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Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications

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Ryter, Stefan W., Jawed Alam, and Augustine M. K. Choi. Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications. *Physiol Rev* 86: 583–650, 2006; doi:10.1152/physrev.00011.2005.—The heme oxygenases, which consist of constitutive and inducible isozymes (HO-1, HO-2), catalyze the rate-limiting step in the metabolic conversion of heme to the bile pigments (i.e., biliverdin and bilirubin) and thus constitute a major intracellular source of iron and carbon monoxide (CO). In recent years, endogenously produced CO has been shown to possess intriguing signaling properties affecting numerous critical cellular functions including but not limited to inflammation, cellular proliferation, and apoptotic cell death. The era of gaseous molecules in biomedical research and human diseases initiated with the discovery that the endothelial cell-derived relaxing factor was identical to the gaseous molecule nitric oxide (NO). The discovery that endogenously produced gaseous molecules such as NO and now CO can impart potent physiological and biological effector functions truly represented a paradigm shift and

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unraveled new avenues of intense investigations. This review covers the molecular and biochemical characterization of HOs, with a discussion on the mechanisms of signal transduction and gene regulation that mediate the induction of HO-1 by environmental stress. Furthermore, the current understanding of the functional significance of HO shall be discussed from the perspective of each of the metabolic by-products, with a special emphasis on CO. Finally, this presentation aspires to lay a foundation for potential future clinical applications of these systems.

I. PERSPECTIVE

The race towards the identification and characterization of a potent vasodilating substance produced by endothelial cells in the 1980s resulted in a new paradigm in biomedical research and human diseases. Research during this era raised the provocative and intriguing notion that the endothelial cell-derived relaxing factor (EDRF) was not a peptide, protein, lipid mediator, or nucleic acid as originally speculated but rather a soluble gaseous molecule. The era of gaseous molecules officially began with the reports of endothelium-dependent vasorelaxation in 1980, which led to the unequivocal identification of EDRF as nitric oxide (NO) (197, 253, 254, 451). The impact of NO in biomedical research and applications to human diseases since the formal discovery of NO has been extraordinary and has arguably generated unrivaled intense interest and passion greater than any other biomolecule in this century. Ironically, we have known for a longer time, some two decades before the discovery of NO, that cells can produce another endogenous gaseous molecule by an enzymatic reaction initially described by Tenhunen and Schmid in 1968: the catalytic breakdown of heme by the microsomal heme oxygenase (HO) enzyme system which releases carbon monoxide (CO) (672, 674). Although HO-derived CO production represents the major intracellular pathway that generates endogenous CO, this process has gone relatively unnoticed by the scientific community during the first 25 years after the discovery of HO. The fact that HO is the rate-limiting enzyme in the breakdown of heme led the drive to better understand this reaction, resulting in a wealth of information on protein structure and reaction mechanism (501). The regulation of its inducible isozyme HO-1, by a broad spectrum of chemical and physical agents, led to a similar intense research effort to understand the mechanisms of gene regulation by environmental stress (19). However, the by-products of this reaction iron, bilirubin, and CO were for many years viewed as obscure waste products, with potential toxicological implications. With regard to CO, the known physiological fact that the administration at high enough concentrations to increase blood carboxyhemoglobin to critical levels can result in tissue hypoxia and subsequent lethality, undoubtedly did not attract many investigators into this field other than those interested in the epidemiology and toxicology of CO poisoning.

In the past decade, the interest in HO isozymes has shifted from their well-defined metabolic function of heme catabolism and erythrocyte turnover to another critical physiological function as a cytoprotective mechanism in numerous models of cellular stress and organ pathology. Even more interestingly, the HO field has recently been absorbed with a passion for understanding the intriguing biological functions of the catalytic by-products of HO-1, in particular CO. Thus the scientific verdict by the jury of investigations on the importance of HO and especially CO has not been rendered as of now. This review presents our current understanding of HO and CO in various physiological and pathophysiological states and their potential for therapeutic applications. We hope that this comprehensive review will prepare the scientific community to address the question of whether the biological importance of CO will attain the same far-reaching proportions as NO and provide a convincing argument that it is worth waiting for the jury to issue its final verdict.

II. HEME OXYGENASE ISOZYMES

A. Enzymatic Activity and Its Measurement

The origin of the colored pigments of the bile, biliverdin and bilirubin, from the degradation of hemoglobin heme, has been known long before the identification of the enzymatic reaction involved (397, 503). The correlation of endogenous CO found in the blood with hemoglobin heme degradation, and the α-carbon selectivity of this process, predates the discovery of heme oxygenase by several decades (116–118, 613–614). Tenhunen et al. (672–674) initially characterized heme oxygenase (HO) (EC 1.14.99.3) as a distinct enzyme system responsible for heme degradation in hepatic microsomes. Their work and the initial debate regarding the involvement of cytochrome *P*-450 in the heme degradation process (671, 583) was resolved when Maines and Kappas (412, 413) demonstrated that heme degradation occurred independently of cytochrome *P*-450.

HO catalyzes the first and rate-limiting step in the oxidative degradation of heme *b* (Fe-protoporphyrin-IX) to form the open-chain tetrapyrrole biliverdin-IXα (672) (Fig. 1). The HO reaction displays regiospecificity for the heme molecule, such that only the α-isomer of biliverdin is produced (489). Biliverdin-IXα (BV) is subsequently converted to bilirubin-IXα (BR) by an NAD(P)H-dependent reductase (675). HO catalyzed heme cleavage re-

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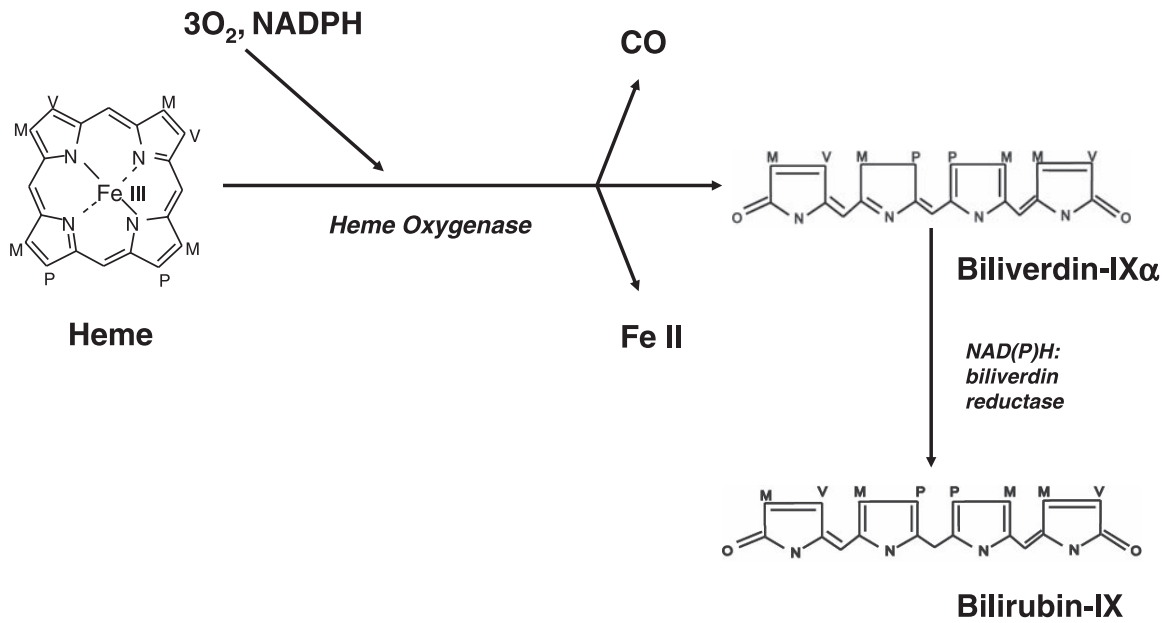

FIG. 1. The pathway of heme metabolism. Heme oxygenase enzymes (HO; E.C. 1.14.99.3; heme-hydrogen donor: oxygen oxidoreductase) catalyze the rate-limiting step in heme metabolism. Both HO isozymes (HO-1 and HO-2) oxidize heme (ferriprotoporphyrin IX) to the bile pigment biliverdin-IXα. The reaction requires 3 mol of molecular oxygen and NADPH:cytochrome *P*-450 reductase as a source of electrons. The cleavage of the heme ring releases the coordinated iron, as well as the α-methene bridge carbon as carbon monoxide (CO). The principal HO reaction product, biliverdin-IXα, is further metabolized to bilirubin-IXα by NAD(P)H:biliverdin reductase (BVR). M, methyl; V, vinyl; P, propionate.

leases the heme iron in the ferrous form Fe (II) and eliminates the α-methene bridge carbon of the heme as CO (672). The HO enzymatic activity requires three moles of molecular oxygen (O_2) per heme molecule oxidized, and reducing equivalents from NADPH:cytochrome *P*-450 (cytochrome *c*) reductase (NADPH: hemoprotein reductase, EC 1.6.2.4)(491, 672, 674, 764). Despite early reports of an exclusive role for NADPH in this process (672, 759), NADH can also serve as an electron-donating cofactor for HO activity in vitro (415). The ability of NADH to support heme degradation was demonstrated in reconstituted HO reactions consisting of NADPH:cytochrome *P*-450 reductase, biliverdin reductase, and partially purified, solubilized preparations of HO from human or rat liver (9, 411), as well as in microsomes derived from rat spleen, liver, or bone marrow (252, 415). Using reconstitution experiments, Maines et al. (411) initially proposed a role for NADH:cytochrome b_5 reductase in NADH-dependent heme degradation. Subsequent studies provided immunochemical and biochemical evidence for the principle role of NADPH:cytochrome *P*-450 reductase in heme degradation in the presence of either reducing cofactor and demonstrated against the specific involvement of NADH:cytochrome b_5 reductase (239, 489, 765).

Upon binding to the HO apoprotein, the heme molecule serves as both substrate and catalytic cofactor in its own degradation (672). Several current reviews describe the understanding of the HO reaction mechanism and its intermediates (501, 554, 727, 762). The reaction initiates with the NADPH-dependent reduction of the ferric heme-iron in the HO-heme complex, which binds O_2 to form an oxyferrous intermediate that in turn accepts a second electron from NADPH (672, 756, 763–764, 768). The resulting ferric hydroperoxide (Fe III-OOH) intermediate hydroxylates the heme ring at the α-methene bridge carbon, forming α-hydroxy heme (544, 728, 762). Two further oxidation cycles involve the elimination of the α-methene bridge carbon as CO during the formation of verdoheme, and the subsequent formation of a ferribiliverdin-IXα complex (BV-Fe III) (325, 441, 566, 763, 766). The utilization and number of reducing equivalents required during the intermediate steps in the reaction cycle has been a matter of controversy (392, 425, 440, 501, 762). In contrast to the first step in the oxidation of heme, the oxidation of α-hydroxy-heme may not require activation of O_2 at the heme iron center. Instead, the reaction of O_2 appears to occur at the meso-edge of the porphyrin molecule (566, 762). An additional reduction step releases ferrous iron from the biliverdin complex (756, 762). HO activity displays a preference for heme *b*, with activity toward heme *c* and hematoheme also observed (351, 406, 419, 769). Intact hemoproteins such as cytochrome *c* and hemoglobin do not serve as enzymatic substrates (406, 419, 687, 769). Both NO and CO, small gaseous heme ligands, can bind to the heme oxygenase-heme complex (441, 716).

Synthetic metalloporphyrins such as cobalt-protoporphyrin-IX (CoPPIX) (403, 770), tin-protoporphyrin (SnPPIX) (151–152, 770), zinc- or manganese-protopor-

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phyrin-IX (ZnPPIX, MnPPIX) (151, 770), tin and chromium mesoporphyrins (SnMP, CrMP) (150), and iron or zinc deuteroporphyrin-2,4-*bis*-glycol (FeDPBG, ZnDPBG) (99, 449), can act as competitive inhibitors of HO activity in vitro. It should be noted, however, that CoPPIX, albeit an inhibitor of HO activity in vitro, is a potent inducer of HO activity in vivo, and care must be taken in extrapolating in vitro results to in vivo conditions (153). In addition, dual control mechanisms exist for metalloporphyrins as exemplified by SnPPIX, which potently inhibits HO activity while increasing the content of HO protein in the liver (573).

A number of analytical methods have been published for the relative determination of HO activity in protein extracts of cells and tissues. The most common method for determination of HO activity depends on the detection of BR formation by spectroscopy. Essentially, in vitro HO reactions consist of a protein extract incubated with an excess of heme substrate and NADPH in a physiological buffer. BR is quantified by chloroform extraction from the reaction mixture, followed by visible spectroscopy at 464 nm (251–252). Earlier versions of this assay describe NADPH difference spectroscopy on protein reaction mixtures (579, 674, 672). Microsomal fractions can be used as a source of enzyme protein (104,000 *g* pellet), although commonly low-speed supernatants (whole cell extracts) may also be used (556, 581, 672). Because BV is difficult to assay by spectrophotometric methods, the quantification of HO activity depends on the complete conversion of BV to BR by BV reductase (BVR) (581). BVR may be supplied from crude extracts (rat liver 105,000 *g* supernatant fraction) or in partially purified form (350, 675). Protein extracts from some cell types contain sufficient BVR to obviate the need for exogenous supplementation, which may also coprecipitate with membrane preparations, presumably in association with HO-1 (327, 554).

HO assays using isotopically labeled ^{14}C-heme have been described, which rely on the detection of [^{14}C]bilirubin by recrystallization from chloroform extracts (674, 671) or thin-layer chromatographic separation (608). High-performance liquid chromatography (HPLC) has also been implemented for the separation and detection of heme metabolites and for the quantification of HO activity (Fig. 2) (62, 366, 387, 554–556, 560). This method allows the simultaneous detection of BV and BR and heme on a single chromatogram, simplifying quantification and eliminating the need for BVR supplementation (554). Alternatively, CO has been used as an end point in HO activity assays by measuring CO evolution in headspace gas, using a gas chromatograph coupled to a reduction gas detector (88, 710). More sensitive methods for the detection of CO in headspace gas utilizing gas chromatography/mass spectroscopy (GC-MS) have recently been described (3, 297).

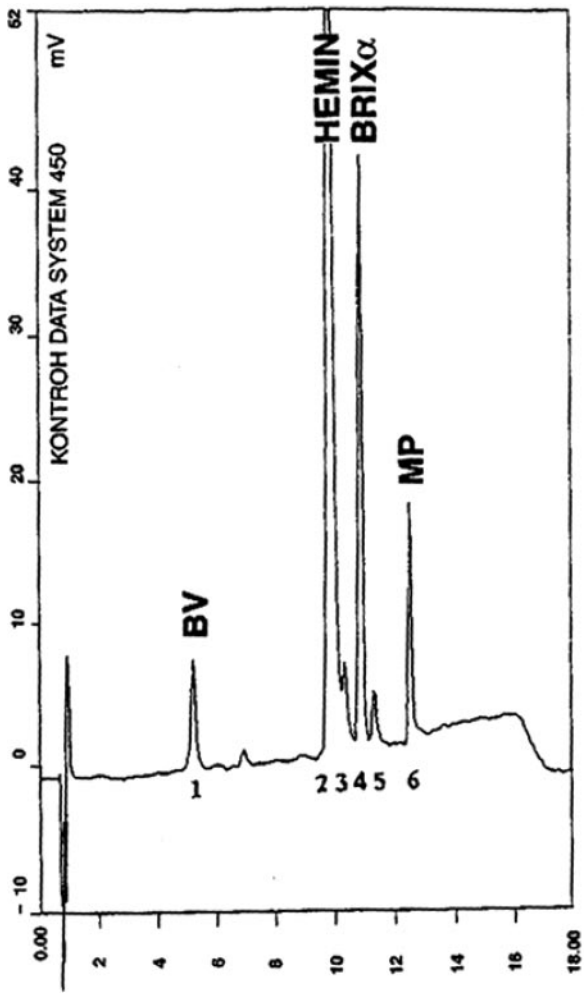

FIG. 2. Analysis of bile pigments by HPLC. HPLC chromatograph showing the resolution of tetrapyrrole standards in an alcohol extract of sonicated microsomal protein solution. Final concentrations were 2 μM biliverdin (BV), 2 μM Bilirubin-IXα (BRIXα), 0.35 μM BRIIIα, 0.27 μM BRXIIIα, 12.5 μM hemin, 0.4 μM mesoporphyrin (MP), corresponding to injected amounts of 240 pmol BV, 240 pmol BRIXα, and 48 pmol MP. The tetrapyrroles eluted with the following typical retention times: *1*) biliverdin (BV) 5.29 min, *2*) hemin 9.8 min, *3*) bilirubin isomer (IIIα or XIIIα) 10.39 min, *4*) bilirubin-IXα (BRIXα) 10.9 min, *5*) bilirubin isomer (IIIα or XIIIα) 11.38 min, and *6*) mesoporphyrin (MP) 12.54 min. Optical density was detected at 405 nm. [From Ryter et al. (554).]

A limitation of HO activity assays in the present form includes their inability to discriminate between HO-1 and HO-2 activity in a protein extract, although inducible activity is typically assumed to represent HO-1. Furthermore, these enzymatic assays provide a relative quantification of the active enzyme protein in a cellular protein extract, in the presence of an excess of exogenous substrate and cofactors; thus they do not necessarily represent activity profiles in vivo. The field may benefit from the development of analytical methods to measure the intracellular formation of heme metabolites in situ, including BR, and CO. In one example of such an attempt, a novel assay measuring endogenous CO production in

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vascular cells by mid-infrared laser absorption spectroscopy has been described (452).

B. Biochemical Properties

Two genetically distinct isozymes of HO have been characterized: an inducible form, heme oxygenase-1 (HO-1), and a constitutively expressed form, heme oxygenase-2 (HO-2) (407, 419, 687). HO-1 proteins (~32 kDa) were first purified to homogeneity from the livers of $CoCl_2$ or heme-induced rats and from porcine spleen (411, 757, 758). The resulting HO-1 proteins bind heme in a 1:1 complex and display hemoprotein characteristics similar to methemoglobin, with characteristic absorption maxima at 405 nm for the oxidized form of the heme-HO complex (758). CO, as well as the heme ligands azide and cyanide, form complexes with the ferrous and ferric forms, respectively. The purified rat HO-1 protein displays functional HO activity in reconstituted microsomal systems in the presence of heme and NADPH:cytochrome *P*-450 reductase (758). Other reports have described the purification of HO-1 (~31–33 kDa) from the hepatic microsomes of the human (5, 6, 9), bovine (770), chicken (61), and rabbit (689).

The resolution of the crystal structure of human HO-1 has revealed a flexible bihelical structure surrounding the heme pocket (357, 591). A conserved histidine (His-25) imidazole acts as the heme iron ligand. The bound heme additionally contacts two glycine residues (Gly-139/Gly-143) in the distal helix domain (591). Biochemical evidence supports the formation of HO complexes with BVR and NADPH:cytochrome *P*-450 reductase (357, 717, 771). From predicted polypeptide sequences, the mouse HO-1 contains one cysteine (free thiol group), while HO-1 proteins from other species (pig, human, and rat) do not contain cysteine (296, 465, 601, 651, 761). The bovine HO-1 was initially reported to contain four titratable cysteines, but nevertheless was not itself subject to redox regulation of activity by sulfhydryl reagents or metal ions (770). Cysteine does not appear in current bovine HO-1 sequence data on record (accession no AAX08985; XB_590873; and Ref 582). Reports of apparent inhibition of HO activity by sulfhydryl reagents refer to reconstituted microsomal systems and apparently result from the inhibition of BVR and/or cytochrome *P*-450 reductase, which have critical sulfhydryl groups susceptible to modification by these compounds (411, 770).

A distinct isozyme of heme oxygenase, HO-2, has been identified in rat liver, spleen, brain, and testes (65, 418, 687–689). HO-2 was initially purified to homogeneity from rat testes and has an apparent molecular mass of 36 kDa (687). Human HO-2 displays 88% amino acid homology to rat HO-2 (429). A larger form of HO-2 (~42 kDa) was partially purified from rabbit testes (689).

Both HO-1 and HO-2 catalyze the identical biochemical reaction, in that they efficiently transform heme into biliverdin-IXα with similar substrate specificity and cofactor requirements. However, in a comparative analysis of rat HO-1 and HO-2, differential properties with respect to enzyme kinetics and substrate K_m values have been reported (0.24 and 0.67 μM, respectively), as well as differences in apparent thermostability and immunoreactivity (407, 419, 687). In HO-2, the His-45 serves as the proximal heme ligand, with an accessory role for His-151 (265, 431).

The rat HO-1 and HO-2 share 43% amino acid homology (548). A highly conserved sequence of 24 amino acid residues has been identified in common to both HO-2 (rabbit and rat) and HO-1 (rat, mouse, human) (549). Furthermore, both HO-1 and HO-2 share similar hydrophobic regions at the extreme COOH terminus that serve to anchor the proteins in cellular membranes (266, 431, 601).

HO-2 contains functional domains not present in HO-1. These domains, termed heme regulatory domains, which contain a conserved Cys-Pro motif, provide additional heme binding sites distinct from the heme catalytic domain (429). The functions of these additional heme centers of HO-2 suggest a heme-dependent function for HO-2 distinct from heme degradation. The additional heme centers of HO-2 have been proposed to act as a sink for both NO and CO (142, 247, 416, 429).

The second step in heme metabolism, NAD(P)H:biliverdin reductase (EC 1.3.1.24) (BVR), subsequently converts the water-soluble biliverdin-IXα into lipophillic bilirubin-IXα by reduction of the γ-methene bridge carbon (672, 674, 675). BVR was originally purified from rat liver and kidney (350, 675). In addition to the major alpha form (BVαR) found in human liver (34 kDa), a second isotype (21 kDa; BVβR) exists that produces bilirubin-β or fetal bilirubin (176, 337, 338, 741). BVαR may use both NADH and NADPH for activity, while BV-βR displays preference for NADPH. The rat liver BVαR displays differential pH optima for NADH and NADPH cofactors (i.e., ~7.0 and ~8.7, respectively) (350). BVR contains critical -SH groups rendering it susceptible to inhibition by heavy metals and thiol reagents (350, 770). Recent evidence also suggests that human BVαR naturally contains zinc (417).

C. Genetics

HO-1 and HO-2 represent the products of distinct genes (*ho-1*, *ho-2*, also specified as *hmox1*, *hmox2*) (127, 407, 419, 428, 686). The human, mouse, and rat *ho-1* genes (~14 kb) and the rat *ho-2* gene (~12 kb) share similar organization into five exons and four introns (17, 432, 465, 602). The entire genome sequence of the rat BVR gene (~12 kb) has also been described, which also displays an organization of five exons and four introns (427).

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Fluorescence in situ hybridization studies show that the human *ho-1* and *ho-2* genes localize to separate chromatin regions (*ho-1*: 22q12; *ho-2*: 16p13.3) (349). Human BVαR localizes to chromosome 7 (511), while human BV-βR to chromosome 19q13.13→q13.2 (565).

A number of HO-1 corresponding cDNA clones have been described (169, 296, 319, 601, 651). Human HO-1 cDNA clones (1.5 kb) were isolated from heme-induced macrophages, and from sodium arsenite-induced fibroblasts, encoding a 288-amino acid protein with a predicted molecular mass of 32 kDa (319, 761). HO-1 cDNA has been cloned from the rat spleen (1.5 kb) (285 amino acids, 33 kDa) (601). Murine HO-1 cDNA was cloned from sodium arsenite-induced fibroblasts (289 amino acids, 33 kDa) (296). A porcine clone (1.5 kb; 288 amino acids, 33 kDa) and an avian clone (1.25 kb; 296 amino acids, 33.5 kDa) have also been described (169, 651). Human HO-1 cDNA displays a high degree of homology (80%) with the rodent cDNAs, while the avian form displays the lowest sequence homology (62%) (169, 761).

Likewise, several HO-2 cDNA clones have also been described (428, 548–549, 687). Two forms of HO-2 cDNA have been cloned from human and rat representing the products of two alternatively polyadenylated homologous transcripts (1.3/1.7 kb in human and 1.3/1.9 kb in rat). The resulting predicted polypeptides of 313 and 315 amino acids for the human and the rat, respectively, share an 88% amino acid homology (428, 548). Additional HO-2 cDNAs corresponding to single transcripts have been isolated from the rabbit (1.3 kb) (549) and the monkey (1.7 kb) (686).

McCoubrey et al. (430) isolated a cDNA (2.4 kb) encoding a putative third HO isozyme (HO-3) in rat tissues. The predicted polypeptide has a high amino acid sequence homology to HO-2 (~90%) and thus shares many common sequence motifs, including putative heme regulatory domains (430).

The *ho*-3 mRNA transcripts corresponding to the cDNA clone reported by McCoubrey et al. (430) were detected in a number of rat tissues, including the spleen, liver, thymus, prostate, heart, kidney, testis, and brain (430). A recent study reported a distribution pattern of *ho-3* mRNA in the brain in the hippocampus, cerebellum, and cortex (579). When expressed in *E. coli*, the resulting HO-3 protein (~33 kDa) had very little heme degrading activity, and therefore, the function of this protein remains enigmatic (430).

A recent study raises uncertainty on the existence and functional relevance of HO-3 in vivo. Hayashi et al. (231) identified two HO-3-related genes (*ho-3a/b*) in the rat in an attempt to clone the *ho-3* gene corresponding to the cDNA described by McCoubrey et al. (430). The *ho-3a/b* genes are nearly homologous to HO-2 exons 2–5 and contain no introns. Furthermore, Hayashi et al. (231) reported a lack of expression of corresponding *ho-3* mRNA and HO-3 protein in rat tissues, and due to high homology with *ho-2* derived sequences, questioned the previous analysis of *ho-3* mRNA distribution. To address this issue, another investigation utilized the *ho-2*$^{-/-}$ mice to avoid potential homology overlaps and reported no transcripts corresponding to *ho-3* in the mouse brain (789). The presence of stop codons within the coding regions, as well as the lack of detectable mRNA or protein product in rat tissues, led Hayashi et al. (231) to conclude that HO-3*a/b* represent pseudogenes originating from HO-2 transcripts.

D. Tissue Distribution

The inducible form of heme oxygenase, HO-1, occurs at a high level of expression in the spleen and other tissues that degrade senescent red blood cells, including specialized reticuloendothelial cells of the liver and bone marrow. High levels of HO activity are detected in these tissues (672, 673). HO is present in hematopoietic stem cells of the bone marrow (1, 10, 72), where it may inhibit cellular differentiation by lowering the intracellular concentration of heme, a differentiation factor for these cells (1, 10). HO is also present in the liver parenchyma, which is the site of uptake and degradation of plasma heme, hemoglobin, and methemalbumin. Under conditions of hemolysis, HO activity dramatically increases in the liver parenchyma, kidney, and macrophages in response to increased levels of circulating hemoglobin (519, 520, 673).

In most other tissues not directly involved in erythrocyte or hemoglobin metabolism, HO-1 typically occurs at low to undetectable levels under basal conditions but responds to rapid transcriptional activation by diverse chemical and physical stimuli.

The highest expression of HO-2, the constitutively expressed isozyme, occurs in the testes, but the protein is also found abundantly and ubiquitously in other systemic tissues including, but not limited to, the brain and central nervous system, vasculature, liver, kidney, and gut (407, 408, 419, 687, 773). HO-2 does not respond to transcriptional activation by environmental stress but may respond to developmental regulation by adrenal glucocorticoids in the brain (409, 535).

E. Subcellular Localization

Since their initial discovery in 1968, HO-1 enzymes have been characterized as endoplasmic reticulum (ER) associated proteins, due to the abundant detection of HO activity in microsomal (104,000 *g*) fractions. Both HO-1 and HO-2 contain a COOH-terminal hydrophobic domain segment that suggests a general membrane compartmentalization (266, 601, 761). Constitutive expression of the rat HO-1 cDNA in monkey kidney cells indicated an ER

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localization of the expression product (266, 601). Recent studies have raised the possibility of the functional compartmentalization of HO-1 in other subcellular domains beside the ER, including but not limited to the nucleus and plasma membrane (Fig. 3). The potential functional subcellular compartmentalization of HO enzymes raises an intriguing issue of organelle specific function of HO metabolites, for example, CO, which is not yet fully characterized.

A recent study from this laboratory has described a functional association of HO-1 with plasma membrane caveolae in endothelial cells (327). Mouse lung endothelial cells (MLEC) were exposed to several known inducers of HO-1, including lipopolysaccharide (LPS), heme, and hypoxia, and their lysates were subjected to sucrose density gradient fractionation. In the absence of chemical stimulation, the detectable HO-1 in MLEC localized to a single fraction with a density consistent with the predicted ER localization (Fig. 4). Interestingly, the treatment of MLEC with inducers of HO-1 expression not only induced the quantity of HO-1 expressed, but dramatically altered the subcellular distribution of HO-1 among the fractions analyzed. Moreover, some variation in this distribution occurred in an inducer-specific fashion. The inducers heme and LPS caused a dramatic translocation of HO-1 to cytochrome *c*-containing fractions, suggesting a possible mitochondrial localization of HO-1. After stimulation with all three inducers, endothelial HO-1 was shown to localize, in part, to low-density or detergent-resistant fractions that contained caveolin-1, a marker and principle structural component of caveolae. Consistent with the requirement of these factors to constitute the complete heme metabolic pathway, NADPH:cytochrome *P*-450 reductase and BVR also colocalized with HO-1 to the caveolin-1-containing fractions. The combined caveolae fractions from LPS stimulated MLEC contained functionally active HO, by enzymatic activity assays. Interestingly, the downregulation of caveolin-1 by antisense expression resulted in a dramatic increase in HO enzyme activity despite stable protein expression, indicating a possible negative regulatory role of caveolin-1 toward HO activity. Conversely, overexpression of caveolin-1 resulted in a downregulation of LPS-inducible HO activity. These observations were supported by coimmunoprecipitation studies that indicate a physical interaction between HO-1 and caveolin-1. The negative regulation of endothelial NO synthase (eNOS) activity by caveolin-1 has also been demonstrated (180). These observations suggest a possible functional role of HO enzymes in caveolae and also suggest that caveolin-1 may serve as a molecular brake on signaling mechanisms involving small gaseous second messengers that originate in the caveolae (327).

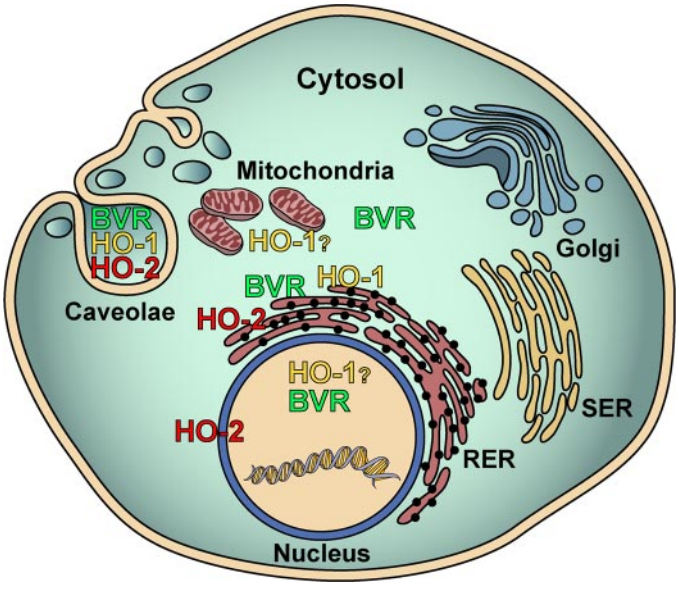

FIG. 3. Subcellular localization of heme metabolic enzymes. The scheme shows the possible subcellular compartmentalization of the principle enzymes in heme degradation based on limited studies. The ER localization of HO-1 has been known for many years (266, 601). The caveolar localization of HO-1 is supported by our recent publication (327). See text for other citations. BVR, biliverdin reductase; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

Relatively little is known about the possible functional compartmentalization of HOs to the nucleus. Preliminary studies indicate that heme stimulates the nuclear translocation of HO-1. Furthermore, HO-2 was detected constitutively in the nucleus of NIH3T3 cells and was proposed to facilitate the entry of HO-1 (P. A. Dennery; see Ref. 485). We have observed diffuse nuclear staining of HO-1 in endothelial cell preparations stimulated with LPS, which coincides with the appearance of HO-1 in high-density sucrose gradient fractions (H. P. Kim, X. Wang, F. Galbiati, S. W. Ryter, and A. M. Choi, personal communication). Nuclear localization of HO-1 was also observed in astroglial cells stimulated with glutamate (393a). Electron microscopy studies of monkey retinal ganglion cells revealed localization of HO-2 to the outer nuclear membrane, in addition to ER localization (403). Interestingly, nuclear localization of BVR has been reported in response to endotoxin stress in the rat kidney (410). Aside from the generally accepted principle function of BVR as a soluble metabolic enzyme, possible functional roles of the BVR in other cellular and nuclear processes have been suggested. These potential activities of the BVR include the regulation of protein phosphorylation, DNA binding activity, and nuclear transcriptional regulation (14, 344, 567).

F. Phylogeny

Recent studies indicate a high degree of evolutionary conservation of the heme degrading enzymes among ani-

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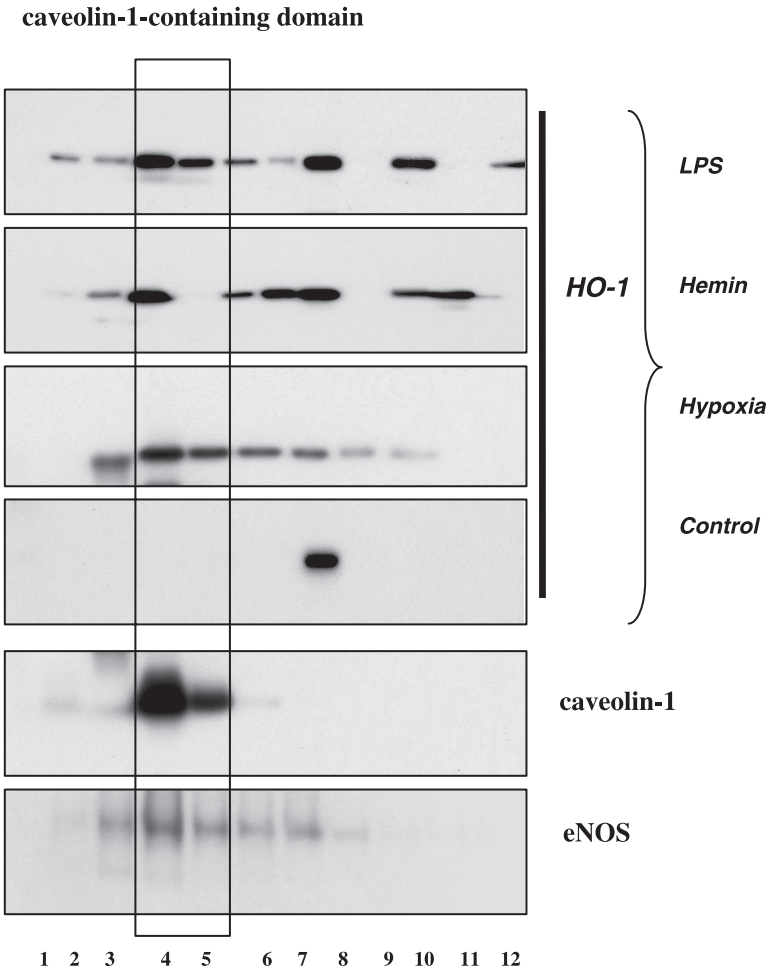

FIG. 4. Caveolae compartmentalization of HO-1 in rat pulmonary aortic endothelial cells (PAEC). Rat PAEC were exposed to stimuli such as hemin (20 μM, for 18 h), LPS (100 ng/ml, for 18 h), or hypoxia (1% O_2, 5% CO_2 mixture, 8 h). Total cell lysates were loaded on the discontinuous sucrose gradients for 18 h (39,000 rpm, SW 41 rotor). Twelve fractions of each were obtained and subjected to Western blot analysis for HO-1, caveolin-1, and eNOS. Note that caveolin-1 was concentrated in fractions 4–5. [From Kim et al. (327).]

mal, plant, and fungal kingdoms. HO-1 appears ubiquitous in higher animals with a high degree of structural similarity and functional identity between enzymes from humans, large mammals, rodents, and birds. Interestingly, a number of HO-1-like proteins and activities have been described in lower organisms including flies, bacteria, fungi, plants, and algae (53, 121, 362, 442, 467, 468, 514, 537, 570, 587, 615, 724, 727, 729, 733, 779, 780, 784, 785).

Among insects, plants, fungi, and bacteria, the basic mechanism of HO-catalyzed heme cleavage appears to resemble that described for mammalian isozymes, in that BV and CO are produced from heme. In most cases, the α-isoform of biliverdin is exclusively produced, with exceptions as noted below. Some differences also arise in the cofactor requirements of HO and BVR homologs, in the processing of BV, as well as in the overall functional significance of heme metabolism among various organisms.

A HO-1 homolog recently isolated from *Drosophila melanogaster* lacks a proximal heme ligand and displays a broader selectivity for heme catalysis (α, β, δ) (780). The pathogenic yeast *Candida albicans* contains a homolog of HO (*CaHMX1*) with a high degree of similarity to mammalian isoforms, which plays a critical role in iron acquisition from heme derived from the host organism (514, 570). No functional HO homolog has been described for the "budding yeast" *Saccharomyces cerevisiae*, although several heme-binding proteins have been characterized (38, 529). The product of the *HMX1* gene facilitates heme utilization in this organism (529).

In higher plants, the *Arabidopsis thaliana HY1* gene, for example, encodes a plant homolog of HO-1 that utilizes ferredoxin as the reducing partner instead of NADPH:cytochrome *P*-450 reductase (467, 468). In general, plants, which lack the equivalent of mammalian BVR, do not produce bilirubin. In *Arabidopsis thaliana*, a ferredoxin-dependent BVR activity (*HY2*) reduces biliverdin-IXα to phytochromobilin (334), which in turn is utilized as a precursor for phytochrome chromophore, a critical component of plant photoreceptors (132, 467, 468). Similarly, the HO homolog of the unicellular red algae *Cyanidium caldarium* generates biliverdin-IXα, which is utilized for phycobillin synthesis instead of bilirubin production (53).

Several soluble HO homologs have been described in various bacterial strains (reviewed in Refs. 189, 727).

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Cyanobacteria, as exemplified by *Synechocystis sp. PCC 6803*, contains two heme oxygenases (*Syn ho-1*, *Syn ho-2*). *Syn ho-1* displays structural and functional similarity with mammalian forms of HO-1 (121, 442, 779). Interestingly the cyanobacterium *ho-1* can complement HO-deficient *Arabidopsis thaliana* mutants (*hy1*) to restore phytochrome biosynthesis (731). Unlike higher plants, *Synechocystis sp. PCC 6803* contains a mammalian-like BVR activity (*bvdR*) that generates bilirubin-IXα that is utilized as a phycobillin precursor (586).

In the Gram-positive bacterium *Corynebacterium diphtheriae*, the *HmuO* gene product produces biliverdin-IXα and CO from heme and requires a bacterial NADPH-dependent reductase (597, 729). *Staphylococcus aureus* contains two HOs (*IsdG* and *IsdI*) of perhaps the smallest known molecular size (13 kDa) (615, 733).

HO enzymes have also been described in Gram-negative bacterial strains including *Neisseria meningitides* (*HemO*) (784, 785) and *Pseudomonas aeruginosa* (*PigA*, *BphO*) (537, 724). The *PigA* gene product differs from mammalian and other bacterial HOs in that it displays an altered regiospecificity for heme cleavage, producing β,δ-biliverdins. The second HO-like isozyme BphO of *Pseudomonas aeruginosa* produces exclusively biliverdin-α for the synthesis of bacterial phytochrome. This organism may have segregated its heme-degrading activities related to iron acquisition and pigment biosynthesis (189). In summary, the HOs of bacterial and fungal pathogens serve a principle functional role as a mechanism for the acquisition of iron from exogenous heme or hemoglobin as an essential nutrient for growth and pathogenesis (189, 587). In plants, photosynthetic, and nonphotosynthetic bacteria however, the HO enzymes also provide the first step in metabolic pathways for the synthesis of pigments: phytochromobillins, phycobillins, and bacterial phytochromes (189).

III. REGULATION OF HEME OXYGENASES

A. Induction of HO-1 by Chemical and Physical Stress

The increased synthesis of the HO-1 protein occurs as a general response to stress in biological systems. The response appears to occur ubiquitously among most tissues tested and also among higher organisms including humans, mammals, marsupials, birds, and fish. While variants of HO have been described in many organisms from bacteria to plants (see sect. II*F*), a discussion of inducible gene regulation in lower phyla is omitted here in a focus on mammalian systems. The general nature of the response and the vast literature now surrounding the phenomenon render it difficult to provide a comprehensive list of all inducing compounds in all cell types and tissues tested. Nevertheless, Table 1 references a selection of classical and recent examples, in favor of omitting cell types in the text. With few exceptions, as described for the response to NO below, the response depends largely on transcriptional activation of the *ho-1* gene and the de novo synthesis of mRNA, regardless of cell type or inducing chemical. This has been verified under a number of conditions using nuclear run-off assays for gene transcription (22, 317, 386, 546) and/or experiments with the transcriptional inhibitor actinomycin D (445, 446, 574, 600). This section will provide an introduction to the classes of stress that may elicit the *ho-1* response. Attention to the mechanisms involved in signaling (see sect. III*B*) and transcriptional regulation (see sect. III*C*) associated with the response to discrete inducers shall be given in the following sections.

HO-1 belongs to a larger family of stress proteins whose transcriptional regulation also responds to adverse environmental conditions. Of the known mammalian stress protein families, the expression of the heat shock proteins (HSPs) (20–30 kDa, 70–73 kDa, 90 kDa, 104–110 kDa) constitutes a global cellular response to protein denaturation associated with hyperthermia (642). The HSPs collectively participate in the subcellular trafficking of unfolded, nascent, or denatured protein and, furthermore, provide an underlying molecular mechanism for the development of acquired cellular thermoresistance (125). HO-1 shares no apparent amino acid homology with HSPs nor displays protein chaperone activity. However, the promoter regions of various *ho-1* genes contain heat shock elements similar to those originally identified in the regulatory regions of various *hsp* genes. HO-1 has sometimes been classified as a heat shock protein (Hsp32) due to its transcriptional responsiveness to hyperthermia, which, however, is apparently somewhat restricted to rodent systems (600, 761). Hyperthermic shock causes accumulation of HO-1 protein and mRNA in various rat organs including liver, heart, and brain (170–172, 174, 533).

A second general class of stress proteins, the glucose regulated proteins (GRPs), constitutes a general response to ER-associated stress, also known as the "ER stress response," or "malfolded protein response." The GRPs, multiple members of several molecular mass classes (75, 78–80, and 94–100 kDa), respond to transcriptional upregulation after glucose starvation, disruption of intracellular calcium homeostasis, protein glycosylation interference, and expression of malfolded proteins (361). Interestingly, HO-1, which normally resides in the ER, also can respond to transcriptional activation in some cell types by nutrient depletion (90, 91, 663). The overlap of the HO-1 response with the GRPs in hepatoma cultures appeared to be restricted to an inducible response to glucose depletion (91). However, induction of HO-1 by a broader spectrum of ER stress-inducing agents has been noted in

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TABLE 1. *Induction of HO-1 by chemical and physical stress*

Inducer	Cell Line	Footnote	Reference Nos.
	ROS generating systems		
H_2O_2	Fibroblasts, dermal (human)	*a,c*	318,319
Menadione	Fibroblasts, dermal (human)	*a*	319
UVA radiation	Fibroblasts, dermal (human)	*a,c*	318,319
Photodynamic therapy	Fibroblasts (Chinese hamster)	*a,c*	207
	Thiol reactive substances		
Sodium arsenite	Embryo fibroblast (mouse)	*a*	296
	Fibroblasts, dermal, colon, lung (human)	*a*	34,319
	Epidermal keratinocytes (human)	*a*	
	Lymphoblastoid (human)	*a*	
	Epithelial cells (mouse)	*a*	
	Fibroblasts (mouse, rat, monkey, marsupial)	*a*	
	Hepatoma (human)	*a*	447
	Melanoma (human, mouse)	*a*	78
	Hepatoma (rat)	*a*	664
	HeLa, HL60 (human)	*a*	665
	Peritoneal macrophages (mouse)	*c*	666
	Embryo fibroblasts (avian)		289
	Embryo hepatocyte (avian)	*d*	572
	Astrocytes (rat)	*a*	177
Phenylarsine oxide	Embryo hepatocyte (avian)	*b*	201,594
Diethylmaleate	Fetal fibroblasts (rat)	*c*	597
	Oocytes (hamster)	*c*	191,577
	Peritoneal macrophages (mouse)	*c*	666
Iodoacetamide	Fibroblasts, dermal (human)	*a*	319
	Melanoma (human and mouse)	*c*	78
	Heavy metals		
Cadmium chloride	HeLa, HL60 (human)	*c*	664
	Hepatoma (human)		447
	Hepatoma (rat)		665
	Peritoneal macrophages (mouse)		666
	Oocytes (hamster)		577
	Melanoma (human and mouse)		78
	Embryo fibroblasts (mouse)		242
	Fibroblasts, dermal (human)	*a*	319
	Hepatoma (mouse)		22
	Hepatoma (human)		445, 447
Cobalt chloride	Embryo hepatocytes (chicken)	*d*	418
	Fibroblasts (Chinese hamster)	*a,b*	209
Stannous chloride	Hepatoma (human)	*a*	445
	Metalloporphyrins		
Heme	Macrophages (human)	*a*	761
	Hepatoma (mouse and human)	*a*	22
	Renal epithilium (rat)	*b*	21
CoPPIX	Hepatoma (human)	*a,e*	445
ZnPPIX	Hepatoma (human)	*a,e*	445
	Nitric oxide and derivatives		
NO donor	Aortic endothelial cells (pig)	*d*	462
	Pulmonary endothelial (sheep)	*d*	749
	Aortic smooth muscle (rat)	*a,c*	157, 229
	HeLa cells (human)	*a,b*	94
	Colon adenocarcinoma (human)		226
NO (gas)	Embryonic lung fibroblasts (human)	*a*	422
	HeLa cells (human)	*a*	
Peroxynitrite	Aortic endothelial cells (bovine)	*c,d*	188
	Colorectal adenocarcinoma (human)	*a,b*	226
Angeli's salt	Cardiomyocyte (rat)	*a,c,d*	479
	Lipid metabolites		
12-PGJ_2	Aortic endothelial cells (pig)	*c*	335
15d-PGJ_2	J774 macrophage (murine)	*c*	368
15d-PGJ_2	HepG2 hepatoma (human)	*a,b,c*	389
15d-PGJ_2	Primary neuronal (mouse)	*c*	789
PGE_2	Raw 264.7 macrophage (mouse)	*c*	96
4-HNE	Hepatocytes (rat)	*a,c*	77
OxPAPC	HUVEC (human)	*a,b*	345
HPODE	Aortic endothelial cells (human)	*a,b,c*	238

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TABLE 1—*Continued*

Inducer	Cell Line	Footnote	Reference Nos.
	Tumor promoters		
TPA	BALB/c 3T3 fibroblasts (mouse)	*a,c*	241–243, 296
	M1 myeloleukemia (mouse)	*a,b*	347
	THP-1 myeloleukemia (human)	*a,b*	469
	HepG2	*b*	20
	O_2 tension		
Hypoxia	Oocytes (Chinese hamster)	*c*	470
	Cardiomyocytes (rat)	*c*	295
H/R	Aortic endothelial cells (bovine)	*c,d*	559
	Pulmonary aortic endothelial (rat)	*a,b,c*	778
Hyperoxia	Pulmonary endothelial cells (human)	*a*	183
	Hepatoma (human)	*a*	659
	Macrophages (mouse)	*a,b*	363
	Antioxidants, flavonoids, and phenolic compounds		
Curcumin	Aortic endothelial cells (bovine)	*a,c,d*	461
Curcumin	Renal epithelial cells (rat, pig)	*b,c,d*	46
Carnosol	Pheochromocytoma (rat)	*a,b,c*	423
Quercetin	Aortic smooth muscle (rat)	*a,c*	385
Resveratrol	Aortic smooth muscle (human)	*a,b*	290
Resveratrol	Primary neuronal cultures	*c*	786
EGb 761	Primary neuronal cultures	*c*	788
PDTC	Aortic vascular smooth muscle (rat)	*a,b,c*	227
	LPS		
	Hepatoma (human)	*a,d*	402
	Macrophages (mouse)	*a,b*	79
	Myeloleukemia (mouse)	*a,b*	348
	Cytokines and growth factors		
IL-1α/β	Endothelial cells (human)	*a*	446, 677, 678
IL-6	Hepatoma (human)	*a*	446
IL-6	Corneal epithelial (rabbit)	*d*	484
IL-10	Macrophages (mouse)	*c*	367
IL-11	Hepatoma (human)	*a*	195
TNF-α	Endothelial cells (human)	*a*	677, 678
TGF-β	Pulmonary epithelial (human)	*a,b,c,d*	488
TGF-β	Corneal epithelial (rabbit)	*d*	484
PDGF	Vascular smooth muscle	*a,c*	158
	Retinal epithelial cells (human)	*a,c*	352
NGF	Pheochromocytoma (rat)	*a,c*	568
IFN-γ	Retinal epithelium (human)	*f*	698
	Hormones		
ANP	Endothelial cells (human)	*c*	322
Angiotensin II	Vascular smooth muscle	*f*	270
Angiotensin II	Renal epithelial cells	*c*	55
ACTH	Adrenocortical cells (mouse)	*a,c,d*	521

Representative examples are provided of the induction of HO-1 by a broad spectrum of chemical and physical stress agents in cell culture models. ACTH, adrenocorticotropic hormone; 4-HNE, 4-hydroxy-2-nonenal; HPODE, 13-hydroperoxyoctadecadienoic acid; H/R, hypoxia/reoxygenation; IL, interleukin; NGF, nerve growth factor; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PDTC, pyrrolidine dithiocarbamate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PGE_2, prostaglandin E_2; 12-PGJ_2, Δ-12-prostaglandin J_2; 15d-PGJ_2, 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β. [a]Induces *ho-1* mRNA transcription and/or accumulation. [b]Induces *ho-1* promoter activity in linked reporter gene assays. [c]Induces HO-1 protein expression. [d]Induces HO enzymatic activity. [e]Inhibits HO enzymatic activity. [f]Inhibits *ho-1* mRNA transcription and/or accumulation.

several studies, such as the Ca^{2+}-ATPase inhibitor thapsigargin, which depletes ER Ca^{2+} stores, or brefeldin A, which interferes with the translocation of glycosylated proteins (391, 203). In addition to interference with the folding and posttranslational processing of nascent polypeptides, interference with the proteolytic degradation of proteins also provokes a stress response. Chemical inhibitors of the proteosome join the list of metabolic inhibitors that can activate *ho-1* in parallel with an ER-stress response (736).

Preceding its unequivocal identification as HO-1 by molecular cloning methods, a distinct low-molecular-weight protein of 32-kDa stress protein (p32) was found to be synthesized following cellular stimulation with a number of cytotoxic agents including sodium arsenite, ultraviolet A (UVA; 320–380 nm) radiation, quinones, sulfhydryl reagents, heat shock, hydrogen peroxide, tumor promoters, and heavy metal salts (78, 296, 318, 319, 664, 665). The term HO-1 will be used hereafter in this review to represent and supercede all references to p32 or Hsp32.

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In early studies of *ho-1* gene activation, various tumor promoters including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), its structural analog phorbol-12,13-didecanoate, as well as structurally distinct indole alkaloid and polyacetate tumor promoters induced HO-1 protein and/or mRNA in cell culture (241–243, 296, 347) (Table 1). TPA mimics the effects of diacylglycerol as an agonist of protein kinase C (PKC) and exerts multiple effects including the stimulation of cellular growth and differentiation programs, associated with the activation of early response genes (324). TPA treatment induced *ho-1* mRNA levels in human and mouse myelomonocytic cell lines in parallel with their TPA-induced differentiation to macrophages (347, 469). In contrast to TPA-induced differentiation programs, HO-1 expression and activity appear to be negatively correlated with the heme-dependent differentiation of erythroid precursors (1, 4, 10, 373).

The *ho-1* gene is also commonly induced by agents and chemicals that produce an oxidative cellular stress involving the generation of reactive oxygen species (ROS). In this regard, HO-1 has been recognized as a general response to oxidative stress. Known examples of ROS generating systems that activate *ho-1* include hydrogen peroxide (H_2O_2), quinones, and related compounds (i.e., menadione) which can generate superoxide anion radical (O_2^-) and/or H_2O_2 through redox cycling, photosensitizers, and UVA radiation (207, 318, 319). UVA radiation generates ROS by photoexcitation of endogenous chromophores. Studies with chemical quenching compounds implied a predominant role for singlet molecular oxygen ($^1O_2$) in the activation of *ho-1* mRNA by UVA treatment (52). Photodynamic therapy generates a similar response from the photoexcitation of synthetic chromophores most commonly based on porphyrin or chlorin structures by application of visible light at discrete wavelengths (148). Phototherapy with dihematoporphyrin ether/ester induced HO-1 expression in rodent tissue culture (207). Exposure to high oxygen tension (hyperoxia) also generates an oxidative stress, by enhancing the leakage of partially reduced forms of oxygen from the mitochondrial electron transport chain (190). Hyperoxic stress strongly induces *ho-1* mRNA and protein expression in macrophages and lung-derived cell lines (137, 363, 659).

HO-1 regulation responds in several models to diminished oxygen (O_2) tension (hypoxia), which can induce its mRNA or protein in several in vivo and in vitro systems that include various animal cell types of vascular or pulmonary vascular origin (i.e., endothelial and smooth muscle) (228, 365, 470, 552, 559, 563). Despite the common end point of *ho-1* activation, the molecular pathway leading to activation may vary in a cell-type specific manner (228). Hypoxia causes transcriptional repression, rather than activation, in several human cell types (331, 477).

Metabolites of bio-oxidation reactions have also been studied as potential inducers of HO-1, including oxidized lipids (238, 345), 4-hydroxy-2-nonenal (4-HNE) (51, 263), oxidized low-density lipoprotein (13, 264, 740), and advanced glycation end products (646, 744). HO-1 has been implicated in the pathologies of a number of diseases associated with oxidative stress including Alzheimer's disease (89, 584, 585, 617, 618, 656, 661), diabetes (11, 131, 141, 390, 530, 696), and atherosclerosis (267, 291, 612, 718).

A separate but related group of inducing chemicals include the thiol-reactive substances, which have the ability to modify reactive -SH groups in protein and in nonprotein thiols such as reduced glutathione (GSH). The formation of mixed disulfides of glutathione, GSSR, by these agents effectively depletes GSH, as such adducts are not reversible by glutathione reductase. Compounds in this family include sodium-*meta*-arsenite (34, 164, 296, 319) and diethylmaleate (DEM) (577). The induction of HO-1 by sodium arsenite represents a general response reproducible in a broad selection of animal cell types (34).

The heavy metal salts potently activate the gene in cell culture systems and produce tissue-specific effects in vivo depending on the compound and route of administration (78, 303, 424, 574, 664, 665). These compound include salts of cadmium (Cd^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}), tin (Sn^{2+}), lead (Pb^{2+}), and mercury (Hg^{2+}). For example, in injection of metal salts into rats, most metals tested produced a hepatic HO-1 induction, with Cd^{2+} and Co^{2+} the most potent. On the other hand, Sn^{2+} or Ni^{2+} and Hg^{2+} displayed a selective induction response in the kidney and heart, respectively (414). Heavy metals form complexes with thiols, such as reduced glutathione (GSH). In vivo, heavy metals cause a characteristic depletion of hepatic GSH followed by a rebound increase from de novo synthesis (414). Prior complexation of metals with thiol complexes diminished their effectiveness at eliciting the HO-1 response in vivo (414).

The complexation of metals with protoporphyrin IX (PPIX), the natural precursor of heme, generates metalloporphyrins. Several metals such as Co^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} can serve as substrates for ferrochelatase, the enzyme that incorporates iron into PPIX in the final step of heme synthesis. The substrate and catalytic cofactor of HO-1, heme (iron-protoporphyrin-IX), acts as an inducer of *ho-1* gene expression and activity (22, 673, 761).

Several metalloporphyrins, including SnPPIX and ZnPPIX, can induce HO-1 transcription while paradoxically acting as competitive inhibitors of HO activity both in vitro and in vivo (573). In contrast, CoPPIX, which is a potent inhibitor in vitro, is a powerful inducer of HO activity in vivo (153, 573).

Free protoporphyrin, in the absence of a central metal chelate, can also activate *ho-1*, albeit only in the context of its photoactivation by intense visible light at discrete wavelengths (561, 562).

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Since the discovery that NO, a gaseous free radical molecule, acts as a potent physiological regulator of many processes, including vascular tone, neurotransmission, inflammation, as well as a bactericidal agent in the macrophage respiratory burst, not surprisingly, intensive investigation has followed on the role(s) of NO in inducible gene regulation. NO produces potent HO-1 induction in many cell types tested, including fibroblasts and many vascular cell types. In addition to direct administration in gaseous form (422), HO-1 induction also follows the application of a number of chemical NO donor compounds in vitro, including sodium nitroprusside and spermine NONOate, nitrosating agents or nitrosonium cation generators such as *S*-nitroso-*N*-acetylpenicillamine, and the peroxynitrite generator 3-morpholinosydnonimine (74, 94, 157, 185, 226, 229, 462, 655, 749). Similarly, various chemical derivatives of NO also elicit the HO-1 response when directly applied to cell culture, including peroxynitrite ($ONOO^-$), *S*-nitrosothiols, nitrosohemes, and the nitroxyl anion (Angeli's salt) (94, 188, 479, 480). A major mechanism for NO-mediated signaling effects involves the activation of soluble guanylate cyclase (sGC), leading to enhanced cGMP formation. The majority of studies to date, however, have demonstrated a general ineffectiveness of cGMP analogs in inducing *ho-1*, in cell types where NO promoted the response (94, 157, 229, 422, 655). Nevertheless 8-BrcGMP can induce *ho-1* in several cell types including hepatocytes and endothelial cells (257, 322). In addition to direct activation of guanylate cyclase, NO and its derivative reactive nitrogen species (RNS) can participate in a multiplicity of redox reactions (732). NO reacts with O_2^- to form the oxidant peroxynitrite ($ONOO^-$). RNS such as nitrosonium cation (NO^+) can modify reactive thiol groups in protein. Thus the generation of metabolites of NO, including peroxynitrite, *S*-nitrosothiols, or nitrosohemes, have been implicated as potential secondary mechanisms underlying *ho-1* activation by NO donor compounds (185, 188, 463, 480). A second generalization from these studies suggests a largely transcriptional activation of *ho-1* by NO and its metabolites (157, 185, 229, 422, 462). However, in human cell types, a major posttranscriptional component has also been observed, whereby NO treatment promoted the stabilization of *ho-1* mRNA (64, 422). The regulation of *ho-1* by NO and its derivative reactive nitrogen species (RNS) has recently been reviewed (463).

A broad class of electrophilic polyphenolic compounds, which also are classified as antioxidants, has recently emerged as potent *ho-1* inducing agents, including caffeic acid phenethyl ester, carnosol, curcumin, and resveratrol (46, 290, 423, 461, 580). Many of these substances are plant-derived antioxidants. For example, the phenolic compounds curcumin and carnosol are derived from the spices turmeric and rosemary, respectively. Resveratrol, from grape skin and seeds, is a common constituent of wine (290). Quercetin is a ubiquitous bioflavonoid found in many plant-derived foods. The latest additions to this list of natural inducers of *ho-1* include the plant-derived chalcones, rosolic acid, and the garlic organosulfur compounds (93, 187). *Ginko biloba* extractum (EGb 761), a complex mixture of phenolic and terpenoid compounds, induces HO-1 expression in neuronal cell culture in association with its cytoprotective activity (788). In general, many phenolic compounds activate *ho-1* through the Nrf-2/Keap1 axis, as discussed in section III*C*, which plays a major role in the transcriptional activation of the *ho-1* gene by electrophillic compounds.

In addition to that of pro-oxidant states and other xenobiotic stress, HO-1 can represent a general molecular marker of proinflammatory states. Thus the role of HO-1 as a general cytoprotectant is not limited to stress from exogenous chemical and physical agents but may also be important during systemic stress caused by injury or infection. HO induction occurs as a component of the hepatic acute phase response (APR), a systemic reaction to injury or infection in the liver characterized by physiological changes including global alterations in hepatic protein synthesis (235). The hepatic APR is strongly induced by bacterial endotoxins (LPS), which stimulates cytokine production from monocytes and macrophages (200). The physiological changes characteristic of the APR are regulated by cytokines, including interleukin (IL)-6, IL-1, and tumor necrosis factor (TNF)-α. Thus a number of proinflammatory agents are potent activators of the HO-1 response. The most notable example is bacterial LPS, which typically produces a robust *ho-1* activation in cell culture (79, 80, 348) and in vivo following injection (545). The intraperitoneal injection of LPS induces HO activity in rat peritoneal macrophages and in hepatic parenchyma and sinusoidal cells (56, 546). The induction of hepatic HO expression (i.e., mRNA or activity) by LPS and other cytokines, in general, could be inhibited by glucocorticoids (82) or thiol antioxidants (545) and augmented by GSH depleting agents (545). More recent studies have reaffirmed that LPS injection induces HO-1 mRNA, protein, and enzyme activity in the liver, lung, and kidney (652).

In addition to LPS, a model of exogenous inflammatory stress caused by infections, HO-1 responds to exogenous stimulation with cell-derived inflammatory mediators, such as the interleukins (IL-1α, IL-1β, IL-6, IL-11) and cytokines such as TNF-α (157, 446, 484, 677, 678). The intraperitoneal injection of TNF-α and IL-1 also induces hepatic HO mRNA expression and/or enzymatic activity in mice (546). In cell culture models, induction of *ho-1* mRNA by TNF-α could be blocked by a variety of inhibitors, including downregulation of PKC by prolonged TPA treatment, Ca^{2+} ionophores, phospholipase A_2 inhibitors, and thiol antioxidants (678). Interestingly, the anti-inflammatory cytokine IL-10 can also stimulate *ho-1* in macro-

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phages (367). HO-1 has been proposed as a downstream effector of the anti-inflammatory action of IL-10 (367). Conversely, IL-10 upregulation has been implicated in the anti-inflammatory effects of CO (504).

Several growth factors also elicit a HO-1 response in a cell type or tissue specific fashion, including transforming growth factor (TGF)-β (in lung and ocular tissues) (352, 484, 488), platelet-derived growth factor (PDGF) (158) (in vascular cells), nerve growth factor (NGF) (in discrete neural cells) (388, 568), and erythropoietin in human bone marrow stem cells (10). In contrast, interferon-γ has been shown in several cell culture models to repress, rather than activate, HO-1 transcription (698).

Early studies on the regulation of HO-1 by hormone-induced systemic stress demonstrated that hypoglycemic shock induced by insulin injection as well as glucagon and cAMP administration induced hepatic HO activity in rats (41). Hormones, eicosanoids, and other circulating mediators have been studied as inducers of HO-1 in discrete cell types (Table 1). These include cyclopentone prostaglandins of the J series such as prostaglandin J_2 (PGJ_2) and its sequential metabolites Δ^{12}-prostaglandin J_2 (12-PGJ_2) (335, 336, 483) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (27, 389, 787, 789), which potently induce cellular differentiation and exert antiproliferative activity. Other prostaglandins implicated in *ho-1* activation include prostaglandin D_2 (PGD_2), the precursor of the J-series prostaglandins as well as prostaglandin A_1 (PGA_1) (389) and prostaglandin E_2 (PGE_2) in distinct cell types (96).

Atrial natriuretic peptide (ANP), a heart-derived cardiovascular hormone, plays a role in the regulation of blood pressure and can stimulate *ho-1* activation in human vascular endothelial cells (322). The induction of hypertension in rats by angiotensin II, a hormone with hemodynamic effects, was associated with increased HO-1 expression in the aorta (269), and also in the heart, liver, and kidney of rats (15, 268, 377). In vitro this hormone produced induction of *ho-1* in rat kidney cell models including renal proximal tubule epithelial cells, although this has not yet been established in human cell models (55, 609).

The HO-1 induction response may occur as a general consequence of exposure to environmental or industrial pollutants, which include some of the aforementioned agents such as heavy metal salts and solar ultraviolet radiation. Observations of *ho-1* induction phenomena have been reported in vitro and during inhalation studies involving cigarette smoke (466), air-borne particulates including diesel exhaust (100), mineral fibers (chrysotile and crocidolite) (650), organic solvents such as benzene and bromobenzene (8, 221), nitrogen dioxide (288), zinc oxide from welding exhaust, (122) and ozone exposure (660, 240, 700). A recent study shows that continuous exposure to ozone in rats (0.8 ppm; 6 days) induces HO-1 predominantly in the skin by direct contact and to a lesser extent in the lung by inhalation (700). Thus HO-1 protein expression could be utilized as a general molecular marker of adverse environmental conditions in fish, birds, wildlife, and humans (66).

The compounds and agents described above represent only a partial list, and due to the generality of the *ho-1* response to any conditions that cause cellular stress, certainly many more will be described. Since the discovery of the *ho-1* transcriptional response, a number of hypotheses have emerged that have attempted to unify the mechanisms between the stimulus (treatment with exogenous inducing chemical) and the response of *ho-1* gene activation. It has become evident that due to the diversity of stimuli and model systems studied, no unifying mechanism can be provided and that likely multiple mechanisms operate with cell-type and inducer-specific variations. One of the earliest of these hypotheses states that a transient increase in intracellular heme content may mediate the induction of HO-1 by certain inducers of the response. This hypothesis rests in part on the fact that heme itself is a potent inducer of the gene in vitro and in vivo, whether applied in free form or in protein complexes. Several inducing chemicals including insulin, epinephrine, diethylmaleate, CS_2, and endotoxin are associated with transient increases in the hepatic heme pool (325). Furthermore, a limited number of hepatotoxins that induce HO-1 in hepatic systems are also associated with cytochrome *P*-450 loss or degradation. For example, CS_2 induces hepatic HO-1 in conjunction with its acceleration of heme release from cytochrome *P*-450 as a consequence of irreversible apoprotein modification (278, 325). In one case, hepatic HO-1 induction coincided with loss of cytochrome *P*-450 as the result of partial surgical hepatectomy (760). Hepatic induction of HO-1 by heavy metals was dissociated from degradation of cytochrome *P*-450 (154). There is only limited evidence in extrahepatic tissues that a transient increase in intracellular free heme or increase in hemoprotein degradation precedes the induction of *ho-1*. In one example, the accelerated degradation of cytochrome *P*-420 was noted in parallel with the UVA-induced activation of HO-1 in human skin fibroblasts (354). This hypothesis is limited by the fact that many *ho-1* inducing chemicals, such as heavy metals and thiol reagents, may not cause appreciable hemoprotein degradation and/or elevations in intracellular heme (325). Although not necessarily a universal intermediate mechanism in the response to xenobiotic inducers of the gene, heme levels play an integral part in *ho-1* regulation by activating the translocation of Nrf2, and concurrently relieving transcriptional repression of *ho-1*, as detailed in section III*C*.

A second general hypothesis that has emerged states that a transient increase in intracellular reactive oxygen intermediates (ROS) mediates the induction of HO-1 in a

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redox-regulated pathway. This hypothesis is based in part on the fact that a large number of inducing agents are oxidants themselves or are associated with the intracellular production of ROS. This hypothesis is also supported by evidence that the induction of HO-1 by many, but not all, inducing chemicals can be inhibited by millimolar concentrations of *N*-acetyl-L-cysteine (NAC), an antioxidant and precursor for GSH (Table 2). For example, the transcriptional upregulation of *ho-1* by H_2O_2 but not that of heme is inhibitable by NAC (18). This hypothesis is compromised by the fact that ROS production by certain agents, especially hypoxia and various cytokines, remains controversial. Furthermore, treatment of cell cultures with nonthiol antioxidants does not universally inhibit *ho-1* activation by various inducers. For example, β-caroteine inhibits *ho-1* activation by UVA radiation, whereas the membrane antioxidant α-tocopherol augments the response in the same cell model (51, 690). The antioxidant caroteinoid lycopene potentiated UVA-induced DNA damage and induction of *ho-1* activation, as a result of the photodegradation of the lycopene to oxidized metabolites (750). Furthermore, many plant-derived phenolic compounds such as quercetin, curcumin, that are naturally occurring antioxidants can induce *ho-1*, rather than inhibit *ho-1* in cell culture models. Thus ROS generating systems produce a strong *ho-1* activation, yet the generation of ROS is not necessarily an intermediate event in the pathway elicited by all classes of inducing chemicals.

The activation of *ho-1* can be inhibited by metal chelating agents such as desferrioxamine (DFO), in a variety of cell culture systems in an inducer-specific fashion. For example, the induction of HO-1 by UVA radiation and H_2O_2 are inhibited by DFO treatment in vitro (320). Other examples of this phenomenon in the context of numerous cellular stress conditions are recapitulated in Table 3. While iron essentially does not exist in biological systems in "free" form, it is thought that potentially reactive iron exists in an intracellular pool in complexes with low-molecular-weight organic molecules (543). The original interpretation of the phenomenon was that metal chelating compounds act by removing a pool of potentially reactive iron, which amplifies the effect of oxidative stress conditions (320). In support of this hypothesis, iron loading has been shown to sensitize endothelial cells to peroxide stress (43). Iron loading potently synergizes the HO-1 activation by hypoxia in endothelial cells (559). A second interpretation of this phenomenon is that the chelating compounds remove a pool of iron that is required for gene transcription. Paradoxically DFO potently activates HIF-1 under normoxic conditions, which is among the factors that govern the transcriptional activation of *ho-1* under hypoxia (365, 593). The prolyl hydroxylase involved in HIF-1α degradation requires iron (275). Recently, it has also been shown that DFO increases the mRNA synthesis of the *ho-1* transcriptional repressor Bach-1. Such a mechanism may account for the inhibitory effects of DFO on *ho-1* activation in a number of systems. Because Bach1 is typically a heme binding protein (see sect. III*C*), the potential role of chelatable iron is not clear (331). Metal-chelating compounds may be of general use in strategies for the pharmacological manipulation of stress responses in vivo.

A third general hypothesis states that alteration of intracellular thiol equilibrium, as expressed by the ratio of intracellular reduced glutathione (GSH) to oxidized glutathione (GSSH), acts as a primary "sensor" for oxidative stress, or at least occurs as a general phenomenon associated with the redox activation of signaling pathways

TABLE 2. *Inhibition of HO-1 expression by N-acetyl-L-cysteine*

Cell or Tissue Type	Inducer	Effect of *N*-acetyl-L-cysteine	Reference Nos.
Cardiomyocytes (rat)	Hypoxia	Inhibits HO-1 protein expression	63
Aortic endothelial cells (bovine)	Hypoxia	Inhibition of HO enzymatic activity	559
Macrophages (RAW 264.7)	LPS	Inhibits *ho-1* transcription and mRNA accumulation	79
Myeloleukemia cells (mouse)	LPS	Inhibits *ho-1* transcription	348
Liver (rat)	LPS	Inhibits *ho-1* mRNA accumulation	545
Vascular smooth muscle (rat)	PDGF	Inhibits HO-1 protein expression	158
Aortic endothelial cells (bovine)	SNP, SNAP	Inhibits HO activity	188
	Peroxynitrite	Inhibits HO activity	185
Vascular smooth muscle (rat)	SNP, SNAP	Inhibits *ho-1* transcription	229
Embryo hepatocytes (avian)	Phenylarsine oxide	Inhibits *ho-1* promoter activity	594
Umbilical vein endothelial cells (human)	TNF-α, IL-1α	Inhibits *ho-1* mRNA accumulation	678
Lung epithelial cells (rat)	$CdCl_2$	Inhibits HO-1 protein expression	211
Premonocytic cells (human)	Cigarette smoke	Inhibits HO-1 protein and *ho-1* mRNA accumulation	178
Macrophages (mouse)	Diesel exhaust	Inhibits HO-1 protein expression	376
Aortic endothelial cells (human)	HPODE	Inhibits *ho-1* mRNA accumulation	238
HepG2 hepatoma (human)	d-15-PGJ_2	Inhibits *ho-1* mRNA accumulation and promoter activity	389

Representative experiments are provided whereby the antioxidant *N*-acetyl-L-cysteine inhibited the induction of HO-1 by various chemical inducing agents. $CdCl_2$, cadmium chloride; HPODE, 13-hydroperoxyoctadecadienoic acid; IL, interleukin; LPS, lipopolysaccharide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 15d-PGJ_2, 15-deoxy-Δ-^{12,14}-prostaglandin J_2; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor-α.

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TABLE 3. *Effect of metal chelation on ho-1 activation*

Cell Type	Inducer	Effect of Desferrioxamine	Reference Nos.
Fibroblasts, dermal (human)	UVA radiation, H_2O_2	Inhibition of HO-1 protein accumulation	320
Fibroblasts, dermal (human)	Hypoxia	Inhibition of *ho-1* mRNA accumulation	411
Aortic endothelial cells (bovine)	Hypoxia	Inhibition of HO-1 protein accumulation and HO activity	559
Pulmonary artery endothelial cells (human); hepatoma (human)	Hyperoxia	Inhibition of *ho-1* mRNA accumulation	183, 659
Renal proximal tubular epithelial cells (rat)	OxLDL	Inhibition of HO activity	13

Representative experiments are provided whereby metal-chelating agents inhibited the induction of HO-1 by various chemical inducing agents.

leading to *ho-1* expression. This hypothesis is based on the observation that many agents, in particular those associated with ROS or RNS generation, or which react directly with free thiol groups, can effectively consume, complex, or reduce the availability of intracellular GSH. These observations can be generalized to the activation of HO-1 by sodium arsenite, H_2O_2, UVA radiation, and NO (185, 358, 463, 577, 666). Induction of HO-1 in vivo has also been associated with GSH depletion in vivo with compounds such as $CoCl_2$ or DEM (173, 686). The depletion of reduced intracellular glutathione (GSH) by agents such as buthionine *S-R* sulfoxime (BSO), an inhibitor of GSH synthesis, sensitizes cells to activation of *ho-1* by oxidative stress or nitrosative stress (358, 479). For example, BSO strongly enhances the activation of HO-1 by oxidants, such as UVA radiation exposure and H_2O_2 treatment in primary human skin fibroblasts, or by LPS and proinflammatory cytokines in rat liver (358, 545). Phorone, which causes hepatic GSH depletion, potently induces *ho-1* in the rat liver (495, 767). The reduction of GSH available for cellular antioxidant capacity would promote an enhanced prooxidant state or render cells subject to increased oxidation events in the presence of ROS-related stimuli. A number of investigators have speculated that such perturbation of intracellular thiol equilibrium would lead to the oxidation of critical thiol groups in a protein or protein(s) responsible for regulation of HO-1. A number of potential intermediate targets of intracellular redox changes have been previously proposed on the pathway to *ho-1* activation. Indirect redox regulation has been proposed for the modulation of phosphoprotein phosphatases, transcription factors AP-1 or NF-κB, and mitogen-activated protein kinases implicated in *ho-1* regulation. Examples of direct redox regulation of transcriptional regulators were until recently only known for bacteria (i.e., OxyR, Hsp33) and yeast (yAP-1) whereby oxidation of a protein factor by disulfide bridge formation leads to an activation of the factor, resulting in either transcriptional activation (OxyR, yAP-1) or chaperone activity (Hsp33) (reviewed in Ref. 553 and references therein). Recent research, however, has suggested that the cytoplasmic factor Keap1 could act as a redox sensor in mammals that regulates the activity of transcription factors potentially involved in *ho-1* regulation (see sect. III*C*). This factor meets this criteria of a redox sensor because *1*) it contains critical -SH groups susceptible to oxidation and *2*) oxidation of the factor leads to activation of the pathway, involving the release of a bound substrate (Nrf2), a transcription factor with critical importance in the activation of *ho-1* (143, 374, 714).

The link between oxidative stress and the regulation of protein phosphorylation/dephosphorylation remains partially understood. As discussed in the following section, it is now generally believed that a great many, if not all, stimuli which induce HO-1 do so by modulating intracellular signal transduction pathways based on protein kinase cascades. In the case of the cytokines and growth factors, these cascades are initiated by binding to cell surface receptors. In the case of xenobiotic inducing agents, the initiation of MAPK is less clear. The precise connection between cellular redox state and the modulation of MAPK activities is also at present not clear. Although several studies have implied that Nrf2 activation in response to discrete inducers requires p38 MAPK and/or other kinases, the interrelationship between MAPK activation and the activation of the Keap1/Nrf2 axis by redox changes remains unclear.

B. Signal Transduction

Accumulating evidence indicates that many inducers of HO-1 activate protein phosphorylation-dependent signaling cascades that ultimately converge on the transcription factors that regulate the *ho-1* gene (Fig. 5). Recent studies have implicated a major role for the mitogen-activated protein kinases (MAPKs) in *ho-1* activation, though other kinases, including tyrosine kinases, phosphatidylinositol 3-kinase (PI3K) and protein kinases A, G, and C have also emerged as potential contributing mechanisms (163, 259).

MAPKs belong to an evolutionary conserved and ubiquitous signal transduction superfamily of Ser/Thr protein kinases that regulate cellular programs such as growth, apoptosis, motility, differentiation, and responses to environmental stimuli. The MAPK superfamily comprises three primary signaling cascades named after their

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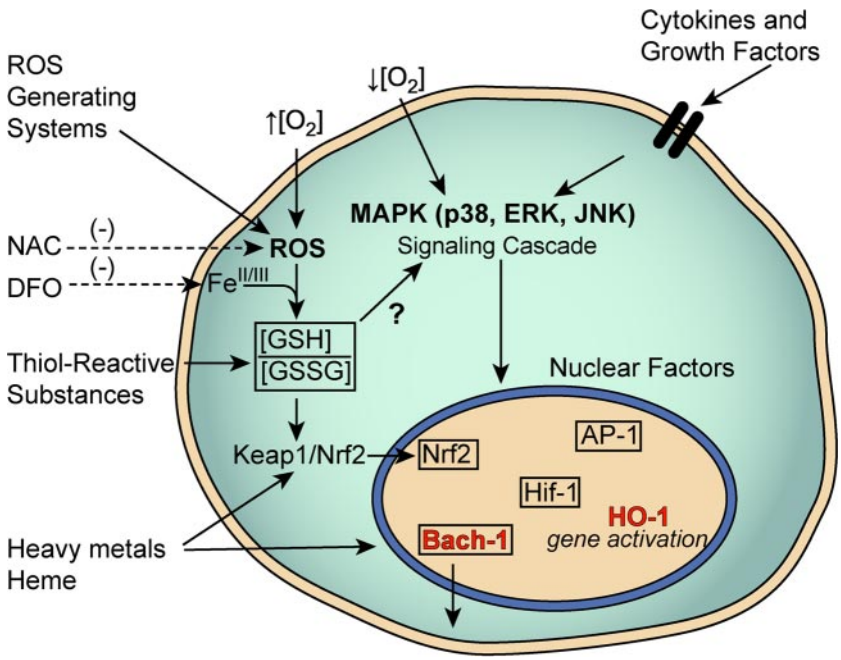

FIG. 5. Signaling pathways leading to *ho-1* activation. The scheme shows the potential interrelationships between intracellular and nuclear signaling events that lead to transcriptional activation of the *ho-1* gene by various inducing agents. Increases of intracellular reactive oxygen species lead to perturbation of intracellular thiol equilibrium, leading to reduction of GSH/GSSG ratio, and redox regulation of Keap1. Treatment with heavy metals and/or heme promotes Nrf2 nuclear translocation and nuclear export of the transcriptional repressor Bach1. Activation of MAPK by environmental stresses and cytokines has also been implicated in *ho-1* activation. The link between redox regulation and MAPK activation is not completely clear. Other nuclear factors involved in certain models include activation of Hif-1 (by hypoxia), AP-1 (by hypoxia, and lipopolysaccharide), and others. Antioxidants *N*-acetylcysteine and metal chelators such as DFO typically inhibit HO-1 activation in many models (see Tables 2 and 3) by respectively preserving thiol status or removing iron (Fe) involved in the amplification of ROS-mediated events. GSH, reduced glutathione; GSSG, oxidized glutathione; MAPK, mitogen-activated protein kinase; Nrf2, NF-E2 related factor-2; Hif1, hypoxia inducible factor; AP-1, activator protein-1; Bach 1, *ho-1* transcriptional repressor; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; DFO, desferrioxamine; HO-1, heme oxygenase-1. [Redrawn from Ryter and Choi (552).]

terminal MAPKs: the extracellular signal regulated kinases (ERK1/2 pathway), the c-Jun NH_2-terminal kinases or stress-activated kinases (JNK/SAPK), and the p38 MAPKs. Each pathway consists of a hierarchy of kinases that sequentially phosphorylate and activate their downstream target kinases. Thus the MAPKs are phosphorylated by the mitogen-activated protein kinase kinases (MKK or MEK), which in turn fall under the regulation of the MEK kinases (MEKK or MAP3K). Because each of these groups consists of many functionally related kinases, this diversity generates a large repertoire of distinct signaling cascades. The terminal, activated MAPKs ultimately phosphorylate a number of target proteins including multiple transcription factors involved in gene regulation (123, 355, 356). The MAPK pathways are subject to counterregulation by the MAPK specific phosphatases (316).

A number of studies have focused on the resolution of MAPK pathways in the activation of *ho-1* in diverse cell types in response to various inducing conditions. Such studies have employed chemical inhibitors (i.e., p38 MAPK: SB203580; MEK1: PD98059, JNK: SP600125) to implicate a role of MAPKs (Table 4). Furthermore, genetic studies have utilized the expression of dominant negative mutants (DNMs) of various MAPK (Table 5) in combination with inducing agents, as well as expression of wild-type and activated forms of MAPK, to further address this question (Table 6). On the basis of these approaches, accumulating evidence supports a role for the MAPK cascades in inducible *ho-1* gene activation.

A variety of structurally and functionally diverse agents that induce the HO-1 response also in parallel can activate one or more of the three major MAPK cascades in multiple cell types. Chemical inducers such as arsenite (164) and cadmium (24), bacterial LPS (223, 225, 531), or changes in oxygen tension associated with I/R stress (761) can activate all three pathways. The activation of a particular MAPK by an inducing chemical does not necessarily imply its direct involvement in *ho-1* induction. For example, treatment of A549 pulmonary epithelial cells with TGF-β1 activates ERK1/2 and p38α MAPKs, whereas pharmacological inhibition of p38α, but not of the ERK pathway, attenuates HO-1 mRNA accumulation (488).

The studies outlined in Tables 4–6 indicate that considerable variation in the specific MAPK(s) utilized for *ho-1* activation occur in an inducer-specific, and also in a cell type- or species-specific manner. The p38 MAPK appears to represent a major MAPK pathway responsible for activation of *ho-1* in several in vitro models, including $CdCl_2$ stimulation of human breast cancer cells (24) and the hypoxic stimulation of rat cardiomyocytes (295). A number of models imply a combined role for more than one MAPK pathway in attaining optimal gene activation. For example, the activation of *ho-1* in response to NO-donating compounds required a combination of p38 MAPK and ERK pathways (94). Both p38 MAPK and ERK pathways were also required for *ho-1* induction by cadmium in human gastric cancer cells (392), whereas only p38 MAPK was required in breast cancer cells (24). Exemplifying species-specific diversity of mechanism, the ERK and p38 MAPKs have been implicated in *ho-1* activation by arsenite in avian hepatocytes (164), whereas a combination of JNK and p38 MAPK were required for the arsenite response in rat hepatocytes (323). In an in vivo

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TABLE 4. *Role of protein phosphorylation cascades in ho-1 regulation: chemical inhibition studies*

Signaling Pathway	Cell Type	Inducer	Inhibitor	Footnote	Reference Nos.
p38 MAPK	MCF-7 (human)	$CdCl_2$	SB, 20–40 μM	*a,b*	24
	MKN gastric cancer (human)	$CdCl_2$, heme	SB, 40 μM	*c*	393
	Lung epithelial (human)	TGF-β	SB, 10 μM	*a*	488
	HeLa (human)	NO donor	SB, 10–50 μM	*a*	94
	HUVEC (human)	OxPAPC	SB, 10 μM	*a,b,c*	345
	Lymphocytes (human)	15d-PGJ_2	SB, 5 μM	*a,c*	27
	Cardiomyocyte (rat)	Hypoxia, <0.5% O_2	SB, 10 μM	*c*	295
	PAEC (rat)	Hypoxia, 1% O_2	SB, 10 μM	*a,d*	563
	PAEC (rat)	Anoxia/reoxygenation	SB, 0.1 μM	*a*	778
	Kidney epithelial (rat)	Curcumin	SB, 20 μM	*b*	46
	Aortic smooth muscle (rat)	Quercetin	SB, 10 μM	*c*	385
	PC12 pheochromocytoma (rat)	Carnosol	SB, 5 μM	*b,c,d*	423
	J774 macrophage (murine)	15d-PGJ_2	SB, 0.1–10 μM	*c*	368
	LMH hepatoma (avian)	Sodium arsenite	SB, 0–20 μM	*a,b*	164
ERK	MKN gastric cancer (human)	$CdCl_2$, heme	PD, 40 μM	*c,e*	393
	HeLa (human)	NO donor	PD, 20–100 μM	*a,c*	94
	HepG2 hepatoma (human)	CYP2E1	PD, 10 μM	*b*	208
	HUVEC (human)	ANP	PD, UO, 50 μM	*c*	322
	WI-38 fibroblasts (human)	Phorone/DEM	PD, 50–100 μM	*a*	493
	HUVEC (human)	PAPC	PD, 10 μM	*a,b,c,e*	345
	PAEC (rat)	Anoxia/reoxygenation	PD, 10 μM	*a,b,e*	778
	PAEC (rat)	Hypoxia, 1% O_2	PD, 10 μM	*a,d*	563
	Raw 264.7 macrophage (mouse)	PGE_2	PD, 10–40 μM	*c,e*	96
	LMH hepatoma (avian)	Sodium arsenite	PD, 30 μM	*a,b,e*	164
JNK	HUVEC	ANP	SP, 10 μM	*c*	322
	Hepatocytes (rat)	Sodium arsenite	SP, 25 μM	*a*	323
PI3K/Akt	Lymphocytes (human)	15d-PGJ_2	LY, 20 μM	*a,c*	27
	Lymphocytes (human)	15d-PGJ_2	W, 0.1 μM	*b*	27
	PC12 pheochromocytoma (rat)	Carnosol	LY, 40 μM	*c*	423
	PC12 pheochromocytoma (rat)	NGF	LY, 40 μM	*a,c*	568
	Macrophage (human)	IL-10	LY, 10 μM	*c*	542
	SH-SY5Y neuroblastoma (human)	Heme	LY, 20 μM; W, 0.1 μM	*c*	476
	Raw 264.7 macrophage (mouse)	LPS	LY, 10 μM	*b,c*	108
Protein kinase A	HUVEC (human)	OxPAPC	H-89, 20 μM	*a,c*	345
	Hepatocyte (rat)	Okadaic acid	KT5720, 1 μM	*a*	256
	Hepatocyte (rat)	Bt_2cAMP	KT5720, 1 μM	*a*	258
	Adrenocortical cells (mouse)	ACTH	H-89, 10 μM		521
Protein kinase C	HUVEC (human)	OxPAPC	Bis1, 10 μM	*a,c*	345
	Cardiomyocyte (rat)	Hypoxia	Chelerythrin, 1 μM	*c,e*	295
	WI-38 fibroblasts (human)	Phorone/4-HNE	Ro-31-8220, 2 μM	*a,b*	492
	Aortic endothelial cells (human)	HPODE	Gö6976, 1–10 μM	*a,c*	238
Tyrosine kinase	HeLa	$NaAsO_2$, Heme, $CdCl_2$	Genistein, 50 μM Herbimycin A, 5 μM	*a,c*	424

Evidence for the participation of kinase-dependent pathways in the regulation of *ho-1*, utilizing specific chemical inhibitors of various kinases. ANP, atrial natriuretic peptide; CYP2E1, cytochrome *P*-450 2E1; DEM, diethylmaleate; 4-HNE, 4-hydroxy-2-nonenal; LPS, lipopolysaccharide; NO, nitric oxide; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PKA, protein kinase A; PKC, protein kinase C; SB, SB203580 (p38α/β inhibitor); SP, SP600125 (JNK1/2 inhibitor); PD, PD98059 (MEK1 inhibitor); p38, p38 mitogen-activated protein kinase; UO, UO126; LY, LY294002; PGE_2, prostaglandin E_2; 15d-PGJ_2, 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2; PI3K, phosphatidylinositol-3-kinase; TGF-β, transforming growth factor-β; TAK1, transforming growth factor-β-activated kinase; W, wortmannin (PI3K inhibitor). [a]Inhibits inducer specific induction of *ho-1* mRNA accumulation. [b]Inhibits inducer specific induction of *ho-1* promoter-reporter gene constructs. [c]Inhibits inducer specific induction of HO-1 protein. [d]Activates *ho-1* mRNA accumulation at the doses tested. [e]Denotes partial response <50% inhibition.

study, the administration of the GSH depletor phorone into rats also led to activation of JNK1, and the phosphorylation of c-Jun, in association with *ho-1* expression (495). In general, the involvement of JNKs in *ho-1* activation appears to arise less frequently than that of ERK1/2 or p38 MAPK among the studies to date.

Further inducer-dependent specificity is demonstrated in a macrophage cell model. Activation of RAW264.7 cells by LPS required p38β MAPK (726), whereas in the same cell type, activation of *ho-1* by the lipid-derived mediator PGE_2 required exclusively ERK MAPK (94). In a rat model of pulmonary I/R injury and corresponding in vitro model of anoxia/reoxygenation (A/R) in pulmonary artery endothelial cells, all three major MAPKs (p38, ERK, JNK) were implicated in the *ho-1* induction response (778). In this study, increases in *ho-1* mRNA accumulation that were activated by A/R were subject to inhibition by both SB and PD compound, implicating both p38 MAPK and ERK in the response. In transient transfection assays, DNMs corresponding to p38 MAPK, JNK1, JNK2, ERK1, and ERK2 all inhibited the activation of *ho-1* promoter constructs by A/R (778).

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TABLE 5. *Role of protein phosphorylation cascades in ho-1 regulation: expression of dominant negative components*

Cell or Tissue Type	Dominant Negative Mutant	Inducing Agent	Footnote	Reference Nos.
MCF-7 (human)	p38α DNM	$CdCl_2$	*a,b*	24
Lung epithelial (human)	p38α DNM	TGF-β		488
PAEC (rat)	p38α DNM	Anoxia/reoxygenation	*b*	778
LMH hepatoma (avian)	Ras DNM	Arsenite	*b*	164
LMH hepatoma (avian)	MEK1 DNM	Arsenite	*b*	
PAEC (rat)	ERK1/ERK2 DNM	Anoxia/reoxygenation	*b*	778
PAEC (rat)	JNK1/JNK2 DNM	Anoxia/reoxygenation	*b*	778
PC12 pheochromocytoma (rat)	Akt1 DNM	Carnosol	*b,c*	423

Evidence is summarized for the participation of kinase-dependent pathways in the regulation of *ho-1*, utilizing corresponding dominant negative mutants (DNM). ERK1/2, extracellular signal regulated kinase-1/2; JNK1/2, c-Jun NH_2-terminal kinase-1/2; MEKK, MAPK/extracellular signal regulated kinase kinase kinase; MKK, mitogen-activated protein kinase kinase; TGF-β, transforming growth factor-β. [a]Inhibits inducer specific induction of *ho-1* mRNA accumulation. [b]Inhibits inducer specific induction of *ho-1* promoter-reporter gene constructs. [c]Inhibits inducer specific induction of HO-1 protein.

The most commonly used pharmacological inhibitor of p38 MAPK, SB203580, inactivates only the α- and β-isozymes but not the other known isoforms (γ, δ). Thus a lack of effect observed with SB203580 does not necessarily exclude the participation of all p38 MAPK isotypes. For example, treatment of rat hepatocytes with SB203580 does not alter *ho-1* mRNA accumulation in response to arsenite, yet the expression of p38γ induces rat *ho-1* promoter activity (323). Pharmacological inhibitors of various MAPK and other kinases typically exert specific inhibitory effects of their molecular targets within narrow dose ranges. At higher concentrations, a more generalized inhibitory profile may occur. For example, wortmannin, which inhibits PI3K at 100 nM, may cross-inhibit p38 MAPK at higher concentrations (>1 μM) (179). Differential dose-response effects of pharmacological inhibitors in some cases may result in activation of the target. In one study, inhibitors of p38 MAPK activated *ho-1* in endothelial cells at 10 μM doses. Under hypoxic conditions (1% O_2), the p38 MAPK inhibitors strongly potentiated, rather than inhibited, the hypoxic activation of *ho-1* in this model (563).

Additional information has been generated from the artificial overexpression of wild-type or activated components of various ERK1/2, p38 MAPK, or JNK pathway members, with respect to their ability to activate *ho-1* mRNA or transcriptional activation of various *ho-1* promoter-reporter gene constructs (Table 6). For example, overexpression of JNK, MKK3, and p38γ MAPK increased, whereas expression of the other p38 isoforms (α, β, δ) inhibited HO-1 expression in rat hepatocytes (323). In the absence of exogenous inducers of the response, the overexpression of several variants of MEKK, including MEKK1, the apoptosis signal regulating kinase-1 and the TGF-β activated kinase induced *ho-1* in hepatoma cells. This effect was further augmented by coexpression with downstream kinases (MKK4, MKK6, and JNK) and abolished by the expression of dominant negative Nrf2 (772).

TABLE 6. *Role of protein phosphorylation cascades in ho-1 regulation: expression of wild-type or activated signaling components*

Pathway	Molecular Species	Cell or Tissue Type	Footnote	Reference Nos.
JNK	MEKK1, TAK1, ASK1	HepG2 (human)	*f*	772
	MEKK1	Hepatocytes (rat)	*f,g*	323
	JNK1	Hepatocytes (rat)		
p38	MKK6*	LMH hepatoma (avian)	*f*	164
	MKK3	Hepatocytes (rat)	*g*	323
	p38γ	Hepatocytes (rat)	*f*	
ERK	Ras	Hepatocytes (rat)	*f,g*	323
	Ras*	LMH hepatoma (avian)	*f*	164
	Raf*	LMH hepatoma (avian)		
	MEK1*	LMH hepatoma (avian)		
	PI3K*	PC12 pheochromocytoma (rat)	*f*	423
PI3K/Akt	Akt-1*	PC12 pheochromocytoma (rat)	*h*	568

Evidence is summarized for the participation of kinase-dependent pathways in the regulation of *ho-1*, utilizing overexpression of wild type or (*) constitutively active mutants. ASK1, apoptosis signal regulating kinase 1; ERK1/2, extracellular signal regulated kinase-1/2; JNK1/2, c-Jun NH_2-terminal kinase-1/2; MEKK, MAPK/extracellular signal regulated kinase kinase kinase; MKK, mitogen-activated protein kinase kinase. [f]Activates *ho-1* reporter gene constructs. [g]Induces HO-1 protein expression. [h]Induces *ho-1* mRNA accumulation.

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In further studies, constitutively activated kinase mutants have been utilized. For example, the overexpression of activated mutant forms of MEK1, Akt, and PI3K stimulated *ho-1* in several cell models (Table 6).

A number of investigations have implicated the involvement of other kinases in the regulation of *ho-1* that are not directly related to the MAPK. A limited number of studies have examined the role of the PI3K cell survival pathway in the context of *ho-1* gene regulation. PI3K, a ubiquitous lipid-modifying enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit, responds to activation by diverse stimuli including growth factors, cytokines, and cytotoxic agents. PI3K activation results in the phosphorylation and activation of the Ser/Thr kinase Akt, leading to the downstream phosphorylation of a number of proteins that affect cell survival, cell cycle regulation, protein synthesis, and cellular metabolism (81, 343, 606). Cells and mice lacking specific members of the PI3K family develop a proinflammatory state, implicating a role of PI3K/Akt in the immunoresponse (340). Specific inhibitors of p38 MAPK and PI3K blocked Nrf2 activation, upon oxidative stress in H4IIE hepatoma cells, leading to a downregulation of phase II detoxifying genes (300). Inhibitors of p38 MAPK and PI3K blocked the activation of *ho-1* by 15d-PGJ_2 in human lymphocytes (27). Stimulation of PI3K/Akt by nerve growth factor and hemin in dopaminergic neuronal cells enhanced *ho-1* gene expression in an Nrf2-dependent fashion and provided protection against oxidative stress (476, 568). A recent study shows that PI3K/Akt mediates the activation of *ho-1* by the natural antioxidant carnosol, associated with downstream activation of p38 MAPK (423). In similar studies using murine macrophages, the PI3K inhibitor LY294002 also blocked *ho-1* activation by LPS (108).

Relatively few studies have implied roles for protein kinases A/G/C in *ho-1* transcriptional regulation. In several cell culture models (i.e., primary rat hepatocytes and vascular smooth muscle), treatment with dibutylated cAMP and other agonists of protein kinase A (PKA) activated *ho-1* transcription (156, 258). The induction of *ho-1* in rat hepatocytes by the NO-donating compounds, or the cell permeable analog of cGMP, 8-bromo-cGMP, could be inhibited by a specific G-kinase inhibitor and required a specific cAMP/AP-1 responsive element located in the context of the rat *ho-1* promoter (see sect. III*C*) (257–259).

Since early studies revealed that *ho-1* responds to activation by the tumor promoter TPA, a PKC agonist, a potential role for PKCs has been implied (18, 241, 242, 296). Consistent with this hypothesis, the diacylglycerol analog 1-oleoyl-2-acetyl-glycerol (OAG) activates *ho-1* in murine fibroblasts (243), and furthermore, the induction of *ho-1* promoter activity by TPA is abolished by chemical inhibitors of PKC (18). Experiments with staurosporine suggested a general role of PKC in the activation of *ho-1* in response to PGJ_2 (483). Furthermore, downregulation of PKC by chronic TPA incubation inhibited the induction of *ho-1* by TNF-α and IL-1α in endothelial cells (678). Less is known, however, of the potential role of specific PKC isoforms in the modulation of *ho-1*. The induction of *ho-1* gene expression by two inducing agents, phorone and 4-HNE, was suppressed by a PKC inhibitor, Ro-31–8220, in human fibroblasts (492). Interestingly, the pathway leading to Nrf-2 and downstream activation of *ho-1* in this model was insensitive to TPA downregulation and linked to activation of atypical PKC (492). In contrast, HO-1 expression was induced by the general PKC inhibitor chelerythrin in human aortic endothelial cells. However, the induction response to organic hydroperoxide was inhibited in the same model by Gö6976, which more selectively inhibits PKC isoforms (α, β_1, and μ) (238). Inhibitor studies have also implied a contributory role for PKCs in the activation of *ho-1* by oxidized phospholipids (345). Further research will no doubt reveal variable isoform specificity of PKC involvement in an inducer- and cell type-specific fashion.

With the use of general inhibitors of protein tyrosine phosphorylation (herbimycin A and genistein), a single report implied the involvement of tyrosine kinases in the regulation of *ho-1* in human cells in response to well-known inducing chemicals such as heme, arsenite, and cadmium (424).

Finally, the role of phosphoprotein phosphatases in *ho-1* regulation remains incompletely characterized. TNF-α, an inducer of *ho-1*, caused a general inhibition of protein phosphatase activity, leading to a kinase-independent upregulation of protein phosphorylation, which was proposed as a mechanism underlying the oxidant-dependent activation of *ho-1* (220). The treatment of rat hepatocytes with okadaic acid, a general inhibitor of Ser/Thr protein phosphatases 1 and 2A (PP1, PP2A), caused a transcriptional induction of *ho-1*. This okadaic acid effect required PKA, as it could be inhibited by a specific PKA inhibitor, and also required a specific cAMP responsive element located in the context of the rat *ho-1* promoter (256). Okadaic acid also potentiated cytokine (IL-1α, TNF-α) inducible *ho-1* mRNA induction in endothelial cells (678).

The discussion thus far has focused on the potential phosphorylation events leading to the transcriptional regulation of *ho-1*. Relatively little is known about the regulation of HO-1 itself by posttranslational mechanisms. Several studies have implicated that HO-1 can undergo phosphorylation at least in vitro (567, 569). A recent study has shown that Akt can phosphorylate HO-1 at Ser-188 in vitro and in cell culture. Phosphorylation of HO-1 by Akt led to moderate changes in HO activity in vitro. These studies suggest that Akt may play a complex role in regulating HO-1 both at the transcriptional and posttranslational levels (569). HO-2 can be activated by phosphor-

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ylation in the context of neuronal regulation by casein kinase-2 (58) (see sect. VI*F*).

In conclusion, the regulation of *ho-1* by multiple forms of cellular stress appears to involve protein phosphorylation cascades that converge on transcriptional activators, or repressors (see sect. III*C*), and possibly to the HO-1 protein itself. However, the specific MAPK and/or other kinases involved appear to vary in an inducer- and cell-specific fashion.

C. Gene/Promoter Regulation

Given the potential physiological importance of HO-1 in mediating cellular homeostasis as a general inducible stress protein response, a considerable research effort has focused on characterizing the molecular mechanisms that regulate the transcriptional activity of the *ho-1* gene. The broad spectrum of chemical and physical agents that induce *ho-1* genes operates on a network of signaling pathways (163, 259, 553, 609, 632) that converge on the activation of an equally complex network of transcriptional regulators (19, 102, 553, 609). Analyses of the *ho-1* genes of various species, including mouse, rat, human, and chicken, have revealed a multiplicity of *cis*-acting DNA sequence elements, or response elements, that serve as potential binding sites for transcription factors. Historically, a number of these potential sequence motifs were initially discovered in the proximal promoter regions (near the transcriptional start site) of the various *ho-1* genes. However, the important functional response elements of *ho-1* genes are now known to occur over a 10-kb expanse of the 5′-regulatory regions. Recent research on the human *ho-1* gene has suggested the possible occurrence of even further distal sequences (238), and also of intragenic sequences that may complement the activity of the 5′-enhancer region (237).

The importance of distal regions in *ho-1* regulation was first observed in deletion mutagenesis studies of the mouse *ho-1* gene. These experiments identified two enhancer regions, the E1 fragment (268 bp) and the E2 fragment (161 bp), located approximately −4 kb and −10 kb relative to the transcriptional start site, respectively (16–20). These enhancer regions mediate the activation of the mouse *ho-1* gene in response to multiple agents including TPA, heavy metals, sodium arsenite, LPS, electrophillic compounds, hydrogen peroxide, antioxidants, and heme (16, 18, 80, 260). Further research has confirmed that in the context of a larger, ~15-kb promoter region, these enhancers are absolutely essential for gene induction (24, 42, 46, 210). Although clear differences exist in the regulation of the mouse and human *ho-1* genes (598, 599, 609), recent studies have also implicated analogous distal regions in the regulation of the human *ho-1* gene (Fig. 6) (95, 237, 238, 305, 331, 345, 662). Though not as extensively characterized as the mouse −4-kb region, the human gene contains sequences between −3500/−4500 that mediate, in part, the induction response to heme and cadmium (237, 662) as well as other disparate inducers such as gold compounds (305), shear stress (95), NO donor compounds (226), cigarette smoke (178), and oxidized phospholipids (345). Sequences between −9.1/−11.6 kb were recently implicated in the response to organic hydroperoxides (238). Taken together, these studies suggest that in mammalian *ho-1* genes, the distal −4-kb region serves as a critical convergence point for a number of transcription factors important in the regulation of the gene.

The dominant sequence element in the E1 and E2 enhancers of mouse *ho-1* is the stress-responsive element (StRE), a 10-bp motif with the consensus sequence of (T/C)GCTGAGTCA. The StRE, which is present in multiple copies in both murine enhancers (−3885/−3876; −3937/−3928; −3983/−3974, and between −9.5/−10.4 kb), is spatially and structurally conserved among the mouse, rat and human *ho-1* genes. In the context of the mouse gene, the StREs mediate transcriptional activation in response to almost all HO-1 inducers thus far tested. The StRE is structurally and functionally similar to the Maf response element (MARE) and the antioxidant response element (ARE), the heme responsive element (GCNNNGTCA) consensus, and also implies an intrinsic TPA responsive element (TRE)/ AP-1 site (20, 260). For simplicity, these elements will be collectively referred to as the StRE for the purposes of this review.

The StREs represent targets of multiple dimeric proteins generated by intrafamily homodimerization or intra- and interfamily heterodimerization of individual members of the Jun, Fos, CREB, ATF, Maf, and the Cap'n'collar/basic-leucine zipper (CNC-bZIP) subclasses of the basic-leucine zipper (b-ZIP) superfamily of transcription factors. Members of each of these families have been implicated in *ho-1* gene activation (19, 24, 46, 164, 322, 323, 345, 493, 495, 598).

Historically, due to sequence similarity of the StRE with the consensus AP-1 binding site, AP-1 factors (i.e., c-Fos/c-Jun heterodimer) were proposed to mediate *ho-1* gene activation in response to regulation by TPA, hyperoxia, LPS, phorone, pyrrolidine dithiocarbamate, and others (20, 79, 80, 227, 363). Accordingly, agents such as LPS, which induce *ho-1* transcription in RAW 264.7 cells, in parallel also can induce in vitro AP-1 DNA binding activity. Furthermore, the entire E2 enhancer rendered an SV40 minimal promoter responsive to activation by TPA, a classical activator of AP-1 factors. This reporter construct also responded to transcriptional activation by the overexpression of c-Fos and c-Jun. Finally, recombinant c-Jun homodimer formed a complex with the StRE in vitro (20).

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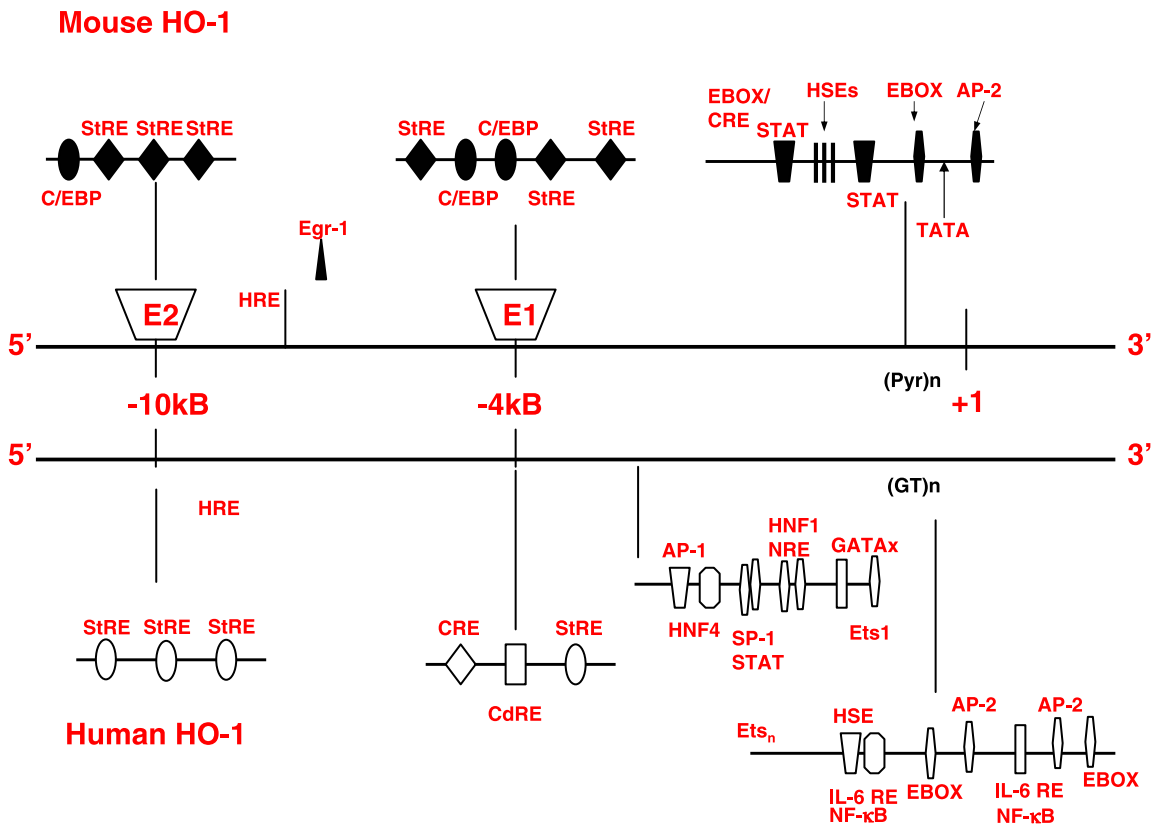

FIG. 6. Regulatory domains of the mouse and human *ho-1* genes. The scheme depicts the known regulatory elements in the mouse and human *ho-1* genes. The −4 and −10 kb regions (E1, E2) are marked for reference. Sequences identified primarily in the context of rat *ho-1* are shown in the hatched boxes. See also Refs. 102 and 609 for promoter diagrams. AP-1, activator protein 1; AP-2, activator protein-2; CdRE, cadmium responsive element; C/EBP, CAAT/enhancer binding protein binding site; CRE, cAMP responsive element; egr1, early growth response factor-1 binding site; ets, ets binding site; GATAx, GATA binding proteins, (GT)n, GT-rich repeat region; HNF1/4, hepatocyte nuclear factor ¼; HRE, hypoxia responsive element; HSE, heat shock element; NF-κB, nuclear factor kappa-B; NRE, negative regulatory element; (Pyr)n, pyrimidine-rich region; SP-1, specificity protein-1; STAT, signal transducer and activator of transcription; StRE, stress responsive element; TATA, TATA box; USF, upstream stimulatory factor.

Not necessarily excluding roles for AP-1 factor binding at these sites, accumulating evidence has led to a current view that CNC-bZIP factors binding to the StREs play a critical and dominant role in *ho-1* gene activation. Mammalian CNC-bZIP proteins include p45, Nrf1, Nrf2, Nrf3, Bach1, and Bach2. CNC-bZIP proteins do not form intrafamily dimers and heterodimerize most prominently with the small Maf proteins including MafF, MafG, and MafK (457). For example, the p45/MafK heterodimer constitutes the erythroid-specific transcription factor NF-E2, a protein that binds to the locus control regions of the β-globin gene. Small Maf proteins do not contain transcription activation domains and, thus, the transcription activity of NF-E2 type factors is derived from the CNC-bZIP subunit. Among the CNC-bZIP factors, the NF-E2 related factor-2 (Nrf2) contains a potent transcription activation domain (19). Nrf2 recognizes and binds to consensus ARE sequences found in the promoter regions of several phase II enzymes involved in xenobiotic metabolism (i.e., NADPH:quinone oxidoreductase, glutathione-*S*-transferase, γ-glutamylcysteinyl synthetase, and others) (23, 271, 701). Nrf2 forms stable heterodimers with MafK, MafF, and MafG (271). Dimerization of Nrf2 with Jun proteins has been implicated in the regulation of several phase II enzymes (701). Transcription factor ATF4 has also been suggested by yeast two-hybrid analysis as potential binding partner to Nrf2 (232). Finally, Nrf2 has also been observed to dimerize with CBP (CREB-binding protein) through two transactivation domains (Neh4 and Neh5) of Nrf2 (306).

Nrf-2 complexes have thus far been associated with the *ho-1* response to multiple agents which include heme, cadmium and cobalt chloride, arsenite, nitroxyl heme, 15d-PGJ$_2$, the plant-derived antioxidants curcumin, caffeic acid, and carnosol; various electrophillic compounds and complex mixtures of phenolic compounds such as diesel exhaust (21, 23, 24, 46, 210, 260, 376, 423, 480).

The link between phosphorylation cascades and Nrf2 activation is not clear. A link between p38 MAPK and Nrf2 activation was elucidated in MCF-7 cells, whereby the p38 MAPK inhibitor blocked the activation of Nrf2 and subsequent *ho-1* activation in response to cadmium (24). While

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phosphorylation of AP-1 constituents is well-documented, the direct phosphorylation of Nrf2 by MAPKs remains to be demonstrated (356, 747). Interestingly, a recent study has demonstrated the direct phosphorylation of Nrf2 at Ser-40 by atypical PKC in response to oxidative stress stimulation.

Under basal conditions a cytoplasmic factor, Keap1, inhibits the activity of Nrf2 by binding to the negative regulatory amino domain (Neh2) of Nrf2 (273, 307, 458,). Keap1 retains Nrf2 in the cytoplasm and promotes its ubiquitination and degradation by the proteosome (274, 332, 436). Recent studies suggest that Keap1 recruits cullin3, a subunit of the E3 ligase complex to Nrf2, to promote its ubiquitination (128, 332, 775). Cadmium, a potent activator of *ho-1*, stabilizes Nrf2, by inhibiting its degradation by the proteosome (22). Induction by various electrophiles releases the Keap1, permitting the nuclear translocation of Nrf2 (272, 273). Keap1 contains critical -SH groups susceptible to oxidation, suggesting that release of Nrf2 by Keap1 is potentially under redox control (143, 374, 613). Thus the oxidative modification of Keap1 and subsequent nuclear translocation of Nrf2 may provide an underlying mechanism for the redox regulation of *ho-1*.

A distinct StRE binding factor, Bach1, plays a critical role in the negative regulation of *ho-1* transcription that also displays a capacity for binding to heme (494, 509, 645, 649). Like Nrf2, Bach-1 acts as a binding partner for maf proteins, which compete against Nrf2/Maf dimers for binding at the StRE. Unlike Nrf2, Bach proteins lack a transactivation domain, and therefore, Bach/Maf dimers function as repressors when bound to target sequences. In transient transfection assays, Bach1/MafK dimers bind to the *ho-1* StREs resulting in a repression of a linked reporter gene. Gene targeting experiments in mice revealed that loss of Bach1 uncoupled *ho-1* from stress-responsive control resulting in constitutive HO-1 expression. Bach1 repressor activity dominates over the activator function of other StRE-binding proteins such as Nrf2, effectively maintaining gene activity at low levels under basal conditions (645).

Bach1 binds with high affinity to heme in vitro (K_D = 140 nM for recombinant Bach1), which inhibits its in vitro DNA-binding activity, and also promotes its nuclear export in intact cells (494, 648). Chromatin immunoprecipitation assays have confirmed that heme also promotes displacement of Bach1 from the *ho-1* enhancers in vivo resulting in the release of transcriptional repression (645).

These observations suggest that transient increases in intracellular heme concentration elicited by heme treatment itself, as well as increased hemoprotein turnover, or heme synthesis, could lead to the release of Bach1-mediated transcriptional repression (494). It remains incompletely understood, however, how nonheme inducers inactivate Bach1. Recent studies indicate that cadmium, like heme, also relieves *ho-1* transcriptional repression by promoting the nuclear export of Bach1 (648, 649). While the export of Bach1 by both heme and cadmium depends on the Crm1/Exportin1 nuclear exporter, these two agents utilize different structural signals within the Bach1 protein. The cadmium-dependent nuclear export signal (NES) coincided with a COOH-terminal cytoplasmic localization signal (CLS). On the other hand, the heme-dependent NES required two functional heme binding motifs or heme regulatory domains. A second isozyme of Bach (Bach2) displays a basal cytoplasmic localization. The cadmium-dependent export signal is conserved between Bach1 and Bach2, such that cadmium also promotes nuclear export of Bach2. The nuclear export of Bach1 in response to cadmium required ERK1/2 MAPK, indicating that MAPK cascades may also culminate in the regulation of repressor activity. The role of these mechanisms in the action of other inducers of *ho-1* remains to be determined.

Analysis of the *ho-1* locus in NIH3T3 fibroblasts indicated that this gene is not repressed by hypoacetylation of the chromatin microenvironment but rather exists in a preactivated state under basal conditions. The H3 histones occurring at the enhancer and the proximal promoter regions exist in a hyperacetylated state, irrespective of gene activity. Thus basal level repression of transcription is mediated primarily by Bach1 binding. Chromatin immunoprecipitation assays revealed that displacement of Bach1 from the StREs by heme was followed by binding of Nrf2 complexes to these elements, suggesting that high-level gene transcription also requires the subsequent action of an StRE-binding protein with activator function. Both cadmium and heme stimulate *ho-1* by a dual mechanism involving the stabilization of Nrf2 by inhibiting its degradation via the ubiquitin-proteasomal pathway, and in parallel, the stimulation of the nuclear export of Bach1 (21, 633). Thus the net effect of these processes will be a relatively greater nuclear abundance of StRE activators (e.g., Nrf2/small Maf dimers) compared with StRE repressors (e.g., Bach/small Maf dimers) resulting in *ho-1* gene activation.

The discussion thus far has focused on StREs, which respond to a multiplicity of inducers by accommodating the potential binding of multiple transcriptional regulators, and thus likely play a dominant role in *ho-1* gene activation in the context of the distal enhancer regions. However, a number of other elements distinct from the StRE, which occur within the defined E1 or E2 distal enhancer regions, have also been described that also potentially participate in gene activation. For example, the murine *ho-1* E1 enhancer contains several copies of CAAT/enhancer binding protein (C/EBP) sites (−3959/−3945; CTCATTTCCTCAGCT) and (−3974/−3960; GTGATTTCCTCACTG) that play an accessory, but not essential, role in mediating transcriptional responsiveness to cadmium. While the promoter regions of the human

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and mouse *ho-1* genes share general similarities in the positioning of the distal enhancer at −4 kb, subtle differences in sequence interpretation in the context of the response to cadmium have been described. The human *ho-1* gene contains an additional 10-bp sequence element, termed the cadmium responsive element (CdRE), occurring within the −4 kb enhancer region (−3947/−3938; TGCTAGATTT) but extrinsic to the StRE sequence homologies, which mediates cadmium chloride induction of the gene (663). Furthermore, the human −4 kb enhancer region contains a binding site preferentially targeted by transcription factor CREB, which participates in the response to oxidized phospholipid (345). The phosphorylation of CREB at Ser-133, as well as the −4 kb enhancer-dependent activation of *ho-1* in response to this agent, was sensitive to multiple inhibitors, including that of PKA/C, p38 and ERK MAPK (345).

Additional response elements occurring in the proximal and distal promoter regions of human, rat, mouse, and avian *ho-1* genes, extrinsic to the defined enhancer regions, have been identified in analyses of the response to single or discrete sets of inducers. While the functional binding of transcription factors has not been characterized in all cases, some of these additional sequence elements respond to unique classes or individual transcription factors. Examples of elements that respond in an inducer-specific or selective fashion include those responsive to hyperthermia (600) or hypoxia (365), which represent the targets of distinct DNA-binding proteins such as heat shock factors (HSF) and hypoxia-inducible factor-1 (HIF-1), respectively.

In the context of the human *ho-1* gene, in addition to regions corresponding to the distal enhancers, a number of proximal promoter elements in the area immediately adjacent to the transcriptional start site (0/−300) have also been identified as potentially important in gene regulation (602, 609, 697). These include E-box motifs potentially recognized by the upsteam stimulatory factor (USF) (−51/−42) and/or basic helix loop helix (bHLH) proteins (−156/−147) (469, 478), ETS binding sites (EBS) (138), as well as potential binding sites for nuclear factor-kappa B (NF-κB), (359–360), STAT-3 (139), activator protein-2 (AP-2) (359–360), and heat shock factor-1 (HSF-1) (602).

Irradiation of human skin fibroblasts with UVA (320–380 nm) radiation resulted in alteration of the binding activity at the USF site (−50/−41) presumably by causing a modification of USF (478). An element occurring at position −156/−147 mediated the induction of human *ho-1* during TPA-induced differentiation of myelomonocytic cell lines (469). This macrophage-specific TPA responsive element (MTE) consists of the sequence (GTCATATGAC), containing an E-box core element (CANNTG). This element was shown to bind proteins in nuclear extracts from monocytic leukemia cells in in vitro gel shift and DNase-1 foot-printing assays.

The human promoter proximal sequence (−1500/+19) contains up to 26 putative ETS binding sites (EBS) of the consensus GGAA/T potentially recognized by transcription factors ETS-1, ERG, and FLI (138).

An NF-κB binding site occurs in the human *ho-1* promoter at position (−166/−156) (359–360). A fragment containing this region was protected from DNase-1 digestion by purified p50 subunit of NF-κB in in vitro footprinting assays. A second NFκB-like sequence (−379/−370) overlaps the putative HSE at position −383/−366 (359, 360). While NF-κB has been described as a transcription factor whose nuclear accumulation and DNA binding activity responds to induction by oxidants such as H_2O_2 in discrete cell lines (590), the role of the proximal NF-κB element in mediating oxidant induction of the *ho-1* gene was not described. Interestingly, the in vitro binding activity of NF-κB to synthetic NF-κB oligonucleotides was induced by heme, the substrate of HO-1 (360). Two putative IL-6 responsive elements (IL6-REa; −386/−360) (IL6-REb; −167/−143) have been identified, the first which overlaps the proximal HSE, both which coincide with the NF-κB consensus sequences (359–360, 448). The IL6-REb constitutively bound a nuclear factor from Hep3B hepatoma cells, which was, however, not IL-6 inducible (446). The IL6-REb overlaps a sequence (−167/−158) also identified as a potential Stat-3 (signal transducer and activator of transcription) binding site (139). Competitive binding of additional factor(s) at this site was proposed to mediate the negative regulation of the IL-6 response by dexamethasone (139). The IL6-RE hexanucleotide motif (CTGGGA) occurs in the promoters of other APR protein genes including rat and human β-fibrinogen and rat α_2-macroglobulin and was originally described as the APR factor, which mediates the transcription of these genes in response to IL-6 (725). Recently, the NF-κB sites in the human *ho-1* promoter have been associated in part with the induction response to two naturally occurring antioxidants, curcumin and resveratrol. The relative significance of NF-κB versus other proteins that may act at these sites remains unclear.

An additional response element-rich region located between position −1976/−1655 contains potential binding sites for hepatocyte nuclear factors (HNE1 and HNE4), AP-1, STAT-x, c-Rel, and GATA-X. This region mediated basal activation of *ho-1* in hepatoma (HepG2) in transient transfection assays but was apparently nonfunctional when tested in HeLa cells (657, 658). This study also described potential negative regulatory region −981/−412 containing several negative regulatory elements that resemble transcriptional silencers originally described in the chicken lysozyme gene (658). An additional potential negative regulatory element specific to the human *ho-1*

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promoter corresponds to a variable GT-rich region (GT_n) (~−243/−198) (658, 739). Longer GT_n sequences in this region have been associated with attenuated ho-1 transcriptional activity. Length polymorphisms in this region have been positively associated with cigarette smoke-induced COPD (739), although independent studies found no association (233), and with coronary artery disease (97, 299) and vascular restenosis (175).

In a comprehensive analysis of the rat *ho-1* proximal promoter, Shibahara and co-workers (465, 498) described consensus heat shock elements (HSE) that mediate the thermal responsiveness of the *ho-1* gene in rat tissues and cells. The HSEs consist of inverted repeats of the core sequence element 5′NGAAN3′ that were first discovered in the regulatory regions of HSP genes. The rat *ho-1* gene contains two HSEs (−290/ −276; HSE1 and −222/−212; HSE2). The HSE-1 contains three inverted repeats of the core element 5′NGAAN3′ in alternating orientation, while the HSE2 consists of only two inverted repeats. The rat HO-1 HSE-1 conferred heat inducibility but not hemin inducibility to reporter gene constructs in transient transfection assays in rat and mouse cells (600). The HSE-1 was required for heat inducibility, whereas HSE2, though not essential, contributed toward the maximal response. Furthermore, both HSE1 and HSE2 bound a heat-inducible nuclear factor from rat glioma cells, potentially a rat homolog of the HSF. HSE2 also formed a complex with a distinct nuclear factor constitutively present in untreated glioma cells, which was designated as the HO promoter binding protein (HOBP) (497). Likewise, the mouse HO-1 promoter also contains three putative HSEs identified by sequence analysis, but these have not been functionally characterized (17). In contrast to the rat, the heat responsiveness of the human *ho-1* gene apparently displays a more restricted cell type specificity. Hyperthermia induces *ho-1* in rat hepatoma and glioma cells cells (600, 664). In human cell lines, *ho-1* was not inducible by hyperthermia in macrophage and glioma cell lines (761) but heat inducible in humans in discrete cell lines such as a hepatoma subtype (Hep3B) (447, 589). The human *ho-1* gene contains a single HSE within the region −383/−366, which failed to confer heat induciblity to similar reporter gene constructs in transient transfection assays, albeit in mouse and rat cell types (600). However, the putative human HO HSE bound a heat-inducible factor in nuclear extracts from heat-treated Hep3B cells (448). Heat shock inhibited the HO-1 induction elicited by chemical inducers in erythroleukemic cells (498). More recent studies have suggested that the human *ho-1* HSE can act as a potential site for transcriptional repression. Overexpression of HSF1 antagonized the induction of *ho-1* by sodium arsenite and 15-D-PGJ_2, and furthermore, this repressor effect depended on an intact HSE at this position (104).

In rodent *ho-1* promoters, several additional sequence elements have been identified as responsive to discrete chemical inducers. The rat *ho-1* gene contains a distinct distal hypoxia response element occurring at position −9551/−9531 from the transcriptional start site, which comprises functional binding sites for the hypoxia inducible factor-1 (HIF-1). The induction of HO-1 by hypoxia in rat aortic vascular smooth muscle cells was associated with increases in HIF1α and -β protein levels and required the hypoxia response element at −9 kb independently of the E1 and E2 distal enhancers (365).

In the context of the murine *ho-1* promoter, a distal element (−9.5 kb) mediated the response to metalloporphyrin induction and resembled a binding site for transcription factor Egr-1 (745). While the induction of mouse *ho-1* by high oxygen tension (hyperoxia) was largely mediated by StRE binding factors at the E1 enhancer, an additional cooperativity in the induction mechanism was provided by STAT proteins binding at two extrinsic positions (−0346/−0338; TTCTGGAAA and −0492/−0484; TTCCGGGAA)(363). Recent studies have uncovered yet another region in the mouse *ho-1* proximal promoter (−117/+74) which mediated the induction response to LPS (108). This region contained two binding sites for transcription factor Ets-2 (−93 and −125). Inhibitor studies implicated a role for PI3K in the phosphorylation and activation of Ets-2. Inhibition by Ets-2 DNM transfection established a role for this factor in the induction of murine *ho-1* by LPS. However, other studies, as previously mentioned, have implied a major role for the StRE sites of the E1 enhancer in mediating *ho-1* activation by LPS in this model, with the potential involvement of Nrf2 or AP-1 transcription factors (80). These apparently conflicting observations are not necessarily mutually exclusive, as it is now apparent that multiple mechanisms may mediate the induction response to a single inducer.

Within the rat *ho-1* proximal promoter region, an element (−0665/−0654; CTGACTTCAGTC) homologous to the consensus cAMP responsive element (CRE) and AP-1 sites, mediates the response to sodium arsenite, okadaic acid, and cyclic nucleotides (cAMP and cGMP) (256, 257, 323) in rat hepatocytes and the response to LPS in macrophages (726). An additional E-box element at position −47/−42 mediated the involvement of p38 MAPK in the sodium arsenite induction response. However, in this series of experiments, only the proximal promoter region was analyzed, while the relative importance of the distal enhancers was not assessed. The E-box consensus CACGTG serves as a potential binding site for bHLH proteins, including Myc/Max complexes. This region overlaps with an element (−51/−35) originally identified as a sequence homology of the adenovirus major late promoter. As initially characterized, this element served as a binding site for a factor termed HO transcription factor

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(HOTF), which was proposed to represent a rat homolog of the USF (576). An additional element residing at position −0678/−0661 of the sequence AGAGCCATGTG-TCCTGAC was identified as responsive to PGJ_2 (336), although the identity of the corresponding transcription factor remains unknown. This element also contains an E-box motif and confers position-independent enhancer activity to an SV40 minimal promoter, in response to PGJ_2.

The proximal promoter regions of rodent *ho-1* genes contain elements similar to the metal responsive elements (MRE) of the consensus (TGCPuCNC), originally identified in the human metallothionein-IIA promoter. In the mouse metallothionein (IA and IIA) genes, metal induction is mediated by an array of MREs, which vary in their affinities for metal responsive transcription factors (MBF-1, MTF-1) (224).

The mouse HO-1 flanking sequence contains two such elements, MREa (TGCACTC) at −697/−690 and MREb (GGAGAGCA) at −733/−726 (17), while the rat *ho-1* promoter contains an MRE (GGGTGCTGCACTC) at position −569/−581 (465), whereas the human gene apparently lacks this homology (602). While similar MREs mediate the metal responsiveness of metallothionein genes, the MREs within the *ho-1* promoter were determined not to contribute to the metal-dependent induction response (16, 17).

Partial characterization of the avian (chicken) *ho-1* gene reveals additional diversity of regulatory sequences. A proximal promoter element mediated the response to sodium arsenite and related compounds (−52/−41) and resembles an E-box (401). Furthermore, a proximal AP-1 site also mediated the induction to arsenicals and $CoCl_2$ (−1584/−1570) (164, 401). A more distal, as yet unidentified element mediated the induction response to heme and metalloporphyrins (595).

In conclusion, these studies taken together have revealed a multiplicity of response elements and corresponding transcription factors potentially important in *ho-1* regulation, not only among different species but also within a single species. This apparent redundancy of mechanisms may account for cell type- and development-specific variations as well as for varying degrees of responsiveness. Furthermore, the important sequence elements in *ho-1* transcriptional regulation are distributed along more than 10 kb of 5′-regulatory sequence and are likely to function in an orientation- and site-independent manner. In some cases, elements differing in sequence structure and spatial positioning have been implicated in gene activation by the same inducer, which may indicate that optimal induction requires the concerted and cooperative action between distinct factors bound at multiple and separate sites. Indeed, several examples of such long-range cooperativity in *ho-1* gene activation have been observed (237, 363, 401). Despite the occurrence of many disparate elements in the 5′-regulatory region(s), current research indicates that the StRE-Bach1-Nrf2 axis plays a critical role in *ho-1* gene regulation.

IV. FUNCTIONAL PROPERTIES OF HEME OXYGENASE-DERIVED IRON

A. HO-Derived Iron and Gene Regulation

Iron that exists in biological systems independently of heme and/or metalloprotein pools resides in complexes with low-molecular-weight organic compounds. This iron occurs in a loosely bound form accessible to metal chelating agents (543). The iron released from heme by HO activity potentially enters such a pool of "labile" or "chelatable" iron, where it may be available for cellular processes that depend on iron (562). Figure 7 outlines several functional outcomes of HO-directed iron release (Fig. 7).

One hypothesis states that the iron released from HO activity is transiently available for the promotion of intracellular ROS production. The potential reactions in which iron may play a catalytic role include but are not limited to the multiplication of lipid peroxidation chain reactions, the NO-dependent nitrosylation of thiols, and the Haber-Weiss reaction, which generates the reactive oxidant hydroxyl radical from the metal catalyzed decomposition of hydrogen peroxide (222). The significance of these reactions in vivo is beyond the scope of the current review. Free heme is also a potentially dangerous pro-oxidant compound, which may promote membrane lipid peroxidation (562; reviewed in Ref. 669).

The removal of heme, and its preclusion in the participation of such oxidative reactions, has been proposed as a possible function for HO activity (635). However, limited evidence from in vitro models describes short-term hypersensitivity to oxidative stress as a result of HO-1 expression (354, 562, 646, 647). These observations occurred primarily under vector-driven HO-1 overexpression in the presence of substrate (heme) loading or hyperoxic challenge. The apparent toxicity of HO-1 in these models was likely related to gene overdosing and/or substrate load conditions resulting in a supraphysiological rise in intracellular iron.

Thus the antioxidative protection afforded by HO activity cannot be explained alone by the conversion of heme in exchange for increased intracellular iron, which is also potentially toxic. This antioxidant protection of HO with respect to iron metabolism is facilitated by further coupling with proteins that either promote the sequestration or export of the liberated iron.

In this regard, the regulation of adaptive gene expression by posttranscriptional mechanisms represents a possible functional consequence of HO-directed iron release. Iron, by binding to iron regulatory proteins (IRPs), can

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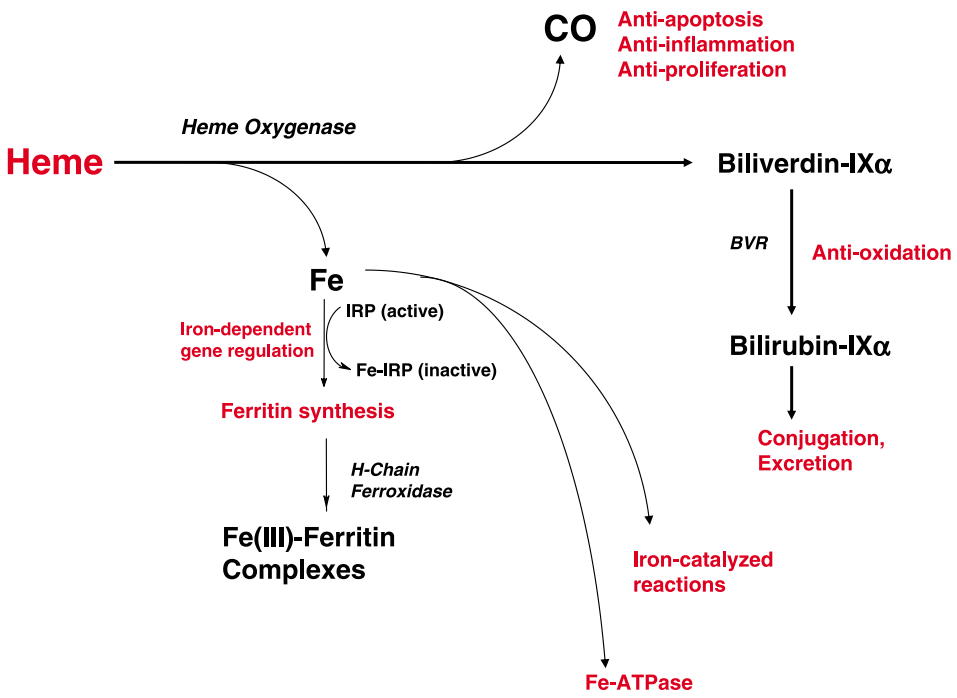

FIG. 7. Functional consequences of HO activity related to the three principle reaction products, bilirubin, iron, and carbon monoxide (CO). Iron released from HO activity triggers pathways involving the activation of the iron regulatory protein (IRP), leading to its sequestration. Competing pathways involve its extracellular efflux and its potential participation in deleterious cellular reactions. Biliverdin and bilirubin produced from HO activity potentially contribute to cellular antioxidant balance prior to their excretion. The downstream signaling events triggered by CO are depicted separately in Fig. 8.

influence the stability of mRNAs corresponding to proteins critical for the processing and trafficking of iron, and thus regulates intracellular iron homeostasis (160). The effect of iron/IRP complexes on mRNA stability can be positive (as in the case of the regulation of ferretin and erythroid δ-aminolevulinate synthase synthesis) or negative (as in the case of transferrin receptor synthesis). For example, the IRP binds to the 5′-untranslated region (UTR) and inhibits the translation of ferritin mRNA under iron-deficient conditions. Cytoplasmic iron, as it becomes available, binds to the IRP, releasing the IRP from ferritin mRNA, and thus allowing its translation to proceed (162). In the case of the transferrin receptor mRNA, binding of the IRP to the 3′-UTR stabilizes the mRNA, whereas the removal of the IRP by iron binding destabilizes the mRNA. Cells that realize an excess of intracellular iron utilize these mechanisms to reduce intracellular iron levels (162, 346). The enhancement of ferritin synthesis by high iron levels increases the iron storage capacity of the cell. Ferritin, a 24-unit oligomeric protein composed of heavy (H: ~21 kDa) and light (L: ~19 kDa) chains in a macromolecular complex of 450 kDa, stores the iron in an internal pocket. The iron thus bound is maintained in the oxidized (ferric) state, by an H-chain ferroxidase activity. The ferritin holoenzyme can accommodate iron up to approximately a 1:4,500 molar ratio. Iron thus bound is unavailable for catalytic reactions until released (679). In a similar paradigm, the increase of erythroid specific δ-aminolevulinic acid synthase, the rate-limiting step in heme synthesis, by increased intracellular iron, promotes a major cellular iron utilization pathway in red blood cells (130). In contrast, elevated intracellular iron depresses the synthesis of the transferrin receptor, leading to a diminished cellular capacity for iron uptake from plasma (86).

Several studies have shown an association between inducible HO activity and the increased synthesis of ferritin (161, 703, 704). This phenomenon was related to a transient increase in chelatable iron caused by HO-mediated heme degradation, which consequently relieved the posttranslational repression of ferritin. For example, the treatment of primary human fibroblasts with heme or exposure to UVA irradiation induced HO activity and subsequently increased ferritin synthesis (24–48 h) posttreatment (161, 703, 704). Increases in ferritin following heme or UVA treatment were inhibited by DFO or by treatment with Sn-PPIX (161, 704).

In addition to these mechanisms, iron is potentially required for other gene regulation events. Recently, it has been discovered that the prolyl hydroxylase activity responsible for basal degradation of HIF-1 requires iron for activity (275, 276).

B. Antiapoptotic Roles of HO-Derived Iron and Ferritin

Ferritin has been implicated as a cytoprotective molecule in a number of in vitro models (44, 45, 382, 703). Thus ferritin synthesized as a consequence of HO activity has been proposed as a contributory mechanism underlying HO-dependent cytoprotection (703). This was initially suggested by in vitro preconditioning experiments where expression of ferritin occurred in association with

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HO-1 expression and in parallel with the development of acquired cellular resistance. For example, the pretreatment of endothelial cells with heme sensitized the cells to subsequent oxidative challenge after short-term incubation, while a longer treatment with heme conferred cellular resistance to subsequent oxidant challenge (44, 45). The treatment of primary human skin fibroblasts with a sublethal dose of UVA irradiation resulted in an acquired protection against membrane damage incurred by a subsequent higher dose of UVA applied 24 h later (703). In both models, the cytoprotection afforded by preconditioning occurred in association with induction of both HO-1 and ferritin synthesis. Antisense oligonucleotides directed against HO-1 mRNA abolished the cytoprotective effect of UVA pretreatment and inhibited both the induction of HO-1 and ferritin synthesis. Iron chelation with DFO also abolished UVA-dependent cytoprotection and inhibited the ferritin synthesis (703). The increase in ferritin in the heme-treated endothelial cells, however, occurred independently of HO-1 induction, since cotreatment with SnPPIX had no effect on heme-induced ferritin synthesis (44). Nevertheless, ferritin accumulation in both models was linked to net cytoprotection. In another example, heme preconditioning conferred cellular resistance to in vitro photodynamic therapy treatments or oxidant challenge applied up to 20 h later, which was reversible by incubation with antisense oligonuleotides against ferritin H-chain mRNA (382). In contrast, exposure of endothelial cells to exogenous NO, a potent inducer of HO-1 expression, inhibited the synthesis of ferritin, possibly by direct interaction with IRP, resulting in a delayed (24 h) sensitization to a subsequent oxidative stress challenge (292, 293). In vivo, the preconditioning of rats with endotoxin provided protection in a model of glycerol-induced renal failure involving rhabdomyolysis. The endotoxin-induced preconditioning was associated with HO-dependent elevations of ferritin in the kidney (706). Pulmonary endotoxin instillation results in coinduction of ferritin and HO-1 in the lung localized to bronchial epithelium and macrophages. However, the induction of ferritin in this model was determined to occur directly as a consequence of endotoxin stimulation, and independently of HO activity, as SnPPIX treatment failed to block the response (84). A recent study has suggested that the adenoviral overexpression of ferritin H-chain can confer resistance to I/R injury associated with rat liver transplantation by reducing apoptosis (54). These studies collectively suggest that ferritin acts as a cytoprotectant following its overexpression or induction by chemical agents in vitro and in vivo. The expression of ferritin by HO-derived iron remains one possible mechanism underlying the cytoprotective action of HO-1.

Recent studies have suggested that iron released from HO activity stimulates increased iron efflux from cells, such that HO activity is inversely related to intracellular iron accumulation (181). The cytoprotective effect of HO-1 was likewise found to inversely correlate to intracellular iron accumulation (181). An ATP-dependent iron pump has been identified which colocalizes with HO-1 in microsomal membranes. This pump may facilitate the transfer of iron across the lumen of the ER, following its generation by HO activity, leading to its transferrin-dependent exocytosis (49). It is not clear whether all the iron generated by HO-1 is destined for export by this mechanism, or whether a portion is conserved for biosynthetic processes. It remains unclear how the iron is transferred from HO-1 to the ATPase, and whether this ATPase in close physical proximity makes direct contact with HO-1. It also remains unclear whether such an iron-dependent ATPase responds to iron-dependent regulation at the transcriptional or posttranscriptional levels.

While recent studies have indicated that the antiapoptotic effects of HO-1 in endothelial cells are related to CO evolution (see sect. VI*E*), an antiapoptotic pathway for HO-1-derived iron has recently been proposed in discrete cell types. The overexpression or chemical induction (i.e., CoPPIX) of HO-1 conferred resistance to anti-Fas antibody-mediated apoptosis in Jurkat T cells. This antiapoptotic effect was linked to iron-dependent NF-κB activation, leading to the expression of NF-κB dependent antiapoptotic mechanisms, including the potential activation of c-Flip. The NF-κB activation and apoptosis protection in HO-1 overexpressing cells were blocked by an iron chelator DFO in Jurkat T cells. These results led the investigators to propose that increased regulatory ROS generated from HO-derived iron primes an NF-κB-dependent pathway leading to compensatory cytoprotection (103). These observations are supported by previous work indicating that iron can stimulate antiapoptotic pathways by modulating p38 MAPK (103). On the other hand, DFO, which removes intracellular chelatable iron, has been shown in the absence of other treatments to stimulate the apoptosis of certain cell types, including murine lymphomas, HL60 cells, human cervical carcinoma cells, human or rat breast cancer cells, and various other transformed cells (215, 279, 326, 339, 611). These experiments indicate that removal of iron required for vital cellular processes including growth and DNA synthesis ultimately results in cell death. Nevertheless, DFO can limit the toxicity and associated HO-1 induction incurred by oxidative stress treatments such as hyperoxia or UVA radiation, where the presence of intracellular iron is expected to exacerbate ROS production (Table 3). The potential role of intracellular iron in modulating HO-1 activation is discussed in section III*A*.

Several in vivo model studies indicate that HO-1 and HO-2 play critical roles in the physiological trafficking of intra- and extracellular iron. The aberrant accumulation of tissue iron and serum iron anemia have been described in *ho-1*$^{-/-}$ and *ho-2*$^{-/-}$ transgenic mice (135, 136, 523).

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The *ho-1*$^{-/-}$ mice were anemic, accumulated nonheme iron in the kidney and liver, but displayed reduced total iron content in the lung, whereas *ho-2*$^{-/-}$ mice accumulated total lung iron without compensatory increase in ferritin levels (135, 136, 523).

These experiments were the first to suggest that iron recycling directed by HO-1 maintains blood iron levels and determines relative levels of tissue iron (522, 523). The mechanisms by which HO-1 deficiency resulted in accumulation of tissue iron remain unclear but may relate to the potential association of HO-1 with intracellular iron pumps. Interestingly, a single reported case of genetic HO deficiency in humans was also associated with hemolytic anemia and accumulations of iron in the kidney and liver (738).

Mice deficient in *ho-1* or *ho-2* display differential sensitivity to oxidant challenge, and this may be related in part to perturbations of iron distribution. For example, *ho-2*$^{-/-}$ mice displayed increased sensitivity to the lethal effects of hyperoxia relative to wild-type mice, which was not compensated for by increases in HO-1, and accumulated iron in their lungs (135). Endothelial cells derived from *ho-1*$^{-/-}$ animals displayed an oxidative stress resistant phenotype, resisting H_2O_2-mediated toxicity in vitro. In contrast, *ho-1*$^{-/-}$ rats were found to resist the lethal effects of hyperoxia in vivo (136). These apparently conflicting results appear to indicate that a basal level of HO activity confers physiological protection against the lethal effects of hyperoxia in vivo, whereas higher levels of HO-1 beyond a critical threshold may exacerbate oxygen toxicity by an iron-dependent mechanism. Clearly more experimentation is needed to resolve these apparent paradoxes. These studies have collectively indicated that animals deficient in either HO-1 and HO-2 display differential sensitivity to oxidative stress conditions relative to wild-type counterparts and also display global alterations in the distribution of intra- and extracellular iron (135, 522, 523).

V. FUNCTIONAL PROPERTIES OF HEME OXYGENASE-DERIVED BILE PIGMENTS

A. Metabolism of Biliverdin and Bilirubin

In exploring the multiple underlying mechanisms of HO-mediated cytoprotection, much attention has focused on the possible functional significance of the bile pigments BV and BR (Fig. 7). BV and BR originate in vivo primarily from the degradation of hemoglobin heme (503). Early experiments using radiocarbon-labeled molecules (i.e., hemoglobin, PPIX) injected into animals established the relationship between bile pigment formation and heme-degradative pathways (250, 503). The evolution of BR from BV has been recognized since the studies of Lemberg in 1936 (371) and confirmed by metabolic labeling experiments (205). BV, the first product of HO-catalyzed heme cleavage, is a soluble greenish pigment. Its enzymatic reduction by BVR produces BR, a hydrophobic yellowish pigment that partitions to the lipid phase. BR produced endogenously is excreted from cells and tissues and passes through the blood serum in association with serum proteins such as albumin. BR is removed from the serum exclusively by the liver, where it is metabolized to mono- and diglucuronides by hepatic phase II UDP:glucuronyltransferases. Glucuronidation solublizes the BR and prevents its intestinal reuptake. Conjugated BR passes from the liver through the bile and feces, where it is further degraded to urobilinogens by the reductive processes of intestinal microorganisms (57, 550).

Like CO (see sect. VI), BR is formally considered as a metabolic waste product with potentially harmful effects. Under normal physiological conditions, BV and BR are processed for rapid elimination. Whether during the course of their elimination these substances provide intrinsic benefit to an organism remains controversial and has provided fuel for recent debate (48, 301, 433, 562). An excess accumulation of unconjugated BR in the plasma, the underlying cause of neonatal jaundice, can lead to dire toxicological sequelae, including neurological encephalopathy. To avoid these potential toxicities, the BR overload is commonly dealt with by the use of phototherapy or, in severe cases, exchange transfusion, performed in a neonatal intensive care unit. These methods are designed to remove preformed BR. Pharmacological inhibition of HO with metalloporphyrins (SnMP) is presently being developed for clinical use (302). This is designed to prohibit the production of BR and is in direct contrast to the present treatment modalities, which are designed to remove preformed BR that is at a level requiring clinical intervention. This is done with the belief that the potential toxicity of BR to the neonate outweighs any potential benefit that could be derived from incidental antioxidant properties of BR. The neurotoxicity of BR has been associated with its lipophilicity and consequent accumulation in discrete neurons and astrocytes in the brain, where it aggregates with phospholipids (109, 471, 472, 667).

Potential antioxidative effects of BR were first noted as early as 1976. BR effectively quenches singlet molecular oxygen ($^1O_2$) in organic solvent and thereby prevents the photooxidation of hydrocarbons (629). BR inhibited the photosensitizer-dependent photooxidation of serum albumin in aqueous solution (513). Both BV and BR can react with enzymatically generated O_2^- in vitro, resulting in bleaching of the pigments (198, 547). In 1986 and thereafter, Stocker and colleagues (486, 487, 636, 637, 639, 640) published a series of experiments establishing that both BV and BR could act as potent in vitro antioxidants with possible physiological implications. These observations provided the basis for subsequent hypotheses that

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bile pigment generation served a physiological antioxidant function in tissues, serum, and bile and, furthermore, possibly mediated an antioxidative function of HO at the cellular level (635). In experiments conducted with phospholipid micelles, the inclusion of BR inhibited chemically induced lipid peroxidation by acting as a chain breaking antioxidant (640). In contrast to BR, which acts as an electron donor in these reactions, BV was proposed to react directly with peroxyl radicals to form an addition product (640). These observations were also extended to other in vitro models of liposomal lipid peroxidation, or plasma protein, lipid, and low-density lipoprotein (LDL) oxidation (486, 487, 636, 637, 734). The protection afforded by conjugated BR or BV was synergistic in the presence of α-tocopherol, possibly by regenerating the α-tocopherol following its oxidation (639).

BR also acts as an efficient scavenger of hypochlorous acid, a macrophage-derived oxidant (638). Finally, BV and BR also display in vitro reactivity toward various forms of RNS, including NO gas, NO donor compounds, and *S*-nitrosothiols, peroxynitrite, or nitroxyl anion, which is prevented by thiol antioxidants (309, 420).

The effects of bilirubin in vitro are not limited to antioxidative and antinitrosative effects, but also include potentially deleterious reactions. For example, the formation of copper complexes with conjugated biliary BR can accelerate copper-dependent lipid peroxidation (636). At micromolar concentrations, BR, like heme, binds to erythocyte membranes and acts as a hemolytic agent (329). High concentrations of BR can also inhibit mitochondrial function and the activities of several metabolic enzymes in vitro (124, 304, 439).

B. Protective Effects In Vitro and In Vivo

Several cell culture model studies have explored the possible capacity of the bile pigments as to act as cellular antioxidants. At micromolar concentrations in cell culture media, BR protected against cytotoxicity induced by H_2O_2 and/or enzymatically generated ROS, in endothelial cells, vascular smooth muscle cells, and cardiac myocytes (110, 462, 735). Furthermore, heme pretreatment conferred resistance to ROS-mediated cytotoxicity in vascular smooth muscle, which correlated with HO-1 expression and BR accumulation (110). In contrast to apparent neurotoxicity at high concentration, nanomolar concentrations of BR-albumin complexes protected primary neuronal cultures against H_2O_2 toxicity in vitro (147).

BR also protected against H_2O_2-mediated cell death in HeLa cells at a 10,000-fold molar ratio of oxidant to antioxidant (48). In this study, the reaction of BR with peroxyl radical generators was shown to result in the oxidation of BR to regenerate its metabolic precursor BV. BVR serves a principle metabolic function of reducing BV produced from heme. The authors proposed a second function of BVR as a regenerator of cellular antioxidant capacity by reducing the BV formed from the reoxidation of BR (48). Cellular depletion of BR by siRNA directed against BVR increased the intracellular levels of ROS and promoted apoptotic cell death in HeLa cells and primary neuronal cultures (48). The increase in cellular pro-oxidant status by BVR suppression exceeded that produced by depletion of intracellular GSH. Thus the authors refuted previous arguments that the intracellular antioxidant capacity of BR is superceded by the millimolar concentrations of GSH that typically occur in cells (438). In support of these observations, a recent study has also shown that BVR silencing sensitizes cells to stress from sodium arsenite, a classical activator of the HO-1 response (444). In endothelial cell culture, preinduction of HO-1 with heme arginate conferred anti-inflammatory protection against a posttreatment challenge of OxLDL and TNF-α. This protection was associated with an attenuation of proinflammatory responses and the restoration of NO tone. With respect to these effects, the addition of BR, but not that of CO, was found to duplicate the effects of HO-1 induction (310).

Potential protective effects of BR have also been determined in animal studies. In a rat model of hyperbilirubinemia, jaundiced Gunn rats displayed a reduced manifestation of plasma biomarkers of oxidative stress following exposure to hyperoxia, than in their nonjaundiced counterparts. These experiments led to the suggestion that a state of jaundice may confer antioxidant benefit to the blood (134). Similar studies have been conducted in Eizai rats, which are hyperbilirubinemic due to mutations in the canilucular multispecific organic anion transporter gene. In this model, the state of hyperbilirubinemia protected against neuronal I/R injury following occlusion of the middle cerebral artery relative to wild-type controls (330). A reduced expression of HO-1 and other damage markers was observed at the site of I/R in hyperbilirubinemic animals.

Irrespective of the metabolic purpose of endogenous bile pigment generation, current research has explored possible therapeutic benefits for the pharmacological administration of bile pigments in various in vivo models. Early experiments in this regard showed that injection of BR into rats protected the animals from hepatic GSH depletion caused by the subsequent administration of $CoCl_2$ or $CdCl_2$ (394, 502). The pharmacological administration of BR protected against hepatotoxicity during experimental endotoxinemia in rats (721). The protective effects of BR in this model were anti-inflammatory in nature, reflected by reduced serum nitrite levels and decreased TNF-α production. In a corresponding in vitro model, BR inhibited LPS-inducible iNOS activation and stimulated prostaglandin production (721). Intravenous injections of BR reduced tissue damage and inflammation

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in a model of bleomycin-induced pulmonary fibrosis in rats (715).

A number of studies have recently explored the potential of BV or BR as potential therapeutic agents in I/R stress models. In an isolated perfused heart model, heme preconditioning conferred protection against myocardial infarction following I/R injury, associated with increased HO-1 expression and BR formation. Administration of exogenous BR at nanomolar concentrations in this model also improved cardiac performance and reduced infarct size and mitochondrial dysfunction following I/R injury (111). In an isolated perfused kidney model, inclusion of BR (10 μM) in the perfusate protected against warm I/R-induced tissue injury and preserved renal function (12). BV provided tissue protection in an ex vivo model of cold hepatic I/R injury. Furthermore, inclusion of BV in the perfusate increased the survivability of rats undergoing orthotopic liver transplantation by preserving liver function (184). This protection afforded by BV occurred in association with the decreased expression of proinflammatory markers, including neutrophil influx, proinflammatory cytokine expression, and iNOS activation (184). BV treatment also improved the survivability of rat cardiac allografts, by reducing leukocyte infiltration and inhibiting T-cell proliferation (743). In independent experiments, little effect of BV or CO was observed individually, whereas the coadministration of BV and CO provided a synergistic tissue protection against transplant-associated cold I/R injury of heart and kidney grafts (474). A general antiproliferative effect of bile pigments has been further demonstrated in cellular and animal models. Exogenous BV administration inhibited neointimal hyperplasia associated with vascular balloon injury in rats (474, 499), while hyperbilirubinemic animals also displayed increased resistance to vascular injury (499). The antiproliferative effect of BV/BR was demonstrated in vascular smooth muscle cell culture, whereby exogenous BV/BR arrested cells in G_1 phase. The authors proposed a mechanism based on the inhibition of p38 MAPK and retinoblastoma protein phosphorylation in vitro (499).

Finally, a general anti-inflammatory effect of BR based on proposed ROS scavenging has been described. In a mouse asthma model, BR administration inhibited VCAM-1-associated airway inflammation and lung leukocyte influx, as well as inhibited leukocyte migration in vitro (315).

The possible antioxidant benefit of serum BR in limiting LDL oxidation and atherogenesis is supported by recent human clinical studies. Schwertner et al. (592) identified serum BR levels as an independent, inverse risk factor for coronary artery disease. These observations were extended to include an association between low serum BR and risk of peripheral vascular disease (68). A large-scale prospective study of British men revealed that subjects in the midrange of serum BR concentration were at the lowest incidence of ischemic heart disease relative to those subjects displaying the lowest or highest fifth of serum BR distribution (67). In healthy subjects, serum BR levels were found to be inversely correlated with two indicators for atherosclerosis, impaired flow-mediated vasodilation and carotid intimal/medial thickening (165).

In conclusion, a physiological antioxidant benefit of the metabolic conversion of heme to the elimination product BR has been suggested to occur in the circulating plasma, which is supported by oxidative stress resistance of hyperbilirubinemic rats. Despite its elimination from cells and tissues, current evidence supports a beneficial role for endogenously produced BR at a cellular level. This mechanism may underlie, in part, the cytoprotective properties of HO-1 in certain models (110, 147, 461, 462). The antioxidant properties of BR are potentially exploited for medicinal purposes. Currently, BV and BR remain viable experimental therapeutic agents for the treatment of several inflammatory disease states including transplant-associated I/R injury and vascular injury (12, 184, 473, 474, 499).

VI. CARBON MONOXIDE

A. Properties, Environmental or Endogenous Sources, and Toxicity

CO, a low-molecular-weight (F.W. 28.01) diatomic molecule, occurs naturally in the gaseous state under atmospheric temperature and pressure (M.P. = −205°C; B.P. = −191.5°C; density =1.250 g/l at 0°C) (708). CO is soluble in aqueous media (2.3 ml/100 ml at 20°C) and organic solvents (75, 539, 708). CO is relatively stable in biological systems relative to NO, a small gaseous molecule of similar structure and molecular size. Unlike CO, which has no unpaired electrons, NO is a free radical with one unpaired electron. NO reacts with other free radicals such as O_2^- (k = 4.3–6.7 × 10^9 $M^{-1} \cdot s^{-1}$) and assumes variable oxidation states by gain or loss of electrons (NO^+, NO^-) (206, 248, 732). CO and NO both function as heme iron ligands and form complexes with a number of hemoproteins and metalloenzymes (117, 408). CO will bind only to reduced (ferrous) iron centers, whereas NO may bind to both ferrous and ferric hemes (500, 754).

As a ubiquitous air pollutant, CO arises primarily from the partial combustion of organic molecules. Its large-scale environmental production results from the oxidation of natural hydrocarbon pools, catastrophic events such as volcanic emissions and forest fires, plant metabolism, and oceanic activity (539). CO also arises from human activities involving the burning of fossil fuels, in household or industrial processes, and as a major component of automobile emissions (708, 539). Individual exposures resulting in morbidity and mortality most com-

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monly occur from the improper use of furnaces, heaters, engines, or flame in inadequately ventilated areas. Due to its invisibility, and lack of odor, CO can present an especially dangerous inhalation hazard (708). Incidence rates of accidental death by CO poisoning have been reported as high as 2,100 per year in the United States (42, 755). The clinical manifestations of CO poisoning include dizziness, drowsiness, vomiting, headache, and loss of motor coordination (212, 515, 723). Prolonged exposures can cause respiratory difficulty, disorientation, chest pain, loss of consciousness, or coma and can ultimately result in death. Chronic exposure to sublethal concentrations may lead to memory loss and other cognitive and neurological complications (723). Symptoms of hypoxic CO poisoning begin to appear at 20% CO-Hb. Death likely occurs in the range of 50–80% CO-Hb (619, 708). Inhalation studies in rats have revealed that CO can cause oxidative damage in the brain, as evident by increased lipid peroxidation, and apoptotic cell death (517, 680, 681).

As first realized by Claude Bernard (ca. 1865), and characterized by Douglas in 1912, CO reversibly binds to hemoglobin, to form carboxyhemoglobin (CO-Hb) (149). The binding affinity of CO for the hemoglobin heme iron is ~240 times that of O_2 and thus CO competes against oxygen for occupancy of four possible binding sites (619). The partial occupation of these binding sites by two CO molecules (half-saturation) inhibits the release of O_2 from the remaining heme groups, evident in a leftward shift of the oxyhemoglobin dissociation curve. This property of CO reduces the O_2-carrying capacity of the blood to deliver O_2, causing the anemic hypoxia that accounts for the asphyxiating properties of CO (515, 619, 708). The CO-Hb complex can be reversible by removal of the CO source in favor of oxygen. In severe cases, oxygen therapy under hyperbaric conditions may be applied as an antidote (212). In addition to hemoglobin, CO can form complexes with reduced forms of metalloproteins including myoglobin (33, 707), sGC (196, 641), inducible NO synthase (631), cytochrome *P*-450 (217, 500), cytochrome-*c* oxidase (cytochrome aa_3) (314, 707), NADPH:oxidase (126), heme oxygenase (441), and other metabolic enzymes such as dopamine-β-hydroxylase (668) and tryptophan oxidase (308). The interaction of CO with cytochrome *P*-450 or cytochrome-*c* oxidase results in inhibition of enzymatic activity (73, 166). These events may represent cellular targets of toxicity at high concentrations of CO, but are superceded by the hemoglobin binding properties of CO as the underlying cause of inhalation-induced mortality (212).

In addition to environmental exposure, a considerable amount of CO arises endogenously as a product of ordinary metabolism. Sjostrand (613, 614) predicted the origination of this endogenous CO pool from the oxidation of the α-methene bridge carbon of heme. Using ^{14}C-heme labeled hemoglobins, Coburn et al. (118) demonstrated the metabolic conversion of hemoglobin to CO in dogs. The rate of endogenous CO production in humans was first estimated by blood carboxyhemoglobin analyses to ~0.42 ml/h (116). The association of endogenous CO production with a distinct enzymatic mechanism, HO, was first described by Tenhunen et al. (672) (see sect. II*A*). As a metabolite of HO-catalyzed heme degradation, CO is formally considered a catabolic waste product. In the absence of significant ambient CO, the majority of blood CO-Hb arises from endogenous production, corresponding to blood CO levels of 0.4–1% (708). These values increase with increasing environmental background. For example, cigarette smokers display an average range of 3–8% CO-Hb levels (539). At least 86% of endogenous CO production originates in heme metabolism, while the remaining fraction may arise from other metabolic processes including lipid oxidation (35, 711, 712) and xenobiotic metabolism (712). While the endogenous production of CO by nonheme sources is poorly defined under normal conditions, it is likely to gain increasing importance under pathological or toxicological situations such as exposure to halogenated hydrocarbons (218, 496, 634). In addition to responding to elevations in the environmental background, blood CO-Hb levels increase under pathological or toxicological conditions that produce global or tissue specific elevations of HO-1. Elevations of systemic HO-1 expression may occur as the product of inflammation, physical stress, or environmental exposure with a variety of agents. Increased levels of CO appear on the exhaled breath of humans in association with several proinflammatory conditions, including asthma and COPD (146, 244, 245, 404, 616, 748).

B. Mechanisms of CO-Dependent Cell Signaling

Recent research has revealed that CO has profound effects on intracellular signaling processes, which culminate in anti-inflammatory, antiproliferative, antiapoptotic, and anticoagulative effects (Fig. 8). The physiological effects of CO have been related to its endogenous production from basal and inducible HO activity. The exogenous application of CO produces similar effects as HO-1 in many models, although it remains unclear in some cases whether the doses under study represent physiological or superphysiological levels.

The physiological signaling effects of CO known to date involve relatively few defined mechanisms; the modulation of sGC activity and subsequent stimulation of cGMP production are the most commonly observed. Other mechanisms include the modulation of various MAPK activation, which, depending on the model, may or may not be linked to the former mechanism and the stimulation of Ca^{2+}-dependent K^+ channel activity. Be-

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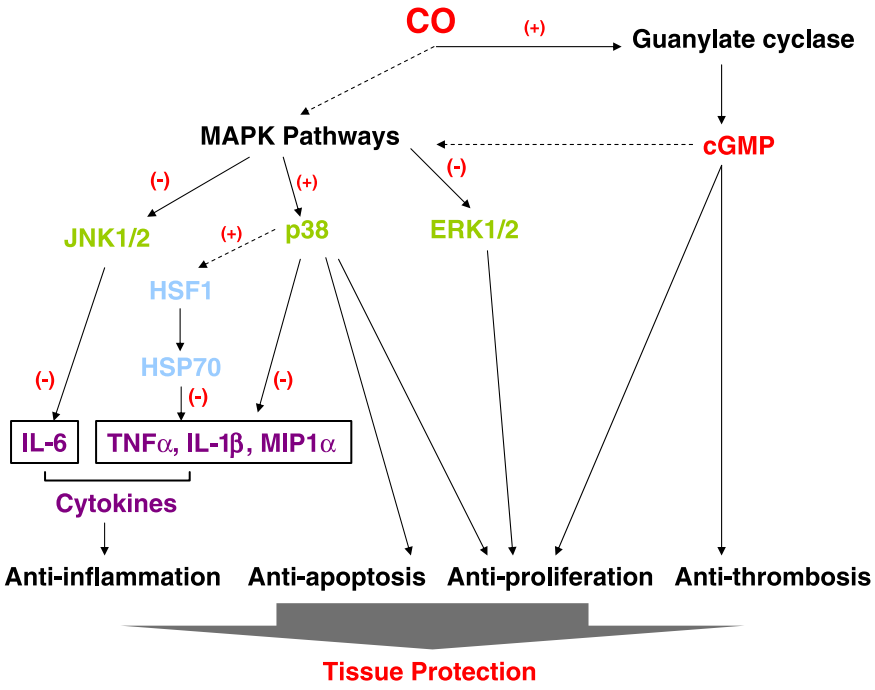

FIG. 8. Potential signaling pathways activated by CO leading to tissue protection. The diagram illustrates the possible signaling pathways affected by CO. CO can activate soluble guanylyl cyclase (sGC) akin to its classical regulator NO, with the resultant production of cGMP. CO can also modulate mitogen-activated protein kinase activities (MAPK), including the p38 MAPK, ERK, and JNK pathways. Most of the effects of CO described in this review depend on modulation of either pathways, which can be dependent or independent depending on the model. The action of CO on unknown heme-dependent targets cannot be excluded. CO may cause a general downregulation of proinflammatory cytokine production through p38 MAPK-dependent pathways leading to anti-inflammatory tissue protection. The heat shock factor 1 and Hsp70 may act as potential intermediates in this pathway (Kim et al., unpublished studies). The potential physiological effects of CO also include tissue-specific inhibition of apoptosis, cellular proliferation, and thrombosis.

cause the MAPK do not provide a binding target for CO, they represent a downstream rather than primary target of CO signaling (reviewed in Ref. 557).

Like the classical agonist NO, CO binds directly to the heme iron of sGC, leading to stimulation of enzymatic activity. The putative vasoactive properties of CO depend on, in part, the stimulation of sGC and subsequent elevation of cGMP levels (196). CO-mediated activation of sGC leads to a severalfold increase in cGMP production, with a potency of enzyme activation ~30–100 times lower than that of NO (196, 298, 408, 641). The importance of CO in sGC activation likely increases in cells or tissues with low endogenous NO production. In the presence of NO, however, CO may even act as a partial antagonist of NO function by competing against NO binding (298). The interaction of CO with the heme of sGC differs from that of NO, in that a hexacoordinate, rather than pentacoordinate, complex is formed without axial ligand displacement (196, 641). The dissociation of CO from sGC, however, proceeds through a pentacoordinate intermediate similar to that of NO binding, and this transition may be responsible for sGC activation by CO (321). The implications of these alternative-binding modes with respect to cell signaling are incompletely understood. The sensitivity of sGC to stimulation by CO can be greatly increased by interaction of the enzyme with the benzylindazole derivative YC-1 (192).

In classical experiments, the CO released from heme by the action of HO activity was shown to regulate cGMP production in vascular smooth muscle cells (VSMC) (454, 455). The treatment of VSMC directly with CO stimulated an increase in cellular cGMP levels (455, 536). Treatment of these cells with hypoxia also increased levels of cGMP. This effect also required HO induction and the production of endogenous CO, but not NO (454).

The role of the sGC/cGMP axis in the cellular and physiological effects of CO are determined experimentally, in addition to direct determination of cGMP production in the presence of CO, by observing a loss of function in the presence of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) or parallel effects of cGMP analogs such as 8-BrcGMP (249). The first approach is imperfect because it assumes a specificity of the chemical inhibitor, while the second approach is largely circumstantial. Experiments with HO-1$^{-/-}$ strains are not generally conclusive, since they cannot directly exclude physiological contributions of BR and/or iron metabolism. The role of this mechanism in various processes is discussed in the subsequent sections. These mechanisms are again discussed in the context of individual disease states in section VII.

C. CO Releasing Molecules: Biochemical Properties

In light of the general protective properties of the HO-1/HO-2 system, and the possible therapeutic applications of CO gas, as detailed for several disease applications in the subsequent sections, Motterlini and colleagues (112, 186, 459, 460, 464) have developed a novel class of chemical compounds designed for the targeted delivery and release of CO in tissues. These compounds provide a promising alternative to proposed therapeutic strategies involving the inhalation of CO and/or potential

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gene therapy approaches in humans dependent on targeted overexpression of HO-1/HO-2. Experiments with these novel compounds, termed "carbon monoxide releasing compounds" (CORM) have also provided further mechanistic insight into the behavior of CO in biological systems. The CORMs are transition metal carbonyls with the capacity to release CO (186, 459, 460, 464). The compounds contain a central metal (i.e., Mn, Bo, Ru, Fe) that is coordinated by carbonyl groups. The first of these to be developed (CORM-1), with the formula [$Mn_2(CO)_{10}$], was limited as a pharmacological agent by its insolubility in aqueous media and by its requirement for photoactivation to release the bound CO (459, 460). A second prototype with a ruthenium metal center, (CORM-2) or [$Ru(CO_3Cl_2)$], also hydrophobic, can release CO in organic solvent, depending on ligand displacement by the solvent (i.e., DMSO) (460). Further research led to improved water-soluble analogs of CORM more readily compatible with biological systems. These are named CORM-3 [$Ru(CO)_3Cl$(glycinate)] and CORM-A1 or boranocarbonate [$Na_2H_3BCO_2$] (112, 186, 464). Both CORM-3 and CORM-A1 spontaneously release CO in aqueous media by exchanging CO for water, with CORM-3 displaying the faster rate of CO release, and CORM-A1 displaying a pH dependence on the rate of CO release (112 186, 464). The CO releasing properties of each CORM have been validated by spectroscopically monitoring the formation of carboxymyoglobin from myoglobin (459, 460). The biological effects of these compounds are validated against an observed lack of effect with the deactivated form of the compound. The specific effects of CORM-derived CO on vascular effects such as dilation and proliferation, and the protective properties of CORMs in disease models including I/R injury, and inflammatory responses are described in additional detail in the subsequent relevant sections in parallel with observations reported for exogenous CO gas.

D. Vasodilation

The vasodilating properties of exogenous CO have long been recognized (115). In 1978, Sylvester et al. (653) demonstrated that CO (11.5% in inspired gas) dilated the pulmonary artery under normoxia and reversed the vasoconstrictive effects of hypoxia in isolated perfused porcine lung. At the time, this was attributed to modulation of cytochrome *P*-450 activity by CO binding, since another cytochrome *P*-450 ligand metyrapone elicited a similar response (653). In isolated perfused rat hearts, McFaul and McGraith (435) observed that the presence of CO (equilibration with 5% CO) in the perfusate significantly decreased coronary flow and also reversed the vasoconstrictive effects of methoxamine (388). The observed effects on CO-induced vasodilation did not depend on intact endothelium or resulted from tissue hypoxia caused by the CO (435). Similary the inclusion of CO in the perfusate dilated precontracted rat thoracic aortas in an endothelium-independent manner (383). Further examples of CO vasodilatory action in large animal vessels include the lamb ductus arteriosis (119), porcine coronary and artery and vein (213), and rabbit aorta and dog coronary artery (196). In a comparative study of the vasoactive effects of NO and CO in isolated rabbit aorta, exogenous CO produced an endothelial-independent vasorelaxant response, albeit with a 1,000-fold less potency than NO under the same conditions (196). The vasodilatory properties of CO in the rabbit aorta were attributed to activation of sGC and generation of cGMP by CO, also with lesser potency than NO (196, 213, 249, 421).

Graser et al. (213) demonstrated the inhibition of CO-dependent vasodilation by methylene blue, a nonspecific inhibitor of sGC and a general vasorelaxant effect of 8-BrcGMP (213). In rabbit aortic rings, CO-dependent vasodilation was abolished by the specific sGC inhibitor ODQ, further validating the requirement for sGC in this system (249). This general mechanism is supported by observations that exogenous or endogenous (HO-derived) CO can elevate cGMP levels in vascular smooth muscle (106, 455, 536). The effects of CO on sGC are not limited to the vascular smooth muscle, but also airway smooth muscle, whereby exogenously administered CO stimulated cGMP-dependent airway bronchodilation in guinea pig trachea following histamine treatment (83).

The widespread distributions of inducible HO-1, and HO-2 in vascular tissues, including endothelium and vascular smooth muscle, are consistent with the proposed roles for endogenous HO-derived CO in vasoregulation (106, 159, 773, 776, 777). A number of studies have observed vasoconstrictive (87, 341, 342, 643, 773) or vasodilatory (282, 370) effects of metalloporphyrin inhibitors of HO, dependent on experimental conditions. In an isolated perfused rat liver model, inclusion of ZnPPIX diminished CO levels detectable in the effluent, and increased perfusion pressure under constant-flow conditions. These effects were reversed by exogenous CO or cGMP analogs in the perfusate (643). In the presence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), the HO inhibitor SnPPIX further attenuated acetylcholine-dependent vasorelaxation in porcine aortic rings (773). Conversely, the endothelium-dependent contractile response to phenylephrine in thoracic aortic rings was augmented in the presence of both ZnPPIX and N^{ω}-nitro-L-arginine (NNA), relative to treatment with NNA alone (87). Thus these effects are dependent on heme degradation and independent of NOS activity or NO generation. In this system, exogenously applied CO also relaxed the aortic rings in a cGMP-dependent fashion. Both CO and heme L-lysinate treatment dilated arterioles in vitro, in the presence of L-NAME. The responses to heme L-lysinate were inhibited

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by metalloporphyrins (i.e., CrMP), leading to the conclusion that HO-derived CO acted as a natural mediator of the heme-dependent arteriolar vasodilation in the absence of NOS activity (341, 370). Treatment of pressurized gracilis muscle arterioles with the HO inhibitor CrMP in the presence of L-NAME amplified pressure-induced vasoconstriction that could be reversed by exogenous CO (342). Further studies with this model demonstrated downregulation of HO-2 with antisense oligonulceotides or CrMP treatment increased pressure-dependent contraction (777).

Additional evidence for a role for HO in vasoregulation is provided by ex vivo models. Overexpression of HO-1 by AdHO-1 infection of the vessels inhibited phenylephrine-dependent vasoconstriction in isolated aortic rings, also in a fashion dependent on heme degradation and independent of NO production (155).

Thorup et al. (683) raised the possibility that CO-dependent vasodilation may involve modulatory roles for NO. In renal resistance arteries at low concentrations (up to 100 nM), CO dose-dependently increased NO release from internal stores as measured by amperometric methods. High concentrations of CO, however, inhibited NO production and eNOS activity in these vessels (683). Recent findings of Johnson and co-workers (282, 283) have indicated that in competition with proposed vasodilatory mechanisms, CO may exert a competing vasoconstrictive effect by inhibiting the endothelium-dependent enzymatic generation of NO. In gracilis muscle CO dilated blood vessels in the presence of L-NAME, or under conditions of NO clamp (L-NAME plus NO donor), but constricted blood vessels in the absence of L-NAME. This effect was lost in endothelium-depleted vessels (282). Under these conditions, CrMP reversed apparent CO-dependent vasoconstriction (282).

Additionally, it has been proposed that SMC-derived CO may exert indirect effects on vasoregulation through paracrine effects on endothelial cells. SMC-derived CO caused the cGMP-dependent downregulation of the expression of mitogen endothelin-1 (ET-1) and PDGF-B in endothelial cells, thus indirectly inhibiting SMC proliferation (453).

Although cGMP appears to play a major role in CO-induced vasodilation in aortas, not all experimental systems have supported a major role for cGMP in CO-inducible dilation (119). In the ductus arteriosis of the lamb, guanylate cyclase inhibitors, while reversing the action of NO donor, exerted marginal effects on CO-dependent dilation (119). Monochromatic light at 450 nm reversed the dilatory effect of CO in this system, leading the authors to conclude a mechanism dependent on a cytochrome *P*-450-derived species. Recently, cGMP-independent mechanisms of vasoregulation by CO have been further characterized in the peripheral vasculature, including pial arterioles of the newborn pig (370), gracilis muscle arterioles (777), and rat-tail artery (719). In these systems CO may dilate blood vessels in part by directly activating calcium-dependent potassium channels (K_{Ca}) (277, 297, 333, 719). Wang et al. (719), using precontracted rat-tail arteries, showed that CO induced an endothelial-independent vasodilation. Partial inhibition by either *R*p-8-BrcGMP or the K_{Ca} inhibitor charybdotoxin indicated that the response depended partially on cGMP and partially on large-conductance K_{Ca} (719). In SMC derived from rat-tail arteries, CO caused hyperpolarization of the cell by increasing outward K^+ current, which in turn inhibited voltage-gated Ca^{2+} channels causing smooth muscle relaxation (720). Furthermore, CO increased the open probability and Ca^{2+} sensitivity of single K^+ channels in SMC (720). In renal intralobar arteries, inhibition of HO activity by treatment with CrMP, or downregulation of HO-2 with antisense oligonulceotides, reduced endogenous CO production (297). Decreased endogenous CO production was in turn associated with a decreased number of open K^+ channels (105 pS K) in intralobar artery smooth muscle cells and with increased vascular contractility. These effects could be reversed by application of exogenous CO (297). Furthermore, inhibition of HO-2 expression by antisense oligonucleotides reduced the EC_{50} of the contractile response to phenylephrine (297). The authors concluded that CO produced endogenously in smooth muscle caused a dilatory response in part by reducing the sensitivity to vasoconstrictor agonists by a K^+ channel-dependent mechanism (297). Similarly, the attenuation of pressure-induced vasoconstriction by CO in gracilis muscle arterioles and the CO-dependent dilation of porcine cerebral arterioles were abolished by the K^+ channel blockers tetraethylammonium or iberiotoxin (370, 777). The vasodilatory action of CO in porcine cerebral arterioles (370) was further attributed to an increase in the effective coupling of calcium sparks to K_{Ca} channels (277). In this system, the Ca^{2+}-release channel blocker ryanodine, which inhibits Ca^{2+} sparks, also inhibited the CO-induced vasodilation (277). Further work with porcine cerebral arterioles has demonstrated a possible accessory role for NOS-derived NO and for cyclooxygenase-derived products in CO-dependent cerebral vasodilation, as respective inhibitors of these pathways also attenuated the response (369). Recent studies have suggested the direct association of HO-2 with large-capacity K^+ channel in the carotid body (730). It remains to be determined whether a similar physical association between HO-2 and K_{Ca} channels occurs in vascular tissue.

Experiments with CORMs further validate a role for CO in vasodilation. CORM-2 exerted potent vasodilatory effects when applied to isolated rat aorta (460). The water-soluble derivatives CORM-3 and CORM-A1 dilated phenylephrine-precontracted isolated aortic rings with a relative effectiveness consistent with the rate of CO release by these molecules. The vasodilation elicted by

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CORM-3 and CORM-A1, similar to observations using CO gas, potentially involved both the elevation of cGMP and the activation of ATP-dependent K^+ channels (186, 464). The vasodilation elicited by CORM-3 required intact endothelium and an accessory role for endogenous NO production (186).

In conclusion, CO exerts a variable and multimodal effect on vasodilation, which involves at least several mechanisms. The dilatory effects of CO are attributed to direct endothelium-independent effects on vascular SMC, including the modulation of cGMP and K^+ channel activity in vascular SMC, and indirect effects on the expression of endothelial-derived vasoconstrictors and myogenic factors. An accessory role for NO mobilization has been proposed in some models of CO-dependent vasodilation, whereas a competing constrictor effect of CO has also been proposed through the inhibition of enzymatic endothelial-dependent NO production. Finally, neural CO may play an indirect role in vasoregulation by signaling in the autonomous nervous system (see sect. VI*F*).

E. Antiapoptotic, Anti-inflammatory, and Antiproliferative Mechanisms

1. *Antiapoptotic effects*

The antiapoptotic potential of CO was first demonstrated in vitro. Exposure of cell cultures to exogenous CO inhibited TNF-α-initiated apoptosis in mouse fibroblasts (516) and endothelial cells (70). A similar in vitro antiapoptotic effect was observed with HO-1 overexpression (516). In the endothelial cell model, the inhibitory effect of CO on TNF-α-induced apoptosis could be abolished with the selective chemical inhibitor SB203580 or a p38 MAPK dominant negative mutant, implying a critical role for the p38 MAPK pathway (70). Furthermore, HO-1 or CO cooperated with NF-κB-dependent antiapoptotic genes to protect against TNF-α-mediated endothelial cell apoptosis (69). CO is not necessarily antiapoptotic in all cell models. A contrary report suggested proapoptotic effects of CO in endothelial cells even at low concentration (682). In Jurkatt T cells, CO treatment increased Fas/CD95-induced apoptosis, associated with the downstream activation of caspases, and the inhibition of antiapoptotic Bcl family members (i.e., Bcl-X_L). In this cell type, the proapoptotic effect of CO in the context of Fas/CD95-induced apoptosis was associated with downregulation of ERK1/2 activation by CO (626).

However, in several models of disease and/or tissue injury, including I/R injury and lung transplantation, a net antiapoptotic effect of low dose CO pretreatment has been observed in vivo (see sect. VII). Nonetheless, higher concentrations of inhalation CO in rodent models cause tissue apoptosis particularly in brain regions, associated with CO poisoning and tissue injury (517, 680, 681).

2. *Anti-inflammatory mechanisms*

Recent studies have determined anti-inflammatory effects of both HO-1-derived CO and exogenous CO in cell culture models and animal models of sepsis (see also sect. VII) (504). In a murine macrophage model (RAW 264.7), an LPS challenge (1 μg/ml) stimulated the production of the proinflammatory cytokines (i.e.. TNF-α). TNF-α production was inhibited in transfected RAW 264.7 macrophage cells overexpressing HO-1, compared with that observed in control transfectants. Exogenously administered CO (250 ppm) also inhibited the production of TNF-α in RAW 264.7 cells in response to LPS treatment, indicating that CO can replace HO activity in mediating these effects (Fig. 9). The pretreatment of RAW 264.7 cells with exogenous CO before LPS treatment inhibited the expression of additional proinflammatory cytokines (i.e., IL-1β and the macrophage inflammatory protein-β, MIP-1β). Conversely, the production of the anti-inflammatory cytokine IL-10 was augmented by the CO treatment. LPS treatment activated the p38 MAPK, ERK1/2, and JNK pathways in RAW 264.7 macrophages. In the presence of LPS, CO increased p38 MAPK activation but did not modulate ERK1/2 and JNK activation. CO treatment of RAW 264.7 cells enhanced the LPS-mediated stimulation of MKK3 and MKK6, two kinases that supercede p38 MAPK (140, 532). In contrast to smooth muscle models, CO treatment did not significantly modulate cGMP production in RAW 264.7 macrophages. Pretreatment of the RAW 264.7 macrophages with a nonhydrolyzable cGMP analog or the NOS inhibitor L-NAME did not alter the anti-inflammatory effects of CO on TNF-α production.

In the same macrophage model (Raw 264.7), additional mechanisms by which CO downregulates the inflammatory response were explored. In contrast to the observations with TNF-α, the downregulation of LPS-inducible IL-6 production by CO treatment required the JNK pathway (456). Mutagenesis experiments demonstrated that the ability of CO to inhibit LPS-inducible IL-6 production required a functional AP-1 site in the IL-6 promoter but did not require nuclear factor κB (NF-κB) or CCAAT/enhancer-binding protein (C/EBP) binding sites. Recent experiments with CORMs provide an additional perspective on the anti-inflammatory properties of CO (578). In stimulated macrophages, application of CORM-2 or CORM-3 downregulated the inflammatory response as evidenced by reduced TNF-α and nitrite production after stimulation with LPS, without affecting iNOS expression. In this system, application of CORMs produced a secondary activation of HO-1 (578). The anti-inflammatory effects of HO-1 expression have also been associated with downregulation of inducible endothelium-derived proinflammatory mediators, including the heme-dependent stimulation of the intracellular adhesion molecule-1

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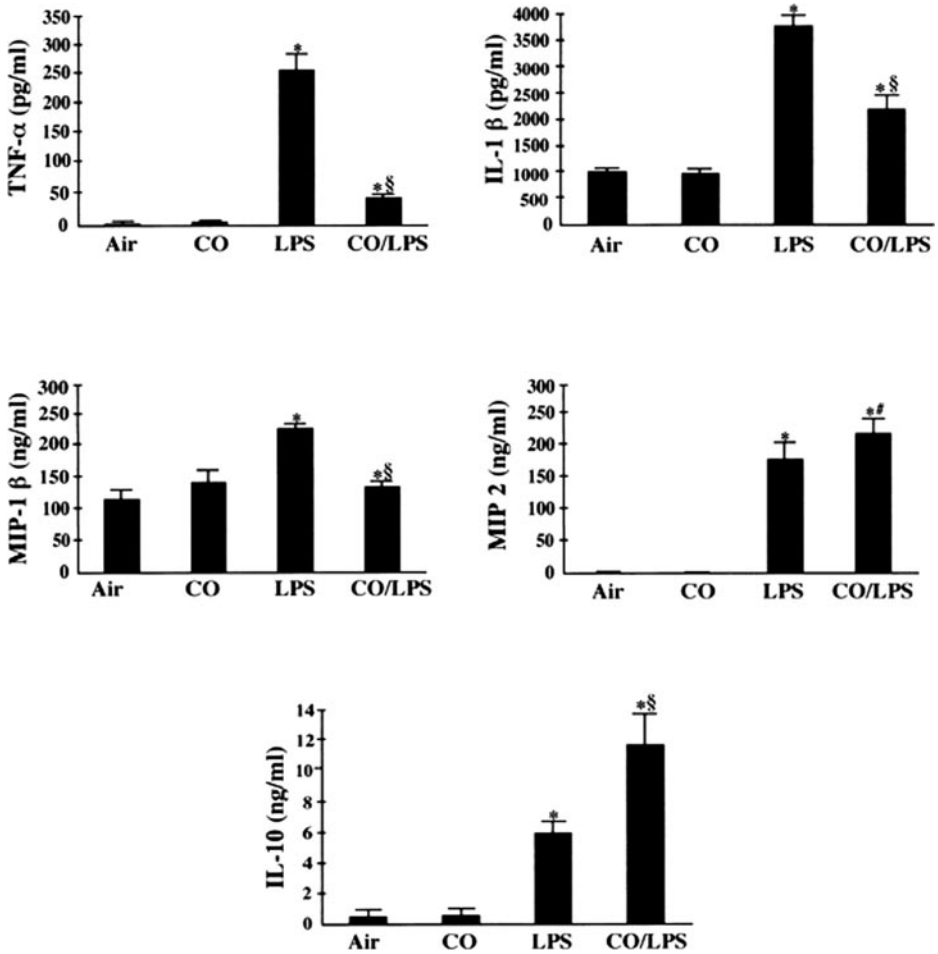

FIG. 9. Effects of CO on LPS-induced cytokine production in vitro. RAW 264.7 macrophages were pretreated with 250 p.p.m. CO before being treated with LPS. Culture media were collected and analyzed by ELISA. *Top left*: TNF-α, $^{\S}P < 0.0001$ compared with LPS. *Top right*: IL-1β, $^{\S}P < 0.001$ compared with LPS. *Middle left*: MIP-1β, $^{\S}P < 0.005$ compared with LPS. *Middle right*: MIP-2, $^{\#}P < 0.3$ compared with LPS. *Bottom:* IL-10, $^{\S}P < 0.05$ compared with LPS. In all panels, $^{*}P < 0.001$ compared with air or CO alone. Data represent the mean values ± SE of 6 independent experiments. [From Otterbein et al. (504).]

(ICAM-1) (713) and the TNF-α-dependent stimulation of E-selectin and VCAM-1 (621).

CO also affected the expression of granulocyte-macrophage colony stimulating factor (GM-CSF) in several models. GM-CSF is a glycoprotein that promotes the proliferation and the differentiation of hematopoetic progenitor cells into neutrophils and macrophages. Besides this function, GM-CSF also plays a critical role in antigen- and complement-mediated phagocytosis and antitumor immunity. Elevated GM-CSF levels appear in chronic inflammatory pulmonary diseases like asthma, COPD, or sarcoidosis and have been linked to pulmonary alveolar proteinosis. Many cell types produce GM-CSF (i.e., fibroblasts, endothelial cells, airway smooth muscle cells, and T lymphocytes). GM-CSF enhances the secretion of proinflammatory cytokines including TNF-α, IL-1, interferon (IFN)-γ, as well as inflammatory mediators (i.e., superoxide anion, E-series prostaglandins, leukotrienes, arachidonic acid, plasminogen activator, and other colony stimulating factors) (541). Macrophages treated with GM-CSF display a higher TNF-α production and cytotoxicity in response to in vitro stimulation with IFN-γ than nontreated cells (60).

RAW 264.7 cells treated with LPS increased the production of GM-CSF, which also could be attenuated by CO pretreatment. CO treatment inhibited the LPS-induced activation of NF-κB, by preventing the phosphorylation and degradation of the inhibitory subunit I-kBα, and this mechanism was associated with GM-CSF modulation, although the relative participation of MAPK and/or cGMP was not established in this model (571). CO also affected GM-CSF production in an in vitro asthma model. Human airway smooth muscle cells (HASMC) were treated with cytomix (TNF-α, IL-1β, INF-γ, and LPS), which stimulated the release of GM-CSF. IL-1β was able to stimulate GM-CSF release alone, while TNF-α and INF-γ stimulated GM-CSF only as components of cytomix. CO inhibited the induction of GM-CSF protein stimulated by cytomix, LPS, and IL-1β alone. Western blotting analysis showed that in IL-1β-stimulated HASMC, the ERK1/2 MAPK pathway was activated, and this activation was blocked with CO. The inhibitory effect of CO on GM-CSF was abolished with the specific cGMP inhibitor ODQ. These results show in HASMC that CO can carry out its anti-inflammatory effect by activating ERK1/2 MAPK in a cGMP-dependent fashion (625).

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3. Antiproliferative mechanisms

The inhibitory effects of CO on cell growth were first examined by Morita et al. (455). CO exerted a general antiproliferative effect in hypoxic smooth muscle cells that could be reversed by chemical inhibitors of sGC (455). CO produced endogenously and released from vascular smooth muscle cells (SMC) as a consequence of hypoxia-inducible HO-1 expression elevated the endogenous production of cGMP of endothelial cells in coculture. The inhibition of vascular SMC proliferation by CO in this model was also associated with a cGMP-dependent downregulation of the expression of endothelial-derived mitogens such as PDGF and ET-1 (453). These investigators were also the first to suggest that CO inhibited cell growth by influencing the expression and/or activation of cell cycle-related factors. Among the initial mechanisms proposed was the inhibition of a cell cycle-specific transcription factor E2F by CO (454). SMC proliferation is regulated by cell cycle-dependent kinases and cyclin complexes, which are under the control of the cell cycle inhibitor $p21^{Waf1/Cip1}$. CO treatment induced $p21^{Waf1/Cip1}$ expression in SMC (508). The antiproliferative effect of CO was inhibited in SMC from the $p21^{Waf1/Cip1}$ knockout mice ($p21^{-/-}$). Although $p21^{Waf1/Cip1}$ is regulated by p53, the absence of the p53 gene in SMC derived from the knockout mice ($p53^{-/-}$) did not affect the antiproliferative effect of CO. Similar to the anti-inflammatory models, CO treatment activated p38 MAPK in SMC. Both the antiproliferative effect and the upregulation of $p21^{Waf1/Cip1}$ by CO treatment depended on the activation of p38α,β MAPK, since these effects were inhibted by SB203580. Treatment of SMC with the guanylyl cyclase inhibitor ODQ abolished the effects of CO on SMC proliferation and activation of p38 MAPK. Furthermore, the nonhydrolyzable cGMP analog 8-BrcGMP activated p38 MAPK and increased $p21^{Waf1/Cip1}$ in SMC. Finally, 8-BrcGMP inhibited proliferation in wild-type but not $p21^{-/-}$ SMC (508). By this mechanism, CO was shown to protect against vascular stenosis associated with vascular transplant and balloon catheter injury (508) (see sect. VII). Further studies suggest that the antiproliferative effects of CO in SMC including the reciprocal modulatory effects on $p21^{Waf1/Cip1}$ and cyclin A expression may be mediated in part by stimulation of caveolin-1 expression by a p38β MAPK-dependent mechanism (328). In experiments using CORM-2, the pharmacological application of CO was also demonstrated to inhibit SMC proliferation. The authors proposed a signaling pathway involving the increased production of mitochondrial ROS and the downregulation of cytosolic ROS as a consequence of NAD(P)H:oxidase inhibition, leading to ERK1/2 downregulation and decreased cyclin D expression (654).

The proliferation of SMC may also play an important role in the pathogenesis of asthma. Airway remodeling determined by SMC proliferation may contribute to airway hyperreactivity. The SMC in the airway respond to inflammatory cell-derived mediators with increased proliferation and release of inflammatory mediators. As in other SMC models, CO treatment suppressed the proliferation of HASMC. Cell cycle analysis showed that CO affected the G_0/G_1 phase of cell cycle. In HASMC, CO exposure increased the expression of $p21^{Waf1/Cip1}$ and decreased cyclin D1. While CO activated ERK1/2 MAPK in this model, ODQ failed to attenuate the CO effect (623). These results directly contrast to the observation that an anti-inflammatory effect of CO in HASMC depended on cGMP-dependent regulation of ERK1/2 (623, 625). The signaling pathways affected by CO apparently can vary in a cell type-specific manner and even in the same cell type in response to different cellular process (e.g., inflammation and proliferation) (623, 625).

The antiproliferative effects of CO were recently also examined in a model of CD3 antibody-induced T-cell proliferation. In addition to their well-known role in apoptosis, caspases can participate in T-lymphocyte activation and proliferation (398). Leukemia cell lines (Jurkat T cells) and primary cultured T lymphocytes increase caspase activity during cellular proliferation. Treatment of lymphocytes with anti-CD3 antibody stimulated cellular proliferation in primary cell cultures. In anti-CD3 activated cells, treatment with CO (250 ppm) caused a marked decrease in cellular proliferation (624). Unlike the model of Fas/CD95-induced apoptosis as described above, CO did not increase cell death in the absence of proapoptotic stimuli. Primary cultured T lymphocytes displayed a diminished activation of caspase-3 and caspase-8 in the presence of CO. Furthermore, CD3 antibody-stimulated T lymphocytes treated with CO displayed an increased expression of the cell cycle inhibitor $p21^{Waf1/Cip1}$ in association with the observed antiproliferative effect. Lymphocytes isolated from $p21^{-/-}$ mice showed a partial decrease in caspase activity. This study suggests that CO can exert antiproliferative effects via the $p21^{Waf1/Cip1}$ pathway in lymphocytes as well as in SMC. Experiments with knockout cells determined that, in the T-lymphocyte model, this effect was independent of either MKK3 or JNK pathways. These experiments illustrate that the pathways mediating CO-dependent antiproliferation may vary in a cell type-specific fashion.

F. Neurotransmission

Accumulating evidence to date suggests possible roles for HO-derived CO as a signaling intermediate in the brain and central nervous system (CNS) in a number of neurological processes that include olfactory signal transduction (702), long-term potentiation (LTP) (630, 790), nonadrenergic noncholinergic (NANC)-dependent muscle

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relaxation (538, 774), cholinergic regulation of circadian rhythms (36), the autonomic regulation of cardiovascular function (284), and oxygen-sensing processes (730). These mechanisms occur independently of, and in addition to, the potential cytoprotective, antiapoptotic, and anti-inflammatory effects of HO/CO, as described for other cell types (see sect. VI*E*) that may, in principle, also apply to neuronal cell types. As in other tissues, CO may act as a mediator of HO-dependent neuroprotection; however, competing mechanisms, such as BR formation, have also been proposed (147). The widespread distribution of HO isozymes in the brain is consistent with a signaling role for HO-derived CO. HO-2 occurs at a high constitutive level in the rat brain (127, 172, 406, 688, 702), with a concentration in olfactory epithelia and olfactory bulb and a general distribution that includes the hippocampus, hypothalamus, thalamus, dentate gyrus, cerebellum, and brain stem (172, 702, 705, 742). In contrast, HO-1 expression is detected under basal conditions in the brain only in discrete neurons of the dentate gyrus, hypothalamus, cerebellum, and brain stem (705). The expression of HO-1 in the brain responds to induction by stress conditions including whole body hyperthermia (170, 171), traumatic brain injury (194), and focal or global cerebral ischemia in rat models (199).

Many of the suggested functions of CO in neurotransmission are linked to the activation of the sGC/cGMP system by CO in the brain. One of the first studies implying such a mechanism demonstrated that the neuronal localization of HO-2 mRNA in brain sections overlaps that of the guanylate cyclase-2 subunit of sGC (702). The distribution of HO-2 mRNA also colocalized with that of NADPH:cytochrome *P*-450 reductase, the essential cofactor for HO activity, and that of δ-aminolevulinic acid synthase, the rate-determining step in heme synthesis (702). The colocalization of HO-2 with sGC occurred in areas lacking neuronal NO synthase (nNOS) expression. Conversely, the neuronal localization of nNOS mRNA or activity only partially overlapped with that of HO-2 and sGC (702, 705). These studies suggested a role for HO/CO in sGC activation on the basis of spatial colocalization of heme metabolic enzymes with sGC. In functional experiments, cultured olfactory receptor neurons (ORN) deficient in nNOS activity elevated endogenous cGMP production when stimulated with odorant chemicals. This effect could be reversed by the NO/CO scavenger hemoglobin and by the metalloporphyrin ZnPPIX, but not by NOS inhibitors, implicating a role for HO/CO in the regulation of neural sGC (702). The endogenous production of CO in ORN was estimated at 4.7 pmol · mg protein^{-1} · min^{-1} in metabolic labeling experiments (263, 264). Furthermore, odorant stimulation of ORN increased the phosphorylation and activity of HO-2, with corresponding increases in cGMP (58). The activation of HO-2/cGMP could be induced by phorbol esters and antagonized by inhibitors of casein kinase II, suggesting that neuronal HO-2 activity and cGMP production are regulated by phosphorylation cascades (58). The exogenous application of CO also stimulated cGMP production in ORN (263, 264). Neurons expressing NOS (i.e., cultured cerebellar granules), however, produced much less CO (3 fmol · mg protein^{-1} · min^{-1}) than that produced by ORN (263, 264). NO acted as the major agonist of cGMP production in cerebellar granules, whereas CO acted as an antagonist of NO-dependent cGMP production (263, 264).

Several reports suggest a role for CO as a retrograde messenger between post- and presynaptic neurons during LTP, a process associated with learning and memory (26, 230, 255, 605, 630, 790). In voltage-clamped CA1 pyrimidal cells in hippocampal slices, tetanic LTP stimulation could be inhibited by ZnPPIX (630). Exogenous CO amplified LTP in hippocampal slices in the presence of tetanic stimulation and reversed the inhibitory effects of metalloporphyrins on LTP (26). The effects of NO/CO on LTP could be duplicated by 8-BrcGMP, indicating relevance to sGC (230, 790). These observations have been challenged by inconsistencies in the interpretation of metalloporphyrin data (437). Furthermore, LTP and its responsiveness to inhibition by metalloporphyrins remained fully intact in *ho-2*$^{-/-}$ mice, refuting a role for HO-2 in this process (524).

CO has been strongly implicated as a neurotransmitter in NANC-dependent smooth muscle relaxation (538, 774). Electrical stimulation of NANC-dependent intestinal smooth muscle relaxation and cGMP formation were diminished in *ho-2*$^{-/-}$ mice, relative to wild-type mice (774). In the internal anal sphincter, CO treatment relaxed smooth muscle by increasing cGMP production, whereas ZnPPIX inhibited NANC-dependent smooth muscle relaxation (538). CO causes smooth muscle relaxation in other models, including ilial, urethral, and airway smooth muscle (32, 83, 699, 774). Experiments from single or double knockout animals for HO-2 and/or NOS suggest an essential role for CO in intestinal smooth muscle relaxation, with a cooperative, but not independent, role for NO (737).

The HO/CO system potentially plays an intermediate role in the autonomic control of cardiovascular tone by glutamatergic signaling (182, 284). Within the nucleus tractus solitarii (NTS), a nexus of nerve endings for cardiovascular chemo- and baroreceptors, the neurotransmitter L-glutamate depresses blood pressure and heart rate by binding metabotropic L-glutamate receptors, which are potentially linked to the HO-2/CO/cGMP axis. The systemic injection of the HO inhibitor ZnDPBG caused arterial vasoconstriction and suppression of the baroreceptor reflex control of heart rate in rats (284, 285). Direct application of ZnDPBG into the NTS exerted a vasopressor response that could be reversed by exogenous CO (284). ZnPPIX antagonized the bradycardic and

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vasodepressor effects of L-glutamate, heme, and other metabotropic L-glutamate receptor agonists (204, 380, 381, 395, 396, 610).

CO potentially plays a role in the control of ventilation by the carotid body, which senses changes in blood gases and signals to respiratory neurons to release neurotransmitters (525). Potassium channel activity in the carotid body, which is inhibited by hypoxia, is inversely proportional to carotid body activity (375). By this mechanism, low arterial P_{O_2} is translated into increased ventilation. A regulatory role for heme-dependent oxygen-sensing molecules has been implicated in the hypoxic regulation of carotid body potassium channels (76, 528). CO apparently displayed an inhibitory role in carotid body activity, while ZnPPIX stimulated carotid body activity. HO-2 localized, by immunocytochemistry, to glomus cells of the carotid body in feline and rodent models (526, 527). A recent study demonstrated that HO-2 directly associates with the large-capacity calcium-sensitive potassium channels in the carotid body (730). Under normoxia, the HO-2-derived CO increases channel activity. Channel activity was reduced by RNA interference against HO-2 but restored by application of CO. Under low P_{O_2}, the inhibition of channel activity by hypoxia was associated with the reduced output of CO from HO-2 (730). This study suggested that the HO-2/K^+ channel complex acts as the principle oxygen sensor in the carotid body.

CO potentially plays a signaling role in cholinergic neurotransmission and in the regulation of circadian rhythms by the hypothalamus (36). Increases of HO activity within the superchiasmatic nucleus of the hypothalamus were found to coincide with the dark phase of the cycle (551). The neurotransmitter acetylcholine regulates the clock through the activation of muscarinic receptors linked to the downstream production of cGMP. Cholinergic effects on circadian clock timing were stimulated by heme and inhibited by metalloporphyrins, whereas a selective NOS inhibitor lowered basal cGMP levels but did not effect cholinergic stimulation of cGMP (36). A mammalian transcription factor that is potentially directly regulated by CO has recently been described. This factor, neuronal PAS domain protein 2 (NPAS2), is a homolog of CLOCK, a transcription factor involved in circadian activity but also present in cells outside the CNS. Binding of CO to the heme domain of NPAS inhibits its heterodimerization and subsequent DNA binding activity (144).

In summary, the evidence surrounding the role of CO in neurotransmission largely depends on enzyme colocalization studies, metalloporphyrin inhibition profiles, loss of function in transgenic knockout animals, and amplification of function by direct instillation of gas or gas donors. Metalloporphyrin data, due to questions of specificity and pleiotropy, have attracted much criticism (85, 216, 437, 540). In general, a potential overlapping and/or competing role for NOS-dependent NO generation has been implied, which may vary in relative importance in a tissue- and process-specific manner. With the exception of LTP, most of the neural processes where CO is implicated have been associated with the activation of HO-2 and the downstream regulation of sGC by CO. The source of heme for HO-dependent neurotransmission remains enigmatic. The identification of a "regulatory" pool of heme in the brain would lend further support for these studies.

VII. PROTECTIVE ROLES OF HEME OXYGENASE-1/CARBON MONOXIDE IN DISEASE MODELS

While the HO-1/CO system has been implicated as a protective mediator in numerous experimental models of disease states, the importance of HO-1/CO in clinical medicine has recently been emphasized by the discovery of a child diagnosed with HO-1 deficiency who exhibited a growth-inhibited phenotype and extensive endothelial damage. The subject suffered from persistent hemolytic anemia characterized by marked erythrocyte fragmentation and intravascular hemolysis, with increased serum haptoglobin, ferritin, and low BR levels. An abnormal coagulation/fibrinolysis system, associated with elevated thrombomodulin and von Willebrand factor, indicated the presence of severe, persistent endothelial damage. Iron deposition was noted in the kidneys and liver (311, 738). By comparison, growth retardation, anemia, tissue iron accumulation, and susceptibility to oxidative stress are similar characteristics observed in *ho-1* gene-deleted mice (522, 523).

In the following sections we describe the potential for endogenous HO-1 expression, its application by gene therapy approaches, and the administration of native or pharmacological CO as mediators of tissue protection in various disease models. We have limited the scope of this discussion here to diseases originating in the cardiovascular and pulmonary systems and to tissue damage related to oxidative or inflammatory stress and/or ischemia/reperfusion (I/R) injury.

A. Inflammatory Diseases

The general anti-inflammatory effect of CO observed in cultured macrophages in vitro (see sect. VI*E*) can also apply to in vivo models of inflammatory diseases, among which include sepsis and asthma.

1. Sepsis

Sepsis, a systematic inflammatory response to infection, is the leading cause of mortality in intensive care units. Until now, therapeutic approaches have failed to

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dramatically reduce the incidence of this inflammatory disease. Approximately 500,000 new cases occur in the United States each year, with a mortality rate of ~35% (512). Sepsis is characterized by tachycardia, tachypnoe, leukocytosis, hyper- or hypothermia, and positive culture for bacteria. On the cellular level, the inflammatory response is mainly mediated by macrophages. Proinflammatory cytokines such as TNF-α and interleukins (IL-1β, IL-6, IL-8) released from macrophages exert direct effects on the organs or activate a multitude of secondary inflammatory mediators that in turn exert overlapping effects on endothelial cell function, vascular function, coagulation, hemodynamics, and the cardiovascular mechanism. Anti-inflammatory mediators such as IL-10 and IL-11 limit the inflammatory process and can compensate for proinflammatory mechanisms in an organ, before sepsis occurs, or during recovery from sepsis. Disruption of the balance between pro- and anti-inflammatory responses due to an overwhelmed immune system, or severe infection, further aggravates the sepsis leading to shock and multiple organ dysfunction syndrome. Only supportive therapy is available to these patients, who have a poor prognosis. Gram-negative bacteria cause ~50% of all sepsis cases, which are more likely to be complicated than Gram-positive infections (129). One of the most investigated sepsis models utilizes Gram-negative bacterial LPS, a constituent of the bacterial cell wall. When administered to cells, rodents or humans, LPS mimics the same inflammatory response as the whole bacterium. LPS activates macrophages, lymphocytes, polymorphonuclear leukocytes, epithelial cells, and complement in different models. Macrophages are the key inflammatory cells in LPS-induced sepsis. LPS binds to their CD14 cell surface protein and toll-like receptor 2/4. The complex activates tyrosine kinases and the major MAPKs (p38, JNK, ERK1/2). In macrophages, the LPS-mediated stimulation of proinflammatory cytokine production involves the activation of the MAPK signaling pathways (105, 223, 225, 531).

The anti-inflammatory effects of CO that were observed in vitro were substantiated in vivo in an experimental model of sepsis. Mice received injections of LPS (1 mg/kg) with or without CO pretreatment (250 ppm). CO dose-dependently inhibited LPS-inducible serum TNF-α levels and increased LPS-inducible IL-10 production. The production of TNF-α in response to LPS treatment appeared downregulated in MKK3$^{-/-}$ mice compared with wild-type mice. CO failed to further downregulate TNF-α levels or upregulate IL-10 levels in LPS-treated MKK3$^{-/-}$ mice. In IL-10$^{-/-}$ mice, CO inhibited TNF-α levels within the first hour of LPS treatment to a similar extent than in wild-type mice, excluding a role for IL-10 in the early anti-inflammatory effects of CO (504).

Wild-type mice subjected to LPS treatment also displayed increased IL-1β and IL-6 levels. In independent experiments, CO pretreatment increased survival after LPS challenge and reduced serum IL-1β and IL-6 levels. Mice deficient in the JNK pathway had decreased serum levels of IL-6 and IL-1β in response to LPS compared with control mice. No effect of CO on these cytokine levels was observed in JNK1$^{-/-}$ or JNK2$^{-/-}$ mice (456). In summary, these studies suggest that CO provides protection in a murine model of sepsis through modulation of inflammatory cytokine production.

The potential relationship between NO and CO in protection against sepsis was also evaluated in a model of acute liver failure. Exposure of hepatocytes in vitro with CO resulted in a rapid upregulation of iNOS followed by subsequent induction of HO-1. The protective effect of CO against TNF-α-induced liver injury in vitro and in vivo required induction of iNOS and NO production, whereas the protective effect of NOS-derived NO required HO-1 activation. As a consequence, the increased HO-derived CO was proposed to drive an amplification cycle leading to further iNOS activation (792). Loss of iNOS/NO function as in *inos*$^{-/-}$ mice or wild-type mice treated with iNOS inhibitor L-Nil resulted in a loss of CO-mediated protection against TNF-α-induced liver failure, which could be compensated by chemical induction of HO-1 with CoPPIX. In *ho-1*$^{-/-}$ mice, regardless of the presence of iNOS/NO, the protection afforded by CO against liver injury was lost. These experiments illustrate a codependence of NO and CO generating systems in mediating cytoprotection specific to the model of liver injury (792).

2. *Asthma*

Asthma, a complex chronic inflammatory disease, affects at least 10 million people in the United States alone (372). Due to the high morbidity of the disease, the pathology of asthma has attracted intense investigation. HO-1 induction has been demonstrated in preclinical asthma models. Furthermore, increased exhaled CO levels were measured in asthma patients (244). These observations have encouraged the application of CO in various asthma models.

Blood eosinophilia has long been known to represent a major characteristic of inflammation in asthma. The BALF of asthmatics contains elevated levels of many inflammatory cells (eosinophils, basophils, lymphocytes, macrophages, and neutrophils) as well as proinflammatory mediators. Activated CD4 T-lymphocyte product T-helper cell type 2 (Th2)-like cytokines (IL-4, IL-5, IL-13, and eotaxin) initiate and maintain inflammation and bronchial hyperreactivity. Mice develop an airway hyperresponsiveness similar to that seen in human asthma, when challenged with aerosolized ovalbumin (OV) after initial sensitization. Animals were treated with aerosol OV, with one group receiving CO before and after aerosol OV treatment. The BALF was collected 24 and 48 h later. Progressive increases in total cell number were detected after 24

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and 48 h of challenge. Differential cell counts showed that OV challenge activates all inflammatory cells. Macrophages, eosinophils, neutrophils, and lymphocytes were detected in the BALF. CO-treated mice displayed a significantly lower increase in total cell number. CO was also able to reduce the number of all inflammatory cell types, especially eosinophils and macrophages in the BALF at 24 h postchallenge. Exogenous CO administration significantly reduced IL-5 production at 24 h, which returned to near control values at 48 h, but did not affect other cytokines. In this model, eicosanoid mediator levels (INF-γ, leukotriene B_4, and PGE_2) were also reduced by CO. It is not clear whether inflammatory cell numbers and mediator levels seen in BALF can accurately reflect the magnitude of inflammation and/or the outcome of the disease. However, the changes in cell count and mediator levels indicate that CO can regulate inflammation in asthma (92).

Recently, CO was shown to attenuate airway hyperreactivity in a mouse model. An acute dose of CO (0.5–1%; 10 min) significantly reduced metacholine-induced airway resistance in OV-challenged C57 mice and also in airway hyperresponsive A/J mice. Repeated administrations of low-dose CO (250–500 ppm) over 5 days in both naive and inflamed A/J mice reduced airway resistance by 50%. Experiments using ODQ and 8-BrcGMP analogs indicated a role for the sGC/cGMP pathway in mediating these responses. These experiments suggested a possible therapeutic use of CO in modulating airway hyperresponsiveness (29).

B. Lung Injury Models

In this section we discuss evidence in support of a therapeutic effect of CO in two acute lung injury models: oxidative lung injury (hyperoxia) and ventilator-induced lung injury (VILI).

Acute lung injury (ALI) is a common disease in medical intensive care units. Although the origins of ALI can be diverse (e.g., infection, trauma, ischemia, hyperoxia, mechanical ventilation), they are all associated with cell damage, cell death, and inflammation (113, 628). An average of 39% of medical intensive care unit patients require mechanical ventilation (167). Many develop VILI (25). Eventually VILI contributes to the development of acute respiratory distress syndrome (ARDS), which has a 40–50% mortality rate (71). A series of clinical trials showed that ARDS/VILI-related mortality could be reduced with lower tidal volume ventilation, positive end-expiratory pressure (PEEP), and more recently with a recruitment maneuver combined with protective ventilator strategies (28, 71, 214). Despite efforts to reduce its mortality, ARDS remains a major complication of mechanical ventilation during critical care. Preclinical animal models have shown that the pro- and anti-inflammatory cytokines released during VILI play a significant role in the pathology of the disease (101, 709, 691).

Mechanical ventilation with high oxygen tension (hyperoxia, >95% O_2) is frequently used in critical care situations as supportive care for acute, severe respiratory failure. Unfortunately, this treatment can cause cell and organ injury, presumably by the increased generation of ROS. The damage occurs predominantly in the respiratory endothelium and epithelium (364). In mice, the lung damage caused by exposure to a hyperoxic environment resembles that of human ARDS (113). Hyperoxia causes cell growth arrest, cell death, and inflammation in various in vitro and in vivo models (50, 114, 722, 783).

1. *Oxidative lung injury*

Rats subjected to a hyperoxic environment developed apoptosis and inflammation in the lung. The increased expression of HO-1 in rat lungs by intratracheal adenoviral mediated gene transfer protected against the development of pulmonary damage during hyperoxia (505). Intratracheal administration of an adenoviral construct containing HO-1 cDNA (Ad5-HO-1) resulted in a time-dependent increase in the expression of HO-1 mRNA and protein in the rat lungs, with diffuse immunohistochemical staining in the bronchiolar epithelium. Rats receiving Ad5-HO-1 before exposure to hyperoxia (>99% O_2) exhibited a reduction in lung injury relative to controls, as determined by pleural effusion volume and histological analyses (significant reduction of edema, hemorrhage, and inflammation). Ad5-HO-1-infected rats had a marked increase in survivability against hyperoxic stress when compared with control-infected rats. Furthermore, rats treated with Ad5-HO-1 exhibited attenuation of hyperoxia-induced neutrophil inflammation and apoptosis (505).

Inhalation CO protects the lung in a model of hyperoxia-induced lung injury, by virtue of its anti-inflammatory action (506). Rats exposed to a low concentration of CO (250 ppm) displayed a marked tolerance to lethal hyperoxia exposures. This increased survival involved the dramatic attenuation of hyperoxia-induced lung injury as assessed by the volume of pleural effusion, protein accumulation in the airways, and histological analysis. The lungs of rats receiving CO treatment in combination with hyperoxia were completely devoid of lung airway and parenchymal inflammation, fibrin deposition, and pulmonary edema relative to rats exposed to hyperoxia alone. Furthermore, exogenous CO also protected against hyperoxia-induced lung injury in rats in which endogenous HO enzyme activity was inhibited with SnPPIX. Rats exposed to CO also exhibited a marked reduction of hyperoxia-induced neutrophil infiltration into the airways and total lung apoptotic index (506).

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When mice were exposed to a hyperoxic environment (>98% O_2), they developed signs of lung injury by 64–72 h and 100% mortality within 90–100 h of exposure. The presence of CO (250 ppm) initiated before the hyperoxia prolonged the survival of mice in the hyperoxic environment, increasing the LD_{50} to 128-h exposure. Similar to the results observed in the rat model, CO inhibited the appearance of histological markers of lung injury associated with hyperoxia, as well as markers of oxidative damage (i.e., lung lipid peroxidation) (507). CO also inhibited the influx of neutrophils into the airways associated with hyperoxia treatment, as measured in bronchoalveolar lavage fluid (BALF). Hyperoxia induced the expression of proinflammatory cytokines including TNF-α, IL-1β, and IL-6 by 84 h of exposure and activated MAPK in lung tissue including ERK1/2, JNK, p38 MAPK, and its upstream kinases MKK3/6. The protection afforded by CO treatment against the lethal effects of hyperoxia correlated with the inhibited release of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the BALF.

$MKK3^{-/-}$ mice, or wild-type mice injected with the selective inhibitor of p38 α/β MAPK (SB203580), displayed the accelerated appearance of tissue damage markers (with the exception of neutrophil influx) and increased susceptibility to the lethal effects of hyperoxia, relative to wild-type mice. Cytokine mRNA (TNF-α, IL-1β, and IL-6) expression in response to hyperoxia appeared earlier in the $MKK3^{-/-}$ mice relative to the wild-type mice exposed to continuous hypoxia. CO did not inhibit the expression of the proinflammatory cytokines in the $MKK3^{-/-}$ mice and furthermore did not confer protection or extend survival against hyperoxia in $MKK3^{-/-}$ mice or in wild-type mice injected with SB203580. In contrast, $JNK^{-/-}$ mice responded like wild-type mice with respect to the anti-inflammatory effects of CO (507). The protective effects of CO in this model were also observed in vitro. CO treatment of A549 lung epithelial cells increased the activation of MKK3, and specifically the β-isoform of p38 MAPK while suppressing that of the α-isoform. CO exposure increased the survival of A549 cells grown in continuous hyperoxia, relative to cells exposed to hyperoxia alone. Treatment with the SB203580 or transient transfection with dominant negative mutants of p38β or MKK3 abolished the cytoprotective effect of CO against hyperoxia. In summary, these experiments demonstrate that CO protects against the lethal and inflammatory effects of hyperoxia in vivo and in vitro, by downregulating the expression of proinflammatory cytokines, through a mechanism dependent on activation of the p38β/ MKK3 pathway (507).

2. *Bleomycin-induced pulmonary fibrosis*

Recent studies have also shown protective effects of HO-1 or CO in a model of bleomycin-induced fibrotic lung injury (693, 791). Mice were treated with bleomycin intratracheally and then exposed to CO or ambient air. Lungs from CO-treated animals had significantly lower hydroxyproline, collagen, and fibronectin levels than air-treated bleomycin-injured controls. The protective effect of CO in this model was associated with an antiproliferative effect of CO on fibroblast proliferation dependent on $p21^{Waf1/Cip1}$ induction and suppression of cyclins A/D expression (791). In a similar model, AdHO-1 treatment, resulting in an increased HO-1 expression in the lung, protected against bleomycin-induced pulmonary fibrosis. The protection was associated with decreased epithelial cell apoptosis and increased IFN-γ production. However, AdHO-1 treatment did not protect against Fas agonistic antibody-induced pulmonary fibrosis. Conversely, AdHO-1 treatment still protected against bleomycin-induced pulmonary fibrosis in mice deficient in the expression of Fas ligand (693). These results indicated an antiapoptotic effect of HO-1 independent of the Fas/Fas ligand pathway. Paradoxically, recent studies indicate that a metalloporphyrin inhibitor of HO activity, ZnDBG, also protected against fibrotic changes in a similar model when administered by subcutaneous injection (37). This result apparently conflicts with the aforementioned studies that HO-1 expression and/or activity is protective in this model, although potential direct antioxidative effects of the metalloporphyrin could not be discounted (37).

3. *Ventilator-induced lung injury*

Recent studies (this laboratory) suggest an anti-inflammatory effect of inhaled CO in a rat model of VILI (145). Rats were ventilated with a relatively injurious ventilator setting (26 ml/kg tidal volume without PEEP), complemented with an intraperitoneal bacterial LPS injection to induce inflammation and injury. One-hour mechanical ventilation with LPS pretreatment significantly increased both the mRNA and the protein expression of HO-1, suggesting that HO-1 may play a role in the defense against VILI. CO was mixed in the ventilator air in different doses (10–250 ppm) and directly applied to the animals. CO (250 ppm) significantly reduced the inflammatory cell count in the BALF at 2–4 h. Furthermore, CO dose dependently (100–250 ppm) attenuated the levels of the proinflammatory cytokine TNF-α in the BALF, while it significantly increased the levels of the anti-inflammatory cytokine IL-10. CO treatment did not alter the arterial blood pressure or blood gas results during ventilation, suggesting that a low dose of CO does not cause significant cardiovascular changes in the animals. Lung tissue extracts displayed increased activation of p38 MAPK following ventilation with CO, similar to findings in other models of ALI. Inhibition of p38 MAPK in vivo with SB203580 attenuated IL-10 production in VILI. On the basis of these findings, it is exciting to speculate

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that CO could represent a potential therapeutic modality in ALI and VILI, although further investigations are needed (145).

C. Cardiovascular Injury/Disease

The expression of HO-1 and/or the application of exogenous CO have been shown to confer protection in several models of cardiovascular injury or disease, including hypertension, atherosclerosis, balloon-catheter injury, and graft rejection.

1. Myocardial infarction

A protective role for HO-1 has been implicated in myocardial ishemia/reperfusion (I/R) injury. Isolated perfused hearts subjected to in vitro I/R treatments displayed increases in cardiomyocyte HO-1 expression (426). Furthermore, HO-1 expression was found to increase in the heart following renal I/R, illustrating a potential cardioprotective response to systemic stress (534). The cardioprotective properties of HO-1 were demonstrated by increased cardiac injury of HO-1-deleted mice (*ho-1*$^{-/-}$) in a model of chronic hypoxia-induced pulmonary hypertension. Chronic hypoxia treatment (10% O_2) increased right ventricular dilation and caused right ventricular infarction with focused mural thrombi, in *ho-1*$^{-/-}$ mice relative to wild-type mice that did not display ventricular infarction (752). In a model of streptozotocin-induced diabetes, the diabetic *ho-1*$^{-/-}$ developed larger myocardial infarcts and increased oxidative stress markers when subjected to coronary artery I/R, relative to diabetic wild-type mice (390). Conversely, the cardiac-specific overexpression of HO-1 conferred resistance to myocardial I/R injury. Isolated hearts from the transgenic mice displayed improved cardiac performance, reduced oxidative stress markers, and diminished infarct size during I/R injury (753). Likewise, cardiac-specific overexpression of HO-1 also protected against I/R injury in diabetic mice (390).

Experiments with CORMs demonstrate the cardioprotective potential of pharmacologically derived CO (112, 219, 627). In isolated hearts, inclusion of CORM-3 in the reperfusion media after ischemia resulted in improved cardiac performance and reduction in infarct size (112). In mice subjected to myocardial I/R, administration of CORM-3 at the time of reperfusion reduced infarct size without exerting hemodynamic effects or elevating blood CO-Hb levels (219). CORM-3 also conferred a delayed cardioprotection similar to the late phase of ischemic preconditioning (627).

2. Systemic hypertension

Increases of vascular HO-1 expression have been observed in several experimental models of hypertension, including angiotensin II-induced hypertension (15, 268, 269, 377), spontaneously hypertensive rats (SHR) (98), Dahl-Rapp salt-sensitive rats (280, 287, 676), and deoxycorticosterone (DOCA) salt-sensitive rats (281). The HO-1/CO system has been associated with antihypertensive effects (reviewed in Ref. 481) as well as pathological roles as in the case of salt-induced hypertension models (280, 281, 676).

SHR display age-dependent increases in blood pressure, gradually leading to hypertension in adults. Administration of exogenous CO or heme derivatives reduced blood pressure in SHR (286). The suppressive effects of heme derivatives on blood pressure were restricted to young SHR with developing hypertension, but not in adult SHR, by upregulating HO-1 (482). Application of the human HO-1 gene by vector-directed gene therapy reduced blood pressure in young SHR (564). Endogenous increases in HO-1 and iNOS expression in aorta occurred during the development of hypertension in SHR and correlated with increases in systolic blood pressure (98). Application of metalloporphyrins (ZnPPIX, CrMP) reversed the antihypertensive effects of heme and also elevated systolic blood pressure in SHR (98, 286, 482). These results implicated HO-1 expression as a compensatory response during the development of hypertension in young SHR. A deficiency or desensitization of downstream cGMP signaling was related to the stable hypertension of the adult SHR (98). Pharmacological application of CO using CORM-2 reduced blood pressure in Lewis rats subjected to experimental hypertension induced with L-NAME (460). In models of angiotension-induced renal hypertension, the expression of HO-1 and/or generation of HO-derived CO was proposed to counterregulate the vasoconstrictive effect of angiotensin (377, 746), in part by inhibiting prostaglandin synthesis (2, 393b).

In contrast to protective roles, a pathological role for HO/CO has been proposed in the development of hypertension and in the impairment of endothelial cell function in Dahl salt-sensitive rats and DOCA-salt sensitive rats rendered hypertensive by a high-salt diet (676). An impaired endothelial-derived NO production is thought to underlie the development of hypertension in these models. In contrast to observations with SHR, the application of CrMP restored endothelial cell function and reduced blood pressure in this model, whereas CO antagonized this effect. CrMP restored deficiencies in acetylcholine-dependent vasodilation in arterioles in both the DOCA and Dahl salt-sensitive models (280, 281). The prohypertensive effects of HO-derived CO in this model were attributed to further impairment of endothelial NO production by inhibition of arteriolar NOS activity. In contrast, a cardioprotective effect of HO-1 was also noted in Dahl salt-sensitive rats, by the promotion of coronary vasodilation (287).

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3. Pulmonary hypertension

HO-1 confers tissue protection in models of pulmonary hypertension. The chemical induction of HO-1 protein prevented the development of pulmonary hypertension in the rat lung in response to chronic hypoxia treatment (107). In chronic hypoxia-induced pulmonary hypertension, transgenic mice with the targeted overexpression of HO-1 in the lung displayed reduced lung inflammation, pulmonary hypertension, and vascular hypertrophy as a function of chronic-hypoxia treatment, relative to wild-type mice (443).

4. Atherosclerosis

HO-1 can be induced in both endothelial and vascular smooth muscle cells by proatherogenic stimuli, including treatments with oxidized low-density lipoprotein, lipid metabolites, shear stress, and angiotension II (612). HO-1 confers protection in animal models of arteriosclerosis, where it may be found in atherosclerotic lesions (718). HO-1 is highly upregulated in the endothelium and in the foam cells of intimal lesions from humans and apolipoprotein E-deficient mice (718). Induction of endogenous HO-1 by heme treatment reduced the formation of atherosclerotic lesions in LDL-receptor knockout mice fed high-fat diets, relative to untreated or SnPPIX-treated controls (267). The adenoviral-mediated transduction of HO-1 into ApoE-deficient mice (apoE$^{-/-}$) inhibited the formation of arteriosclerotic plaques relative to control mice. The mechanism by which HO-1 protects against arteriosclerosis may involve, in part, the inhibition of platelet aggregation by HO-derived CO (291). Additional deletion of HO-1 against an ApoE-deficient background (*ho-1*$^{-/-}$apoE$^{-/-}$) exacerbated arteriosclerotic lesion formation relative to *ho-1*$^{+/+}$, apoE$^{-/-}$ mice (751).

5. Vascular injury

Vascular injury, intimal hyperplasia, and arterioscleosis leading to vessel occlusion can also occur as a consequence of chronic graft rejection after vascular transplant. Infiltrating leukocytes and proliferating SMC play key roles in the pathology of the disease. Leukocytes initiate an immune-mediated injury that activates endothelial cells and disturbs their barrier function. Macrophages and T lymphocytes infiltrate the graft. In the meantime, SMC penetrate to the intima of vessels. The hyperproliferation of SMC produces extracellular matrix depositions leading to luminal stenosis. A beneficial effect of CO in inhibiting SMC proliferation was shown in two models. In a vascular transplant model, Brown-Norway rat aortic segments were transplanted into Lewis rats. The most significant changes were seen after 50–60 days characterized by intimal hyperplasia, the loss of SMC in the medial region of the vessels, and leukocyte infiltration in the adventitia. A group of rats was pretreated with 250 ppm CO for 2 days and maintained in the CO-containing environment until they were killed. These animals displayed significantly less intimal proliferation and a lower magnitude of leukocyte infiltration in the graft. The accumulation of macrophages, helper, and cytotoxic T cells was also reduced in the transplanted aortic ring (508).

Adenoviral-mediated overexpression of HO-1 (AdHO-1) in pigs inhibited vascular cell proliferation and lesion formation in a model of arterial injury. Conversely, HO-1$^{-/-}$ mice subjected to arterial injury displayed increased vascular cell proliferation and developed hyperplastic lesions compared with wild-type controls (155). In a model of intimal hyperplasia, where SMC proliferate uncontrollably following balloon angioplasty of the carotid artery, exposure to CO also completely prevented vascular stenosis (508). Pretreatment with CO (250 ppm) for just 1 h significantly reduced the neointimal proliferation seen at 14 days postballoon angioplasty relative to control rats that did not receive CO treatment (Fig. 10). The expression of HO-1 by adenoviral-mediated gene transfer protected against intimal hyperplasia following vascular balloon injury (694). The mechanism of this effect was attributed to the inhibition of SMC proliferation by CO (155, 454, 508) (see sect. VI).

D. Ischemia/Reperfusion

I/R during lung surgery and lung transplantation or after hemorrhagic or cardiogenic shock leads to tissue injury. The massive cell death associated with I/R limits therapeutic options. Animal models have suggested that apoptosis is a major cause of cell death following I/R trauma in lung, heart, kidney, and brain (30, 193, 234, 604). Anti-inflammatory effects of CO have been demonstrated in models of I/R injury of the heart, lung, kidney, and small bowel (473). CO protected against liver I/R injury via activation of the p38 MAPK (31). Homozygous *ho-1* null mice (*ho-1*$^{-/-}$) displayed increased mortality in a model of lung I/R injury. CO inhalation (1,000 ppm) partially compensated for the HO-1 deficiency in *ho-1*$^{-/-}$ mice and improved survival after I/R (193). In this model, the authors proposed that the protection provided by CO involved the enhancement of fibrinolysis, by the cGMP-dependent inhibition of plasminogen activator inhibitor-1 (PAI-1), a potent smooth muscle cell proliferation activator produced by macrophages (193). Mice treated with an sGC inhibitor, ODQ, were not protected from I/R-induced lethality by CO.

In the mouse lung I/R model, CO exposure protected against I/R-induced lung injury. A left hilar clamp was placed on mechanically ventilated rats for 30 min. After removing the clamp, a 2-h reperfusion was allowed. CO

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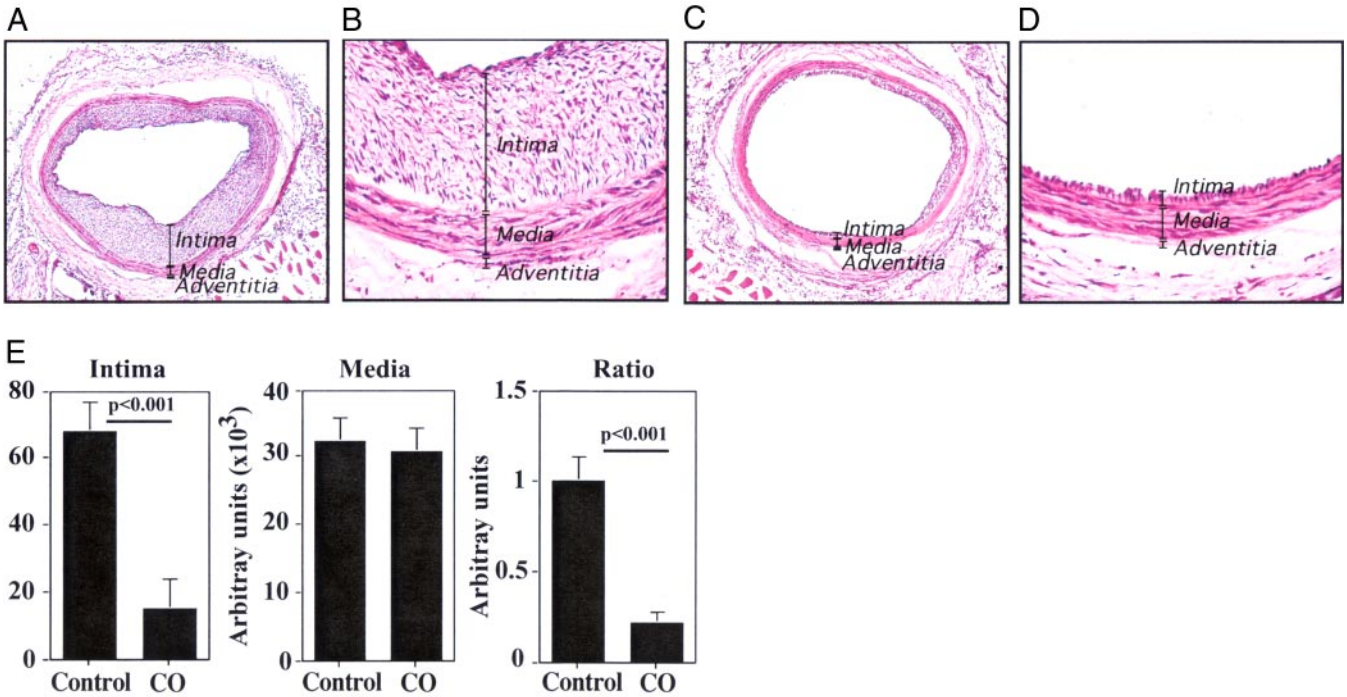

FIG. 10. CO suppresses the development of vascular lesions associated with balloon injury. *A–D:* immunocytochemical analysis of carotid arteries 14 days after balloon angioplasty. Rats were exposed to room air (*A* and *B*) or CO (1 h; 250 ppm) (*C* and *D*) before balloon injury. All animals were exposed to room air following injury. Two weeks after injury, samples were stained with hematoxylin and eosin. Photomicrographs (*A* and *C*) are ×10 and (*B* and *D*) are ×50 magnification. *E*: mean standard deviation ($n = 8$, $P < 0.001$ vs. control) of relative areas corresponding to the intima and media regions from the same experiment as (*A–D*) above. [From Otterbein et al. (507).]

was introduced through the ventilator for a 1-h pretreatment period and throughout the experiment. TUNEL staining showed a decreased number of apoptotic cells in the lungs of CO-treated animals. Chemical inhibition of p38 MAPK activity, or deletion of MKK3 as in *mkk3*$^{-/-}$ mice, abolished the antiapoptotic effects of CO during I/R by preventing the modulation of caspase-3 activity (781, 782). Exogenously applied CO at concentrations starting at 15 ppm inhibited I/R-induced apoptosis in pulmonary artery endothelial cell (PAEC) cultures, associated with the CO-dependent activation of the p38β MAPK isoform and its upstream MAPK kinase (MKK3), and with the suppression of ERK and JNK activation. Inhibition of p38 MAPK with SB203580 inhibited the protective effect of CO in this model. The antiapoptotic effect of CO also involved inhibition of Fas/FasL expression and other apoptosis-related factors including caspases (-3, -8, -9), mitochondrial cytochrome *c* release, Bcl-2 proteins, and poly (ADP-ribose) polymerase (PARP) cleavage (781, 782). These studies confirmed a link between p38 MAPK and the downregulation of caspase-3 activity by CO, as previously described for Fas-mediated apoptosis (294). In addition to I/R, apoptotic pathways play a central part in many models of disease. A better understanding of cytoprotection provided by CO in vivo could lead to future therapeutic solutions in other illness.

E. Organ Transplantation

Increasing survival rates in organ transplantation represent one of the successes of modern medicine. However, graft rejection limits its efficacy especially following lung transplantation. The frequency and severity of acute rejection episodes is a predominant risk for chronic graft rejection. Expression of the stress protein HO-1 in rodent allografts (kidney, heart, and liver) and xenografts (heart) correlated with long-term graft survival in several models of transplantation (39, 120, 133, 575, 620, 695). In a rodent model of renal transplantation, HO-1 expression increased in the allograft in response to immune injury (39). The reduced expression of HO-1 in chronic rejection compared with acute rejection may represent an inadequate response to injury or a consequence of prior injury that sensitizes further tissue response to immune attack (39). HO-1 gene therapy protected against rejection in rat liver transplants (312). The upregulation of HO-1 protected pancreatic islet cells from Fas-mediated apoptosis in a dose-dependent fashion, supporting an antiapoptotic role of HO-1 in transplantation models (518, 684). In a rat liver allograft model, HO-1 may confer protection in the early phase after transplantation by inducing Th2-dependent cytokines such as IL-4 and IL-10, while suppressing IFN-γ and IL-2 production (313). HO-1 gene therapy in rats

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undergoing liver transplantation resulted in protection against I/R injury and improved survival after transplantation, possibly by suppression of Th1-cytokine production and decreased apoptosis after reperfusion (30).

Protective effects of HO-1 have also been documented in xeno-transplantation models, where HO-1 gene expression correlates with xenograft survival (575, 620). In a mouse to rat cardiac transplant model, the effects of HO-1 expression could be mimicked by CO administration, suggesting that HO-derived CO suppressed the graft rejection (575). The authors proposed that CO suppressed graft rejection by inhibition of platelet aggregation, a process that facilitates vascular thrombosis and myocardial infarction. The ability of CO to suppress inflammation is likely involved in xenograft transplant models in which 400 ppm CO for 2 days prevented rejection for up to 50 days (575). The effects of CO on platelet aggregation, vasodilation, and proinflammatory cytokines all potentially contribute to the favorable outcome in xenograft transplantation (508).

Lung transplantation has become an accepted treatment modality for end-stage lung disease. After lung transplantation, there remains a persistent risk of acute and chronic graft failure, as well as of complications of the toxic immunosuppressive regimen used (246). Compared with other solid organ transplants, the success of lung transplantation has been severely limited by the high incidence of acute and chronic graft rejection. The frequency and severity of episodes of acute rejection are the predominant risk factors for chronic airway rejection, manifested as obliterative bronchiolitis (OB) (47, 202). Data from rodent allograft studies as well as from clinical lung transplantation show that the lung, compared with other solid organs, is highly immunogenic. Despite advances in immunosuppression, the incidence of acute rejection in lung graft patients can be as high as 60% in the first postoperative month (607, 692). OB, which may develop during the first months after transplantation, is the main cause of morbidity and death following the first half-year after transplantation despite therapy. Once OB has developed, retransplantation remains the only therapeutic option available (168).

Until recently, only very limited research data were available on the possible role for HO-1 in allograft rejection after lung transplantation. Increased HO-1 expression has been detected in alveolar macrophages from lung tissue in lung transplant recipients with either acute or chronic graft failure compared with stable recipients (399). The level of HO-1 mRNA and protein expression correlated with the acute rejection grade scores in lung fibroblasts taken from biopsies from a lung transplant patient (622). The effects of CO were also examined in a rat model of lung transplantation. Orthotopic left lung transplantation was performed in Lewis rat recipients from Brown-Norway rat donors. HO-1 mRNA and protein expression were markedly elevated in transplanted rat lungs at 4 days posttransplantation compared with sham-operated lungs. Animals were exposed to continuous inhalation CO (500 ppm) or air. Transplanted lungs developed severe intralveolar hemorrhage and intravascular coagulation. In the presence of continuous CO exposure, however, the gross anatomy and histology of transplanted lungs showed dramatic preservation relative to air-treated controls, with marked reduction in hemorrhage, fibrosis, and thrombosis on the sixth day after transplantation. Furthermore, transplanted lungs displayed increased apoptotic cell death compared with the transplanted lungs of CO-treated recipients, as assessed by TUNEL and caspase-3 immunostaining. IL-6 expression corresponds to the magnitude of injury during transplantation (400, 588). CO exposure inhibited the induction of IL-6 mRNA expression in lung and serum caused by the transplantation. Gene array analysis revealed that CO also downregulated other proinflammatory genes, including MIP-1α and macrophage migration inhibitory factor (MIF), and growth factors such as PDGF, which were upregulated as a result of transplantation (622). In organ transplantation, the I/R injury that occurs leads to rapid endothelial cell apoptosis. The loss of endothelial cells in the vessels serving the organ results in a rapid cascade of events including thrombosis that can ultimately result in the rejection of the organ. These data suggest CO limits lung graft injury by maintaining cell viability and suppressing inflammation

VIII. CONCLUSIONS AND FUTURE DIRECTIONS

A. Summary

The saga of the HO enzyme system has undergone interesting historical transformations, from the study of a key biochemical reaction mechanism involved in the degradation of heme to bile pigments to an important stress response molecule critical in maintaining cellular and tissue homeostasis in response to environmental insults. The HO enzymatic reaction has now been extensively characterized in biochemical and genetic terms.

Section II has described the pioneering work of Tenhunen and Yoshida as well as the investigations led by Ortiz de Montellano and others that have elucidated a three-part reaction mechanism dependent on serial reduction and oxidation steps, leading to the cleavage and insertion of oxygen into the heme ring. The groundwork of Maines and co-investigators has characterized the major isoforms of HO including constitutive (HO-2) and inducible isoforms (HO-1). Remarkably, the HO enzymes display a high degree of evolutionary conservation and have representative counterparts now characterized in numerous organisms from bacteria to plants to humans.

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Recent studies also point to a more complex subcellular distribution of HO enzymes than originally thought, with potential implications in cellular signaling.

Section III has described how HO-1 induction responds to a vast array of chemical, physical, and physiological stimuli in countless cell and tissue models. The mechanisms of cell signaling leading to the transcriptional regulation of the *ho-1* gene are highly variable in a cell-type and inducer-specific fashion, though they involve the major participation of protein phosphorylation cascades. Similarly, the mechanisms of transcriptional regulation at the *ho-1* gene vary in a model-specific fashion, although the extensive research of Alam et al., and others, has implied a dominant role for distinct transcriptional enhancer regions (E1, E2) containing the StRe/ARE consensus (16–18). The relevant transcription factors also vary in an inducer-specific fashion; however, the Nrf2/Keap axis is perhaps the most widely utilized mechanism. After years of speculation, the recently characterized Nrf2/Keap axis may also provide a long-sought mechanism for the redox regulation of the *ho-1* gene.

In sections IV–VI, we have examined how each of the HO reaction products, iron, bile pigments, and CO may influence cellular functions. These mechanisms may operate, depending on the model, individually or in concert, to provide underlying mechanisms for the complex cytoprotection offered by HO proteins. We have seen how HO-1 may play a critical role in the distribution of intracellular, plasma, and tissue iron. The bile pigment products of HO, though once viewed as metabolic waste, show promise as natural pharmaceuticals with a variety of applications. Recently, we have also witnessed an exciting period of investigation in the cellular functions of an invisible colorless and odorless gaseous molecule CO. In section VII, we have highlighted critical functions of HO-derived and exogenous CO in numerous biological and pathophysiological models of tissue injury from lung/cardiovascular disease to organ transplantation.

B. Conclusions and Future Directions

In addition to well-characterized roles as a metabolic enzyme specialized in the breakdown of heme, with implications in differentiation, hemopoiesis, erythrocyte turnover, and iron trafficking, HO-1 is now generally accepted as a mediator of cyto- and tissue protection against a wide variety of injurious insults. While methods for detecting HO-1 involvement in various systems have evolved, further progress may depend on the development of new tools for the measurement and/or visualization of HO reaction products such as CO in situ. The trend of current and future research continues to focus on methods of translating the apparent protective properties of the endogenous HO-1 stress response into therapeutic strategies for the treatment of human disease. Along these lines, a number of approaches have been proposed over the years. For example, experimentation with natural antioxidants and pharmaceuticals has raised the possibility that HO-1 induction in tissue could be stimulated by agents selected for their capacity to invoke the response without eliciting tissue damage. To bypass the need for exogenous inducing agents altogether, which may cause unknown side effects, the "gene therapy" approach relies on the introduction of the *ho-1* gene into tissues by viral-directed overexpression. Because excessive expression of HO-1 at nonphysiological levels may have a demonstrated counterprotective effect in some models, this approach would require additional strategies to control the artificial level of expression, such as inducible or tissue-specific vectors. Currently such gene therapy approaches remain of limited clinical applicability. A third general strategy has focused on the pharmacological application of HO-derived end products. While the possibilities for modulating tissue iron levels have not been as extensively explored, possibilities exist for the application of bile pigments (i.e., biliverdin and bilirubin) and/or CO for therapeutic gain in a variety of disease states. For example, exogenous application of bile pigments has demonstrated effectiveness in alleviating organ ischemia/reperfusion injury, transplant rejection, and hyperproliferative disorders. Inhalation CO has been demonstrated to be effective in animal models of inflammation, hypertension, organ transplantation, vascular injury, and ventilator-induced lung injury among others. Human trials underway have yet to place a final verdict of whether inhalation gas strategies can be used as therapies for inflammatory diseases of the lung. The pharmacological administration of CO to tissues using CO-RM and related molecules may present a viable alternative to the inhalation of gas. Questions remain as to the safety and optimal use of these compounds for human delivery. The future will no doubt see the development of improved versions of these agents, as well as the continuing development of other safe and effective methods for harnessing the protective potential of the HO/CO system. Thus this review has laid a foundation to prepare the researcher for the critical future challenges of translating these current discoveries into new therapeutic modalities to combat human diseases.

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