions would be restricted during periods of subsequent oxidative stress.

The role of HO-2 in cells is not as well understood; however, it is becoming apparent that HO-2 may have an important role in epidermal cells, germ cell development, and signal transduction in neural tissues. There is great variability in the tissue distribution of the two isoforms of HO and the basal levels of HO-2 protein vary considerably from tissue to tissue. HO-2 is unaffected by light stress (Kutty et al., 1995) and is moderately expressed in normal rat retina compared with the brain and testes, which have the highest levels of HO-2 expression. It has been reported that HO-2 mRNA levels are low in human dermal fibroblasts but high in epidermal keratinocytes (Applegate et al., 1995), and it has been suggested that keratinocytes benefit from constitutively high levels of ferritin and that this is regulated by iron released as a result of the activity of the constitutive HO isozyme, HO-2.

C. Heme Oxygenase and the Heme Pool

Both intracellular heme and iron levels play an important role in the regulation of many cell functions. Heme levels are maintained and regulated by either the synthesis of heme or the degradation of heme by HO. Synthesis and degradation of heme are thus closely linked. Figure 2 depicts the heme biosynthetic pathway and regulation of the heme pool.

The rate-limiting synthetic enzymes are believed to be ALA synthase and, in part, porphobilinogen deaminase. Both enzymes exist in erythroid and nonerythroid forms. In nonerythroid cells such as liver, ALA synthase essentially plays a housekeeping role, maintaining intracellular heme levels. High levels of heme thus repress the synthesis of nonerythroid ALA synthase while stimulating heme degradation through the induction of HO. In erythroid cells, however, it appears that excess heme may stimulate cellular proliferation and differentiation and increase erythroid ALA synthase mRNA levels and enzyme activity. Furthermore, excess heme enhances the synthesis of globin mRNA. Thus, high heme levels in erythroid cells appear to stimulate the synthesis of hemoglobin. An iron-binding element has been located on the 5′ untranslated region of the erythroid, ALA synthase, so it is possible that the enzyme is actually regulated by intracellular levels of iron. Thus, an increase in heme may induce HO, increasing the levels of free iron which in turn stimulate the formation of erythroid ALA synthase mRNA. The regulatory role of heme in erythroid ALA synthase has been reviewed previously (Abraham et al., 1983).

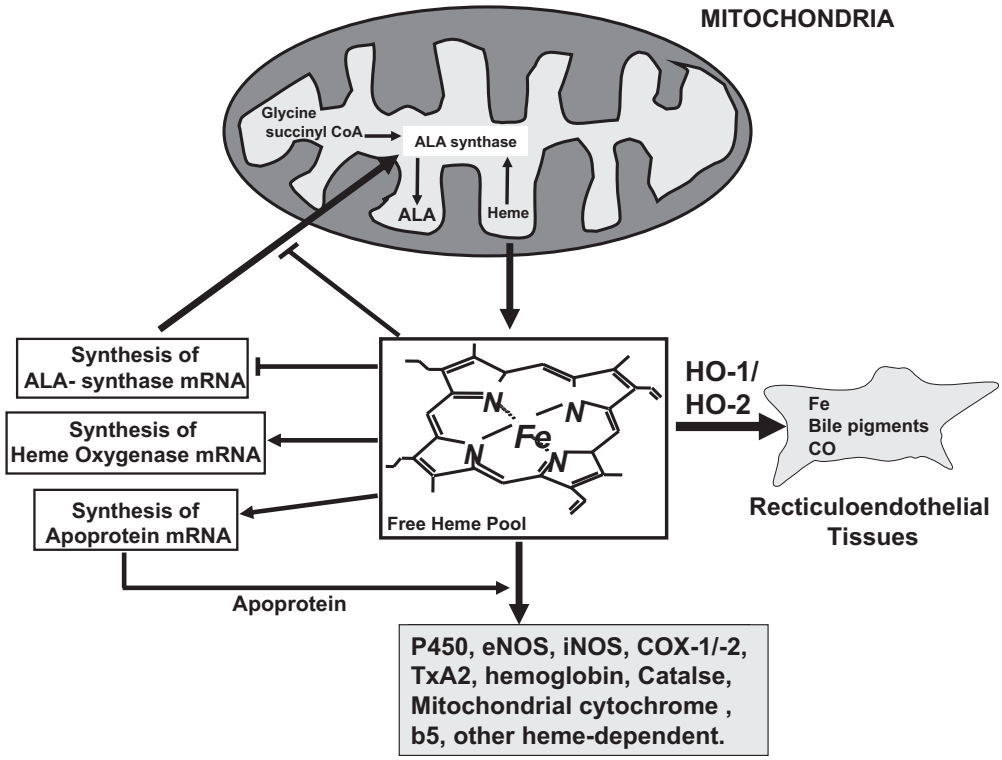

FIG. 2. Heme is regulated by the rate of synthesis and rate of degradation via the rate-limiting enzymes ALA synthase and HO-1. When heme is released by the mitochondria to the cytoplasm and exceeds the physiological need, it will cause suppression of ALA synthesis and/or inhibit ALA transport to the mitochondria; however, if heme is further increased it will cause induction of HO-1 and enhance the rate of its degradation.

D. Heme Oxygenase-NADPH Biliverdin Reductase

Heme degradation occurs mainly by oxidative cleavage of the α-methylene bridge of the molecule, eventually leading to the formation of the biliverdin IXα isomer along with trace amounts of IXβ, IXδ, and IXγ isomers. Kappas' group (Yoshinaga et al., 1982c) and others (Kutty and Maines, 1982) have demonstrated that heme and different substituents, such as heme C after its proteolytic cleavage from cytochrome *c*, become substrates for HO and are converted to biliverdin.

HO was partially purified from pig spleen, and it was reported that the enzyme could not use NADH as a reducing equivalent (Yoshida et al., 1974). HO was also isolated and purified from rat liver microsomes (Maines et al., 1977). However, several investigators (Maines et al., 1977; Hino and Minakami, 1979; Abraham and Levere, 1980) provided evidence that NADH can replace NADPH for the HO reaction in rat liver and bone marrow, but it is less efficient as an electron donor. Yoshinaga et al. (1982a) purified bovine spleen HO to homogeneity with a molecular weight of 31,000. The bovine spleen enzyme had a K_m values of 23 μM and 2 mM for NADPH and NADH, respectively. Sn-, Co-, Zn-, and Mn-protoporphyrin strongly inhibited the HO reaction whereas Mg-, Ni-, and Cu-protoporphyrin had little effect on heme degradation in vitro. In the reconstituted system, composed of purified enzyme, NADH replaced NADPH, but at a concentration higher than 5 μM NADPH was found to be a potent reducing agent, which is in agreement with earlier findings with rat bone marrow HO (Abraham and Levere, 1980).

Biliverdin reductase (EC 1.3.1.24), the enzyme that catalyzes the conversion of biliverdin to bilirubin (Singleton and Laster, 1965), uses NADPH rather than NADH as the preferred cofactor. The purified enzyme from liver has K_m values of 1 to 2 μM in assays with NADH and 0.2 μM in assays with NADPH. O'Carra and Colleran (1971) purified biliverdin reductase from guinea pig liver. This enzyme has a molecular weight of approximately 30,000 to 60,000. It does not contain flavin, heme, or metal and is inhibited by sulfhydryl reagents (Yoshinaga et al., 1982b). The ability of biliverdin reductase to bind to NADH or NADPH is dependent on the concentration of biliverdin (Singleton and Laster, 1965).

Recently, biliverdin reductase was reported to have a critical role in linking heme metabolism to cell signaling (Maines et al., 2007). Biliverdin reductase was shown to be induced and localized in the nucleus of rat kidney and, as such, is related to the chain breaking antioxidant activity of bilirubin, the inhibition of superoxide (O_2^-) formation by biliverdin and the modulation of the signal transduction pathway (Maines et al., 2001). These properties were associated with the unique characteristics of the dual pH/cofactor dependence of biliverdin reductase. Human biliverdin reductase was shown to undergo autophosphorylation and that phosphorylation was required for its activity (Salim et al., 2001). More recently, a role in the signaling cascade for AP-1 complex activation in the HO-1 oxidative stress response, as a novel regulator of activating transcription factor 2, has been proposed for biliverdin reductase, (Kravets et al., 2004) and of protein kinase (PKC) βII (Maines et al., 2007). These results indicate a much more complex role for biliverdin reductase than previously thought.

III. Pathophysiology of and Clinical Role of Heme Oxygenase-1/Heme Oxygenase-2: Reaction Products

A. Increased Levels of Heme Oxygenase-1 and Heme Oxygenase Activity

The degradation of heme is now considered critical in cellular defense for two contrasting reasons. First, the pro-oxidant heme is removed. Second, the increased production of bilirubin and CO is now regarded as beneficial and critical to cellular defense mechanisms. Iron, which can stimulate free radical formation, is immediately bound by ferritin. Thus, CO and bilirubin are seminal to the protection that occurs from elevated levels of HO-1 protein and HO activity.

Chronic induction of HO-1 protein can have both beneficial and detrimental effects. The role of HO, for example, in the acquisition of resistance to H_2O_2 and hemin toxicity was evaluated in renal epithelial cells. Adapted by long-term exposure to H_2O_2, renal epithelial cells showed a 2-fold increase in basal HO activity and increased levels of HO-1 protein, which was beneficial. Acute treatment with H_2O_2 and hemin, however, produced an increase in HO-1 that was associated with a decrease in the viability of the renal epithelial cells. Long-term exposure to both stressors resulted, however, in the acquisition of some resistance to further acute challenges of oxidant stress in these cells (da Silva et al., 1996). Thus, it appears that resistance built up, suggesting, that, to be effective, increased levels of HO-1 protein must be present before the onset of chronic disease.

1. Acute Effects. The acute induction of HO-1 has been shown to have a beneficial effect because of the rapid decrease in undesired heme. Sacerdoti et al. (1989) first reported the benefits of an acute effect, describing the fact that treatment with stannous chloride ($SnCl_2$) prevented the development of high blood pressure. Subsequently, others reported that acute and chronic expression of HO-1 decreased vasoconstrictors, such as 20-HETE (Laniado-Schwartzman et al., 1992; da Silva et al., 1994), thromboxane synthase activity (Sessa et al., 1989), and COX-2 activity (Abraham, 2003). Heme arginate, or heme, which is used clinically for the treatment of porphyria (Kordac et al., 1989), has been shown to have a beneficial effect on acute induction of HO-1 and lowers blood pressure in hypertensive rats (Schwartz-

man et al., 1990; Martasek et al., 1991; Ndisang et al., 2003; Wang et al., 2006b). Hemin, a critical component of hemoglobin, is an active ingredient of a biological therapeutic agent approved by the U.S. Food and Drug Administration for the treatment of acute porphyrias.

2. Chronic Effects. Chronic induction of HO-1 protein can have both beneficial and detrimental effects on cellular metabolism. The role of HO in the acquisition of resistance of H_2O_2 and hemin toxicity was examined in renal epithelial cells. Adapted by long-term exposure to H_2O_2, renal epithelial cells had a 2-fold increase in basal HO activity and increased levels of HO-1 protein which appeared to be beneficial. In contrast, acute treatment with H_2O_2 and hemin produced an increase in HO-1 protein that was associated with a decrease in the viability of renal epithelial cells. Long-term exposure to both stressors resulted, however, in the acquisition of resistance to acute challenges of oxidant stress in these cells (da Silva et al., 1996). Thus, it appears that resistance increased with time of exposure, suggesting that, to be effective, increased levels of HO-1 protein must be present before the onset of chronic disease. The results described above lead to the question, can chronic induction of HO lead to adverse effects in vivo? In cell cultures, chronic induction of HO activity by $SnCl_2$ resulted in a time- and dose-dependent decrease in heme and cGMP levels, attributable to the limitations of heme and culture media and the limitation in the heme synthesis (Abraham et al., 2002b). However, chronic induction of HO-1, using iron as an inducer, did not affect cellular heme or cytochrome P450 content because of the rapid increase in heme turnover (Abraham et al., 1983). Wang et al. (2006b) have elegantly shown that heme used for chronic treatment led to the continuous expression of HO-1, lowered blood pressure, and modestly decreased cellular heme. Heme administration has been shown to reduce NADPH oxidase and administration of the HO inhibitor tin mesoporphyrin (SnMP) reversed this effect and increased NADPH (Datla et al., 2007). These authors also showed that inhibition of NADPH oxidase was minimized by bilirubin (Datla et al., 2007).

In contrast, inducers such as CoPP or $CoCl_2$ will, at high concentrations, inhibit heme synthesis and decrease cytochrome P450 (Drummond and Kappas, 1982b; Lin et al., 1990). Investigators have administered high concentrations of CoPP or $CoCl_2$, which inhibit ferrochelatase and heme synthesis and may be toxic owing to the severe decrease in heme P450 (Abraham et al., 1983, 1988a). However, low concentrations of CoPP, administered once a week in experimental diabetes, moderately decreased cellular heme and cytochrome P450 (unpublished results) and increased eNOS, pAKT, and cell survival (Ahmad et al., 2005; Turkseven et al., 2005). Chronic administration of small doses of CoPP attenuated the coronary constrictor response to ischemia-reperfusion (L'Abbate et al., 2007). This modest and chronic expression of HO-1 resulted in a significant increase in serum adiponectin (Li et al., 2008). The HO-1-mediated increase in adiponectin provides the heart and vascular system with tolerance and resistance to oxidative stress generated not only in diabetes, but also to other types of vascular stress (Li et al., 2008). One can speculate that in anemias or when a genetic or environmental decrease in heme synthesis occurs, chronic induction of HO activity would exacerbate the disease state. Similarly, in porphyrias, in which the synthesis of heme is impaired, chronic increases in HO activity would only serve to worsen the disease through the depletion of heme. Thus, a careful review of the individual circumstances is necessary before one undertakes chronic manipulation of HO activity.

B. Role of Carbon Monoxide

CO has long been considered to be a toxic air pollutant because of its strong affinity for hemoglobin, more than 200-fold greater than that of oxygen. There are two major sources of CO in biological systems (Rodgers et al., 1994): one is HO-dependent. The other is HO-independent, due to the photo-oxidation and the auto-oxidation of organic molecules, phenols, and flavenoids and the peroxidation of lipids as a result of severe stress, which may not be achieved under physiological conditions (Fig. 3). However, the rapid increase in CO that occurs in vivo is solely due to either the induction of HO-1 or the constitutive HO-2 (Abraham et al., 1983). Because the major source of CO in animals is the degradation of heme by HO, it is now becoming apparent that CO serves as an important cellular signal molecule (Maines et al., 1993; Verma et al., 1993). Nitric oxide (NO) is generated by nitric-oxide synthase (NOS), a heme containing enzyme. It has been proposed that certain NO effects can be duplicated by CO, specifically; the action of certain neurotransmitters and muscle relaxants can be regulated by both molecules.

Physiological and immunocytochemical studies have demonstrated the presence of HO-2 in the carotid body glomus cell and suggested that the endogenous genera-

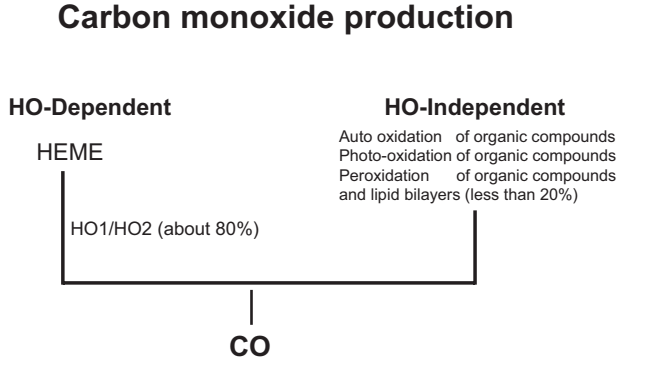

FIG. 3. CO is produced in vivo by two distinct routes. The HO-dependent route produces CO as a result of the enzymatic reaction of HO-1/HO-2 and the HO-independent route results from the random nonenzymatic cleavage of a variety of compounds producing a broad spectrum of products, including CO.

tion of CO appears to be a regulator of carotid body sensory activity (Prabhakar et al., 1995). Furthermore, inhibition of HO activity with ZnPP increases carotid body sensory activity and CO reverses this effect, suggesting that CO may be a neurotransmitter in this sensory organ. Direct evidence for the role of CO in vascular response was presented when it was shown that a reduction in CO generation resulted in increased vascular resistance in rat liver (Suematsu et al., 1994). Subsequent studies have clearly shown that HO-1-derived CO and bilirubin result in a vasorelaxant effect not only via cGMP-dependent but also via cGMP-independent (Ollinger et al., 2007; Li et al., 2008) stimulation of certain K channels (Wang and Wu, 1997; Wang et al., 1997, 2001; Dong et al., 2007) and an increase in adiponectin levels (L'Abbate et al., 2007). Furthermore, it is considered unlikely that CO and NO represent redundant messenger molecules, even though both are active in the vessel wall. Recent studies have shown that HO-derived CO may regulate or inhibit NOS and can contribute to hypertension and endothelial dysfunction in Dahl salt-sensitive rats (Teran et al., 2005). The measurement of increased levels of HO-1 protein is not an indication of increased total HO activity. Increased HO-1 protein in Zucker rats did not result in increased total HO activity. Additionally, total HO activity was restored by antioxidants and peroxynitrite scavengers such as ebselen (Kruger et al., 2006). Therefore, the measurement of HO-1 protein or describing an increase in HO-1 protein levels does not mean an increase in HO activity, i.e., increased CO and bilirubin production. The measurement of total HO activity is essential. Inducers of HO-1 gene expression restored the activity of preexisting inactive HO-1 to active HO-1, increased the levels of CO and bilirubin, restored eNOS, decreased blood pressure, and normalized kidney function (Kruger et al., 2006). CO is an activator of guanylyl cyclase, and it has been suggested that it may be a major physiological regulator of cGMP levels in the brain (Rattan and Chakder, 1993). In the brain, HO-2 has been found to be closely associated with guanylyl cyclase, ALA synthase, cytochrome P450 reductase, and NOS (Maines, 1993). It has been suggested that the role for HO-2 in the brain is to produce CO, which could then function as a messenger. However, this role has not been clearly defined. The function of CO produced by HO in tissues other than the brain is also unclear, although it appears that CO can act as a smooth muscle relaxant (Patel et al., 1993). Although CO, like NO, is considered a vasorelaxant via stimulation of cGMP, the relative potency of CO is very small compared with that of NO. However, the potency of the CO-mediated increase in cGMP may be greatly increased by other agents, (Friebe et al., 1996; Friebe and Koesling, 1998). These authors show that the administration of the benzyloid derivative YC-1 potentiates CO-mediated cGMP formation to a level similar to that seen for NO (Friebe et al., 1996).

Although numerous reports show that HO-1-derived CO mediates vasorelaxation in the aorta (Furchgott and Jothianandan, 1991), hepatic vein (Pannen and Bauer, 1998), piglet mesenteric artery (Villamor et al., 2000), pial arterioles (Leffler et al., 1999), and pulmonary artery (Ahmad et al., 2005), the vasorelaxant effect of CO appears to be dependent on unknown factors. For example, it was reported that CO causes vasodilation in the hepatic vein (Pannen and Bauer, 1998), but Coceani et al. (1984) showed that hepatic arteries are resistant to vasodilation. In support of HO-1-mediated vasodilation, inhibition of HO activity increased blood pressure in spontaneously hypertensive rats (SHR) (Ndisang and Wang, 2002). The vasodilator effect of CO in various vessels may be due to a CO-mediated decrease in the cytochrome P450-dependent generation of vasoconstrictors (Coceani et al., 1988; Wang, 1998). Cytochrome P450 metabolizes arachidonic acid to the very potent vasoconstrictor, 20-HETE (Harrison et al., 1993; Schwartzman et al., 1996). Initially, the systemic inhibition of HO activity with ZnDPBG was reported to be associated with an increase in mean arterial pressure and a decrease in the gain of the baroflex. In addition, an increase in blood pressure was reported after either unilateral or bilateral injection of ZnDPBG into the nucleus of the tractus solitarius (NTS). This was reversed by direct injection of saline-saturated CO, suggesting that decreased levels of CO were responsible for the pressor actions of ZnDPBG. However, the same author later reported that both exogenous and endogenous CO caused vasoconstriction (Johnson and Johnson, 2003). These contradictions remain to be clarified.

Another important mechanism by which CO plays a major vasodilator role is activation of the calcium-activated potassium channel. Wang and associates (Cannon, 1929; Wang and Wu, 1997, 2003; Wang, 1998; Ollinger et al., 2007; Li et al., 2008) have presented a series of elegant papers showing that CO-mediated relaxation was partially blocked by inhibitors of BK_{Ca} channels without affecting Ca homeostasis. Other investigators have shown that CO and CO-releasing molecule (CORM)-3 stimulate BK_{Ca} channels in HEK-293 cells (Williams et al., 2004; Xi et al., 2004). CO-mediated relaxation is not dependent on the cGMP pathway but is dependent on activation of BK_{Ca} channels (Barkoudah et al., 2004) and is abolished by blockers of BK_{Ca} channels (Leffler et al., 1999). Therefore, the vasodilatory effect of CO may be dependent on the level of NO, BK_{Ca} activities, and soluble guanylate cyclase (sGC), among other factors. CO inhibits NO-mediated relaxation in conditions in which there is an elevation of NO levels. In contrast, CO stimulates sGC and cGMP in conditions in which NO is diminished. Therefore, CO may act as a partial agonist for sGC activation to regulate vascular function and response (Ishikawa et al., 2005; Suematsu et al., 2005; Hangai-Hoger et al., 2007). CO acts as a

guardian against hepatic dysfunction and regulates NO-mediated activation of cGMP (Suematsu et al., 2005; Wu and Wang, 2005). Wu and Wang (2005) have provided an excellent review on the beneficial effect of CO in stimulation of K channels and vascular relaxation independent of cGMP. Exposure of endothelial cells to CO-releasing molecules restores GSH levels, which are diminished when cells are treated with HO-1, siRNA, or HO inhibitors (Li et al., 2007c). This effect was due to the increase in glutamate cysteine lipase, the rate-limiting enzyme in GSH biosynthesis. The HO-1-mediated increase in GSH levels was not limited to endothelial cells but to renal cell primary cultures (Quan et al., 2004b).

HO-derived CO has been identified as playing a role in many processes related to vascular control and tissue viability (Fig. 4). Clearly the benefits of CO have dominated much of the recent literature regarding the cytoprotective role of HO during pathological conditions. However, one must be aware (in accordance with the admonition of Paracelsus) that whereas a little may be beneficial, a lot may be harmful, as discussed earlier. High levels of exogenously administered CO will bind to hemeproteins, thereby disrupting mitochondrial transport which leads to release of superoxide anions, and become toxic. In addition, excessive CO will competitively remove NO from hemeproteins, which in a ROS-rich environment will lead to the production of $ONOO^-$ and tissue damage (Thom et al., 1999). Therefore, administration in the form of CO donors or CORMs (Motterlini et al., 2002., 2005) must be carefully titered.

In recent years, CORMs have been developed to facilitate CO delivery and prolong its bioactions, especially in vascular protection (Motterlini et al., 2002, 2005). The first generation of these molecules contained a central metal (CORM-3). Although the compounds were effective in delivering CO, preventing renal injury, and attenuating diabetes-mediated endothelial cell death in vivo (Vera et al., 2005; Rodella et al., 2006), they also were effective in inducing HO-1 protein levels (Vera et al., 2005). CO donors or HO-1 overexpression blocks interleukin-18-mediated NF-κB-phosphatase and tensin homolog deleted on chromosome 10-dependent human cardiac endothelial cell death (Zabalgoitia et al., 2008). CO or HO-1 expression blocks diabetes-mediated human and animal endothelial cell death (Abraham et al., 2003a; Rodella et al., 2006). The next generation of CO-releasing molecules to be developed, having long-term action and lacking the ability to induce HO-1, may hold additional options for clinical benefit.

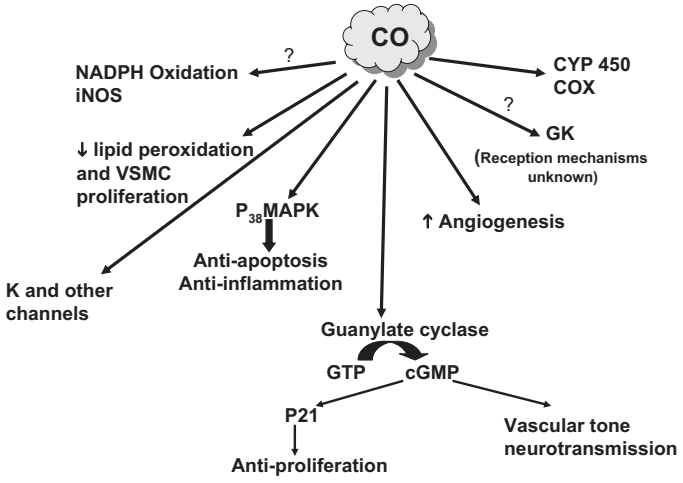

FIG. 4. Schematic representation of the role of CO in biological systems. Perturbations in the levels of CO influence a broad spectrum of metabolic pathways. Although CO, like NO, acts to stimulate cGMP, it differs from NO in many functions. For example, CO produces vasorelaxation by stimulating K channels (at the α subunit, but NO acts at the β subunit), increases angiogenesis, and is antiapoptotic and anti-inflammatory. CO may stimulate NO release from the NO binding sites of the heme molecules.

C. Role of Bilirubin/Biliverdin

Biliverdin produced by the HO-1 catabolism of heme is rapidly converted to bilirubin, which at high concentrations has been shown to be a CNS-toxic agent in newborns (Kappas, 2004). Kernicterus, the severe form of bilirubin neurotoxicity, became extremely rare with the advent of phototherapy. However, in recent years, as a result of the early discharge of mothers and infants after delivery, there has been a reemergence of this irreversible neurological syndrome. At normal concentrations in mammals, unconjugated bilirubin is an efficient scavenger of singlet oxygen and acts as a reducing agent for certain peroxidases, including horseradish peroxidase and prostaglandin H synthase, in the presence of hydrogen peroxide and organic hydroperoxides. Thus, biliverdin and bilirubin are reducing molecules and hence potential antioxidants. Although the protective effects of these bile pigments in vitro and in vivo are noteworthy, it remains to be clearly established whether these compounds can be developed as a therapeutic modality with a protective role.

The biological actions of bilirubin may be especially relevant to the prevention of oxidant-mediated cell death (Kushida et al., 2002). Bilirubin at a low concentration scavenges ROS in vitro, thereby reducing oxidant-mediated cellular damage and attenuating oxidant stress in vivo (Stocker et al., 1987). The roles of biliverdin and bilirubin in counteracting oxidative and nitrosative stress have been reviewed previously (Foresti et al., 2004; Morse and Choi, 2005). In a recent study, using a rat model of lipopolysaccharide (LPS)-induced shock, exposure to biliverdin was shown to provide a defense against lung injury (Sarady-Andrews et al., 2005). In addition, biliverdin provided a defense, systematically, against lethal endotoxemia and effectively abrogated the inflammatory response (Fig. 5). Biliverdin therapy has also been shown to protect the liver of rats from ischemia and reperfusion injury (Fondevila et al., 2004). The use of siRNA to silence human biliverdin reductase, but not HO-1, has been reported to attenuate the arsen-

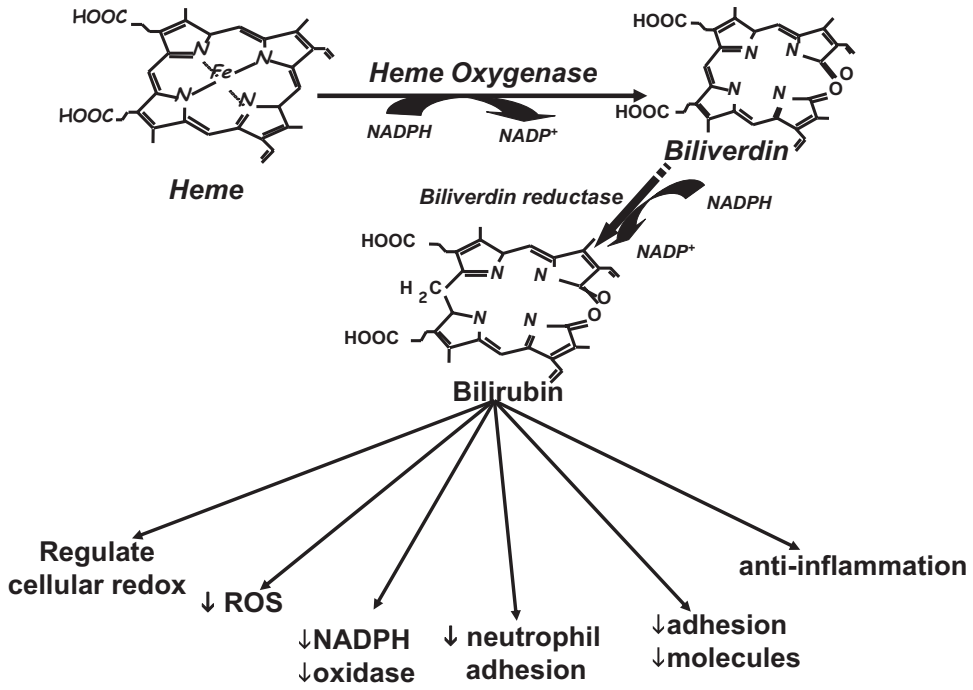

FIG. 5. Schematic representation of the role of bilirubin in biological systems, representing the antioxidant properties of the bile pigment. Bilirubin is produced from the reduction of biliverdin by the cytosolic enzyme biliverdin reductase. Bilirubin, like all compounds, is toxic at high concentrations, e.g., kernicterus in newborns. However, bilirubin has been shown to prevent adhesion molecule expression and neutrophil adhesion and inhibits ROS and NADPH oxidase activity.

ite-mediated induction of cell injury and to increase apoptosis in 293A kidney cells (Miralem et al., 2005). This is the first demonstration that biliverdin per se affords cytoprotection. It should be remembered that biliverdin is rapidly converted to bilirubin by biliverdin reductase in mammals and, thus, in vivo, is short-lived. However, recent studies by Snyder and colleagues (Baranano et al., 2002; Sedlak and Snyder, 2004) found that bilirubin was capable of protecting cells from a 10,000-fold increase in oxidative stress generated by hydrogen peroxide. Furthermore, they identified an amplification process for bilirubin bioaction whereby bilirubin, acting as an antioxidant, is itself oxidized to biliverdin and then recycled by biliverdin back to bilirubin, a process that could readily afford 10,000-fold amplification.

Bilirubin inhibits NADPH oxidase (Kwak et al., 1991) and PKC activity (Sano et al., 1985). Both enzymes have been shown to mediate angiotensin II-induced vascular injury (Rajagopalan et al., 1996; Ishizaka et al., 2000). Recently, biliverdin and bilirubin have been shown to preserve endothelial cell integrity (Sedlak and Snyder, 2004), prevent endothelial cell death and sloughing, enhance vascular reactivity, and prevent restenosis (Rezzani et al., 2003; McClung et al., 2004). Bilirubin is also implicated in reducing oxidative stress in experimental diabetes, in part, by increasing the bioavailability of NO needed for endothelial cell integrity. Bilirubin-mediated inhibition of PKC and NADPH oxidase (Fig. 5) may be one mechanism by which HO-1 attenuates the diabetes-mediated generation of oxidants and the uncoupling of eNOS. Glucose enhances O_2^- production, leading to increased vascular formation of the NO/O_2^- reaction product, $ONOO^-$. Peroxynitrite oxidizes the active NOS cofactor tetrahydrobiopterin to cofactor inactive molecules, such as dihydrobiopterin. This process uncouples the enzyme, which then preferentially increases O_2^- production over NO production (Milstien and Katusic, 1999).

HO-1-derived bilirubin has also been shown to display cytoprotective properties in the cardiovascular system (Clark et al., 2000; Hill-Kapturczak et al., 2002). Increased levels of pulmonary HO-1 protein are a potential biomarker of chronic silicosis. Inflammation was found to be suppressed by treatment with hemin, an inducer of HO-1, and enhanced by ZnPP. HO-1 suppressed ROS activity and the subsequent pathological changes, thereby attenuating silicosis-induced lung injury and disease progression (Sato et al., 2006). Bilirubin has been shown to increase tolerance to islet allograft by a mechanism that includes its anti-inflammatory and antioxidative properties (Wang et al., 2006a; Lee et al., 2007b). These properties, including its antiproliferative effect in smooth muscle cells (Ollinger et al., 2005), may underlie its beneficial effect in the treatment of atherosclerosis (Ollinger et al., 2007). Indeed, having high normal levels of bilirubin is associated with less atherosclerosis compared with that in individuals with low normal levels (Ollinger et al., 2007).

D. Role of Iron and Ferritin

Plasma iron is bound to transferrin, which can transfer iron to the intracellular milieu of endothelial cells via cell surface receptor binding. The up-regulation of HO-1 increases ferritin synthesis to sequester the iron released from the HO-1 pathway (Eisenstein et al., 1991). Ferritin is a high-capacity, low-affinity protein responsible for binding most intracellular iron, and its synthesis is rapidly up-regulated when free iron is present (Ferris et al., 1999). Under normal conditions, very minute amounts of iron exist in free metal form; thus, the concentration of ferritin is an efficient indicator of intracellular iron levels (Ponka et al., 1998). The most abundant source of iron is heme, which can release iron during metabolism by HO or via the oxidative degradation of this molecule (Nagababu and Rifkind, 2004). Whereas this transition metal is essential to biological processes, iron can be extremely toxic if intracellular concentrations are not tightly regulated; thus, maintenance of cellular homeostasis relies on the up-regulation of ferritin when HO-1 transcription is increased.

Iron is known to lead to the generation of ROS, potentially resulting in damage to various cellular components. Iron can become integrated into the phospholipid bilayer and act to oxidize cell membrane constituents (Balla et al., 1991; Valko et al., 2005). Perhaps the most well known of these reactions is the Fenton reaction, which generates hydroxyl radical (OH^-) from hydrogen peroxide (H_2O_2) and iron. Iron(III) is first reduced to iron(II) by molecular oxygen. The combination of these two steps is known as the Haber-Weiss reaction (Lloyd et al., 1997). The hydroxyl radical can oxidize lipids and damage virtually all molecules, including DNA and proteins; however, iron generated from the HO-1/HO-2 catalyzed degradation of heme is sequestered by ferritin induced by the release of iron. The increased iron concentration produced by HO-1 activity is believed to cause the increased expression of ferritin and ferritin synthesis, which serves to sequester iron, a potent oxidant for cells (Paller and Jacob, 1994). Ferritin has been shown to protect endothelial cells from oxidized low-density-lipoprotein (LDL) and iron-induced oxidative stress (Matsumi et al., 2002) and from UV light (Vile and Tyrrell, 1993). Ferritin has been shown to be a cytoprotective antioxidant of endothelium (Balla et al., 1992; Ren et al., 2007), presumably due to antiapoptotic effects (Berberat et al., 2003). For a review of the role of ferritin in cell protection, see Ponka et al. (1998).

IV. Heme Oxygenase in Disease

A. Hypoxia and Ischemia

Ischemia has been found to induce increased expression of HO-1 mRNA in the rat brain (Takeda et al., 1994). In this study, HO-1 expression was investigated by Northern blot and in situ hybridization analyses of rat brain after 20 min of forebrain ischemia. The level of HO-1 mRNA was undetectable in the cerebral cortex of sham control animals, but increased after ischemic insult. After 12 h of reperfusion, increased HO-1 mRNA levels were observed in both neuronal and glia-like cells distributed in the neocortex, hippocampus, and thalamus. HO-1 is thought to play an important role in protective mechanisms during critical conditions, such as ischemia. Hypoxia is readily produced in the ocular system in tight lid closure and reperfusion ischemia. The interruption of blood supply to an organ can result in a wide variety of metabolic derangements and possible cell necrosis. If the blood supply can be restored (reperfusion) within a certain period of time, cells may recover. However, the process of reperfusion itself can be deleterious, and reperfusion injury has been extensively documented (Forman et al., 1991). Reperfusion injury has been defined as the conversion of reversibly injured cells (myocardial, endothelial, and others) to irreversibly injured cells (Forman et al., 1989) and is mediated by a burst of free radical generation as the previously hypoxic cells are flooded with oxygen (Ferrari et al., 1991; Flaherty, 1991). HO induction due to hypoxia has also been observed in vitro. Murphy et al. (1991) showed an increase in HO mRNA in Chinese hamster ovary cells during chronic exposure to a hypoxic environment.

Hypoxia can aggravate heart disease, resulting in a pressure overload to the right ventricle. The distribution of HO-1 in heart tissue indicates that the levels of HO-1 in the atrium and right ventricle are higher than those in the left ventricle. This finding is in agreement with the distribution of cytochrome P450 in heart tissues (Abraham et al., 1987b). Heart HO activity is inhibited by several synthetic metalloporphyrins. In addition, a $SnCl_2$-citrate formulation causes a massive cardiac induction of HO-1 mRNA and HO activity (Neil et al., 1995a). To better understand the role of HO in the pathogenesis of this type of heart stress, rats were exposed to a hypoxic environment (10% O_2). HO-1 mRNA increased in both the right and left ventricles within 1 h of exposure to hypoxia. Maximal HO mRNA levels were achieved after 3 days and remained elevated for 14 days in the right ventricle, whereas left ventricle levels returned to basal levels by the 7th day of exposure. There was no increase in the mRNA levels of HSP 70, HO-2 and the heme synthetic enzymes, nor were there any increases of HO-1 mRNA in other tissues besides the heart (Katayose et al., 1993). These authors postulated that HO may be induced as a protective mechanism in the heart, as it has been observed that CO, a product of HO activity, relaxes coronary and aortic smooth muscles (Maines et al., 1993). It is conceivable that HO may help improve cardiac function during conditions of hypoxia by dilating the coronary arteries (Katayose et al., 1993). Prostaglandin synthesis (PGA) is stimulated in muscle during contraction in the heart in response to ischemia. PGA is a potent inducer of HO expression in myoblastic cells, which suggests that induction of HO by PGA in

myoblasts is a protective mechanism during stress and hypoxia (Rossi and Santoro, 1995).

Hyperoxia is used clinically in severely ill patients, and prolonged use is associated with respiratory failure, multiorgan failure, and death. In an animal model of endothelial cell injury, the overexpression of HO-1 or STAT-3 conferred protection against injury. This effect was lost in STAT-3-deficient mice or in the presence of STAT-3 siRNA, indicating that HO-1 requires STAT-3 for protection (Zhang et al., 2005). In hepatocytes, the regulation of HO-1 expression by IL-6 is mediated by activation of the Janus tyrosine kinase/STAT pathway (Tron et al., 2006). IL-10-induced expression of HO-1 requires the activation of STAT-3, but not p38 mitogen-activated protein kinase (Ricchetti et al., 2004). Alam and colleagues (Alam and Cook, 2003, 2007; Mingone et al., 2006) have done pioneering work on the antioxidant, anti-inflammatory, and signaling properties of the reaction products of the HO-1 gene under a variety of conditions of cellular stress.

B. Corneal Inflammation

Processes or agents that produce oxidative tissue injury and inflammation, such as exposure to and absorption of the most energetic wavelengths of direct sunlight, atmospheric oxygen, and a variety of chemical agents known to generate ROS, constantly threaten the corneal epithelium. A common property of many of these agents is their ability to enhance cellular hemeprotein prooxidant systems. The absence of blood vessels in the normal cornea poses a further risk to this tissue because it denies the corneal epithelium access to circulatory plasma-based antioxidant systems. HO in the corneal epithelium may thus participate in protective mechanisms against ocular injury and inflammation. Present knowledge in this field is derived primarily from early studies that show the presence of HO activity and HO-1 protein in human and rabbit corneas and that HO-1 protein levels increasing after oxidative stress in vitro and hypoxic injury in vivo (Abraham et al., 1987a, 1995a; Conners et al., 1995; Neil et al., 1995b; Laniado-Schwartzman et al., 1997; Bonazzi et al., 2000). These studies culminated with the seminal finding that induction of HO-1 alleviates hypoxic injury-induced ocular surface inflammation and point to a potentially important function for the ocular HO system (Conners et al., 1995; Laniado-Schwartzman et al., 1997). However, the physiological function of this enzyme is yet to be understood. It has been suggested that oxidative stress, resulting from various stimuli such as light exposure or free radicals, may be alleviated by enhanced HO activity having an antioxidant effect. A reverse transcription polymerase chain reaction assay showed mRNA for HO-1 as well as for HO-2, the noninducible form, in the retina. Retinal samples from animals exposed to high-intensity visible light for 12 or 24 h and from unexposed control animals were analyzed. HO-1 mRNA was markedly increased in the retina after light exposure (Kutty et al., 1995). Increased HO-1 levels are thought to be a cellular defense against oxidative damage. This mechanism may play an important role in protecting the retina against light damage.

Hypoxia-induced inflammation is readily produced in the cornea after contact lens use, especially if combined with tight lid closure (Bron et al., 1985; Holden, 1989; Sack et al., 1992). This profound hypoxia creates a severe inflammation characterized by neovascularization of the cornea, which is promptly reversed by hyperoxia (Nishida et al., 1991). Free radical generation is another consequence of hypoxia. For example, in oxidative stress and hypoxia, high-energy ATP molecules are sequentially dephosphorylated and, degraded to nucleosides, yield hypoxanthine. Hypoxanthine is oxidized to xanthine and uric acid by xanthine oxidase and the generation of a superoxide radical, which, in turn, is dismuted first to hydrogen peroxide and then to the highly reactive hydroxyl radical in the Fenton reaction (Halliwell and Gutteridge, 1984). In addition, hemeproteins can also be involved in the generation of ROS because of the presence of iron in the porphyrin ring. For example, the ocular hemeproteins, cytochrome P450, COX, and thromboxane synthetase, are responsible for the generation of proinflammatory agents such as the prostaglandins and 12(*R*)-hydroxy-5,8,10,14-eicosatetranoic acid, 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid, and thromboxane, respectively. 12(*R*)-hydroxy-5,8,10,14-eicosatetranoic acid, a potent inhibitor of Na^+-K^+-activated ATPase, promotes conjunctival vasodilation, tissue infiltration of inflammatory cells, and neovascularization (Schwartzman et al., 1987; Masferrer et al., 1991; Edelhauser et al., 1993). These compounds are products of the cytochrome P450-dependent pathway of arachidonic acid metabolism in the corneal and conjunctival epithelium. Manipulation of the ocular cytochrome P450 enzymatic pathway provides a means of determining the importance of the pathway in hypoxic inflammation induced by contact lens use (Davis et al., 1992). The contact lens model of corneal hypoxia was used to examine whether HO-1 is inducible in corneal epithelium and whether its induction lowers the inflammatory response (Conners et al., 1995); these studies confirm this hypothesis. In addition, it was found that the corneal epithelium suffered less inflammation after contact lens wear when the lens was presoaked in $SnCl_2$.

The postulated mechanisms of action of HO-1 induction, i.e., down-regulation of the inflammatory cytochrome P4B1-derived eicosanoids, led to the current examination of whether a deficiency in HO activity exacerbates ocular surface inflammation. The effect of CO and bilirubin in the prevention of inflammation and hypoxic injury to the cornea and ocular system remains to be evaluated. Drugs that may up-regulate HO-2 in the

ocular system could be of great therapeutic potential as ocular anti-inflammatory agents.

C. Hyperbilirubinemia

Bilirubin, the product of heme catabolism, is poorly soluble and is thus transported in the circulation tightly, but reversibly, bound to albumin. Bilirubin is then rapidly extracted from the circulation by the liver and bound to cytosolic proteins, which prevent the efflux of the bile pigment back into the circulation. The enzyme UDP-glucuronosyltransferase, an integral membrane protein of the endoplasmic reticulum, conjugates bilirubin with glucuronic acid to form bilirubin-monoglucuronide and bilirubin-diglucuronide, both of which are then excreted in the bile.

1. Neonatal Jaundice. In newborn humans, the rate of bilirubin production is severalfold greater than that in adults. Peak bilirubin levels in the plasma occur 3 days after birth in full-term infants and are delayed in preterm infants. This increase in plasma bilirubin levels is due, in large part, to the combination of the rapid degradation of fetal hemoglobin in the first few days of life and the immaturity of the hepatic bilirubin-conjugating system, leading to an increase in unconjugated bilirubin. If the levels of unconjugated bilirubin become too high, bilirubin may cross the blood-brain barrier, resulting in bilirubin encephalopathy or kernicterus (Cashore and Oh, 1982). There is much debate on what constitutes a toxic level of bilirubin and on the methodology of treatment (Newman and Maisels, 1992; Dodd, 1993). Phototherapy is the current method of choice to lower serum bilirubin levels. Visible light is known to produce photoisomers of bilirubin, which are more water-soluble than bilirubin IXα (the product of HO and biliverdin reductase activities) (McDonagh et al., 1982; McDonagh and Lightner, 1985). However, there are increasing concerns regarding the safety of phototherapy, including the possibility of DNA and erythrocyte membrane damage, loss of glucose-6-phosphate dehydrogenase and glutathione reductase activities, and retinal damage (Cohen and Ostrow, 1980; Sissen and Vogel, 1982; Rosenstein and Ducore, 1984; Moseley and Fielder, 1988). For some, there is also the psychological effect of prolonged separation of mother and child (Dodd, 1993). Because phototherapy acts via the skin, only 15% of bilirubin can be photoisomerized at any given time, making phototherapy less efficient for non-neonatal cases of jaundice, such as in children or adults with Crigler-Najjar syndrome. The management of jaundice by inhibition of HO with metalloporphyrins has thus created much interest.

2. Clinical Use of Heme Oxygenase Inhibitors. The tin porphyrins, tin protoporphyrin (SnPP) and SnMP, are potent competitive inhibitors of HO activity. SnPP and SnMP have been shown to control serum bilirubin levels in normal volunteers (Berglund et al., 1988; Galbraith and Kappas, 1989). There is also substantial clinical experience using SnPP and SnMP to control serum bilirubin levels in patients with hereditary porphyria (Galbraith and Kappas, 1989; Dover et al., 1991), liver disease (Kappas et al., 1984; Berglund et al., 1990) or Crigler-Najjar type I syndrome (Galbraith et al., 1992) and in newborns with ABO incompatibility (Kappas et al., 1988) or glucose 6-dehydrogenase deficiency (Valaes et al., 1998; Kappas et al., 2001b). The results of extensive randomized clinical trials using SnPP and SnMP in particular to control neonatal jaundice (Valaes et al., 1994) indicate that the use of SnMP within 24 h of birth in premature newborns substantially moderates the development of hyperbilirubinemia and markedly reduces the requirement for phototherapy by approximately 75% in inhibitor-treated infants compared with control subjects. The only side effect observed with SnMP use was a higher incidence of erythema in the SnMP-treated newborns receiving phototherapy compared with the control group. However, the erythema was not dose-dependent and was transient, disappearing without sequelae. Because SnMP is a photosensitizer, special phototherapy lamps (Philips F20T12BB) were used in the premature newborns to minimize erythema. The use of a nonphotosensitizing inhibitor of HO would avoid this problem. In addition, because the increased levels of bilirubin are due to increased HO-1 expression and activity, other specific inhibitors of HO-1 could be developed for clinical use. Other trials in newborns of a greater gestational age showed that the longer after the birth that SnMP was administered, the less the requirement for phototherapy. Indeed, when administered at the appropriate time to near-term and term newborns with hyperbilirubinemia, SnMP can entirely eliminate the need for phototherapy to control this problem (Kappas et al., 1995). The use of SnMP to control hyperbilirubinemia in newborns has been comprehensively summarized by Kappas (2004).

D. Heme Oxygenase-1 in Diabetes and Obesity

Hyperglycemia, a major cause of kidney disease, is manifest by the development of hypertension and the risk of diabetic neuropathy. Hyperglycemia, defined as elevated levels of serum glucose, produces oxidative stress through elevated levels of ROS, leading to the derangement of cellular physiology. In addition it plays a critical role in the pathogenesis of diabetic complications including cell survival. The impairment of vascular responses to the formation of the super anion radical, O_2^-, represents the major contributor to vascular injury and the clinical complications of diabetes (Fig. 6) (Abraham and Kappas, 2005). The perturbations in heme metabolism are manifest by increased levels of HO-1 protein, HO activity, and increased production of CO, iron, and biliverdin/bilirubin. Their role in the regulation of oxidative stress and cell survival will be discussed relative to diabetes.

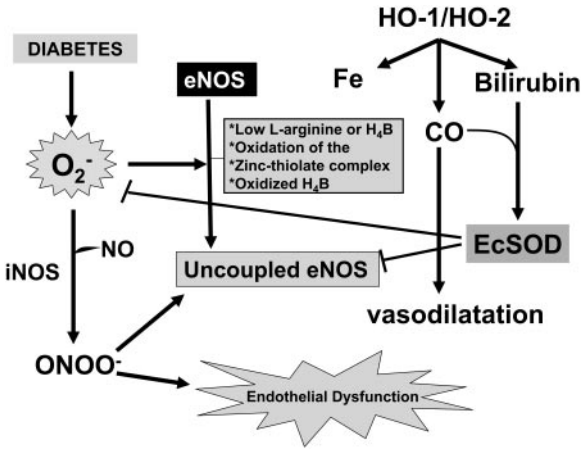

FIG. 6. Schematic representation of HO-1-derived CO and bilirubin in the regulation of oxidative stress and cell survival. Up-regulation of HO-1 by pharmacological agents or by gene transfer or as a result of stress leads to an increase in heme degradation and the generation of CO and bilirubin. This process increases heme turnover with a resultant effect of decreasing inducible enzymes such as iNOS, but not eNOS. Simultaneously, CO or bilirubin or both enhance antiapoptotic, antioxidant, and signaling molecules. HO-1-derived CO or bilirubin may directly increase EC-SOD or, via their activation, transcriptional factors. EC-SOD scavenges O_2^-, decreases formation of $ONOO^-$, and limits oxidative and nitrative stress.

In type 1 diabetes, insulin deficiency provokes high blood glucose levels and alterations in lipid metabolism. This disease evolves with the development of premature micro- and macrovascular complications, the pathogenesis of which is linked to oxidative stress (The Diabetes Control and Complications Trial Research Group, 1993; Wolff, 1993; Giugliano et al., 1996; Rabinovitch et al., 1996). Increased ROS generation contributes to β-cell damage and vascular dysfunction through a variety of mechanism (Baynes, 1991; Wolff, 1993; Tesfamariam, 1994; Li et al., 2007b). In children with diabetes, puberty may trigger microvascular complications that later result in tissue damage, disability, and death. The exact mechanism of hyperglycemia-associated toxicity is unclear. However, recent studies have implicated ROS and its ability to promote the formation of cytotoxic lipid peroxides (Tesfamariam, 1994; Ihara et al., 1999; Pileggi et al., 2001; Bottino et al., 2002). ROS destruction of β-cells, whether induced by oxidants administered exogenously or cytokines elicited, occurs through changes in the equilibrium between apoptotic and antiapoptotic processes (Kaneto et al., 1995; Chervonsky et al., 1997; Itoh et al., 1997; Kurrer et al., 1997; O'Brien et al., 1997; Di Noia et al., 2006). It is apparent from these studies in animal models of diabetes that an intrinsic cardiovascular sensitivity to oxidative stress exists, a property that, in all likelihood, extends to humans.

The experimental basis for chronic oxidative stress as an underlying mechanism for glucose toxicity in β-cells has been examined and shown to be a major problem in diabetes (Robertson et al., 1992; Robertson, 2004). Hyperglycemia-mediated local formation of ROS is considered to be a major contributing factor to endothelial dysfunction. This includes endothelial cell apoptosis, abnormalities in cell cycling due to lack of HO-1 induction and increased O_2^- formation (Abraham et al., 2003a), and delayed replication (Zou et al., 2002). Some of these perturbations can be reversed by antioxidant agents or by increased levels of antioxidant enzymes (Ceriello et al., 1996). A reduction in antioxidant reserves has been related to endothelial cell dysfunction in diabetes (Baynes, 1991; Zou et al., 2002). Increased levels of HO-1 through gene transfer in hyperglycemic rats resulted in a decrease of endothelial cell sloughing (Abraham et al., 2004). Delivery of the human HO-1 gene to endothelial cells attenuated glucose-mediated oxidative stress, DNA damage, and cell death (Sacerdoti et al., 2005). The ability of increased levels of HO activity to attenuate the production of ROS has been attributed to both the degradation of heme and increased production of bilirubin and CO. Increased levels of HO-1 protein attenuated endothelial cell apoptosis and decreased O_2^- formation in experimental diabetes (Turkseven et al., 2005). HO-1 induction has been shown to provide vascular cytoprotection against oxidative stress via mechanism(s) that involve an increase in mitochondrial function (Turkseven et al., 2005; Di Noia et al., 2006). The finding that HO-1 increases EC-SOD is considered seminal, as a decrease in superoxide will limit the formation of peroxynitrite and inactivation of eNOS, thus leading to an increase in NO bioavailability (Kruger et al., 2005, 2006; Turkseven et al., 2005; Peterson et al., 2007). Hyperglycemia is also known to increase the levels of cellular heme and O_2^- as a result of a decrease in HO activity (for review, see Abraham and Kappas, 2005). Thus, HO-1 plays a pivotal role in mitigating the detrimental effects of hyperglycemia. It should be noted, however, that by using hyperglycemic HO-$2^{+/+}$ mice, HO-2 deficiency was found to contribute to a diabetes-mediated increase in superoxide ion levels and renal dysfunction. HO-2 deficiency causes major renal morphological injury and impairs renal function (Goodman et al., 2006) suggesting that diabetes-induced renal impairment is markedly exacerbated. Increases in HO-1 expression in diabetic obese mice were paralleled by increases in serum adiponectin levels, insulin sensitivity, decreases in visceral and abdominal fat content, and decreased plasma TNFα, IL-6, and IL-1β levels (Li et al., 2008). Induction of HO-1 also resulted in decreased adipocyte superoxide production. Up-regulation of HO-1 by CoPP treatment caused a decrease in adipogenesis in bone marrow, both in vivo and in vitro in cultured mesenchymal stem cells and in increases in secretion of adiponectin in the culture media (Li et al., 2008). In Zucker rats, the antidiabetic/antiobesity effect of HO-1 induction causes an increase in adiponectin (Kim et al., 2008).

Cardiac mitochondrial damage, such as that seen in type 1 diabetes, is the result of a decrease in reduced glutathione and STAT-3 of the mitochondrial respiration system (Shen et al., 2004). A deficiency in the de-

oxynucleotide carrier has been associated with abnormal brain growth (Rosenberg et al., 2002), and a deficiency in carnitine-acylcarnitine was shown to cause muscle weakness and cardiomyopathy (Stanley et al., 1992). Diabetic complications have been related to abnormalities in mitochondrial function (Shen et al., 2004; Hsieh et al., 2005; Sparks et al., 2005) as well as to increased endothelial cell death and detachment (Detaille et al., 2005). Therefore, up-regulation of HO-1 in the mitochondria or in the vicinity of mitochondrial membranes may be essential to modulate the redox state in favor of antioxidants and to enhance mitochondrial transport of substrates and metabolites. Restoration of six mitochondrial carriers, i.e., carnitine, citrate, phosphate, deoxynucleotide, ATP, and dicarboxylate, as a result of an increase in HO-1 protein and HO activity in diabetic rats, has been reported (Di Noia et al., 2006; Turkseven et al., 2007). Specific human HO-1 gene transfer to diabetic rats has also resulted in the restoration of mitochondrial carriers, including ADP/ATP and decarboxylate (Di Noia et al., 2006). The increase in HO-1 in diabetic rats is associated with increased eNOS and pAKT (Li et al., 2007a,b; Peterson et al., 2007). An increase in AKT phosphorylation is critical to cell survival in diabetes (Tsang et al., 2005; Varma et al., 2005). Increases in AKT phosphorylation and Bcl-xL levels have been shown to prevent the loss of β-cells in diabetes (Dai et al., 2003; Kowluru, 2005). It is interesting to note that the alteration in mitochondrial function in vitro and in vivo correlates with the levels of activation of AKT and the Bcl-2 family of proteins (Bojunga et al., 2004; Chen et al., 2004; Huang et al., 2005b; Sun et al., 2005). A decrease in Bcl-2 family members has been reported to contribute to apoptosis and the translocation of cytochrome *c* from the mitochondria to cytosol (Srinivasan et al., 2000; Bojunga et al., 2004; Sun et al., 2005). Activation of AKT has been shown to augment ATP synthesis (Huang et al., 2005c) and promote the association of hexokinase with the voltage-dependent anion channel and, in so doing, promote voltage-dependent anion channel closure, thus blocking release of cytochrome *c* (Gottlob et al., 2001).

The interdiction of the diabetic state in NOD mice by sustained induction of HO-1 suggests a possible role of CO and bilirubin. This was associated with a decrease in infiltrated $CD11C^+$ dendritic cells (Li et al., 2007b). HO-1 up-regulation has proven to be capable of providing cytoprotection to vascular function (Abraham et al., 2003a; Kruger et al., 2005) and to pancreatic β-cells in vivo (Pileggi et al., 2001). An increase in HO-1 levels has a salutary effect by modulating the pancreas phenotype, as reflected by the increases in the antiapoptotic proteins, AKT and Bcl-xL. This effect renders β-cells resistant to oxidant stress and, hence, prevents the development of type 1 diabetes (Li et al., 2007a). These novel findings provide a link between the increase in HO-1 and a decrease in infiltrated $CD11c^+$ dendritic cells and suggest that the induction of HO-1 activity can be used to enhance cell survival and moderate the diabetic state (Li et al., 2007a). Similarly, others have shown that HO-1 slows the progression to overt diabetes in prediabetic NOD mice by down-regulating the phenotypic maturity of dendritic cells and Th1 effector function. CO appears to mediate, at least partly, the beneficial effect of HO-1 in this disease setting (Hu et al., 2007). Hyperglycemia is also known to increase the levels of cellular heme and O_2^- as a result of a decrease in HO activity (for reviews, see Abraham and Kappas, 2005; Abraham and Drummond, 2006).

Type 2 diabetes mellitus (T2DM) is common and is characterized by hyperglycemia, insulin resistance, and a relative impairment in insulin secretion. More than 7% of adults in the United States are known to have diabetes, and the number increases each year. The dramatic increase in T2DM over the last decade correlates well with increased levels of obesity and physical inactivity in the US population (Sullivan et al., 2005). Abdominal obesity, specifically, is associated with resistance to the effects of insulin on the utilization of fatty acids and on peripheral glucose levels. Insulin resistance is integral in the pathogenesis of T2DM and is frequently accompanied by hypertension, high serum LDL, low serum high-density lipoprotein, and high serum triglyceride levels, which promote the development of atherosclerotic cardiovascular disease (DeFronzo and Ferrannini, 1991). The majority of diabetic patients succumb to cardiovascular disease and, thus, the control of risk factors leading to atherosclerosis is a major challenge in the management of these patients (Fonseca, 2007).

It has been reported that the PPARδ agonist pioglitazone improves insulin secretory capacity and prevents a reduction in the mass of pancreatic β-cells in obese diabetic *db*/*db* mice (Ishida et al., 2004). HO-1 expression is transcriptionally regulated by both PPARα and PPARδ agonists, implying that the mechanism by which PPAR provides anti-inflammatory effects is an increase in HO-1-derived CO and/or bilirubin (Krönke et al., 2007). The recent report of the parallel increase in HO-1 protein levels and HO activity and the levels of serum adiponectin, a protein hormone that can modulate a number of metabolic processes including glucose metabolism, gives an insight into possible mechanisms involving PPARδ. The identification of HO-1 as a target gene for PPAR widens the options for the development of drugs for the management of cardiovascular disease.

Circulating adiponectin has been shown to down-regulate the generation of classic and proinflammatory risk factors that mediate vascular dysfunction (Touyz, 2005; Altinova et al., 2007). Adiponectin (AcRP30, adipo-Q) appears to play a critical role in vascular disease as increases in serum adiponectin have been shown to be associated with the amelioration of vascular dysfunction (for review see Hopkins et al., 2007). Reduced adiponec-

tin levels have been implicated in the development of obesity-linked diseases, including diabetes, vascular inflammation, and cardiovascular disease (Touyz, 2005; Bahia et al., 2007; Hopkins et al., 2007; Minoura et al., 2007). In addition, reduced plasma adiponectin levels have been documented in patients with coronary artery disease and diabetes, presumably as a result of increases in ROS (Bakkaloglu et al., 2006; Ohashi et al., 2006; Haider et al., 2007). Diabetes and hyperglycemia-mediated local formation of ROS is considered to be the major contributing factor to endothelial cell dysfunction, leading to endothelial cell death and abnormalities in cell cycling (Lorenzi et al., 1987; Baumgartner-Parzer et al., 1995; Abraham et al., 2003a). The importance of ROS production in adipocytes and the associated insulin resistance and changes in serum levels of adiponectin have recently been demonstrated (Furukawa et al., 2004; Lin et al., 2005), suggesting that the increases in ROS are associated with concomitant decreases in adiponectin. Induction of HO-1 decreased superoxide and ROS generation and increased adiponectin with a subsequent elevation in tolerance to ROS in the heart (L'Abbate et al., 2007). More recently, overexpression of HO-1 in obese diabetic mice resulting in an increase in serum adiponectin improved insulin sensitivity and reduced adiposity (Li et al., 2008).

A question arises as to whether vascular endothelial cell damage in diabetes can be prevented or salvaged by altering the balance between the pro- and antiapoptotic pathways regulated by the mitochondria. It has been reported that the overexpression of HO-1 enhances cell proliferation through activation of AKT (Salinas et al., 2003). Similarly, a lack of HO-1 or HO-2 in either transgenic mice or in humans significantly increases apoptotic cell death (Nascimento et al., 1993; Poss and Tonegawa, 1997; Dennery et al., 1998; Quan et al., 2001, 2004a). The report that HO-1 regulates mitochondrial transport carriers and function (Di Noia et al., 2006) suggests that HO-1, by activating Bcl-2 and Bcl-x_L, prevents cytochrome *c* release and the activation of caspases. These results suggest that it may be possible to favorably modulate the balance between pro- and antiapoptotic mechanisms. However, the therapeutic utility of regulating either HO-1 activity or bilirubin and CO has yet to be tested in the treatment of diabetes.

The beneficial effect of cytosolic HO-1 in vascular protection may be considered to be a result of the activation of EC-SOD (scavenging O_2^-), enhancing NO bioavailability and preserving endothelial function (Fig. 6). In addition, several reports suggest that β-cell destruction caused by elevated intracellular levels of ROS, including superoxide radicals, hydrogen peroxide, and NO, is a process that occurs through both apoptotic and necrotic mechanisms (Rabinovitch et al., 1992; Kaneto et al., 1995; Chervonsky et al., 1997; Itoh et al., 1997; Kurrer et al., 1997; O'Brien et al., 1997). T-cell-mediated infiltration of the pancreas leads to the generation of ROS and proinflammatory cytokines. The HO-1 system has been shown to regulate T-cell proliferation and immune response (Pae et al., 2004; Choi et al., 2005). Studies have shown that $CD4^+$ T cells express HO-1 in response to CoPP and that the lack of HO-1 modulates T-cell proliferation and maturation (Chauveau et al., 2005; Chen et al., 2005). Thus HO-1 appears to be clinically important as a focal point in the development of strategies to reverse the detrimental effects of diabetes.

E. Heme Oxygenase-1 in Atherosclerosis

Considerable evidence has accumulated to suggest that the HO-1/CO system plays a beneficial role in atherosclerosis. Oxidized LDL, a major determinant in the pathogenesis of atherosclerosis, is a potent inducer of HO-1 in vascular cells (Agarwal et al., 1996). In vascular endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages, HO-1 is markedly up-regulated by oxidized LDL (Fig. 7), whereas HO-1 is not increased in vascular endothelial cells or in smooth muscle cells when exposed to native LDL (Yamaguchi et al., 1993; Ishikawa et al., 1997). The component in oxidized LDL responsible for HO-1 induction appears to be oxidized arachidonic acid-containing phospholipids, such as 1-palmitoyl-2-isoprostanoyl-*sn*-glycero-3-phosphorylcholine (Ishikawa et al., 1997) and linoleyl hydroperoxide (Agarwal et al., 1998). HO-1 expression is observed throughout the development of atherosclerotic lesions, from the early fatty streaks to advanced complex lesions in human aortic endothelial and smooth muscle cells (Wang et al., 1998). Induction results in the attenuation of monocyte chemotaxis, resulting from the treatment with mildly oxidized LDL in vitro and in vivo (Ishikawa et al., 2001b). HO-1

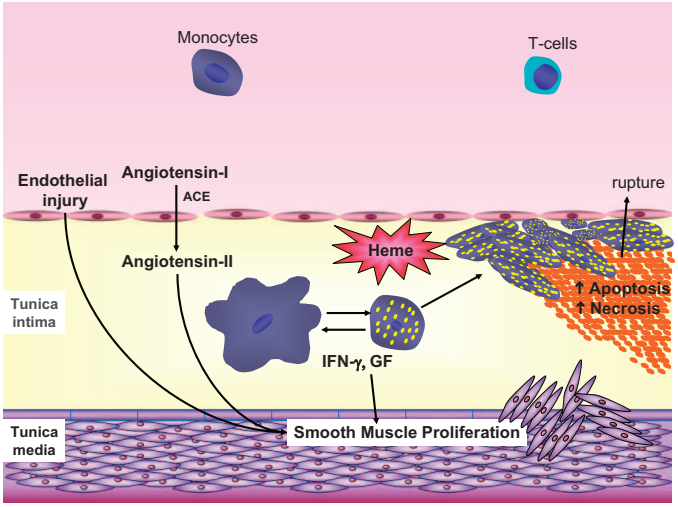

FIG. 7. A proposed protective role for HO-1 in the regulation of NO and iNOS in atherosclerosis. HO-1 inhibits ROS and iNOS and subsequently inhibits various steps in the pathogenesis of atherosclerosis, including endothelial cell activation, migration of leukocytes, oxidation of LDL, angiotensin- and inflammatory cytokine-mediated increases in smooth muscle cell proliferation, foam cell formation, and plaque formation.

induction reduces atherosclerotic lesion size in Watanabe heritable hyperlipidemic rabbits (Ishikawa et al., 2001a) and in LDL-receptor knockout mice (Ishikawa et al., 2001b). In addition, transgenic mice deficient in HO-1 in an apolipoprotein E null background (Yet et al., 2003) exhibited accelerated and more advanced atherosclerotic lesion formation in response to a Western diet compared with controls, despite similar elevations in total plasma cholesterol levels.

Both the pharmacological induction of HO-1 and the adenovirus-mediated gene transfer of HO-1 decrease lesion formation in murine models of atherosclerosis whereas the inhibition of HO-1 promotes lesion development (Juan et al., 2001). In 1999, the first known case of HO-1 deficiency in humans was reported: a 6-year-old boy suffering from severe growth retardation, persistent hemolytic anemia, and an abnormal coagulation/fibrinolysis system with the presence of severe persistent endothelial damage and early atherosclerotic changes in the vasculature, as reflected by the presence of fatty streaks and fibrous plaques (Yachie et al., 1999). This case further added to our understanding of the protective role of HO-1 in atherosclerosis and endothelial dysfunction.

Genetic polymorphism of the HO-1 gene may indicate the potential importance of HO-1 in the pathogenesis of cardiovascular and pulmonary diseases. Yamada et al. (2000), in examining a $(GT)_n$ dinucleotide repeat in the 5′-flanking region of the human HO-1 gene, described the polymorphism of HO-1 and reported that the larger the size of the $(GT)_n$ repeat in the HO-1 gene promoter the greater the chance of reducing HO-1 inducibility by reactive species in cigarette smoke, resulting in the development of emphysema. A cohort study was carried out in patients to evaluate HO-1 gene promoter polymorphisms and the risk for restenosis after percutaneous transluminal angioplasty (Exner et al., 2001). Patients with short ($<$25 GT) dinucleotide repeats in the HO-1 gene promoter on either allele had restenosis significantly less often that patients with longer ($\geq$25 GT) dinucleotide repeats. These data imply that up-regulation of HO-1, associated with shorter dinucleotide repeats, may be a protective factor after balloon angioplasty. It is believed that shorter $(GT)_n$ repeats, compared with longer $(GT)_n$ repeats, have higher transcriptional activity and thus higher HO-1 expression levels. In a Chinese population, type 2 diabetic patients, shown to carry longer ($\geq$32) $(GT)_n$ repeats, have higher levels of oxidative stress and increased susceptibility to the development of coronary artery disease and atherosclerosis (Chen et al., 2002). Conversely, in a Japanese population of patients with significant risk factors (hyperlipidemia, diabetes, and smoking) for coronary artery disease, shorter ($<$27) $(GT)_n$ repeats were associated with less disease (Kaneda et al., 2002). In addition to $(GT)_n$ dinucleotide-length polymorphism, a single nucleotide polymorphism in the HO-1 promoter T($-$417)A was shown to correlate with a reduced incidence of ischemic heart disease in a Japanese population (Ono et al., 2004).

In contrast with the above findings for HO-1 genetic polymorphism, 1807 patients were studied and no clinically relevant association of a HO-promoter polymorphism and ischemic events after coronary stenting was reported (Tiroch et al., 2007). In support of this finding, no evidence of a protective effect for short alleles, i.e., low $(GT)_2$ repeat, for graft or recipient survival in clinical renal transplant was seen (Courtney et al., 2007). In a study of 3104 patients with vascular disease, restenosis after percutaneous coronary intervention was associated with angiotensin II-type l receptor 116 A/C polymorphism but was not associated with polymorphism of HO-1 (Wijpkema et al., 2006). These studies both advocate and/or contradict the role of the HO-1 gene in genetic polymorphism and atherosclerotic processes. Thus, more research is required to clearly define the role, if any, that genetic polymorphism plays in the susceptibility for developing cardiovascular disease.

1. Endothelial Dysfunction and Vascular Injury. For a long time, endothelial cells were considered to be a passive monolayer covering the inner part of vascular walls. The role of these cells was regarded as a mechanical barrier between circulating blood and vascular structures. Now, after a series of biochemical and experimental studies, the endothelium is considered an organ, covering approximately 700 m^2. Endothelial cells produce NO as a result of either higher blood pressure or a growing demand for oxygen from the amino acid L-arginine by eNOS, which has vasodilating, anti-inflammatory, and healing actions in the vasculature. In contrast, vasoconstrictors, such as endothelin 1, angiotensin II, and thromboxane A, are produced in the vascular wall by endothelial cells, acting as opponents to NO. These molecules impart a vasoregulatory function to the endothelium. Endothelial dysfunction is a well-established response to cardiovascular risk factors, including diabetes, and precedes the development of atherosclerosis (Fig. 8).

Endothelial activation by oxidized LDL and TNFα is considered to play an essential role in the development of atherosclerotic lesions (Chen et al., 2006a; Korobowicz, 2006; Gao et al., 2007). Endothelial dysfunction is involved in lesion formation by promoting both early and late mechanisms of atherosclerosis, including upregulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced LDL oxidation, platelet activation, cytokine elaboration, and VSMC proliferation and migration (Hadi et al., 2005). Endothelial HO-1 overexpression significantly attenuates the production of inflammatory mediators and reverses the decrease in eNOS by oxidized LDL and TNFα (Kawamura et al., 2005). In addition, HO-1 overexpression also improves the impaired vasodilatory responses of aortic segments treated with oxidized LDL (Kawamura et al., 2005). The

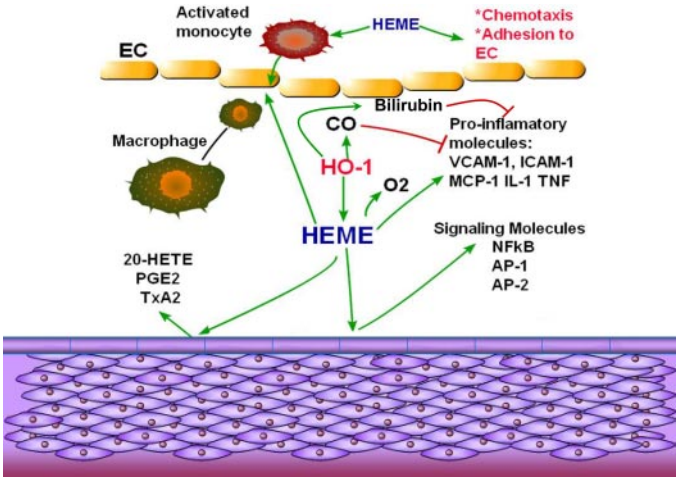

FIG. 8. Schematic representation of the role of HO in diabetes. Diabetes is manifested by increases in metabolic risk factors and oxidative stress and the derangement of cellular physiology. The increase in metabolic risk factors, such as TNF, IL-1, and IL-6, stimulates adhesion molecule expression (intercellular adhesion molecule-1, VECAM-1, and MCP-1). The adhesion of monocytes to vessel walls causes the stimulation of VSMC proliferation and vasoconstrictors, such as 20-HETE, thromboxane A_2, and PGE_2. An increase in HO-1 will attenuate production of these factors via the increase in CO and bilirubin or the increase in adiponectin levels and decrease in inflammatory cytokines.

induction of HO-1 and EC-SOD by D-4F administration enhances the ability of high-density lipoprotein to protect LDL against oxidation in atherosclerotic animals (Kruger et al., 2005). HO-1 stimulates cell cycle progression and proliferation in the vascular endothelium (Dulak et al., 2004; Bussolati and Mason, 2006). Transduction of the HO-1 gene into endothelial cells promotes their growth and the development of capillary-like tube structures (angiogenesis), whereas inhibition of HO-1 activity blocks cell growth and tube formation (Suzuki et al., 2003). However, additional studies are needed to directly determine whether HO influences angiogenesis and the underlying mechanism(s) of action. The ability of HO-1 to stimulate endothelial cell regrowth at the site of arterial injury is another mechanism designed to limit lesion formation because re-endothelialization of the vessel wall is believed to maintain the underlying smooth muscle cells in a quiescent state. Thus, HO-1 as an intrinsic antioxidant plays an important role against endothelial dysfunction and atherogenesis (Fig. 8).

2. Inflammation and Vascular Injury. Oxidative stress and inflammation are accepted as major factors in the pathogenesis of atherosclerosis. The presence of inflammation-related molecules, such as IL-1, TNFα, CD-40 ligand, TNFγ, growth factors (platelet-derived growth factor and fibroblast growth factor), plasma C reactive protein, fibrinogen, IL-6 complement, thrombin, and heat-shock proteins, further implies the pivotal role of inflammation in the pathogenesis of atherosclerosis (Alvaro-Gonzalez et al., 2002). It has been suggested that CO contributes significantly to the anti-inflammatory properties of HO-1. CO inhibits the lipopolysaccharide-mediated expression of proinflammatory cytokines, such as TNFα, IL-1β, and macrophage inflammatory protein-1β, while simultaneously increasing the expression of the anti-inflammatory cytokine, IL-10, in both endothelial cells and macrophages (Otterbein et al., 2000). Furthermore, HO-1/CO activation down-regulates the inflammatory response by blocking the release of NO from iNOS and expression of the granulocyte-macrophage colony-stimulating factor from macrophages and smooth muscle cells (Zhan et al., 2003; Sawle et al., 2005). The activation of both sGC and p38 MAPK has been implicated in the suppression of inflammatory cytokines by HO-1/CO activation (Otterbein et al., 2000; Zhan et al., 2003). Despite similar total plasma cholesterol levels in response to hypercholesterolemia, HO-$1^{-/-}$apoE$^{-/-}$ mice have accelerated and more advanced atherosclerotic lesion formation compared with HO-$1^{+/+}$apoE$^{-/-}$ mice. In addition to greater lipid accumulation, the advanced lesions from HO-$1^{-/-}$apoE$^{-/-}$ mice contained macrophages and smooth muscle α-actin-positive cells (Yet et al., 2003). Moreover, the expression of HO-1 in atherosclerotic plaques (Ameriso et al., 2005) and the decrease in experimental atherosclerosis after HO-1 up-regulation (Ishikawa et al., 2001b; Juan et al., 2001) further establishes the protective role of HO-1 against atherosclerosis.

3. Smooth Muscle Cell Proliferation and Vascular Injury. Smooth muscle cell proliferation and monocyte recruitment are essential steps for the development of atherosclerosis. Concomitant with hypoxia-mediated induction of VSMC HO-1, endothelial cell production of endothelin-1, platelet-derived growth factor B, and VEGF was inhibited via a smooth muscle, CO-dependent mechanism (Morita and Kourembanas, 1995). The inhibition of these factors by CO led to a decrease in VSMC proliferation. In addition, smooth muscle cell-derived CO directly decreased VSMC growth by inhibiting E2F-1, a transcription factor that participates in the control of cell cycle progression from the G_1 to the S phase (Morita et al., 1997). HO-1 overexpression, or the exogenous administration of CO, arrests cultured smooth muscle cells in the G_0/G_1 phase of the cell cycle (Duckers et al., 2001; Tulis et al., 2005). This inhibition of cell cycle progression is associated with a marked decrease in cyclin-dependent kinase 2 (cdk2) activity, a critical event required for *S*-phase entry and DNA synthesis (Tulis et al., 2005). The ability of CO to block cdk2 activity is probably mediated via its ability to modulate the expression of key regulatory proteins. In particular, CO suppresses the expression of the cdk2 activators, cyclin A and D1, while stimulating the expression of the cdk2 inhibitor, p21 (Taillé et al., 2005; Tulis et al., 2005). The sGC/cGMP pathway mediates the antiproliferative action of CO as inhibitors of either guanylate cyclase or protein kinase G restore smooth muscle cell growth (Duckers et al., 2001; Otterbein et al., 2003).

Bilirubin exerts its growth suppressor functions in VSMC, at least in part, by modulating the p38 MAPK signaling pathway (Zhao et al., 2002; Ollinger et al., 2005). More information is needed to establish the role of bilirubin/biliverdin in relation to VSMC proliferation and in its prevention, but for now the protective role of HO-1 against VSMC proliferation in atherosclerosis is attributed to CO. These reports suggest that HO and its byproducts (bilirubin and CO), by limiting VSMC growth, may act as key mediators in the body's compensatory response to vascular remodeling and limit the damage caused by atherosclerosis.

4. Vasodilatation and Vascular Function. Similarities between NO and CO suggest that CO may have a physiological role (Marks et al., 1991); both behave as messenger and signaling molecules. Also both CO and NO are capable of inducing the relaxation of blood vessels through vasodilation and the inhibition of VSMC proliferation (Stanford et al., 2003). Earlier studies suggested that CO might bind to and activate guanylate cyclase, thereby increasing intracellular levels of cGMP, as has been demonstrated for NO (Verma et al., 1993; Morita et al., 1995; Maines, 1997). Like NO, HO-derived CO influences the sGC and cGMP pathways, which serve to regulate both blood pressure and vascular contractility (Ndisang et al., 2004); sGC acts to increase cGMP, which in turn serves as a vasodilator to lower blood pressure levels. However, the physiological relevance of CO as a vasodilator is controversial. Indeed, overexpression of HO-1 in arteries was reported to stimulate vascular relaxation mediated by sGC and cGMP independently of NO. Leffler and others (Barkoudah et al., 2004; Leffler et al., 2005; Kanu et al., 2006) have examined the role of CO in cerebral circulation and shown that endogenously produced CO dilates cerebral arterioles by activating Ca^{2+}-activated K^+ channels and that CO is a major component in the regulation of cerebrovascular circulation (Kanu et al., 2006). Thus, the potency of CO in these vessels appears greater than that in rabbit aorta where it is low. CO is more chemically stable than NO but is manyfold less potent than NO as a relaxing agent (Furchgott and Jothianandan, 1991); thus, the biological availability of NO and CO may differ (Maines, 1997).

CO probably acts via multiple mechanisms, including the direct modulation of cGMP levels and K^+ channels in smooth muscle cells and the indirect modulation of endothelium-dependent vasoconstrictors and myogenic factors (Mingone et al., 2006). Some reports suggest that CO may act as a physiological regulator of vascular tone through cGMP-mediated responses in large vessels (e.g., aorta) (Sammut et al., 1998; Kozma et al., 1999), whereas others suggest that CO may dilate the smaller renal arteries or pial arterioles via activation of Ca^{2+}-activated potassium channels (Leffler et al., 1999; Kaide et al., 2001). Other reports have hypothesized that CO may play a role in blood pressure regulation in acute hypertension (Motterlini et al., 1998; Sabaawy et al., 2001). Additionally, CO has been reported to exert cGMP-independent actions on Ca-dependent potassium channels (Wang and Wu, 1997; Kaide et al., 2001). CO directly activates Ca-dependent potassium channels, leading to vascular relaxation through increases in intracellular calcium (Zhang et al., 2001; Wu et al., 2002). The antiproliferative effects of CO on VSMCs after balloon injury are mediated by the p38 MAPK pathway, including MAPK 3 and 6 (Amersi et al., 2002). p38 MAPK pathway activation by CO has been observed to protect endothelial cells from TNFα-mediated apoptosis (Brouard et al., 2002). p38 MAPK pathways act differently in VSMCs than in endothelial cells. Thus, the actions of CO via cGMP-dependent and cGMP-independent pathways may explain a number of the potential actions of CO regarding the pathogenesis of cardiovascular diseases.

F. Heme Oxygenase-1 in Myocardial Ischemia-Reperfusion Injury

The discovery of cytochrome P450-dependent monooxygenase in intimal cells of the hog aorta and identification of HO and cytochrome P450 in the rabbit heart (Abraham et al., 1985c, 1987b) was a breakthrough in the investigation of the role of this enzyme in heart function. Cardioselective overexpression of HO-1 protein exerts a cardioprotective effect after myocardial ischemia-reperfusion in mice, and this effect is probably mediated via the antiapoptotic action of HO-1 (Vulapalli et al., 2002). Transgenic mice expressing cardiac-specific HO-1 were generated, and it was reported that the heart showed improved recovery in contractile performance during reperfusion after ischemia as well as protection against ischemia, evident by decreased infarct size, in a HO-1 dose-dependent manner (Yet et al., 2001). Hemin injected into rats before induction of ischemia to upregulate HO-1 decreased left ventricular pressure during ischemia and reperfusion, whereas end-diastolic pressure, coronary perfusion pressure, and coronary resistance increased (Kukoba et al., 2003).

The HO-CO pathway is involved in ischemic vasodilation in the coronary microcirculation (Nishikawa et al., 2004). The occurrence of severe right ventricular enlargement after chronic hypoxia was shown in HO-1-deficient compared with wild-type mice (Yet et al., 1999) and the cardiac-specific expression of HO-1 protected against ischemia and reperfusion (Yet et al., 2001). Furthermore, HO-1 gene expression has been shown to reverse neointimal hyperplasia (Kong et al., 2004) and ischemic heart injury (Guo et al., 2004) and prevent vascular dysfunction in experimental diabetes (Kruger et al., 2005; for review, see Abraham and Kappas, 2005).

There are numerous possible mechanisms by which the HO-1/HO-2 pathway may improve vascular function. It has been reported that HO-2 activation occurs in ischemic hearts in dogs and that inhibition of the HO

system inhibits vasodilation during ischemia in the presence of NO and COX inhibitors (Nishikawa et al., 2004). CORM-3 prevents ischemic damage (Guo et al., 2004). CO also protects isolated hearts against ischemia-reperfusion (Clark et al., 2003). Patients with ischemic and nonischemic cardiomyopathy exhaled CO levels lower than those for healthy control subjects at rest and after exercise, suggesting lower HO activity in this group than in control subjects. CO was able to reduce infarct size (Guo et al., 2004). Increased CO levels have a beneficial effect on vascular relaxation and prevent endothelial cell death (Di Pascoli et al., 2006; Rodella et al., 2006). In addition, up-regulation of HO-1 may improve vascular function by increasing superoxide dismutase and catalase activity and reducing tissue O_2^- levels (Kruger et al., 2005; Turkseven et al., 2005).

Hyperglycemia and ischemia enhance endothelial O_2^- production, leading to increased vascular formation of the NO/superoxide reaction product, peroxynitrite (Kossenjans et al., 2000; Di Filippo et al., 2004; Moreira et al., 2007). Peroxynitrite oxidizes the active NOS cofactor, tetrahydrobiopterin, to cofactor inactive molecules, such as dihydrobiopterin (Milstien and Katusic, 1999). This uncouples the enzyme, which then preferentially increases O_2^- production over NO production (Wever et al., 1998; Milstien and Katusic, 1999). The HO-1 gene expression-mediated decrease in O_2^- may, in turn, lead to the protection of eNOS from uncoupling. Bilirubin may have vascular protective effects as well. A higher serum bilirubin level is related to a decrease in lipid peroxidation and is associated with a decrease in the risk for coronary artery disease in humans (Sano et al., 1985; Vasa et al., 2001).

Induction of HO-1 was associated with a parallel increase in the serum levels of the antidiabetic, anti-inflammatory, and antiatherogenic adipocytokine, adiponectin (L'Abbate et al., 2007). Adiponectin has been ascribed antioxidative properties (Jung et al., 2006); it improves endothelial function in nondiabetic patients with metabolic syndrome (Bahia et al., 2007) and also improves the beneficial effects of antihypertensive agents in hypertensive patients (Yilmaz et al., 2007). These observations serve to define some of the key mechanisms by which HO-1 is involved in the maintenance of microvascular tone and to offer a possible approach as to how these mechanisms might be therapeutically manipulated.

Pharmacological induction of HO-1 significantly reduces infarct size and the incidence of reperfusion arrhythmias after myocardial ischemia-reperfusion whereas cardiac tissue damage is exacerbated by HO inhibitors (Clark et al., 2000; Hangaishi et al., 2000; Masini et al., 2003). The products of increased HO activity are protective in rodent models of ischemia-reperfusion injury, allograft and xenograft survival, intimal hyperplasia after balloon injury, or chronic graft rejection (Otterbein et al., 2003). Up-regulation of HO-1 during heart failure serves to mitigate pathological left ventricular remodeling and reduce myocardial hypertrophy, oxidative stress, and inflammatory activation (Kawamoto et al., 2004). Up-regulating HO-1 also has the potential of attenuating cardiac hypertrophy in genetically hypertensive rats (Seki et al., 1999). Gene delivery of HO-1 by adeno-associated viruses several weeks in advance of coronary ligation led to marked myocardial protection in a rat model of acute ischemia-reperfusion injury (Melo et al., 2002). Chronic expression of HO-1 increases the heart's tolerance and resistance to ischemia-reperfusion by decreasing lactate formation and increasing adiponectin (L'Abbate et al., 2007). An increase in adiponectin was associated with increased heart eNOS and pAKT levels, both of which increase resistance to oxidants and cell survival. These data demonstrate that increased HO-1 gene levels by either pharmacological or genetic means produce therapeutic, cardioprotective benefits by reducing oxidative stress and associated inflammation and cell death. Considerable evidence supports a protective role for the HO-1/CO system in coronary artery disease states.

Isolated hearts from heterozygote HO-1 knockout mice also demonstrate an increased susceptibility to ischemia-reperfusion relative to controls (Yoshida et al., 2001). A maladaptive response, consisting of enhanced ventricular dilatation, infarction, and thrombosis, has also been reported in HO-1 null mice during hypoxia (Yet et al., 1999). Moreover, the preemptive delivery of HO-1 has been shown to inhibit remodeling after a myocardial infarction and to restore ventricular function after ischemia-reperfusion (Liu et al., 2006b). A study of ischemia-induced myocardial injury demonstrated that rats, having undergone successful HO-1 gene administration, had a dramatic reduction in left ventricular myocardial infarction after coronary artery ligation and release (Coito et al., 2002). Autologous atrial cardiomyocytes are a readily available cell source for infarct repair; however, they readily undergo apoptosis, precluding their use as cellular repair grafts (Kawamoto et al., 2004). This study demonstrated that preconditioning with HO-1 acts to retain functional viability in vivo in adult cardiomyocyte cellular grafts after implantation, which, in turn, may prove effective in repairing infarcted myocardium. These findings indicate that gene therapy and/or HO-1 overexpression may be an effective approach in infarct repair.

G. Cardiovascular Drugs and Drug Developments Targeting Heme Oxygenase-1 Gene Expression

A spectrum of compounds has been used to up-regulate HO-1 expression and HO activity. $SnCl_2$ has been reported to lower blood pressure in 7-week-old SHR (Sacerdoti et al., 1989); however, care must be taken with the chronic use of $SnCl_2$ as it may result in nephrotoxicity. $CoCl_2$, a short-term inducer of HO-1 expression and HO activity, has been reported to have exhib-

ited antihypertensive effects in the same animal model described above for $SnCl_2$ (Sacerdoti et al., 1989). Metalloporphyrins, such as heme, heme arginate, and CoPP, are also commonly used to induce HO-1 expression and activity and have been used to normalize blood pressure in animals and humans (Kordac et al., 1989; Levere et al., 1990; Martasek et al., 1991). The side effects associated with their use are discussed elsewhere is this review. The ability of this compound to increase HO activity in vitro and increase HO-1 and HO activity in vivo is not unique.

In the treatment of atherosclerosis, hypertension, and vascular injury in humans, HO-1 has potential of being either directly delivered as a gene or pharmacologically induced. However, in discovering the ideal pharmacological drugs, one must consider the dose and time of HO-1 induction. Therefore, most of the pharmacological inducers of HO-1, such as hemin and heavy metals, used in experimental studies may show cellular and tissue toxicity if used at high concentrations. Thus, the adverse and long-term effects of HO-1 expression and its effect on the heme synthesis pathway must be elucidated before clinical application. Nonetheless, recent studies have revealed that some well-known and commonly used drug agents modulate HO-1 expression in vascular cells as described in Fig. 9.

1. Aspirin. Aspirin is known to reduce the incidence of thrombotic occlusive events, such as myocardial infarction and stroke, by inhibiting platelet COX-2 activity. Aspirin increased HO-1 protein levels and HO activity in a dose-dependent manner in cultured endothelial cells derived from human umbilical vein. Pretreatment of cells with aspirin or bilirubin protected endothelial cells from H_2O_2-mediated toxicity (Grosser et al., 2003). HO-1 is a cGMP-sensitive endothelial gene and established a causal relationship between HO-1 induction and endothelial protection by the cGMP/NO system (Polte et al., 1997). Aspirin has also been shown to increase ferritin synthesis in endothelial cells, presumably as a result of HO-1 induction and iron release, suggesting a role in the prevention of endothelial injury during atherogenesis. Induction of HO-1 activity is a novel mechanism by which aspirin prevents cellular injury under inflammatory conditions and in cardiovascular diseases.

It has been recently reported that aspirin-triggered lipoxin induced endothelial HO-1 protein expression in a time- and concentration-dependent manner. It is believed that this mechanism is mediated by the activation of the G protein-coupled lipoxin A_4 receptor (Nascimento-Silva et al., 2005). It has been suggested that aspirin-triggered lipoxin induces HO-1 in human endothelial cells and that this increase in HO-1 protein is responsible for the anti-inflammatory activity of these lipid mediators (Becker et al., 2003; Nascimento-Silva et al., 2005). It should be noted, however, that the dose of aspirin used in the studies described above is higher than that used clinically.

2. Statins. Statins, the widely used lipid-lowering agents, substantially decrease cardiovascular morbidity and mortality in patients with and without coronary

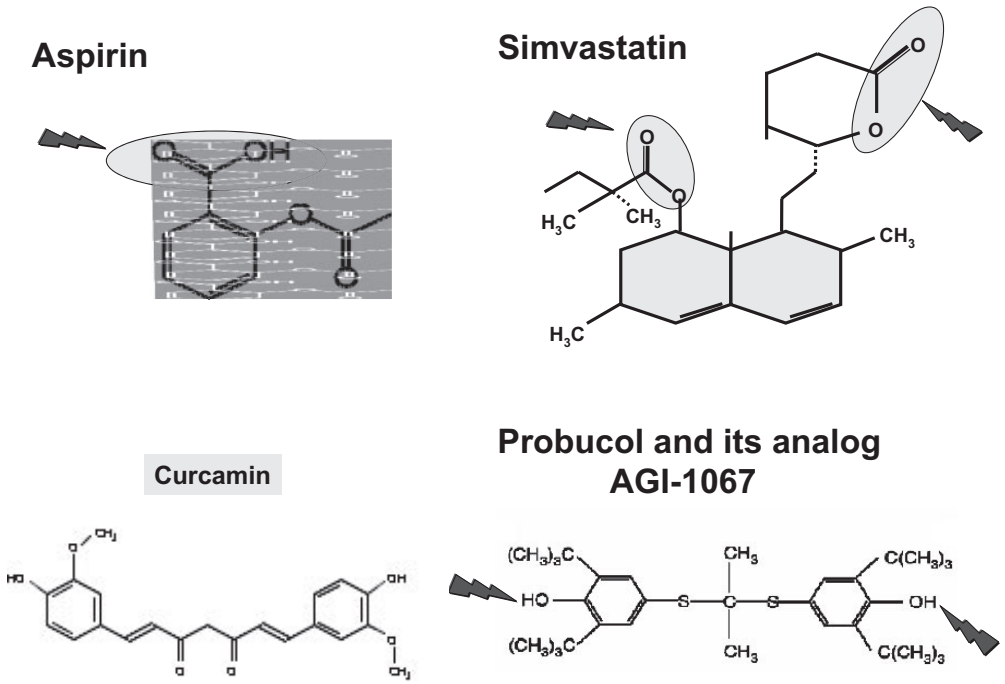

FIG. 9. Cardiovascular drugs that increase HO-1 expression and HO activity. These represent a broad spectrum of chemical structures, illustrating the disparity of compounds that induce HO-1 (there is, however, a commonality among the structures, such as the phenol ring, which may be considered as a major activator of the HO-1 promoter region) and increase HO-1 mRNA, proteins, and total HO activity. Other drugs are not included in the scheme, such as CoPP with a 4-carbon and nitrogen ring. In addition, sildenafil citrate (Viagra) and tadalafil (Cialis) either increase total HO-1 activity and CO levels.

disease. Simvastatin and lovastatin increase HO-1 mRNA levels in cultured endothelial cells derived from human umbilical vein (Oberle et al., 2003; Grosser et al., 2004a,b; Lee et al., 2004). Increased HO-1 transcriptional expression by statins is associated with elevated HO-1 protein levels and a reduction in free radical formation. Simvastatin activates HO-1 in VSMCs in vitro and in vivo and suggested the involvement of p38 MAPK and the p13K-Akt pathway in HO-1 induction (Lee et al., 2004). These results may explain the pleiotropic antioxidant, anti-inflammatory, and antiatherogenic actions of statins in that they function by maintaining elevated levels of HO-1 and thus the reciprocal levels of free radicals.

A series of statins (simvastatin, lovastatin, atorvastatin, and rozuvastatin) administered orally to mice increased HO-1 expression and HO activity in a statin- and tissue-specific manner with all statins increasing heart and lung enzyme activity (Hsu et al., 2006). The authors concluded that, in this animal model, HO-1 induction is statin- and tissue-specific and that statins confer antioxidant and anti-inflammatory actions in the vascular and extravascular systems (Chen et al., 2006b). Recently, statins have been reported to activate protein kinase G in a murine macrophage system, eliciting the activation of extracellular signal-regulated kinase (ERK) and p38 MAPK, which results in the induction of HO-1 (Hsu et al., 2006), suggesting a novel anti-inflammatory mechanism. These observations provide the basis for an interesting clinical trial in which *n*-tidal CO is measured to verify whether HO activity is increased.

3. Amino Acid Apolipoprotein A-I L-4F and D-4F Mimetic Peptides. The development of compounds such as 4-F is well documented (Navab et al., 2005) and these peptides display equal efficacy whether synthesized from D-or L-amino acids (Van Lenten et al., 2007). Recent studies show that rats made diabetic by streptozocin treatment exhibited increased aortic oxidative stress and endothelial sloughing that was ameliorated by administration of D-4F. D-4F increased levels of aortic HO-1 protein, HO activity, and extracellular superoxide dismutase while decreasing superoxide levels (Kruger et al., 2005; Peterson et al., 2007). These results were extended in a mouse model of obesity and diabetes (*ob/ob* mice) to show that L-4F increased HO-1 protein and adiponectin levels, decreased superoxide generation, and improved insulin sensitivity and glucose tolerance. In addition, L-4F administration resulted in a reduction in fat content and a significantly smaller increase in body weight (Fig. 10) in these animals despite no change in food intake. L-4F decreased adipogenesis in mouse bone marrow and in cultures of human bone marrow-derived mesenchymal cells (Peterson et al., 2008). The mechanism by which peptides such as L-4F mediate these changes could be related to their ability to bind and remove proinflammatory phospholipids from tissue (Buga et al., 2008). These compounds have been reported to play a role in a number of chronic inflammatory conditions (Berliner and Watson, 2005). The remarkable properties of D- and L-4F described above suggest a potential clinical role in the treatment of obesity, insulin resistance, the metabolic syndrome, and diabetes.

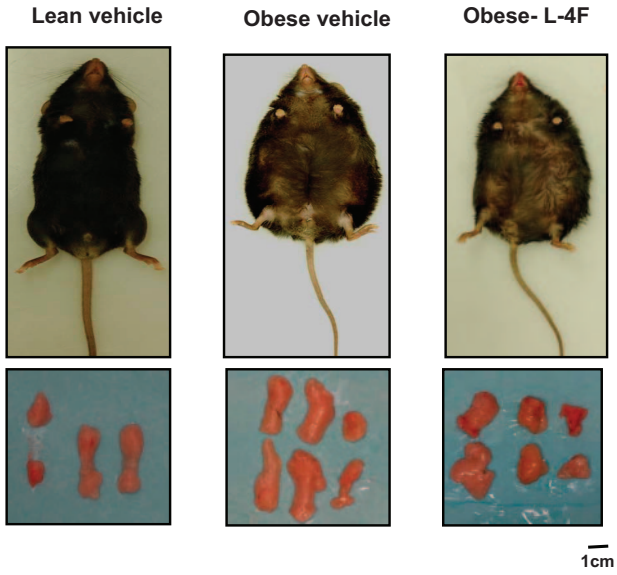

FIG. 10. The effect of L-4F on *ob/ob* mice after 6 weeks of treatment. Visceral fat removed from the abdomen of lean, *ob/ob* mice and L-4F-treated obese mice. The appearance of L-4F-treated *ob/ob* mice was consistent with decreased weight gain in L-4F-treated mice compared with vehicle-treated *ob/ob* mice (Peterson et al., 2008).

4. Probucol. Probucol, an antioxidant drug, reduces the risk of restenosis (Heinecke, 2006). The protective effect of probucol depends not only on its ability to inhibit lipid oxidation but also on its ability to induce HO-1. Probucol inhibits macrophage accumulation, stimulates re-endothelialization, and inhibits vascular smooth muscle cell proliferation. These processes are mediated via the induction of HO-1, an activity not shared by vitamin E (Choi et al., 2004; Wu et al., 2006), which, along with other antioxidants, has failed to protect against atherosclerotic disease. A striking exception is probucol, which retards atherosclerosis in carotid arteries and restenosis of coronary arteries after angioplasty (Wu et al., 2006). These findings indicate the contribution of HO-1 in the actions of probucol.

AG1067, an agent capable of inducing HO-1, is in clinical trials (Stocker and Perrella, 2006). The ability of this compound to induce HO-1 is presumably due to the presence of phenol moieties, and it may work through the activation of the HO-1 promoter at the transcriptional level. Statins, which contain phenol groups, could induce HO-1 protein levels and increase HO activity by acting in a similar manner.

5. Losartan. HO-1 is expressed in medial smooth muscle and adventitial cells in normotensive rat aorta and is markedly increased in adventitial and endothelial cells in angiotensin II-induced hypertensive rat aorta

(Ishizaka et al., 1997). This up-regulation in blood pressure, and thus HO-1, was reduced to levels comparable to those of controls by treatment with losartan (Ishizaka et al., 1997). Losartan markedly reduced pulmonary pressure and inhibited vascular remodeling in volume-overloaded left-to-right shunt rats, resulting in the down-regulation of HO-1 mRNA expression (Yuan et al., 2005). Unique renoprotective properties have been proposed for angiotensin receptor blockers independent of blood pressure lowering but related to decreased oxidative stress, correction of chronic hypoxia, and inhibition of advanced glycation end product formation and of abnormal iron deposition (Izuhara et al., 2005).

6. Paclitaxel. The antiproliferative effects of paclitaxel on VSMCs have been reported to be due to the induction of the HO-1 gene. Treatment with paclitaxel resulted in a marked time- and dose-dependent induction of HO-1 mRNA, followed by corresponding increases in HO-1 protein and HO activity (Choi et al., 2004).

7. Rapamycin. Rapamycin, a macrolide antibiotic, blocks cell cycle progression at the G_1 phase and rapamycin-coated coronary stents have been shown to reduce coronary restenosis (Morice et al., 2002). Visner et al. (2003) reported that rapamycin induces HO-1 and suppresses platelet-derived growth factor-dependent VSMC growth., 2003). It has been suggested that HO-1, induced by rapamycin in smooth muscle cells, shows an antiproliferative effect, resulting in the reduction of the restenosis rate (Visner et al., 2003).

8. Immunosuppressive Drugs. Cyclosporin is a powerful stimulator of oxidative stress signaling, leading to transforming growth factor-β production, NO degradation, endothelial dysfunction, hypertension, and post-transplant nephropathy. Down-regulation of HO-1 expression by cyclosporin-A could be one mechanism underlying cyclosporin-A-induced toxicity (Rezzani et al., 2005b) and induction of HO-1 can prevent this (Rezzani et al., 2005a). Carvedilol-mediated induction of HO-1 has been shown to reduce oxidative stress and to correct altered cellular signaling mediated by oxidative stress in cyclosporin-A-induced post-transplant hypertension (Calò et al., 2002). A similar protective role for HO-1 has been demonstrated in cisplatin-induced toxicity (Agarwal et al., 1995; Schaaf et al., 2002).

9. Curcumin. Curcumin, a widely used spice and coloring agent in food, possesses antitumor and anti-inflammatory properties and has been recently reported to induce HO-1 protein levels in vascular endothelial cells (Scapagnini et al., 2002). Increased HO activity is an important component in curcumin-mediated cytoprotection against oxidative stress (Motterlini et al., 2000). The ability of curcumin to induce HO-1 is presumably due to the presence of phenol moieties, similar to that seen in AG1067, and may also work through the activation of the HO-1 promoter at the transcriptional level. These findings indicate the existence of a novel class of natural substances as potent inducers of HO-1, which could be used for therapeutic purposes in the protection of tissues against inflammatory and neurodegenerative conditions.

10. Resveratrol. Resveratrol is an important component in certain varieties of red grapes and may underlie the cardioprotective effects thought to be obtained from moderate consumption of red wine. Recent studies have demonstrated that resveratrol can pharmacologically precondition the heart through an HO-1-dependent, NO-mediated mechanism. Resveratrol causes phosphorylation of p38 MAPK and Akt, which is completely reversed with SnPP treatment (Das et al., 2006). Pretreatment with resveratrol has markedly reduced infarct size 24 h after myocardial infarction and increases capillary density in the peri-infarct myocardium along with better left ventricular function compared with controls (Kaga et al., 2005). These results indicate that resveratrol generates cardioprotection by preconditioning the heart via a HO-1-mediated mechanism (Huang et al., 2005a). It is important to note that resveratrol is an important but not a major component of red grapes. Indeed, red wine is composed of a great number of components, any of which, acting singly or in conjunction with other compounds, could be responsible for cardioprotection.

It is important to note that despite the wide disparity in mechanism(s) among the pharmaceutical agents described above, the common thread of HO-1 induction exists. This highlights the pivotal role that this enzyme plays in providing protection against metabolic insults in humans and offers an obvious target for designing compounds with widespread clinical applications in a broad spectrum of disease states.

H. Heme Oxygenase-1 and Cardiovascular Disease: Therapeutic Rationale

The recognition that many drugs, including aspirin, statins, rapamycin, paclitaxel, and others, are inducers of HO-1 and are used to treat cardiovascular disease has added a new dimension to the use of HO-1 induction as a therapeutic modality. Apart from toxicity, a possible concern with the use of pharmacological inducers of HO-1 relates to the GT length in the HO-1 promoter. Patients with long GT repeats are more resistant to HO-1 induction, which may make such an approach difficult. Increasing HO-1 expression via viral-mediated delivery of HO-1 circumvents this problem and provides for a more selective approach in targeting this gene to specific tissues. Changes in CO and bilirubin formation and in heme content as a result of increased HO-1 protein expression via genetic intervention are modest and less abrupt or volatile than those obtained after bolus administration of chemical inducers. Genetic interventions result in a steady change in HO activity and heme content. The delivery of the human HO-1 gene and other genes to rats has proved successful for achieving long-term overexpression and decreases blood pressure (Wang et al., 1999; Raizada et al., 2000; Sabaawy et al., 2001; Sellers et al., 2001; Quan et al., 2002; Yang et al.,

2004). Human HO-1 gene transfer has been shown to provide vascular protection, attenuating the hyperglycemia-mediated increase in circulating endothelial cells, superoxide formation (Abraham et al., 2003a) and urinary isoprostane (8-epi) output (Abraham et al., 2004). In contrast, the underexpression of rat HO-1 magnified these effects (Abraham et al., 2003a, 2004), substantiating a significant role for HO-1 as part of the cellular antioxidant defense system against oxidant-mediated damage in vascular endothelium. These and other findings demonstrate an inverse relationship between the HO system and the vascular complications observed in experimental models of diabetes (Abraham et al., 2003a, 2004).

This review is intended to stimulate interest in the long-term effects of increased HO-1 expression in the early development of vascular disorders. The goal is to attenuate cardiovascular complications by replacing long-term treatment with pharmacological agents with one injection of a viral vector. The continuing search to provide this vascular protection is motivated by our findings, accumulated over the past decade, in which we have shown that adenoviral-mediated HO-1 gene therapy prevented hemoglobin toxicity and the generation of inflammatory molecules (Abraham et al., 1995a,b, 2000). Recently, HO-1 gene transfer has been shown to inhibit liver fibrosis (Tsui et al., 2005) and to prevent inflammation (Jian et al., 2005), neointimal hyperplasia (Kong et al., 2004), and ischemic heart injury (Tang et al., 2004). HO-1 gene targeting specifically to endothelial cells, not vascular smooth muscle cells, has been shown to protect endothelial cells from hyperglycemia and TNF-mediated cell death (Asija et al., 2007). These benefits have, thus far, been of short duration. However, we believe that retroviral-mediated HO-1 gene therapy may provide long-term vascular protection and thus alleviate or significantly delay vascular complications.

One concern regarding the use of gene therapy is that it could increase the incidence of cancer. Fang and colleagues (Fang et al., 2005; Davis et al., 2006; Guo and Fang, 2006; Huang et al., 2006a) have examined this aspect in a series of studies that have also included investigation of the potential beneficial use of gene therapy in oncology. In recent studies, they reported that ectopic expression of factor VIII in platelets by lentivirus-mediated bone marrow transduction/transplantation may be a promising strategy for gene therapy in human hemophilia A. The use of lentivirus-based gene transfer offers a safe means of gene transfer. A lentiviral vector-mediated HO-1 gene has been developed for long-term expression (Abraham et al., 2007).

I. Acute Kidney Injury and Heme Oxygenase-1/Heme Oxygenase-2

The mechanism by which various drugs, such as transforming growth factor-β, IL-1, endothelin, and TNF, cause acute kidney injury will not be covered in detail in this review. (The reader is referred to the reviews on acute kidney injury by Nath 2006, 2007.) However, the role of HO-1 and HO-2 in attenuating renal injury will be covered herein. The presence of HO isoforms segmented within the kidney and along the nephron has been demonstrated (Botros et al., 2001).

The cyclosporine suppressive effect on HO-1 may contribute to renal injury and therefore its use may become limited. Atrial natriuretic peptide (ANP)-mediated up-regulation of HO-1 has been shown to ameliorate the effect of cyclosporine on renal function, presumably via a cGMP-dependent mechanism (Polte et al., 2000). Treatment with ANP induced HO-1 in renal and endothelial cells (Kiemer et al., 2003) and suppressed the levels of CYP4A and COX-2 proteins in a tissue-specific manner, thereby suggesting a new direction in the immunoprotection of renal function during the use of cyclosporine. Rifampin is another immunomodulator, and its beneficial effect has been attributed to its ability to enhance levels of the HO-1 gene (Visner et al., 2003).

HO-1 induction has been shown to exert a protective effect on renal function in animal models of rhabdomyolysis, cisplatin nephrotoxicity, and nephrotoxic nephritis (Nath, 2007). Furthermore, the products of heme degradation provide a protective role in acute kidney injury and hypertension. HO-1-derived CO increased blood carboxyhemoglobin levels, renal blood flow, and glomerular filtration (Arregui et al., 2004). Heme-induced renal vasodilation, which increases renal blood flow, is a COX-dependant response whereas heme-induced diuresis and natriuresis are HO-dependant responses, involving inhibition of tubular reabsorption of water and sodium (Rodriguez et al., 2003).

In a rat model of radiation-induced nephropathy, elevated glomerular HO-1 protein levels were prevented by treatment with AT_1-receptor antagonists, which block the up-regulation of HO-1 (for review, see Nath, 2006). Renal responses to repeated exposure to endotoxin causes acute kidney injury; its prevention, by preconditioning the cytoprotective pathways, is presumably via HO-1 induction (Fig. 11) (Nath, 2007). HO-1 is considered a major determinant of the cytoprotective pathways in the kidney as well as other tissues. Angiotensin II was reported to be a mediator of HO-1 induction in a study of angiotensin II-mediated tubulointerstitial injury (Nath, 2006) and the resultant salt-sensitive hypertension (Pradhan et al., 2006). Protection against paraquat injury is mediated by the induction of metallothionine-1 and HO-1 (Tomita et al., 2006). The elevated level of HO-1 in the renal proximal tubule of rats treated with angiotensin II was associated with increased HO activity (Haugen et al., 2000). In another study (Nath et al., 2000), angiotensin II infusion decreased glomerular filtration rate and increased proteinuria, which led to hypertension. HO-1 up-regulation provided a cytoprotective effect. Overexpression of HO-1 significantly attenuated the pressor responsiveness to angiotensin

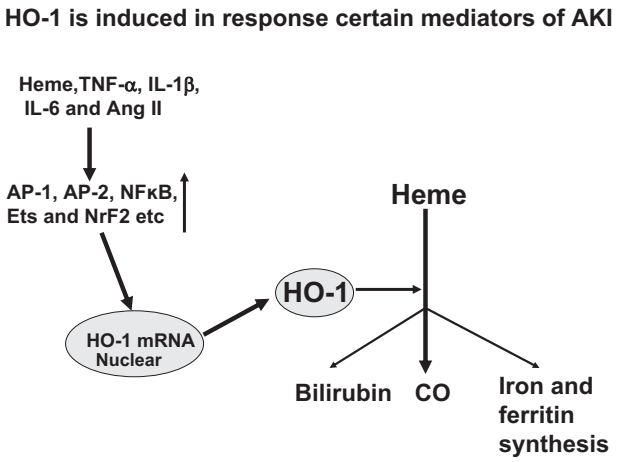

FIG. 11. Acute kidney injury (AKI) is enhanced by several factors, including drugs and ischemia, all of which result in increased levels of IL-1, IL-6, TNFα, and angiotensin II. These factors activate a number of signaling factors, including AP-1, AP-2, NF-κB, Ets, and Nrf2, leading to a rapid increase in HO-1 mRNA levels. Drugs that increase HO-1, such as CoPP, gene transfer or gene targeting to specific renal structures, have been shown to attenuate acute kidney injury.

II in rats transduced with retroviruses containing the human HO-1 gene (Yang et al., 2004). CO has been shown to exert cytoprotection against tubulointerstitial fibrosis, the hallmark of chronic progressive kidney disease leading to end-stage renal failure via the MKK3 pathway (Wang et al., 2008).

Other studies have documented the induction of vascular, cardiac, and renal HO-1 in response to angiotensin II in vitro and in vivo. Additionally, the systemic administration of an HO inhibitor has been shown to increase blood pressure in Sprague-Dawley rats. The administration of HO inhibitors directly into the NTS of rats also increased blood pressure; an effect reversed by ipsilateral microinjection of CO into the NTS. CO from HO-2 activity is not only a dilator but also can act as a tonic regulator against NO-dependent vasodilation in the cerebral microcirculation (Ishikawa et al., 2005). Collectively, these findings suggest that the HO/CO system in the NTS helps to maintain normal blood pressure by suppressing the activity of the sympathetic nervous system. Furthermore, the up-regulation of HO-1 has been shown to provide protection against renal injury after unilateral urethral obstruction; this effect is dependent on modulation of the antiapoptotic pathway by HO-1 expression (Kim et al., 2006b). Renal injury as a result of ischemia was exacerbated in HO-1-deficient mice as reflected by marked induction of IL-6 and renal dysfunction (Fig. 11) (Tracz et al., 2007). Radiocontrast agents are major players in activation of the proapoptotic pathway and caspase 3 and caspase 9 (Goodman et al., 2007). Up-regulation of HO-1 increases kidney tolerance to radiocontrast-mediated injury and renal dysfunction by the suppression of the mitochondrial proapoptotic pathways and increased expression of the inactive Bax (Goodman et al., 2007).

The effect of genetic polymorphism may play a role in acute kidney injury. A small number of patients (472) with advanced peripheral artery disease were followed for a median of 21 months for the occurrence of coronary events. Patients with short (<25) $(GT)_n$ repeats appeared to have a lower incidence of peripheral artery disease (Dick et al., 2005). However, no significant differences for cerebrovascular events and mortality were found (Dick et al., 2005). Similarly, the presence of long $(GT)_n$ repeats (>30) for HO-1, low HO-1 gene transcription, was associated with high arteriovenous fistula failure in patients undergoing hemodialysis (Lin et al., 2006).

J. Heme Oxygenase-1 and Hypertension

1. Blood Pressure Regulation. Up-regulating the HO/CO system lowers blood pressure in young (8 weeks old) SHR (Sacerdoti et al., 1989; Levere et al., 1990) but not in adult animals (20 weeks old) (Sacerdoti et al., 1989). In young SHR, blood pressure elevates and continues to increase with aging whereas adult SHR have established hypertension (Sacerdoti et al., 1989; Ndisang and Wang, 2003). If the HO/CO system is defective in young SHR, then HO-1 inducers could enhance the activity of this system. If, however, the HO/CO system is already enhanced as a compensatory reaction, then HO-1 inducers might be unable to up-regulate this system (Sacerdoti et al., 1989). Previous studies have demonstrated that either acute or chronic administration of an HO-1 inducer was able to normalize blood pressure in SHR. Heme administration decreased blood pressure in SHR (Levere et al., 1990; Martasek et al., 1991; Botros et al., 2005). Treatment with HO inhibitors produced an increase in systemic arterial pressure, even in normotensive rats, and magnified myogenic tone in gracilis muscle arterioles. HO-2-derived CO appears to be involved in the regulation of the basal vascular tone of resistance blood vessels (Leffler et al., 1999, 2001). CO produced from heme metabolism in blood vessels is reported to elicit relaxation (Wang et al., 1997; Kaide et al., 2001) through elevation of cGMP levels (Morita et al., 1995) and potassium as well as other channels (Wu and Wang, 2005).

Furthermore, CO has been reported to inhibit the activity of cytochrome P450 (Mitani et al., 1989) and the generation of vasoconstrictive substances, such as 20-HETE (Fig. 12), thus ameliorating the development of hypertension (Omata et al., 1992). Therefore, it appears that the antihypertensive effect of HO activity enhancement may be due, in part, to blunting the vasoconstrictor action of 20-HETE (Zou et al., 1994a,b, 1996). HO inhibitors have been shown to decrease renal blood flow acutely, implying that the renal HO system supports renal circulation via formation of CO (Johnson et al., 1996; Rodriguez et al., 2003, 2004).

A certain amount of confusion has arisen because of the diverse nature of the animal models used to study this phenomenon and is compounded by the use of met-

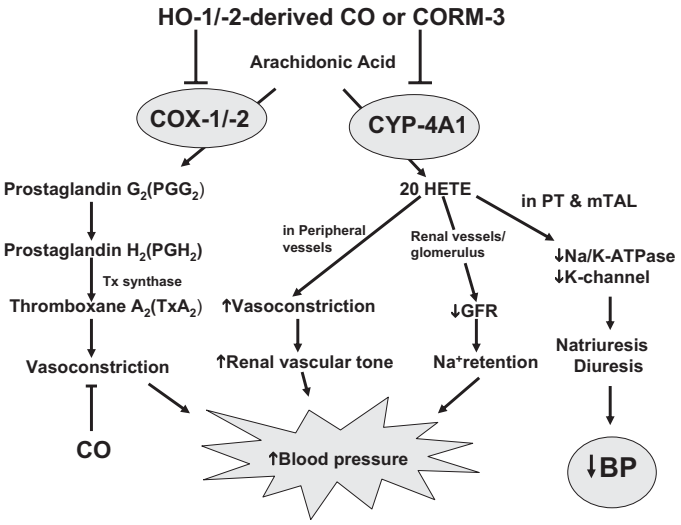

FIG. 12. Schematic representation of the role of HO-1 in hypertension. Induction of HO-1 leads to perturbations in renal arachidonic acid metabolism, including inhibition of the heme-containing thromboxane A_2 (TxA_2) synthase and CYP4A enzymes that are responsible for the formation of TxA_2 and 20-HETE, respectively; both are potent renal vasoconstrictors whose bioactivity leads to increased renal vascular resistance, vascular tone, and blood pressure. Alternatively, HO-1 induction leads to increased production of CO, which, in addition to its ability to inhibit heme-containing enzymes, also acts as a vasodilator and regulator of ion channels, thus contributing to antihypertensive mechanisms, including increased vasodilation and natriuresis.

alloporphyrins to manipulate the animal models under study. It has been suggested that HO-1 deficiency does not lead directly to the development of hypertension in mice (Wiesel et al., 2001). Moreover, treatment of normotensive rats with metalloporphyrins to inhibit HO activity resulted in an increase in systemic arterial pressure accompanied by an increase in peripheral resistance (Johnson et al., 1995). This result led to the conclusion that a decrease in CO production as a result of the inhibition of HO activity was responsible because biliverdin and iron did not induce vasorelaxation (Johnson et al., 1996). The daily injection (for 4 days) of a low dose of chromium mesoporphyrin increased the elevated blood pressure in 8-week-old SHR, whereas blood pressure in age-matched normotensive Wistar-Kyoto rats was unaffected (Ndisang et al., 2002). These findings led the authors to conclude that different blood pressure responses to the inhibition of HO activity in different strains of rats reflected a strain-specific contribution of the HO/CO axis to the regulation of blood pressure. This hypothesis requires further study to elucidate the mechanism(s) involved as suggested in Fig. 12.

Angiotensin II is systematically and/or locally elevated in many forms of hypertension and is associated with increased vascular O_2^- production (Laursen et al., 1997; Raizada et al., 2000). Increased O_2^- has been shown to contribute to the vascular and renal effects of angiotensin II (Ortiz and Garvin, 2002). Previous studies have documented the induction of vascular, cardiac, and renal HO-1 in response to angiotensin II in vitro and in vivo (Haugen et al., 2000; Ishizaka et al., 2000). HO-1 protein was shown to be markedly increased in aortic adventitial and endothelial cells from rats with angiotensin II-induced hypertension; however, treatment with losartan, a selective AT_1-receptor antagonist, blocked the up-regulation of HO-1 (Ishizaka et al., 1997). Angiotensin II increases renal oxidant stress and HO activity caused by up-regulation of HO-1 in renal proximal tubules (Haugen et al., 2000). In a rat model of radiation-induced nephropathy, elevated glomerular HO-1 protein levels were prevented by treatment with AT_1-receptor antagonists, suggesting that angiotensin II may mediate HO-1 induction (Datta et al., 2001). In contrast, treatment of rat VSMCs with angiotensin II decreased HO-1 mRNA levels and this decrease was blocked by losartan (Ishizaka and Griendling, 1997). It is conceivable that angiotensin II-mediated up-regulation of HO-1 subserves mechanisms that counteract the actions of angiotensin II.

An increase in HO-1 gene levels may interrupt the vasoconstrictor pathway and attenuate the inflammatory aspect of the microcirculation in hypertension, i.e., oxidative stress, leukocytes/endothelial interaction and apoptosis (Suematsu et al., 2002), by increasing bilirubin and CO, which are of overriding importance in the pathogenesis of vascular injury. Thus, the ability to up-regulate HO-1 offers a unique therapeutic approach to the control of hypertension.

2. Renovascular Hypertension. The products of the arachidonic acid metabolic pathway, mediated by the hemoproteins COX and cytochrome P450, have been reported, in animal models, to be responsible for hypertension. HO has been implicated as a major regulator of several cytochrome P450s, including those responsible for the formation of 20-HETE. HO acts by limiting the amount of available heme and/or by producing CO, which binds strongly to the heme moiety of cytochrome P450, resulting in inhibition of cytochrome P450-mediated reactions (Abraham et al., 2002c). Such interactions may play an important role in the regulation of renal function. For example, selective induction of cortical and outer medullary HO-1 is associated with a decrease in 20-HETE, a potent vasoconstrictor, and normalizes blood pressure in SHR (Fig. 12). This suggests a critical role for HO-1 in the regulation of urine volume, electrolyte excretion, and blood pressure (Abraham et al., 2002a).

With the use of cultured renal endothelial cells, erythropoietin-induced HO-1 expression provided cytoprotection against oxidative stress (Katavetin et al., 2007a). A study using clipped and nonclipped kidneys from two-kidney one-clip hypertensive rats reported induction of HO-1 and increased HO activity as well as increased levels of the antiapoptotic molecules Bcl-2 and Bcl-xL and decreased levels of the apoptotic molecules caspase 3 and caspase 9 (Botros et al., 2007). The induction of HO-1 has been shown to lower blood pressure and superoxide production of angiotensin in hypertensive mice

(Vera et al., 2007). The induction of HO-1 also attenuates the development of hypertension and renal injury, leading to a decrease in angiotensin II-induced injury and salt-sensitive hypertension (Pradhan et al., 2006). Moreover, the induction of HO-1 before rapamycin treatment of transplant kidneys appears to limit the acute toxicity associated with rapamycin use (Gonçalves et al., 2006). Up-regulation of HO activity by gene transfer results in the normalization of blood pressure and increased expression of the antiapoptotic molecules Bcl-2, Bcl-xL, Akt, and pAkt in two-kidney one-clip renovascular hypertension (Olszanecki et al., 2007). This study further emphasized the antiapoptotic action of the HO system as an important protective mechanism in kidney pathology and suggested that this pathway could be a specific target in the treatment of hypertension.

3. Pulmonary Hypertension. Alterations occurring with the HO/CO system in pulmonary arteries in hypertension were examined (Ndisang and Wang, 2003), leading to the conclusions that an impaired HO/CO-sGC/cGMP system in the pulmonary arteries of young and prehypertensive SHR might be indicative of the pathogenesis and development of hypertension. It has been proposed that the mechanism of pulmonary hypertension and pulmonary artery structural remodeling, induced by high pulmonary flow, might be associated with changes in the endogenous CO/HO pathway (Li et al., 2006). Chronic hypoxia causes pulmonary hypertension with smooth muscle cell proliferation and matrix deposition in the wall of pulmonary arterioles. Hypoxia also induces a pronounced inflammation in the lung before the structural changes of the vessel wall occur. HO-1 transgenic mice are protected from the development of pulmonary inflammation, as well as hypertension, and vessel wall hypertrophy induced by hypoxia (Minamino et al., 2001). These findings suggest an important protective role for the enzymatic products of HO-1 catabolism in pulmonary hypertension (Ito et al., 2007).

In pulmonary arterial hypertension, IL-10 gene transfer significantly improved survival rates of monocrotaline-induced pulmonary hypertension, which contributed to the IL-10-mediated increase in HO-1 (Ito et al., 2007). Results from the European Community respiratory health survey in France showed that long HO-1 gene promoter $(GT)_n$ repeats in heavy smokers are associated with susceptibility to develop lung dysfunction and airway obstruction (Guénégou et al., 2006). Lung dysfunction as a result of lung cancer was seen in Japanese patients with long GT repeats of HO-1; however, this effect was limited to male patients (Kikuchi et al., 2005).

4. Portal Hypertension. The role of HO-1 in oxidative stress, inflammation, angiogenesis, and splanchnic hemodynamics was examined in rats with portal hypertension (induced by partial portal vein ligation). The authors found that HO plays an important beneficial role in attenuating oxidative stress and inflammation by regulating VEGF (Angermayr et al., 2006). In another study, a recombinant adenovirus carrying rat HO-1 (rAAV/HO-1) was generated, and the virus was injected through the portal vein (Tsui et al., 2005). These data showed that portal hypertension was markedly diminished in rAAV/HO-1-transduced animals compared with controls. Other reports (Fernandez et al., 2001; Gonzales et al., 2006) have also suggested a beneficial role for HO-1 overexpression in portal hypertensive rats.

K. Hepatic Injury

Inhibition of HO-1 was shown to protect against tissue injury in carbon tetrachloride (CCl_4) exposed livers (Eipel et al., 2007). Animals pretreated with hemin and then exposed to CCl_4 demonstrated a reduction in leukocyte response. Elevated HO activity harmed liver tissue, most probably due to interference of the HO pathway with tetrachloride-dependent metabolism via cytochrome P450 and heme overload-associated toxicity (Eipel et al., 2007). Liver-specific HO-1 induction by recombinant human interleukin-2 was shown to prevent carbon tetrachloride-induced hepatoxicity (Kawakami et al., 2006). Hepatocyte apoptosis and liver injury were both attenuated in the livers of rats pretreated with hemin.

These results suggest that the HO system has a potent protective effect on acute liver injury induced by CCl_4 in rats. Induction of HO-1 protein inhibited the progress of hepatic damage, probably due to the alleviation of lipid peroxidation, reduction of caspase 3 activity, and inhibition of TNFα production (Wen et al., 2006). Penta-*O*-galloyl-β-D-glucose up-regulates HO-1 by stimulating Nrf2 nuclear translocation in an ERK-dependent manner. HO-1 up-regulation by penta-*O*-galloyl-β-D-glucose may serve as one of the important mechanisms for its hepatoprotective effects (Pae et al., 2006). The pharmacologically induced up-regulation of HO-1 by pyrrolidine dithiocarbamate conferred protection against subsequent ischemia-reperfusion injury to the rat liver. Pretreatment with pyrrolidine dithiocarbamate attenuated the disturbance of hepatic microcirculation but not parenchymal injury, in the early phase of ischemia-reperfusion injury (Heiman et al., 2006). Ischemic preconditioning blunted the activation of STAT-3 and stress-responsive genes, such as NF-κB, AP-1, and HO-1, during reperfusion. Ischemic preconditioning modulates the activity of transcription factors and triggers IL-6 production, which may prevent hepatic ischemia-reperfusion damage in an oxidative stress-dependent way (Tacchini et al., 2006). Induction of HO-1 in hypoxic preconditioning plays a protective role against hepatic ischemia-reperfusion injury (Lai et al., 2004). Induction of metallothionein and HO-1 with Maotai (a Chinese drink containing 53% v/v alcohol) could be important adaptive responses to reduce alcoholic liver injury (Liu et al., 2006a).

Basal HO-1 levels appear more critical than the ability to up-regulate HO-1 in response to ischemia-reper-

fusion injury and may also predict the success of pharmacologically induced cytoprotection (Tsuchihashi et al., 2006). CoPP-induced HO-1 up-regulation suppresses the type-1 interferon pathway downstream of the Toll-like receptor (TLR) 4 system in a hepatic ischemia-reperfusion injury model (Tsuchihashi et al., 2005). Hepatoprotection appears to involve the rapid degradation of heme by HO, with subsequent alterations in hepatic microvascular resistance and heme accumulation. Rhabdomyolysis attenuates a detrimental response in the hepatic parenchyma and microcirculation mediated by the rapid degradation of hepatic heme via CoPP-induced HO activity (Dorman et al., 2005). Tris(methoxymethoxy) chalcone (TMMC) induced the expression of HO-1 in hepatic stellate cells (Lee et al., 2006). The inhibitory action of TMMC on platelet-derived growth factor-induced proliferation was mediated by HO-1. GSH depletion produced by TMMC activated ERK, which led to c-Fos expression and transactivation of AP-1 and HO-1 gene expression in the hepatic stellate cells. HO-1 expression was thus reported to be responsible for the antiproliferative effect of TMMC on hepatic stellate cells (Lee et al., 2006).

In steatotic livers, HO-1 preconditioning and geranylgeranylacetone treatment (which are responsible for HO-1 induction) increased protein kinase C activity. Induction of HO-1 by CoPP protected both liver types. Preconditioning reduced p38 MAPK and c-Jun N-terminal kinase (JNK), resulting in HSP 72 induction, although HO-1 remained unmodified. Like HSP 72, both p38 and JNK appeared not to be crucial in preconditioning, and inhibitors of p38 and JNK were less effective against hepatic injury than were HO-1 activators. These results provide new data regarding the mechanisms of preconditioning and the development of new pharmacological strategies in liver surgery (Massip-Salcedo et al., 2006).

β_1-Adrenoceptor-dependent hepatic up-regulation of HO-1 resulted in better maintained hepatocellular function after hemorrhagic shock in animals pretreated with dobutamine (Raddatz et al., 2006); because of the up-regulation of HO-1, improved hepatocellular function may be mediated, in part, by CO. The development of micronodular cirrhosis was significantly inhibited in rAAV/HO-1-transduced animals compared with controls (Tsui et al., 2006). In addition, portal hypertension was markedly diminished with no significant changes in systolic blood pressure. This decrease was accompanied by improved liver biochemistry, fewer infiltrating macrophages, and fewer activated hepatic stellate cells in rAAV/HO-1-transduced livers. Thus, increased HO activity in the liver suppressed the development of cirrhosis. Isolation of different types of cells from CCl_4-induced fibrotic livers showed the predominant expression of the transgene in hepatic stellate cells after rAAV/HO-1 administration. In addition, HO-1-transduced stellate cells showed reduced transcript levels of type 1 collagen and impaired proliferative ability compared with control animals, suggesting a new approach in the treatment of liver fibrosis using adeno-associated virus-mediated gene transfer (Tsui et al., 2005).

Acetaminophen injection markedly augmented intrahepatic gene expression of iNOS and HO-1; moreover, neutrophils expressed iNOS, whereas HO-1 was mainly expressed by macrophages (Ishida et al., 2006). Acetaminophen caused more exaggerated liver injury in CXCR2-deficient mice, which was associated with reduced macrophage infiltration and HO-1 gene expression, compared with that in neutropenic wild-type mice. Inhibition of HO activity significantly increased acetaminophen-induced mortality, implicating HO-1 as a protective molecule for acetaminophen-induced liver injury. CXCR2 may regulate the infiltration of both iNOS-expressing neutrophils and HO-1-expressing macrophages, and the resultant balance may determine the outcome of acetaminophen-induced liver injury. Hepatic HO-1 mRNA levels were up-regulated in animals subjected to hemorrhagic shock. The vasodilator, dihydralazine, increased nutritive portal and hepatic microvascular flow and limited liver injury after hemorrhagic shock (Schmidt et al., 2006). Treatment with L-arginine increased the expression of HO-1, suggesting that L-arginine could prevent oxidative damage during hepatic surgery (Lanteri et al., 2006).

Exogenous CO treatment suppressed early proinflammatory gene expression and neutrophil infiltration and efficiently ameliorated hepatic ischemia-reperfusion injury (Kaizu et al., 2005). The possible mechanism by which CO protects the liver against cold ischemia-reperfusion appears not to be associated with the down-regulation of the NF-κB-signaling pathway. ROS generation subsequent to reoxygenation inflicts tissue damage and initiates a cellular cascade leading to inflammation, cell death, and, ultimately, organ failure. Accruing evidence suggests that Kupffer cells and T cells mediate the activation of neutrophil inflammatory responses. Activated neutrophils infiltrate the injured liver in parallel with the increased expression of adhesion molecules on endothelial cells. The HO system is among the most critical of the cytoprotective mechanisms activated during cellular stress, exerting antioxidant and anti-inflammatory functions, modulating the cell cycle, and maintaining the microcirculation. The activation of TLRs on Kupffer cells may provide the triggering signal for proinflammatory responses in the ischemia-reperfusion injury sequence. Dissecting the signaling pathways linking HO-1 and Toll-like receptor activation may be important in devising novel therapeutic strategies for combating ischemia-reperfusion injury (Kupiec-Weglinski and Busuttil, 2005).

Chronic hepatitis C virus (HCV) infection leads to increased oxidative stress in the liver. Hepatic antioxidant enzymes provide an important line of defense against oxidative injury. Although the expression of HCV-nonstructural proteins leads to increased oxidative

stress as well, the antioxidant enzymatic responses are different. The overexpression of HCV-nonstructural proteins increased levels of antioxidant enzymes (MnSOD and catalase), HO-1, and GSH, indicating different mechanism(s) of pro-oxidative activity than HCV-core protein. In a recent study, the expressions of iNOS, an inducer of oxidative stress, and HO-1, a cytoprotective protein, were higher in leptin-deficient *ob*/*ob* mice compared with lean mice after chronic alcohol feeding. However, LPS-induced HO-1 but not iNOS expression was attenuated in *ob*/*ob* mice compared with lean mice. These results imply that the increased sensitivity of alcoholic fatty liver to LPS occurs without the up-regulation of TLR2 or TLR4 genes and may be related to an imbalance of proinflammatory/oxidative and cytoprotective mechanisms (Romics et al., 2005).

Bilirubin treatment improved survival and attenuated liver injury in response to LPS infusion in a rat model of endotoxemia (Wang et al., 2004). These data suggest a cytoprotective role for bilirubin that is mediated, at least in part, through the inhibition of iNOS expression and, potentially, through stimulation of local PGE_2 production. Inhalation of CO during HO inhibition significantly reduced microcirculatory deficits, hepatic inflammation, and injury in response to hind limb ischemia-reperfusion, suggesting that HO-derived hepatic protection is mediated by CO (Ott et al., 2005). Inhalation of low concentrations of CO may represent a novel therapeutic approach to prevent remote organ injury during systemic inflammatory response syndrome (SIRS). Adenovirus-HO-1 pretreatment attenuated remote liver injury during normotensive SIRS, induced by bilateral hind limb ischemia and reperfusion, indicating that gene transfer of inducible HO is an effective method to protect the liver during SIRS. The anti-inflammatory benefits afforded by increased HO activity within the liver appear to involve the control of sinusoidal diameter and volumetric blood flow rather than altered adhesion molecule expression during the early stages of SIRS (Wunder et al., 2004). ANP-preconditioned livers are protected from ischemia-reperfusion injury. ANP-treated organs showed increased protein levels of HO-1. The cGMP-mediated attenuation of iron regulatory protein binding activity by ANP resulted in increased hepatic ferritin levels. This change in iron regulatory protein binding activity was independent of ANP-induced HO-1 and reduced protein kinase C activation (Kiemer et al., 2004).

Gender influences the hepatic expression of HO-1 after trauma and hemorrhage. Trauma and hemorrhage induced a 2-fold increase in hepatic HO-1 expression in proestrus females compared with males (Toth et al., 2003). Hepatic expression of HO-2 was unaffected by sex or trauma and hemorrhage. SnPP abolished the gender differences caused by diverse HO-1 expression. SnPP also elevated portal pressure, decreased bile production, and increased alanine transaminase to similar levels in proestrus females and males after trauma and hemorrhage. The enhanced induction of HO-1 expression and activity in females after trauma and hemorrhage may attenuate hepatocellular dysfunction and injury by maintaining microcirculation via the increased production of CO (Toth et al., 2003). These data highlight the role of elevated levels of HO-1 in protecting against liver damage, largely through the beneficial properties of CO and biliverdin/bilirubin.

L. Hormonal Regulation

Hormonal effects were among the first facets of HO to be studied. In 1972, the effects of certain hormones and starvation on HO activity were examined. Epinephrine and glucagon increased hepatic HO activity by 5.6-and 2.3-fold, respectively, whereas thyroxine (T_4) and hydrocortisone had no effect. Arginine, which releases endogenous glucagon, resulted in a 5-fold increase, and a 3-fold increase was observed with the administration of cAMP. Fasting resulted in a 3-fold increase in hepatic HO activity after 72 h after which a gradual decline occurred. Hypoglycemia, induced by injection of insulin or mannose, was a powerful stimulator of HO (7-fold increase). The coinjection of insulin and glucose abolished this effect (Bakken et al., 1972).

3,5,3′-L-Triiodothyronine (T_3) enhances both hepatic HO (Smith et al., 1982) and ALA synthase (Smith and Drummond, 1988) activity in a dose- and time-dependent manner while also markedly depleting cytochrome P450 content. This activation is stereospecific for T_3 in that reverse T_3 (3,3′,5′-L-triiodothyronine) is inactive and thyroxine is less potent. In thyroidectomized rats, T_3 enhances hepatic HO activity by 72% and ALA synthase by 251% while depleting cytochrome P450 and heme levels (Smith and Drummond, 1991). Retinoic acid alone had no effect on either of these enzymes, but resulted in a moderate depletion in the levels of cytochrome P450 and heme. However, retinoic acid coadministered with T_3 enhanced the T_3 stimulation of HO activity in a synergistic manner, but failed to influence the effect of T_3 on ALA synthase or cytochrome P450 and heme levels (Smith and Drummond, 1991).

Estrogen was reported to be ineffective in altering HO activity in rat testis (Sadler et al., 1986). Testicular HO activity increased after one or two doses of human chorionic gonadotropin every 12 h. Thereafter, even with repeated human chorionic gonadotropin administration, enzyme activity gradually declined, but remained above control levels for the 9-day study period. A sex difference in adrenal HO activity has also been reported, suggesting a repressive regulatory role for testosterone in this tissue (Veltman and Maines, 1985). Adrenalectomy enhanced by 2-fold the induction of hepatic HO by $CoCl_2$ and impaired ALA synthase activity (Sardana et al., 1980); administration of hydrocortisone reversed these effects. Pregnancy failed to alter the response of hepatic HO to $CoCl_2$, but greatly reduced the response of ALA

synthase to 2-allyl-2-isopropylacetamide (Sardana et al., 1981).

In certain cell lines, the prostaglandin, deoxy-$\Delta^{9,12}$-13,14-dihydro-prostaglandin D_2 (Δ^{12}-PGJ_2), is a potent inducer of growth inhibition (Ohno et al., 1986) and cell differentiation (Santoro et al., 1979). The inhibitory effect of Δ^{12}-PGJ_2 involves the induction of a 68-kDa protein, which has been identified as a heat-shock protein (Ohno et al., 1988). Δ^{12}-PGJ_2 has also been found to induce a p31 protein; since identified as HO in porcine aortic endothelial cells (Koizumi et al., 1991, 1992). The function of HO in response to Δ^{12}-PGJ_2 remains unclear. Δ^{12}-PGJ_2 is known to conjugate readily with glutathione (Atsmon et al., 1990); thus, HO may be induced in response to a change in the antioxidant/oxidant ratio of the cell (Koizumi et al., 1992). PGA_1 is known to be a potent inducer of HO mRNA in myoblastic cells (Rossi and Santoro, 1995).

The synthetic glucocorticoid, dexamethasone, inhibits the induction of HO (Lutton et al., 1992). Dexamethasone and heme are thought to modulate HO through the activation and suppression of NF-κB, respectively, and the formation of an inhibitory unit by dexamethasone (Lavrovsky et al., 1994). Estradiol treatment induced increased HO-1 mRNA expression, HO-1 protein levels, and HO activity in cardiac and hepatic tissue compared with vehicle-treated trauma-hemorrhage rats. The administration of chromium mesoporphyrin prevented the estradiol-induced attenuation of shock-induced organ dysfunction and damage (Szalay et al., 2005). Thus, the salutary effects of estradiol administration on organ function after trauma-hemorrhage are mediated, in part, via up-regulation of HO-1 expression and activity.

M. Stromal/Mesenchymal Stem Cells and Hematopoiesis

Heme synthesis and degradation play pivotal roles in the regulation of growth and differentiation of erythroid and nonerythroid cells (Abraham, 1991). Suppression of erythropoiesis may result from either insufficient levels of one of the heme biosynthetic enzymes or from increased heme degradation. During in vitro erythroid colony development, HO is elevated in the early phases of culture, after which it progressively declines as ALA synthase increases (Lutton et al., 1991). Thus, a decrease in erythroid HO activity may be an important feature in the differentiation process. In patients with X-linked sideroblastic anemia and ineffective erythropoiesis, HO activity was found to be increased by 40 to 60% above normal (Abraham, 1991). Cell growth and differentiation in these patients may be hindered by the absence of a specific initiation factor, which may control HO levels (Kutty et al., 1994). Excess HO-1 is an important regulator of hematopoietic stem cell differentiation into various lineages (Abraham et al., 1983; Abraham, 1991).

The effect of exogenous hemin/HO-1 on cellularity and the hemopoietic clonal potential of cells maintained in murine long-term bone marrow stromal cultures has been studied. Hemin was reported to produce mobilization of hemopoietic cells and committed precursors from adherent mesenchymal stem cells into suspension (Abraham et al., 1991a; Loewy et al., 1992; Abraham, 1996). Furthermore, supplementation with hemin in adherent stromal/mesenchymal stem cells significantly increased the myeloid progenitor compartment and longevity of the culture without altering the erythroid compartment, and prevented AZT toxicity (Chertkov et al., 1991; Harrison et al., 1993; Greene, 2004). Anemia and neutropenia, occurring as a result of AZT treatment, were not as pronounced in the presence of hemin (Loewy et al., 1992; Abraham et al., 1993; Harrison et al., 1993; Moqattash et al., 1994). This is due to the beneficial effect of heme on stromal/mesenchymal stem cells, providing an excellent environment for hematopoiesis and subsequently increasing the number of red blood cells. Furthermore, colony forming unit-fibroblast levels were significantly reduced in the presence of low concentrations of AZT. This result may indicate that a major target site of bone marrow toxicity is the stromal/mesenchymal stem cell microenvironment, which is responsible for maintaining short- and long-term hematopoiesis (Abraham et al., 1991a, 1993; Loewy et al., 1992; Abraham, 1996). The additive effect of heme and erythropoietin to stromal/mesenchymal stem cells in long-term bone marrow cultures was seen in increased red cell production, as manifest by increases in BFU-E and colony forming unit-spleen production. Thus, heme exerts a significant protective effect on hematopoietic progenitors in vivo and may be of potential clinical use in combination with erythropoietin in the promotion of effective erythropoiesis during AZT therapy (Loewy et al., 1992; Abraham et al., 1993; Harrison et al., 1993).

These results show that HO-1 expression is essential for mesenchymal stem cell growth and stem cell differentiation (Abraham, 1991; Chertkov et al., 1991, 1992, 1993). The direct effect of HO-1 on mesenchymal stem cells was demonstrated when the mesenchymal stem cell-mediated immunosuppressive effect was shown to be dependent on the levels of HO-1 (Chabannes et al., 2007). The essential role of HO-1 in restoration of bone marrow-derived stem cells and prevention of type 2 diabetes has been described (Abraham et al., 2008).

Erythropoietin, the primary regulator of red cell formation, is synthesized predominantly in the kidney and to a small extent in the liver. The erythropoietin gene is expressed in response to hypoxic stress (Schuster et al., 1989; Fandrey and Bunn, 1993). EPO has been shown to exert cytoprotective effects on erythroid progenitor cells as well as various nonerythroid cells and the renoprotective effects of EPO in various acute and chronic renal injury models are due to increases in HO-1 (Katavetin et

al., 2007b). EPO has been shown to increases HO-1 in human and mice bone marrow stem cells (Abraham et al., 1985b, 1989b; Abraham, 1991).

The HO-1 system has been shown to regulate T-cell proliferation and immune responses (Pae et al., 2004; Choi et al., 2005). Studies have shown that $CD4^+$ T cells express HO-1 and that the lack of HO-1 modulates T-cell proliferation and maturation (Chauveau et al., 2005; Chen et al., 2005). HO-1 up-regulation has proven to be capable of providing cytoprotection to vascular function (Abraham and Kappas, 2005) and to pancreatic β-cells both in vivo (Pileggi et al., 2001) and in vitro (Ye and Laychock, 1998). Recently, HO-1 up-regulation has been shown to dramatically decrease dendritic cell infiltration into pancreatic tissues in diabetic mice (Li et al., 2007a). Furthermore, HO-1 up-regulation decreases the levels of ROS and iNOS in diabetic rats via an increase in biliverdin/bilirubin production and CO generation and an increase in EC-SOD levels (Kruger et al., 2005; Turkseven et al., 2005). Under these conditions, HO-1 increases bone marrow stem cell-derived endothelial progenitor cell lineages (Peterson et al., 2007). Up-regulation of HO-1 gene levels in the early development of diabetes in NOD mice results in the acquisition of a new pancreatic phenotype as reflected by increases in anti-apoptotic signaling proteins, thus preventing β-cell destruction and delaying the development of diabetes over the study period (Li et al., 2007a,b). CoPP-mediated increases in HO-1 protein levels and HO activity were associated with the prevention of $CD11c^+$ dendritic cell infiltration into pancreatic tissues and an improvement in insulin secretion (Li et al., 2007a,b). More importantly, the euglycemic effect of HO-1 induction and prevention of $CD11c^+$ dendritic cell infiltration were reversed when NOD mice were treated with SnMP. Up-regulation of HO-1 protein in hematopoietic cells and/or in pancreatic tissues changed the pancreas from a naive to a defensive phenotype by producing a robust increase in pAKT, RSK, and Bcl-xL. The antiapoptotic Bcl-2 family proteins, such as Bcl-xL, prevent the release of apoptotic proteins from mitochondria. Similarly, increased HO-1 protein is associated with increased pBAD levels, resulting in increased cell survival (Olszanecki et al., 2007; Turkseven et al., 2007). One of the important substances released from mitochondria during apoptosis is cytochrome *c* (Liu et al., 1996). Released cytosolic cytochrome *c* binds to Apaf-1, inducing a conformational change in Apaf-1 (Jiang and Wang, 2000). Binding of Apaf-1 to cytochrome *c* triggers its oligomerization to form the apoptosome, which recruits procaspase-9 (Zou et al., 1999). Furthermore, the HO-1-mediated increase in RSK is important for the prevention of cell death. RSK phosphorylates Bad at Ser^{112} and prevents the proapoptotic effect of Bad (Tan et al., 1999). Recently, it has been reported that IL-10 regulates inflammatory and immunosuppressive responses in a HO-1-dependent manner (Chen et al., 2005). Thus, the anti-inflammatory and immunosuppressive effect of CoPP on dendritic cells, as seen in NOD mice, is achieved by the up-regulation of HO-1 via IL-10-mediated suppression. Multiple lines of evidence suggest that $CD4^+CD25^+T_{regs}$ are important in preventing an immunoresponse to oxidants in which IL-10 and/or other cytokines play a critical role (Herman et al., 2004).

N. Infectious Disease and Heme Oxygenase-1

Infection is associated with a steady and global increase of nonheme iron in the cortex, particularly in neuronal cell bodies of layers II and V, and in capillary endothelial cells. An increase in nonheme iron was associated with the induction of HO-1 in neurons, microglia, and capillary endothelial cells whereas HO-2 levels remained unchanged, suggesting that the nonheme iron increase might be the result of HO-1-mediated heme degradation (Ren et al., 2007). Indeed, treatment with SnPP (which completely blocked the accumulation of bilirubin detected in HO-1-positive cells) prevented the infection-associated nonheme iron increase. Early HO-1 expression may modulate the systemic inflammatory response and limit end-organ injury in a model of endotoxic shock (Tamion et al., 2006). HIV infection caused induction of HO-1 by approximately 20- to 50-fold in peripheral blood mononuclear cells (Levere et al., 1991, 1993), which resulted in depletion of various heme proteins needed for the immune system. The mechanism by which HIV increases HO-1 is not proven but HIV-mediated increases in proinflammatory cytokines and oxidative stress have been attributed to up-regulation of HO-1 in the blood of patients with AIDS (Abraham et al., 1991b; Levere et al., 1993). However, an increase in heme levels caused inhibition of HIV reverse transcriptase (Staudinger et al., 1996; Mingone et al., 2006). Heme causes the enhancement of peripheral blood stem cell progenitors in patients with HIV infection and normal subjects (Abraham et al., 1991b; Moqattash et al., 1994).

In contrast to the HIV-mediated induction of HO-1 described above, hepatitis C virus core protein reduces HO-1 levels in human hepatocyte cell lines (Wen et al., 2008) by decreasing a negative regulator, Bach-1 (Ghaziani et al., 2006). Heme has been reported to induce a host-defense against HIV-1 infection via HO-1 induction (Devadas and Dhawan, 2006).

AIDS is associated with hematological abnormalities such as anemia, leukopenia, and thrombocytopenia. Heme has been shown to protect bone marrow cells against cytotoxicity produced by the AIDS drug AZT (Abraham et al., 1989a; Levere et al., 1991). Treatment of human peripheral blood leukocytes from AZT-sensitive patients with 10 μM heme resulted in a 70% inhibition of HIV replication (Levere et al., 1991; Mingone et al., 2006). Although the clinical significance of this observation remains to be determined, it was reported that the level of HO mRNA was elevated in peripheral blood

adherent cells from patients with AIDS compared with those from normal subjects (Levere et al., 1993). SnPP decreased HIV infection in cell cultures (Neurath et al., 1991). Thus, inhibition of HO activity in patients with AIDS may decrease heme catabolism and lead to the preservation of cellular heme levels needed for hemoglobin synthesis and induction of the erythroid differentiation process. In addition, peripheral blood mononuclear cells from patients with AIDS retain the capacity to generate erythroid precursors such as BFU-E in the presence of erythropoietin. Hemin specifically enhances growth of BFU-E colonies obtained from both peripheral blood and bone marrow cells (Mingone et al., 2006).

Huh-7 cells expressing HCV proteins show significant up-regulation of the HO-1 gene and reciprocal down-regulation of the Bach-1 gene (Ghaziani et al., 2006). Exogenous oxidative stressors and antioxidants can modulate the expression of these genes. These and other results suggest a key role for the down-regulation of Bach-1 and the up-regulation of HO-1 in diminishing the cytotoxic effects of HCV proteins in human hepatocytes. Others suggested that the survival pathway during *Escherichia coli* infection might be activated by the HO-1-derived production of CO (Chen et al., 2006c). The $CD16^{high}CCR2^{-}$ subpopulation was found to be increased in Kawasaki disease and influenza virus infection; HO-1 mRNA was significantly increased by mononuclear cells in these illnesses. The above results indicate that The $CD16^{high}CCR2^{-}$ subpopulations are of a distinct lineage from The $CD16^{low}CCR2^{+}$ monocytes. More importantly, they may represent a monocyte subpopulation with a unique functional role to regulate inflammation by producing HO-1 in steady state in vivo (Mizuno et al., 2005).

Severely impaired heme metabolism has been shown to result from malarial infection (Chatterjee, 1979). During the intraerythrocytic stage of infection, the parasite uses the host's hemoglobin, discarding heme as a waste product. Upon completion of schizogony, red blood cells rupture, releasing large amounts of heme and hemoglobin into the bloodstream, resulting in anemia. Organs rich in reticuloendothelial cells sequester the heme and hemoglobin and acquire the characteristic gray-black pigmentation of malarial infection. Studies in mice infected with the malarial parasites *Plasmodium yoelii nigeriensis* (Sahni et al., 1991) and *Plasmodium berghei* (Srivastava et al., 1993) showed significant increases in the levels of hepatic HO activity and heme; these rose in conjunction with an increase in parasitemia. In severely infected animals, hepatic HO activity was increased more than 4-fold. This level of enzyme activity in the liver was so high that the administration of $CoCl_2$ did not effect further change. After treatment with the antimalarial drug chloroquine, parasitemia was quickly eliminated, but HO activity and circulatory heme levels remained elevated for 2 to 3 weeks, followed by a steady decrease over the next 4 to 6 weeks, reaching normal levels by 7 to 8 weeks. Changes in hepatic microsomal membrane function, as a result of parasitical infection, have also been noted in infection with the trematode *Fasciola hepatica* (Galtier et al., 1994). At 3 to 9 weeks after infection, the juvenile larvae migrate through the liver, causing extensive damage, and the adults then settle in the bile ducts, causing obstructive jaundice. A significant decrease in hepatic HO activity occurs 3 to 6 weeks after infection, as does a decrease in total microsomal cytochrome P450 and certain cytochrome P450-dependent monooxygenases. These results suggest that HO-1 induction may have therapeutic potential against these inflammatory insults.

O. Cancer and Heme Oxygenase-1

The role of HO-1 in cancer stems from the demonstration that HO-1 is a potent regulator of cell growth and angiogenesis. CO signaling has been established in the promotion of angiogenesis in human microvessel endothelial cells, presumably by increasing the levels of HO-derived CO (Deramaudt et al., 1998, 1999a,b; Li Volti et al., 2005). HO is responsible for prolactin-mediated cell proliferation and angiogenesis in human endothelial cells (Malaguarnera et al., 2002). In addition, HO-1 has been shown to accelerate tumor angiogenesis in human pancreatic cancer (Sunamura et al., 2003). Stimulation of HO-1 protein levels by hypericin-photodynamic therapy is a cytoprotective mechanism governed by the p38 MAPK and phosphoinositol 3-kinase pathways, probably through the control of the nuclear availability of the Nrf2 pool (Kocanova et al., 2007). Mice lacking Nrf2 are more susceptible to dextran sulfate sodium-induced colitis, suggesting that Nrf2 plays an important role in protecting intestinal integrity through regulation of proinflammatory cytokines and the induction of phase II detoxifying enzymes (Khor et al., 2006). Transcriptional activation of Nrf2/antioxidant responsive element is critical in the sulforaphane-mediated induction of HO-1, which can be modulated, in part, by the blockade of the p38 MAPK signaling pathway. In addition, p38 MAPK can phosphorylate Nrf2 and promote the association between Nrf2 and heap1 proteins, thereby potentially inhibiting nuclear translocation of Nrf2 (Keum et al., 2006). Constitutive overexpression of Nrf2-dependent HO-1 confers resistance to apoptosis induction by epigallocatechin 3-gallate; therefore, its inactivation may be a target for overcoming the resistance to chemoprevention and chemotherapy (Kweon et al., 2006).

The up-regulation of phase II detoxifying and stress-responsive genes is believed to play an important role in cancer prevention, and many natural compounds have been shown to be potent inducers of these genes. The antioxidant-responsive elements found in these genes can be bound by the transcription factor Nrf2 and are responsive to activation by chemopreventive compounds and by oxidative stress. Nuclear Nrf2 activates antioxi-

dant-responsive elements and induces the expression of stress-responsive genes, including HO-1 (Xu et al., 2006b). Nrf2 and HO-1, regulated by Nrf2, were not expressed in skin tumors from mice of either genotype whereas HO-1 expression in $Nrf2^{+/+}$ mice was much higher than that in $Nrf2^{-/-}$ mice in nontumor skin samples (Xu et al., 2006a). These results demonstrate that $Nrf2^{-/-}$ mice are more susceptible to skin tumorigenesis and that the chemopreventive effects of sulforaphane, a dietary isothiocyanate, are mediated, at least in part, through Nrf2. HO-1 overexpression increased the viability, proliferation and angiogenic potential of melanoma cells, augmented metastasis, and decreased survival in tumor-bearing mice, suggesting that the induction of HO-1 may be detrimental in the treatment of melanoma (Was et al., 2006).

SNP increased the expression of HO-1 and ferritin in IHOK and HN12 cells in a concentration-dependent manner. NO-induced cytotoxicity was also inhibited by hemin (an HO-1 agonist) but was enhanced by ZnPP (Lee et al., 2007a). On the basis of these results, it was concluded that HO-1 plays a major role in mediating cytoprotection and iron homeostasis against NO toxicity in immortalized and malignant oral keratinocytes. The down-regulation of HO-2 expression with siRNA constructed against HO-2 (siHO-2) caused the induction of HO-1 mRNA and protein in HeLa and HepG2 cells (Ding et al., 2006).

β-Carotene combined with cigarette smoke condensate (***TAR) regulates HO-1 via its transcriptional factor Bach-1 and modulates cell growth. The role of HO-1 repression in increasing cell growth was also confirmed in Mv1Lu cells by the knockdown of the Bach-1 gene, thus demonstrating HO-1 repression as a conserved mechanism by which cells can react to oxidative stress (Palozza et al., 2006). Although the protective effect of NO against apoptotic cell death has been attributed, in several model systems, to an increase in the expression of HO-1, HSP 70, or Bcl-2, this was not the case after photodynamic therapy (Gomes et al., 2002). These results show that NO decreased the extent of apoptotic cell death after photodynamic therapy through a PKG-dependent mechanism upstream or at the level of caspase activation. Cysteamine was shown both in vitro and in situ to rapidly induce various heat shock proteins in astrocytes long before granulation occurred. Cysteamine treatment resulted in increases in HSP 27, HSP 90, and HO-1 at both the protein and mRNA level. In addition, C6 glioma cells, unlike primary astrocytes, constitutively express HSP 27, HSP 90, and HO-1 at low levels (Chopra et al., 1995).

A relationship between malignancy and perturbations in HO exists in cancerous conditions. Angiogenesis is necessary for the continued growth, invasion, and metastasis of tumors, and several studies have shown that HO-1 plays an important role in angiogenesis (Cisowski et al., 2005; Li Volti et al., 2005; Loboda et al., 2005). As such, HO-1 can be considered a target for antitumor therapy because the growth of most tumors depends on the activity of this enzyme. Fang and colleagues have studied the effect of inhibition of HO-1 activity on tumor growth (Fang et al., 2004a) and demonstrated that inhibition of HO activity by ZnPP significantly reduced tumor growth in a rat model (Fang et al., 2003, 2004b; Tanaka et al., 2003). The effect of human HO-1 gene modulation on angiogenesis, tumor growth, and metastasis was examined in a mouse model of pancreatic carcinoma (Sunamura et al., 2003). Increased levels of human HO-1 protein accelerated tumor growth, stimulated the early stages of angiogenesis, and increased the occurrence of lung metastasis. Conversely, the inhibition of HO activity, was shown to be beneficial in controlling angiogenesis and both the growth and the spread of tumors (Malaguarnera et al., 2003). ZnPP was reported to inhibit HO-1 (Regehly et al., 2007), resulting in suppressed biliverdin/bilirubin production accompanying lowered antioxidative capacity. As a consequence, significant suppression of tumor growth in vivo was demonstrated. In addition, prolactin-mediated angiogenesis and cell proliferation are dependent on HO-1 gene expression (Malaguarnera et al., 2002). These findings suggest that HO-1 is an attractive target for chemotherapeutic intervention.

The effects of HO-1 induction on cell survival were examined in a human colon cancer cell line, Caco-2 (Busserolles et al., 2006). Serum deprivation-induced apoptosis, reduced Akt and p38 phosphorylation, and increased p21(Cip/WAF1) levels. HO-1 induction resulted in resistance to apoptosis, activation of Akt, reduction in p21(Cip/WAF1) levels, and modification of the Bcl-2/Bax ratio toward survival. This study shows the antiapoptotic effect of HO-1 in colon cancer cells, which was mediated through the formation of bilirubin and biliverdin. It also supports an antiapoptotic role for HO-1 in these cells and provides a mechanism by which increased levels of HO-1 may promote tumor resistance to stress in conditions of limited nutrient supply. These observations were extended to show that the effects are independent of p38, but are mediated via the Akt pathway (Busserolles et al., 2006).

In a recent study, the proteasomal inhibitor MG-132 was shown to cause oxidative stress, as indicated by the up-regulation of HO-1 and the appearance of oxidized proteins (Goldbaum et al., 2006). Activation of the mitochondrial pathway was involved in the apoptotic process, mitochondrial membrane potential was disturbed, and cytochrome *c* was released from the mitochondria. Concomitantly, the death-related caspases 3 and 9 were activated and poly(ADP-ribose)-polymerase cleavage occurred. In an earlier study (Berberat et al., 2005), the targeted knockdown of HO-1 protein level led to a pronounced growth inhibition of pancreatic cancer cells and resulted in tumor cells being significantly more sensitive to radiotherapy and chemotherapy. This may be a new

option in pancreatic cancer therapy and may be used as a sensitizer to chemotherapy and radiotherapy.

The alteration in the gene expression pattern in the squamous cell carcinoma cell line A-431 was analyzed after endogenous protoporphyrin IX photodynamic treatment (Verwanger et al., 2002). Increased levels of HO-1 protein, after dark incubation, were not further increased by irradiation and were, therefore, probably caused by the need for heme degradation. Presumably, HSP 70 and HO-1 are required for cell protection and growth. Similar results have been found with photodynamic treatment using external porphyrin-based photosensitizers (Verwanger et al., 2002). The differential effect of HO-1 in cancer may also be related to HO-1 genetic polymorphism. A microsatellite polymorphism promoter is also associated with the risk for melanoma (Okamoto et al., 2006). Higher HO-1 levels may be associated with a higher risk for malignant melanoma and a higher tendency for the melanoma to be resistant to apoptosis, i.e., patients with low $(GT)_n$ repeat genotypes for HO-1 are at risk for drug resistance (Okamoto et al., 2006). In agreement with this study, it was found that lymphoblastoid cell lines from patients with short allele $(GT)_n$ repeats (<27) are more resistant to oxidant-induced apoptosis than those cells obtained from patients with longer $(GT)_n$ repeats (>33), i.e., less HO-1 expression (Hirai et al., 2003). Thus, HO-1 may be an ideal target to control cancer growth; in fact, enhancement of the chemotherapeutic response of tumor cells was achieved by an HO inhibitor, ZnPP, in vivo and in vitro (Fang et al., 2003, 2004a). A high-loading nanosized micelle of copoly(styrene-maleic acid), ZnPP has been shown to be effective in delivering HO inhibitors (Iyer et al., 2007) and may be of use in cancer chemotherapy.

P. Aging, Parkinson's Disease, and Alzheimer's Disease

The ontogenesis of liver HO has been examined, and high HO activity levels were observed during fetal development (Maines and Kappas, 1975b; Lin et al., 1989, 1990). During development and aging, the transcriptional response to oxidative stress decreases, and HO-1 protein levels do not increase progressively during aging. These phenomenon may be explained by a decreased transcriptional ability to respond to stress rather than by a reduction in oxidative stress (Patriarca et al., 2007). HO-1 responds to known inducers when administered to young rats, but induction of HO-1 in old animals (24-months of age) did not change the levels of cytochrome P450 compared with the perturbations seen in young rats (Abraham et al., 1985a). Glutathione depletion enhances oxidative stress markers, including HO-1. Decreased GSH promotes multiple apoptotic pathways, contributing at least partially to motor neuron degeneration in amyotrophic lateral sclerosis (Chi et al., 2007). Functional inhibition of HO-1 abrogated capillary dilation, decreased functional capillary density, and aggravated tissue necrosis (comparable with that observed in senescent mice). It appears that aging is associated with an increased susceptibility to tissue necrosis, which is due to a loss of vascular reactivity to endogenous HO-1 levels, rather than to a reduction in ischemic tolerance (Harder et al., 2007). Independent of its PPARγ activity, Δ^{12}-PGJ_2 was shown to protect retinal pigment epithelial cells from oxidative stress by elevating GSH and enhancing MAPK activation (Qin et al., 2006). HO-1 was strongly induced by Δ^{12}-PGJ_2 but was not associated with protection.

Aging and age-related disorders, such as Alzheimer's disease, are usually accompanied by oxidative stress as one of the main mechanisms contributing to neurodegeneration and cognitive decline. Combination therapies, such as those that include an antioxidant-fortified diet and exercise, which improve the antioxidant reserve system and significantly increase the protein levels of HO-1, can result in a reduction in oxidative stress and an improvement in cognitive function (Opii et al., 2008). In the developing mouse cortex, HO-1 was observed to be progressively down-regulated in an age-related manner (Zhao et al., 2006). The authors concluded that HO-1 gene expression in the cortex is developmentally regulated and that methylation of the HO-1 CpG island was not associated with down-regulation of the gene.

Up-regulation of HO-1 has been demonstrated in experimental models of neurodegeneration, subarachnoid hemorrhage, cerebral ischemia, and traumatic brain injury (Fig. 13). HO-1 increases appeared more prominent in cerebrospinal fluid from infants compared with older children after traumatic brain injury (Cousar et al., 2006). Increased HO-1 concentration was associated with increased injury severity and unfavorable neurological outcome. Brain expression of HO and NOS may participate in the pathogenesis of hypertension-related neuronal disorders. The understanding of altered HO/CO

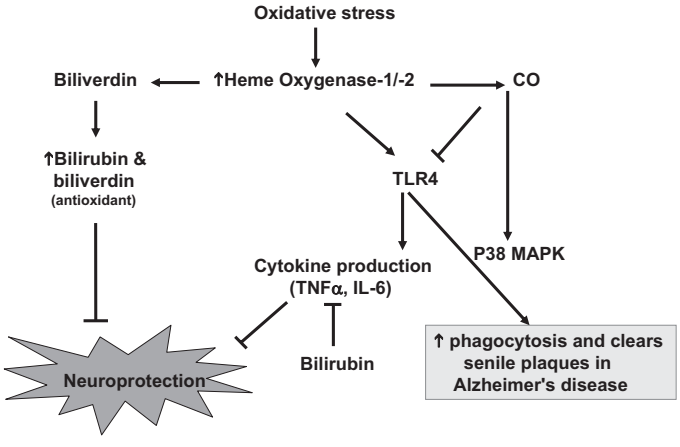

FIG. 13. Schematic representation of the role of HO-1 regulation in the brain. Heme metabolism in the brain is under separate control from heme metabolism in other tissues. The brain is unique, and oxidative stress and hypoxia have been shown to be involved in neurodegenerative processes. HO-1 induction will contribute to the inhibition of oxidative stress, a known factor in numerous neurodegenerative diseases, including Alzheimer's disease.

and NOS/NO systems may shed light on the pathogenic development of memory deficits associated with vascular dementia (Huang et al., 2006b). The transcriptional factor NF-κB is among the key cellular components that are exquisitely sensitive to oxidative stress. Age-associated NF-κB activation up-regulates the NF-κB targeting genes including HO-1, iNOS, and COX-2 (Abraham and Kappas, 2005). The increased expression of these genes is inhibited by the antioxidative properties of baicalin, a flavonoid (Kim et al., 2006a).

The brain and heart are uniquely vulnerable to hypoxic conditions and oxidative stress, with hypoxia being implicated in aging and neurodegenerative diseases (Fig. 13). Significantly higher levels of HO-1 and HSP 70 are among the changes in gene expression induced by ambient hypoxia at altitude (Appenzeller et al., 2006). Hypercholesterolemia is commonly associated with an impaired vascular relaxation response and augmented vasoconstriction; however, animals with hypercholesterolemia are less vulnerable to seizures and have enhanced resistance to systemic hypoxia. Patients with hypercholesterolemia have been shown to have a better outcome after stroke. This paradoxical effect is associated with a concomitant down-regulation of cerebral VEGF expression (Xi et al., 2006). Hypercholesterolemia increases brain levels of amyloid-β and iron and is thought to be a potential trigger in Alzheimer's disease. Iron preferentially accumulates around amyloid-β plaques in the cortex. Amyloid-β is accompanied by apoptosis, DNA damage, brain-barrier disruption, and dysregulation in the levels of the iron regulatory proteins, ferritin, and HO-1 (Ghribi et al., 2006).

The HO-1 gene is redox regulated and its expression is modulated by redox-active compounds, including nutritional antioxidants (Calabrese et al., 2006a). Dietary supplementation with curcumin and ferulic acid, two powerful antioxidants and inducers of HO-1 (Motterlini et al., 2000; Hill-Kapturczak et al., 2001), could conceivably be considered as a novel approach to reducing oxidative damage and amyloid pathology in Alzheimer's disease (Calabrese et al., 2006c; Mancuso et al., 2007). The redox modulation of heat shock protein expression by acetylcarnitine was shown to induce HO-1 as well as HSP 70 and superoxide dismutase-2 (Calabrese et al., 2006b). This effect was associated with the up-regulation of GSH levels, prevention of age-related changes in mitochondrial respiratory chain complex expression, and a decrease in protein carbonyls and nitration and may represent an approach to decreasing the risk of neurodegeneration. Current evidence suggests that resveratrol, a polyphenol stilbene, stimulates HO-1 expression and could act as a signaling molecule within brain tissues and cells to modulate the expression of genes and proteins (Doré, 2005).

The intense HO-1 immunostaining in the substantia nigra of patients with Parkinson's disease suggests its involvement in the pathogenesis of this neurodegenerative disease. Glial cell line-derived neurotrophic factor, a potent neuroprotective factor for dopaminergic neurons, negatively modulates HO-1 expression in rat postnatal cell cultures (Saavedra et al., 2005). Glial HO-1 is overexpressed in the CNS of subjects with Alzheimer's disease, Parkinson's disease, and multiple sclerosis. On the other hand, glial HO-1 hyperactivity may contribute to cellular oxidative stress, pathological iron deposition, and the bioenergetic failure characteristics of degenerating and inflamed neural tissues and thus may constitute a rational target for therapeutic intervention in these conditions (Song et al., 2006). Up-regulation of HO-1 in the substantia nigra of patients with Parkinson's disease supports the view that the affected tissue is experiencing chronic oxidative stress. Excessive cellular levels of heme-derived free iron and CO, resulting from HO-1 overactivity, may contribute to the pathogenesis of Parkinson's disease (Schipper et al., 1998, 2006). Evidence from whole animal and in vitro studies indicates that enhanced HO-1 activity may either ameliorate or exacerbate neuronal injury, depending upon factors such as the duration and intensity of HO-1 induction (Schipper, 2004b).

The advent of one component of the triad of oxidative stress, augmented iron deposition and mitochondrial insufficiency in the aging and degenerating CNS, obligates the appearance of the others. HO-1 has a pivotal role in consolidating this triad in brain iron deposition and the free radical-mitochondrial theory of aging (Schipper, 2004a). Aberrations in iron homeostasis arise primarily from aberrations in heme metabolism. In contrast, others (Chen and Regan, 2004) have suggested that HO-1 and, perhaps, HO-2 in astroglia may be responsible for the abnormal pattern of brain iron deposition and mitochondrial insufficiency documented in various neurodegenerative disorders (Schipper, 1999). Because HO-1 is increased in Alzheimer's disease, mitochondria turnover, mitochondrial DNA, and cytochrome *c* oxidative activity are all increased. The reduction in microtubule density in neurons in Alzheimer's disease suggests that mitochondrial dysfunction, acting in concert with cytoskeletal pathology, serves to increase redox-active heavy metals and initiates a cascade of abnormal events culminating in Alzheimer's disease pathology (Castellani et al., 2004). Oxidative damage, Lewy body formation, and decreased mitochondrial complex I activity are consistent pathological findings in Parkinson's disease. HO-1 is an important cytoplasmic constituent of Lewy bodies (Yoo et al., 2003). Downstream response genes, such as HO-1, can be useful biomarkers in monitoring the pathological condition of dopaminergic neurons under neurotoxic insult (Chun et al., 2001). Increased HO-1 levels contribute to the pathological iron deposition and mitochondrial damage documented in age-related neurodegenerative disorders. Paradoxically, HO-1 mRNA levels are markedly suppressed in peripheral lymphocytes of patients with early sporadic Alzheimer's

disease and thus may provide a useful biological marker (Schipper, 2000).

Activation of the HO-1-MnSOD axis may play an important role in the pathogenesis of Alzheimer's disease, Parkinson's disease, and other free radical-related neurodegenerative disorders. In these conditions, a compensatory up-regulation of MnSOD may protect mitochondria from oxidative damage accruing from heme-derived free iron and CO liberated by the activity of HO-1 (Frankel et al., 2000). A $(GT)_n$ repeat in the human HO-1 gene promoter region was found to be highly polymorphic (Kimpara et al., 1997), although no particular alleles were associated with Alzheimer's or Parkinson's disease. However, this newly identified genetic marker could allow for the study of the possible involvement of HO-1 in other human diseases.

Oxidative stress specifically targets the cytoskeleton in a variety of neurodegenerative disorders characterized by abnormal filament accumulation. Immunoreactivity to HO-1 was demonstrated in ballooned neurons, Pick bodies, neurophil threads and glial inclusions (Castellani et al., 1995). HO-1 immunolabeling of Pick bodies was closely associated with the abnormal filaments comprising the inclusions. Unaffected neurons showed only background levels of HO-1 immunoreactivity. The HO-1 suppressor activity of α_1-antitrypsin may curtail the HO-1-dependent derangement of cerebral iron homeostasis and account for diminished HO-1 expression in peripheral tissues in Alzheimer's disease (Maes et al., 2006). Heme is a common factor linking several metabolic perturbations in Alzheimer's disease, including iron metabolism, mitochondrial complex IV, HO, and bilirubin. A model of Alzheimer's disease pathobiology has been proposed in which intracellular amyloid-β complexes with free heme, thereby decreasing its bioavailability and resulting in functional heme deficiency (Atamna and Frey, 2004). Although disturbed heme metabolism causes mitochondrial decay, oxidative stress, and iron accumulation, all of which are hallmarks of aging, heme has been little studied in age-related disorders such as Alzheimer's disease. Only a small fraction of the porphyrins synthesized from succinyl-CoA are converted to heme; the rest are excreted out of the body together with the degradation products of heme (e.g., bilirubin). Aging and Alzheimer's disease are also associated with hypometabolism, an increase in HO-1, loss of complex IV, and iron accumulation (Atamna, 2004). Heme is a common denominator in all of these changes, suggesting that heme metabolism may be altered in age-related disorders.

1. Alzheimer's Disease. ROS, even if secondary to other initiating causes, are deleterious and part of a cascade of events that can lead to neuron death (Markesbery, 1997). Neuronal oxidative stress occurs early in the progression of Alzheimer's disease, most notably before the development of pathological hallmarks, such as neurofibrillary tangles and senile plaques. Oxidative stress was correlated with neuropsychological functions, neurofibrillary pathology, and mild cognitive impairment (Schipper et al., 2006). Therefore, therapeutic efforts aimed to mitigate the deleterious affects of ROS or prevent their formation may prove beneficial.

The involvement of the HO pathway is crucial to attenuate the degenerative effect mechanisms operating in Alzheimer's disease. Heat-shock proteins, such as HSP 90, HSP 70, and HSP 32, induce the production of IL-6 and TNFα and increase the phagocytosis and clearance of amyloid-β peptides mediated by TLR4 activation (Kakimura et al., 2002). Exogenous HO-1 protein administration was shown to induce the production of cytokines, TNFα and IL-6, and facilitate the phagocytosis and clearance of amyloid-β peptides in rat and mouse microglia (Schipper, 2000). These results suggest that HO-1 may have a role to play in neuroprotection in the brain.

The relationship between HO-1 and tau, using neuroblastoma cells transfected with sense and antisense HO-1 constructs as well as with vector alone, has been examined (Takeda et al., 2000). In transfected cells overexpressing HO-1, the level of tau protein was dramatically decreased compared with that in the antisense HO-1 transfected cells. The decrease in tau was almost completely reversed by inhibition of HO activity. The spatial distribution of HO-1 expression in neurodegenerative disease is essentially identical to that of tau (Takeda et al., 2004). HO-1 expression or its translocation into the mitochondria is protective through a concomitant decrease in caspase activity and cytochrome *c* release (Fig. 14). For example, the antiapoptotic Bcl-2 family proteins, such as Bcl-xL, prevent the release of

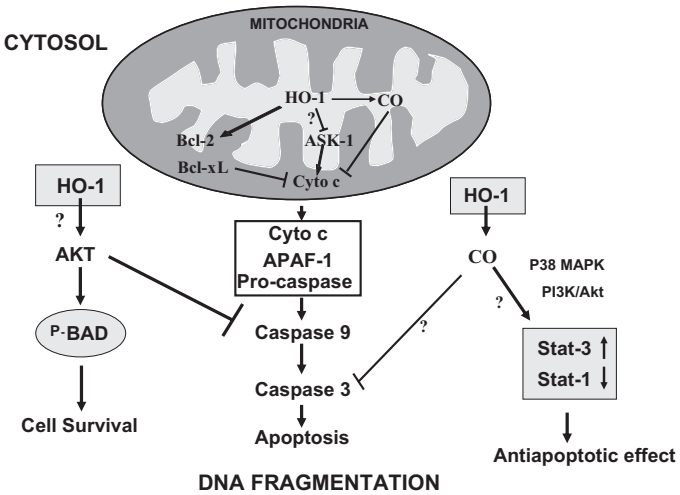

FIG. 14. Mitochondrial HO-1. Schematic representation of the effects of HO-1 induction on the caspase pathway leading to apoptosis, in which HO-1 induction decreases caspase 3 and 9. In addition, increased HO-1 levels result in increased CO production and activation of p38 MAPK and the STAT-3 pathway. Similarly, increased HO-1 expression increases pBAD levels, resulting in increased cell survival and mitochondrial function (Olszanecki et al., 2007; Turkseven et al., 2007).

apoptotic proteins from mitochondria. Similarly, increased HO-1 expression increases pBAD levels, resulting in increased cell survival (Olszanecki et al., 2007; Turkseven et al., 2007). One of the important substances released from mitochondria during apoptosis is cytochrome *c* (Liu et al., 1996), which is inhibited by increases in HO-1 (Olszanecki et al., 2007; Turkseven et al., 2007). Released cytosolic cytochrome *c* binds to Apaf-1, inducing a conformational change in Apaf-1 (Jiang and Wang, 2000). Binding of Apaf-1 to cytochrome *c* triggers its oligomerization to form the apoptosome, which recruits procaspase-9 (Zou et al., 1999). Furthermore, the HO-1-mediated increase in RSK is important for the prevention of cell death. RSK phosphorylates Bad at Ser^{112} (Tan et al., 1999) and prevents the proapoptotic effect of Bad. IL-10 regulates inflammatory and immunosuppressive responses in a HO-1-dependent manner (Chen et al., 2005). Thus, it appears that the expressions of tau and HO-1 are regulated by oxidative stress in a coordinated manner and may play a pivotal role in the cytoprotection of neuronal cells. The constitutive expression of stress proteins is very low in the course of normal brain aging compared with the profound overexpression of HO-1 protein in neuropathologically proven Alzheimer's disease, which indicates that the changes occurring in Alzheimer's brains are not merely due to aging (Schipper, 2000). APP and an amyloid precursor-like protein bind to HO with the resultant inhibition of HO activity, thus contributing to neuronal cell death in Alzheimer's disease (Takahashi et al., 2000). This finding suggests that a pharmacological approach designed to block the interaction of APP and HO might be of clinical benefit.

Familial Alzheimer's disease has been associated with distinct mutations of APP, which exhibit increased HO inhibition (Takahashi et al., 2000). Sporadic Alzheimer's disease reflects multiple etiologies and pathogenic mechanisms. Free radical-induced oxidative stress has been implicated in the pathophysiology of sporadic Alzheimer's disease (Uberti et al., 2002). Also increased oxidative damage (Nunomura et al., 2001; Praticò et al., 2002), iron accumulation (Schipper, 2004a). and HO-1 induction (Schipper et al., 1995) have been reported. Understanding the physiological and pathophysiological implications of these associations will provide valuable insights leading to reversal of the processes of neurodegeneration in Alzheimer's disease.

2. Parkinson's Disease. Oxidative stress is also a primary pathogenic mechanism of nigral dopaminergic cell death in Parkinson's disease. Oxidative damage (lipid membranes are significantly damaged), Lewy body formation, and decreased mitochondrial complex I activity are the consistent pathological findings. HO-1 is an important cytoplasmic constituent of Lewy bodies, a pathological hallmark of idiopathic Parkinson's disease (Schipper et al., 1998; Thompson et al., 2003). Parkinson's and Alzheimer's diseases are associated with elevated iron accumulation relative to the amount of ferritin that is present in the brain. The accumulation of more iron than can be adequately stored in ferritin creates an environment of oxidative stress. A mouse model was developed that mimics the protein profile for iron management seen in Parkinson's and Alzheimer's diseases and shows evidence of oxidative stress (Thompson et al., 2003). This mouse model may be useful in elucidating the role of iron management in neurodegenerative disorders and for testing antioxidant therapeutic strategies. The regulation of HO-1 may reflect some fundamental aspect of the pathophysiology of Parkinson's and implicate HO-1 as a potentially useful biological tool for the treatment of this condition.

Q. Brain Hemorrhage and Heme Oxygenase System

1. Subarachnoid Hemorrhage. Delayed cerebral vasospasm after subarachnoid hemorrhage remains a significant cause of morbidity and mortality. Hemoglobin causes the contraction of cerebral arteries and is also believed to cause vasospasm after subarachnoid hemorrhage. Increased levels of HO-1 inhibit arterial contractions induced by hemoglobin and can reduce vasospasm after experimental subarachnoid hemorrhage (Ono et al., 2002). Activation of the HO/CO pathway is an intrinsic protective mechanism against cerebral ischemic injury after subarachnoid hemorrhage (Ono et al., 2002; Sun et al., 2006). Adenovirus-expressing HO-1 was injected into the cisterna magna of rats, producing a significant increase in the expression of HO-1 mRNA protein and HO activity in the basilar artery. Compared with vehicle, this increased the baseline diameter of the basilar artery (measured directly via a transclival window) and brainstem cerebral blood flow (measured by laser Doppler flowmetry). Contraction of the basilar artery was significantly inhibited after the addition of hemoglobin, and intrathecal administration of antisense HO-1 oligodeoxynucleotide aggravated vasospasm, suggesting that HO-1 gene induction has spasmolytic effects. These results demonstrate that overexpression of HO-1 inhibits arterial contractions induced by hemoglobin and can reduce vasospasm after experimental subarachnoid hemorrhage.

2. Intracerebral Hemorrhage. Hemoglobin degradation products produce brain injury after intracerebral hemorrhage. The development of intracerebral hemorrhage-induced hemispheric edema elevates intracranial pressure and can cause death. HO-1 induction is temporally associated with increased tissue heme and is considered a marker for heme-mediated oxidative stress in intracerebral hemorrhage (Chen and Regan, 2007). In survivors, edema-related white matter injury can lead to life-long neurological deficits. At present, there are no scientifically proven treatments for intracerebral hemorrhage. In high concentrations, iron and bilirubin can be toxic to cells but, in models of cerebral ischemia, the metalloporphyrins that inhibit HO activity reduce

edema and infarct size. SnMP reduced edema development after experimental intracerebral hemorrhage (Gong et al., 2006) and reduced intracerebral mass by decreasing both hematoma and edema volumes (Wagner et al., 2000). These results suggest that inhibition of HO activity might have potential in the development of new therapies for intracerebral hemorrhage.

3. Stroke. The overexpression of HO-1 has been shown to be neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. It was suggested that pharmacological stimulation of HO-1 activity constituted a novel therapeutic approach to the amelioration of ischemic injury during the acute period of stroke (Panahian and Yoshiura, 1999). More recently, increased HO-1 expression, via adenoviral gene transfer, was shown to protect astrocytes from heme-mediated oxidative injury (Teng et al., 2003), and the efficacy of this treatment in injury to other CNS cell types is currently being investigated. Recent studies have demonstrated that HO participates in the regulation of nociceptive (neuropathic and incisional) signal transmission in spinal cord tissue and that inhibitors of HO activity may be viable as analgesics in this setting (Li and Clark, 2000, 2003; Li et al., 2004; Liang et al., 2004). However, the induction of HO-1 where there is no ferritin synthesis may be harmful to the site of brain injury. Therefore, it is essential to understand the site of injury before using a HO-1 inducer. Therapeutic gene induction and HO-1 gene transfer could be a novel strategy for the prevention and treatment of hemoglobin-induced pathological conditions, including delayed cerebral vasospasm, hemorrhage, and stroke, but also dependent on the site of injury.

V. Summary

The last decade has witnessed an explosion in the elucidation of the role that the HO system plays in human physiology. This system encompasses not only the heme degradative pathway, including HO and biliverdin reductase, but also the products of heme degradation, CO, iron, and biliverdin/bilirubin. Their role in diabetes, inflammation, heart disease, hypertension, transplantation, and pulmonary disease are areas of burgeoning research. Research has focused not only on heme itself but also on its metabolic products as well as endogenous compounds involved in a vast number of genetic and metabolic processes that are affected when heme metabolism is perturbed. The use of pharmacological agents and genetic probes for manipulating HO has led to new insights into the complex relationship of the heme-HO system with the biological and pathological systems under investigation.

It should be noted, however, that although the use of CO and biliverdin/bilirubin as therapeutic agents has been successful, these agents can be toxic at high levels in tissue, e.g., kernicterus. Care must be used to ensure that when these compounds are used as therapeutic agents their deleterious effects are minimized or avoided. On balance, however, the strategies to target HO-1 as described in this review offer promising therapeutic approaches to clinicians for the effective management of a number of disease states, including inflammation, diabetes, obesity, hypertension, and heart disease. The approaches detailed in this review provide a window into the development of new therapeutic strategies against diseases that have, in the past, proved difficult to treat.

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