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Comprehensive Invited Review

Heme Oxygenase-1 and the Vascular Bed: From Molecular Mechanisms to Therapeutic Opportunities

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Abstract

Heme oxygenase-1, an enzyme degrading heme to carbon monoxide, iron, and biliverdin, has been recognized as playing a crucial role in cellular defense against stressful conditions, not only related to heme release. HO-1 protects endothelial cells from apoptosis, is involved in blood-vessel relaxation regulating vascular tone, attenuates inflammatory response in the vessel wall, and participates in blood-vessel formation by means of angiogenesis and vasculogenesis. The latter functions link HO-1 not only to cardiovascular ischemia but also to many other conditions that, like development, wound healing, or cancer, are dependent on neovascularization. The aim of this comprehensive review is to address the mechanisms of HO-1 regulation and function in cardiovascular physiology and pathology and to demonstrate some possible applications of the vast knowledge generated so far. Recent data provide powerful evidence for the involvement of HO-1 in the therapeutic effect of drugs used in cardiovascular diseases. Novel studies open the possibilities of application of HO-1 for gene and cell therapy. Therefore, research in forthcoming years should help to elucidate both the real role of HO-1 in the effect of drugs and the clinical feasibility of HO-1–based cell and gene therapy, creating the effective therapeutic avenues for this refined antioxidant system. *Antioxid. Redox Signal*. 10, 1767–1812.

I. Introduction

Heme oxygenase-1 (HO-1) catalyzes the oxidative degradation of heme to biliverdin, carbon monoxide, and iron (415). However, removing toxic heme is not the only function of HO-1, and its products have been recently recognized to play important roles in numerous organs. The significance of HO-1 in vascular biology was particularly emphasized by the discovery of the only known case of human HO-1 deficiency (460). Moreover, experiments with HO-1 knockout mice have revealed the importance of HO-1 in iron reutilization and protection against oxidative stress (340, 341). HO-1 is evolutionarily conserved, and it has been detected in bacteria, plants, fungi, and animals (454). This confirms that HO-1 is critically important for the proper functioning of different organisms.

Nowadays, the protective effect of HO-1, exerted by its products and probably protein itself (247), is well known and widely accepted, although the opposing data also are published. Therefore, in this review, we not only describe in detail the role of HO-1 in the vascular wall but also provide a critical evaluation of its significance in the vascular bed.

II. Heme Oxygenases: Genes, Expression, and Activity

In the late 1960s, heme oxygenase (HO) was demonstrated to participate, as the first and rate-limiting enzyme, in the conversion of heme into a bile pigment, bilirubin (414). The sources of heme for this reaction are hemoglobin and other heme-containing proteins such as myoglobin, cytochromes, peroxidases, and respiratory burst enzymes. Heme degradation is energy consuming, and NADPH donates electrons through the cytochrome P450 system. Three moles of molecular oxygen (O_2) are consumed for the liberation of iron from the porphyrin ring of heme, the release of carbon monoxide (CO), and the formation of biliverdin (264, 415, 471) (Fig. 1).

Biliverdin is quickly reduced to bilirubin. Recent discoveries have clearly shown that biliverdin reductase (BVR) not only converts biliverdin to bilirubin, but also has more functions not related to its reductase activity. BVR was first characterized as a serine/threonine kinase, capable of autophosphorylation (362), that is activated by oxygen radicals and translocates to the nucleus in response to cyclic guanosine 3′,5′-monophosphate (cGMP) and oxidative stress (9, 267). More recently, it was recognized that BVR can also act as a tyrosine kinase (233). This observation characterizes BVR as one of a rare group of dual-specificity kinases, which have the ability to autophosphorylate on all three hydroxy amino acids.

A. HO isoforms

Two isoforms of heme oxygenases have been identified so far: an inducible form, HO-1, and a constitutive form, HO-2 (265). Moreover, HO-3, a pseudogene derived from an HO-2 transcript, was found only in rats (276).

HO-1 and HO-2 are the products of different genes (262, 276, 385), and in rat and human, they share 45% and 43% homology in amino acid sequences, respectively (276, 357).

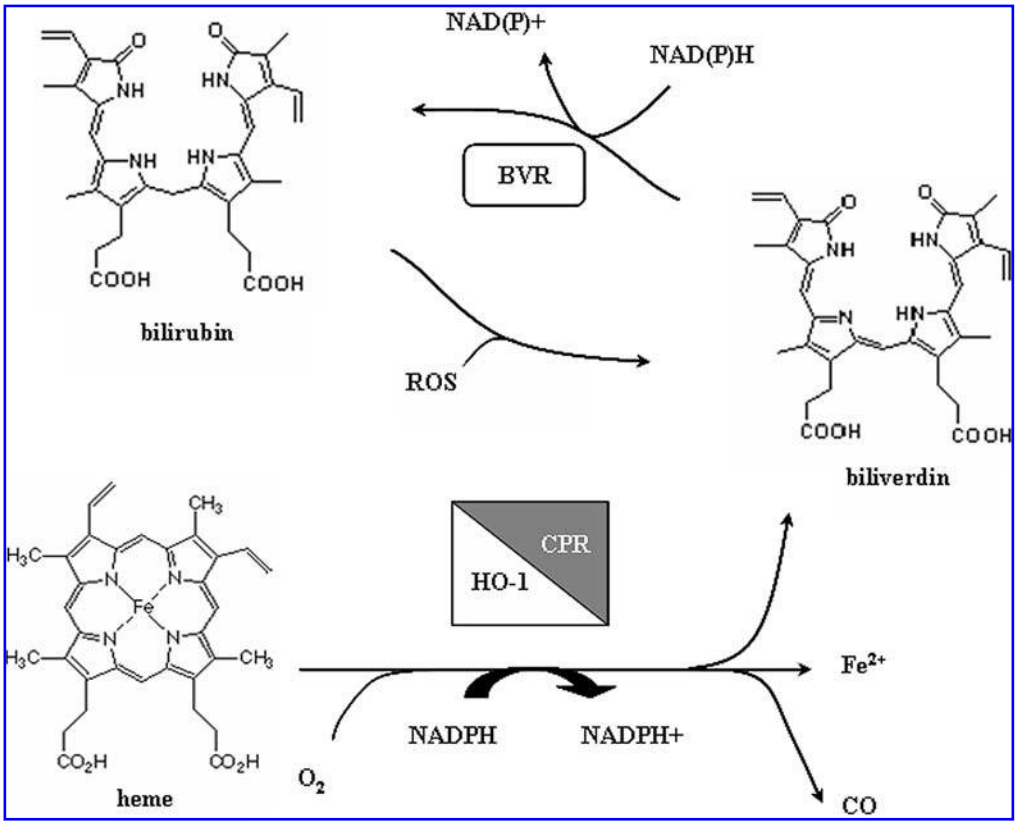

FIG. 1. Heme-degradation pathway. HO, cooperating with NADPH cytochrome P450 reductase (CPR), degrades heme to produce three products: free iron, carbon monoxide, and biliverdin, which is rapidly converted to bilirubin by biliverdin reductase (BVR). Reactive oxygen species (ROS) can be scavenged by bilirubin, protecting the cell from oxidative stress. ROS are suggested to oxidize bilirubin to biliverdin, which can be then reduced to bilirubin by BVR, completing a catalytic cycle.

Although they require similar substrates and cofactors for heme oxidation, the kinetics of this reaction differs, as the K_M for HO-1 is 0.24 μM, and for HO-2, it is 0.67 μM (265).

HO-1, a 32-kDa protein also known as the stress protein HSP32 (203), is considered a protective, early stress-response gene, the expression of which is generally not detected in normal tissues, apart from the spleen, where it is the predominant form even under normal, unstressed conditions (415). Its low basal expression can be strongly upregulated by a wide variety of stimuli causing oxidative stress, including heme, cobalt protoporphyrin (CoPPIX), heavy metals, cytokines, lipopolysaccharide (LPS), hydrogen peroxide (H_2O_2), growth factors, heat shock, and ultraviolet (UV) light (Table 1). Recently, CO, a product of HO-1, has also been demonstrated to induce HO-1 expression in endothelium (208). Conversely, HO-1 seems to be constitutively expressed in renal inner medullary cells (482), Kupffer cells in the liver (33), Purkinje cells in the cerebellum (304), and $CD4^+/CD25^+$ regulatory T cells (326).

HO-2, a 36-kDa protein (357), is present mostly in the brain and, at lower levels, also in testes, endothelium, distal nephron segments, liver, and myenteric plexus of the gut, with subcellular localization in mitochondria. HO-2 expression is generally constant and can be augmented only by a limited number of factors, such as dexamethasone in human primary epithelial cells (103) or corticosterone (266) in fetal rat brain. However, depending on the cell type and microenvironment, its expression can be both upregulated and downregulated by hypoxia (150). For instance, transient decrease of HO-2 in the liver and elevation in the heart has been observed during the acclimatization of mice to normobaric hypoxia (150). Recently, HO-2 was suggested to act as an O_2 sensor. It is noteworthy that HO-2–deficient ($HO\text{-}2^{-/-}$) mice survive normally for at least 1 year (342); however, they show mild hypoxemia and a blunted hypoxic ventilatory response with a normal hypercapnic ventilatory response (7). HO-2 is also suggested to maintain placental blood flow and pregnancy (384). Accordingly, the reduced HO-2 expression has been reported in abnormal pregnancies, such as preeclampsia and spontaneous abortion. However, as the $HO\text{-}2^{-/-}$ mice are apparently healthy, a compensatory mechanism has evolved, which is at least partially dependent on induced HO-1 expression. Indeed, experiments with siRNA silencing of HO-2, demonstrating the induction of HO-1, suggest that HO-2 may regulate the expression of HO-1 by modulating the cellular heme levels. HO-2 is also involved in calcium signaling (41) and neuroprotection (105) (see also *HO-1 and stroke).*

The HO-3 gene was initially suggested to encode a 33-kDa protein in different organs (277). However, on the basis of recent findings, HO-3 is regarded as a pseudogene derived from the HO-2 transcript, and it cannot be considered the functional enzyme (156).

Table 1. Inducers of HO-1 Expression/Activity

Stimulus	*Species*	*Cell line*	*References*
Metalloporphyrins			
Heme	Human	Keratinocytes	185
		Microvascular endothelial cells	386
		Macrophages	472
	Rat	Vascular smooth muscle cells	114
		Pancreatic cells	457
	Mouse	Aortic endothelial cells	81
		Macrophages	428
Cobalt protoporphyrin	Human	Colon cancer cells	54
		Microvascular endothelial cells	256
		Hepatoma	378
	Rat	Neonatal cardiomyocytes	171
		Auditory cells	206
Heavy metals			
Cobalt chloride	Human	Microvascular endothelial cells	386
	Rat	Hepatoma	175
		Glioma cells	211
		Lung cancer cells	165
	Mouse	Macrophages	238
Stannous chloride	Human	Hepatoma	136
		Femoral artery endothelial cells	241
Cadmium chloride	Human	Breast adenocarcinoma	18
		Hepatoma	409
	Rat	Hepatoma	175
	Mouse	Peritoneal macrophages	410
Lipid metabolites			
15d-PGJ2	Human	Lymphocytes	21
	Mouse	Endothelial microvascular cells	195
		Breast cancer cells	205
		Macrophages	229
oxLDL	Human	Aortic smooth muscle cells	24
	Mouse	Peritoneal macrophages	177
4-HNE	Human	Fibroblasts	311
	Rat	Hepatocytes	57
	Mouse	Peritoneal macrophages	177
Cytokines			
IL-1β	Rat	Vascular smooth muscle cells	114
IL-6	Human	Macrophages	352
		Hepatoma	426
	Rat	Hepatocytes	426
IL-10	Human	Monocytes	352
	Rat	Macrophages	352
TNF-α	Human	HUVECs	416
Growth factors			
SDF-1	Human	Aortic endothelial cells	99
TGF-β1	Human	Retinal pigment epithelial cells	223
		Lung epithelial cells	246
		Renal proximal tubular cells	424
VEGF	Human	HUVECs	111
	Bovine	Endothelial cells	55
PDGF	Rat	Aortic smooth muscle cells	117
Gases and derivatives			
Nitric oxide (NO donors or gas)	Human	HeLa cells	66, 269
		Embryonic lung fibroblasts	269
	Rat	Vascular smooth muscle cells	118, 252
	Mouse	Macrophages	19
Carbon monoxide	Human	HUVECs	208
	Rat	Pheochromocytoma cells	237
Peroxynitrate	Rat	Pheochromocytoma cells	236
ROS			
H_2O_2	Human	Keratinocytes	81
		Hepatoma	136
		Fibroblasts	203
	Rat	Vascular smooth muscle cells	81
	Mouse	Fibroblasts	81

(*continued*)

TABLE 1. INDUCERS OF HO-1 EXPRESSION/ACTIVITY (CONT)

Stimulus	*Species*	*Cell line*	*References*
UVA	Human	Fibroblasts	203
Photodynamic therapy	Human	HeLa cells	214
		Urinary bladder carcinoma	
	Mouse	Colon adenocarcinoma	310
Oxygen tension			
Hypoxia	Rat	Glioma cells	211
		Vascular smooth muscle cells	290
Anoxia	Rat	Cardiac monocytes	123
Hyperoxia	Mouse	Macrophages	227

B. Regulation of the HO-1 gene

The human HO-1 gene *(Hmox-1)* consists of five exons and four introns, spanning a 14-kb region at human chromosome 22q12 (383). As in the rat *Hmox-1* (299), no typical TATA or CAAT boxes are present in the 5′-flanking region of the human gene. However, a TATA-like sequence, ATAAATG, is located 21 bp upstream of the transcription initiation site (383). Analyses of the *Hmox-1* genes of various species, including mouse (16), rat (299), chicken (260), and human (383), revealed discrete differences between their promoters, which can be at least partially translated to species-dependent *Hmox-1* regulation at the transcriptional level.

Shibahara and co-workers (383) stressed the importance of the proximal promoter regions, located near the transcription start site. They found a potential heat-shock element (HSE) (CTGGAACCTTCTGG, nucleotide residues −401 to −368) (383) in the human *Hmox-1*, similar to the rat *Hmox-1* gene (299) (Fig. 2). It seems, however, that the human HSE is not functional and is consistent with observations that human HO-1 is not induced by heat shock (472). Apart from HSE, many other positive regulatory elements have been found in the HO-1 promoter, such as a stress-responsive element (StRE) (176), cadmium-responsive element (CdRE) (407), SMAD-binding element (SBE) (424), consensus binding sites for activating protein-1 (AP-1) (13), nuclear factor-κB (NF-κB) and AP-2 (226), STATx (227), and upstream stimulatory factor (USF) (307). Moreover, the region located between position −1976 and −1655 contains a potential binding site for c-Rel, hepatocyte nuclear factor-1 (HNF-1), HNF-4, and GATAx (406) (Fig. 2).

The importance of the distal regions that are involved in HO-1 regulation was first described in mouse *Hmox-1*. Two such sequences have been discovered: the 268-bp E1 enhancer fragment and the 161-bp fragment located approximately −4 and −10 kb relative to the transcriptional start site, respectively (13, 16, 17). These regions are extremely important for heme, heavy metals, H_2O_2, and sodium arsenite–mediated regulation of transcription (15–17).

Some distal sequences of the human *Hmox-1* gene have been identified, mostly close to the −4- and −10-kb regions (162, 163) (Fig. 2). An internal enhancer located in the human *Hmox-1* gene, which is specific for heme and cadmium-mediated transcription but does not function for other known HO-1 stimuli, such as transforming growth factor-β (TGF-β), H_2O_2, or 13-hydroperoxyoctadecadienoic acid (13-HPODE) has been identified (163) (Fig. 2). Additionally, the regulatory sequences responsible for 13-HPODE– and TGF-β–mediated human HO-1 induction (424) have been found close to the -10-kb region (between 9.1 and 11.6 kb) (162).

Among the positive elements, a dominant role is played by StRE, a 10-bp motif with the consensus sequence of

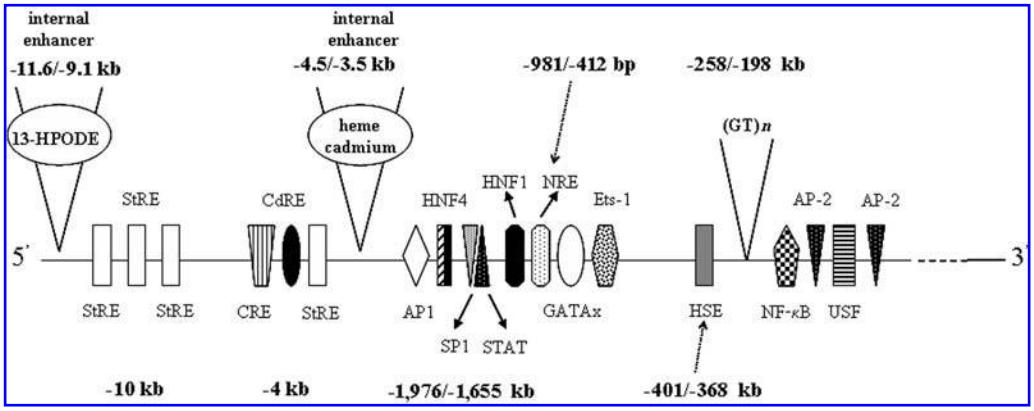

FIG. 2. Human HO-1 gene promoter organization. The locations of several transcription factor consensus binding sites are indicated, as well as regulatory regions in the 5′-proximal and -distal regions. AP-1,2, activator protein 1, 2; CdRE, cadmium-responsive element; GATAx, GATA-binding protein; (GT)*n* region, variable length (11–40) polymorphic GT repeat sequence; HNF1/4, hepatocyte nuclear factor 1/4; HSE, heat-shock element; NF-κB, nuclear factor kappa-B; NRE, negative regulatory element; SP-1, specificity protein-1; STAT, signal transducer and activator of transcription; StRE, stress-responsive element; USF, upstream stimulatory factor.

(T/C)GCTGAGTCA (176) (Fig. 2). The StRE is structurally and functionally similar to the Maf response element (MARE) and the antioxidant response element (ARE) (176). This site appears to play a crucial role in nuclear factor-erythroid 2–related factor 2 (Nrf2)-mediated induction of HO-1. Many different proteins (both hetero- and homodimers) can bind to the StRE, including Jun, Fos, CREB, ATF, Maf, and the cap 'n' collar/basic leucine zipper (CNC-bZIP) subclasses of the basic-leucine zipper (b-ZIP) family of transcription factors.

In contrast to the various positive elements described earlier, only two negative regulatory elements (NREs) are present in the promoter of the human HO-1 gene. One contains consensus binding sites for NRE boxes and is located between position −981 and −412 bp (406) (Fig. 2). Another one (situated between −258 and −198) corresponds to a polymorphic microsatellite DNA region consisting of 11–40 GT repeats (406). Longer (GT)*n* sequences (>25) have been associated with weaker transcription of HO-1 in response to oxidative stimuli (164). A consequence of the attenuated HO-1 expression could be the higher incidence of some diseases in patients with the longer (GT)*n* fragment (461) (see also *Polymorphism of HO-1 promoter).*

1. Inducers and inhibitors of HO-1 expression. Different factors inducing HO-1 mRNA transcription and/or accumulation, protein expression, or enzymatic activity are listed in Table 1 (see also Fig. 3). The effect of heme, both a substrate and an inducer of HO-1 expression, has been widely studied in many cell types, including macrophages (472), keratinocytes (185), and endothelial cells (386). HO-1 also is upregulated by reactive oxygen species (ROS), UVA radiation, inflammatory cytokines, growth factors, heavy metals, oxidized lipids, nitric oxide (NO) donors, and many others. In contrast to this large number of inducers, only some inhibitors of HO-1 expression are known. The repression of HO-1 expression by hypoxia, desferrioxamine, or interferon-γ (IFN-γ) appears to be restricted to human cells (211, 305, 405). Similarly, heat shock induces HO-1 only in rodent cells (383, 472) (see *Species differences in regulation of HO-1 gene expression*). Tin and zinc protoporphyrins are widely used inhibitors of HO-1 activity; however, they concomitantly cause an increase in HO-1 mRNA and protein expression (287, 364, 464) (Table 2).

2. Involvement of Nrf2 in regulation of HO-1 gene expression. Nrf2 plays a special role in HO-1 regulation, distinct from other transcription factors (Fig. 3). Nrf2 belongs to a cap 'n' collar–related basic region leucine-zipper factor family (284), which includes p45 NF-E2 (22), Nrf1 (59), Nrf2 (285), and Nrf3 (212). In addition, two distantly related proteins are termed Bach1 and Bach2 (323).

Nrf2 is a key regulator of antioxidant-responsive genes and phase II detoxifying enzymes, including HO-1, γ-glutamylcysteine synthetase (γ-GCS), glutathione S-transferase (GST), and NAD(P)H:quinone reductase. It typically exists as a heterodimer with a protein of the Maf family (MafK, MafG, and MafF) (294) and recognizes MARE (Fig. 3). The importance of this transcription factor was made clear by work with knockout animals. Although in heterozygous Nrf2 mice, the expression of detoxifying enzymes is not dis-

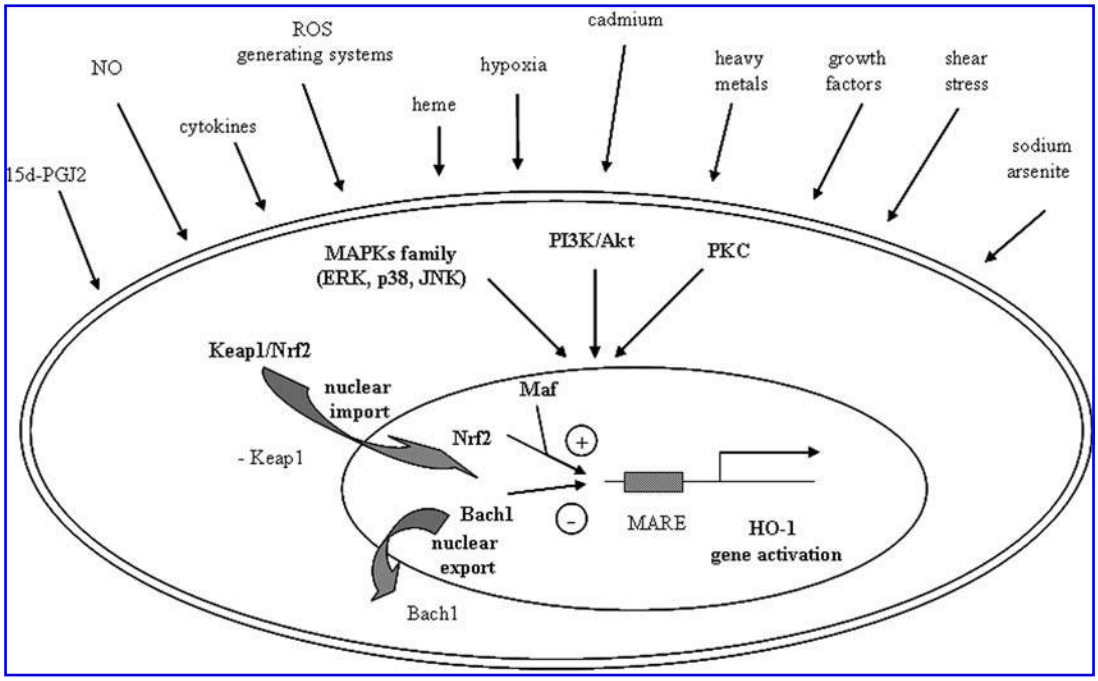

FIG. 3. Induction of HO-1 by Nrf2 transcription factor. Various agents can influence HO-1 transcription *via* activation of different kinases, and Nrf2 transcription factor. Keap1 sequesters Nrf2 in cytoplasm; however, after stimulation with thiol-modifying compounds, the SH-groups in cysteines of Keap1 are oxidized. Nrf2 undergoes nuclear translocation. At the same time the nuclear export of the transcriptional repressor Bach1 occurs. In some human cells (*e.g.*, endothelial), hypoxia inhibits the expression of HO-1 by induction of Bach1, which, by binding to MARE, blocks the HO-1 transcription. See details in text.

Table 2. Inhibitors and Noninducers of HO-1 Expression in Human Cells

Inhibitor	*Cell line*	*References*
Hypoxia	Lung cancer cells	211
	HUVECs, astrocytes, CAECs	305
Desferrioxamine	HUVECs, glioblastoma	211
Cytokines	Retinal epithelium	431
IFN-γ	Glioblastoma	211
Heat shock	Macrophages, glioma cells	472

turbed, *Nrf2*-null mutant animals have an impaired xenobiotic inductive response involving phase II detoxifying enzymes (184).

In unstimulated cells, the entrance of Nrf2 to the nuclei is blocked by its inhibitor, Kelch-like ECH-associated protein 1 (Keap-1) (Fig. 3). Keap1, a cytoplasmic protein, was identified as an inhibitor of Nrf2 and the expression of phase II detoxifying-enzyme genes is increased significantly in homozygous Keap1 knockout animals (441). In nonstimulated cells, Keap1 recruits Nrf2 into the Cul3-containing ubiquitin ligase complex for ubiquitin conjugation and subsequent proteosomal degradation. Stress-induced thiol modification of cysteine residues in Keap1 releases Nrf2, which can bind small Maf1 protein and activate the ARE in genes encoding antioxidant enzymes (213).

A great number of HO-1 inducers act through Nrf2. One of them, 15d-PGJ_2, synthesized *via* prostaglandin H_2 (PGH_2) from arachidonic acid by the action of cyclooxygenase (COX), very potently augments HO-1 expression in different cell types (137, 183, 479). In mesangial cells, the effect of PGJ_2 is connected with direct interaction with Keap1 (479) (Fig. 3). 15d-PGJ_2 was shown covalently to bind Keap1. The modification of its cysteine residues causes the translocation of Nrf2 from the cytoplasm to the nucleus (183). Similar to PGJ_2, PGA_1 also induces the nuclear accumulation of Nrf2 (183).

Keap1 is a cysteine-rich protein and has 25 cysteines that can be possibly modified by some electrophilic intermediates. Five of these residues are extremely important for Nrf2 activation. Electrophilic compounds can attack cysteine residues, leading to conformational change of the Keap1-Nrf2 association motif and dissociation of Nrf2 from Keap1 (440). The mechanism of NO-mediated regulation of Nrf2 hypothetically can also involve S-nitrosylation of "oxidant sensing" cysteine residues in Keap1, and this might promote dissociation of Nrf2 from the complex and translocation into the nucleus to initiate ARE-mediated gene transcription. The same mechanism was shown for 15d-PGJ_2, a nucleophilic prostaglandin (225).

Nrf2 appears to be involved in the maintenance of gene expression in endothelial cells under physiologic conditions of blood flow (72, 168, 193, 448). Human coronary aortic endothelial cells (HAECs) kept in static culture or subjected to laminar flow with shear stress at 20 dyn/cm^2 show a different expression of HO-1 as well as other ARE-regulated genes, including ferritin [heavy (H) and light (L) chains], quinone oxidoreductase-1 (NQO1), microsomal epoxide hydrolase (mEH), GST, and γ-GCS (72). In human microvascular endothelial cells (HMEC-1s), exposure to laminar flow caused a dramatic increase in the ARE-driven HO-1 promoter activity, which was diminished when the ARE sequence was mutated (72).

It should be remembered that in the vasculature, some areas have different blood flow. Nonlaminar, oscillatory, or low fluid shear stress (5 dyn/cm^2) is proatherogenic, whereas laminar flow and high fluid shear stress (20 dyn/cm^2) is atheroprotective. Interestingly, laminar flow induced the expression of NQO1 by fivefold, and HO-1, by eightfold. In contrast, oscillatory flow did not change the expression of those genes; however, both pro- and antiatherogenic flows induced the nuclear accumulation of Nrf2 to comparable levels (168). It is supposed that the differences observed in NQO1 and HO-1 expressions are due to oscillatory flow-mediated inhibition of Nrf2 binding to ARE in its target genes. The authors suggested the existence of a special ARE-binding factor that is inducible by oscillatory flow or is inhibited by laminar flow and that influences DNA binding activity by Nrf2. This hypothesis remains to be confirmed.

The potent endogenous inducer of HO-1 expression is NO (53, 101). Its production in endothelial cells can be elevated by blood flow, which affects the expression of endothelial nitric oxide synthase. Accordingly, Warabi *et al*. (448) evaluated the role of reactive nitrogen species in the shear stress–mediated upregulation of antioxidant gene expression in human umbilical vein endothelial cells (HUVECs) (448). However, although NO production was increased after laminar stress, neither the inhibitor of endothelial NO synthase (eNOS) nor siRNA against eNOS affected the expression of Nrf2-regulated genes (448). Instead, oxygen species (mostly $O_2^{\bullet -}$) and modifications caused by them, like lipid peroxidation products, played a pivotal role in this process.

3. Bach1–dependent regulation of HO-1. Apart from Keap1, which sequesters Nrf2 in the cytoplasm, another inhibitor of Nrf2-induced activation of HO-1 was found to act in human cells. Bach1 is a heme-regulated transcriptional repressor for the HO-1 gene and plays an important role in the feedback regulation of HO-1 expression (211, 398). Moreover, another related transcription repressor, Bach2, has been discovered to be expressed in the brain (323) and B lymphocytes (301); however, no data are available regarding the role of Bach2 in endothelial cells.

The involvement of Bach1 in regulation of HO-1 has been shown in studies on Bach1-knockout animals. In Bach1$^{-/-}$ mice, HO-1 protein was expressed at much higher level in various organs, including thymus, heart, and lung, but not in the spleen, in comparison to the wild-type mice (398). As

a consequence of the increased amount of HO-1, Bach1-knockout mice are resistant to proatherosclerotic and ischemic stimuli (398).

Bach1 has two functions that are important for HO-1 regulation. First, similar to Nrf2, it can bind Maf proteins and then acts *via* the MARE of the HO-1 promoter; however, in contrast to Nrf2, it represses HO-1 transcription (398). Second, Bach1 binds heme with high affinity, and this Bach1–heme interaction prevents Bach1 binding to MARE (313); therefore, heme abrogates the repressor function of Bach1. Heme also has the ability to regulate the nuclear export of Bach1 (401).

Bach1 appears to be extremely important for the hypoxia-induced HO-1 repression observed in some human cells (211). It was shown that concomitant with hypoxic downregulation of HO-1 expression, augmentation of Bach-1 expression occurs in human glioblastoma cells, lung cancer cell line, and umbilical vein endothelial cells (211). Additionally, upregulation of Bach1 expression caused decreased HO-1 promoter activity (211). However, in the D407 retinal pigment epithelium cell line, hypoxia induces both HO-1 and Bach-1 mRNA (332). The lack of opposite regulation of HO-1 and Bach-1 suggests that, at least in the D407 cell line, Bach-1 may not be a key determinant for the hypoxia-mediated modulation of HO-1 expression.

Similar to hypoxia, desferrioxamine and IFN-γ also induce the expression of Bach1, leading to downregulation of HO-1 in human glioblastoma cells (211). Interestingly, potent HO-1 inducers have been reported to inhibit Bach1. In human liver cells, CoPPIX reduced the Bach1 level *via* increased degradation of Bach1 protein ($t_{1/2}$ was reduced from 19 h to 2.8 h) (378). Additionally, tin mesoporphyrin SnMPPIX, a competitive HO inhibitor that concomitantly induces both HO-1 mRNA and protein expression, was shown to accelerate Bach1 degradation, enhancing HO-1 expression in the NIH3T3–HO-1-luc cells (1).

4. AP-1 family. Recent studies indicate, however, that regulation of HO-1 expression, ascribed to Nrf2, can be more complicated. Accordingly, Hock *et al*. (166) performed a comprehensive evaluation of the regulatory regions of the human HO-1 gene by using DNase I hypersensitivity and *in vivo* DMS footprinting in human renal epithelial cells. Four constitutive hypersensitive sites (HSs) within the HO-1 promoter were identified. One proximal region, HS-1, mapped to ~2.2 kb proximal to an *Hin*dIII site, was shown previously to be an E box (166). Three distal sites (HS-2, -3, and -4), corresponding to approximately −4.0, −7.2, and −9.2 kb, respectively, of the HO-1 promoter, were present in both vehicle and hemin-treated cells (Fig. 2). *In vivo* DMS footprinting of the HS-2 region revealed six individual protected guanines. Single mutations (adenine substituted for guanine) of each of these six guanines revealed that only one of the substituted guanine regions (G-5) decreased HO-1 promoter activity by ~50% in both hemin- and cadmium-treated cells as compared with the wild-type 4.5-kb promoter construct. This protected guanine (G-5) is located within a classic AP-1 sequence (TGACTCA) that extends to form an ARE sequence. When two mutations within HS-2, the G-5 single-point mutation plus deletion of a region that resides in a consensus DNA-binding motif for cAMP response element (CRE), were combined with a mutation of the proximal E box in HS-1 (shown to prevent USF binding), hemin- and Cd-driven HO-1 promoter activation was abolished. These recently published studies suggest that these three sites (G-5 and CRE in HS-2, and an E box in HS-1) synergize for maximal HO-1 gene expression (166, 167).

Antibody supershift experiments were performed with anti-Nrf1, anti-Nrf2, and anti-Jun pan (recognizing c-Jun, JunB, and JunD) antibodies. Only the Jun pan antibodies produced a reduction in band-shift intensity and a small supershift in nuclear extracts from uninduced (control) or hemin-stimulated HK-2 cells. Both Nrf1 and Nrf2 antibodies failed to show a supershift or signal reduction of the protein–DNA complex. Because Jun proteins bind to the ARE in the HS-2 region *in vitro*, their association with the HO-1 promoter *in vivo* was examined by chromatin immunoprecipitation assays. In the proximal promoter region, a significant signal with JunD in the vehicle-treated lane was detected, but not after hemin induction. In the −4.0-kb region, a significant association with JunB (in vehicle and hemin-treated samples) was observed. JunB and JunD overexpression studies demonstrated that JunB activated whereas JunD repressed HO-1 promoter activity and HO-1 expression in human renal epithelial cells, results that were corroborated in junB$^{-/-}$ and junD$^{-/-}$ cells. Taken together, these results demonstrate that JunB and JunD have opposing effects; JunD is a repressor, whereas JunB is an activator of HO-1 expression. Those interactions, remaining to be confirmed in cells of the vascular bed, may be relevant in terms of the regulation of angiogenic genes. Interestingly, JunD, which represses HO-1, is antiangiogenic, whereas Jun B, which is proangiogenic, upregulated HO-1 expression (167).

5. Species differences in regulation of HO-1 gene expression. Although many similarities exist between *Hmox-1* regulation in different species, a growing body of evidence clearly indicates that human and mouse *Hmox-1* genes are regulated in opposite ways under particular circumstances. The main differences between human and animal HO-1 regulation include (a) the interspecies variations in the hypoxic modulation of the HO-1 gene, (b) the differences between heme- and cadmium-mediated HO-1 expression, (c) the existence of HO-1 promoter polymorphism, and (d) the identification of an internal enhancer in regulation of the human HO-1 gene (163). Moreover, human HO-1 is not induced by heat shock, although HSE was identified in the human promoter 5′ flanking region (383). Thus, the term heat-shock protein is not valid for human HO-1, and it better corresponds to rodent HO-1, which is highly upregulated after heat shock (299).

6. Hypoxia and HO-1. Hypoxia, the state of low oxygen concentration, induces the expression of many genes, of which the most important are those responsible for proper oxygen and nutrient supply. Hypoxic regulation of transcription of numerous genes is dependent on hypoxia-inducible factor-1 (HIF-1) stabilization. HIF-1 consists of two subunits, α and β, the first of which is degraded at normal oxygen concentration, in a process initiated by the hydroxylation of specific proline residues by three prolyl hydroxylases (PHDs 1–3). PHDs need the presence of oxygen, iron, and 2-oxoglutarate, so their activity can be diminished by

hypoxia, iron chelators, or 2-oxoglutarate analogues (for reviews, see 345, 455).

The effect of hypoxia on HO-1 expression seems to be species and cell-type dependent. Bach1 acts as a hypoxia-inducible regulator that represses the transcription of the HO-1 gene in some human cells. Although the hypoxia-responsive element (HRE)-like sequences were found in the promoter regions −1,249 to −1,255 and −36 to −43 of human *Hmox-1* (98), it is still not clear whether they correspond to typical HRE (*e.g.*, TACGTG sequence), present in regulatory elements of vascular endothelial growth factor (VEGF) or erythropoietin genes, which are strongly upregulated in hypoxic conditions. Nevertheless, the effect of hypoxia on HO-1 expression is inhibitory in many investigated human cell types (258, 305). In contrast, HO-1 is induced by hypoxia in rat, bovine, mouse, and monkey cells (211).

Hypoxia (1% O_2) decreased the expression level of HO-1 mRNA and protein in HUVECs, despite the functional activation of HIF-1 (305). Additionally, hypoxic repression of HO-1 mRNA was observed in A549 human lung cancer cells (211), human astrocytes, and coronary artery endothelial cells (305).

However, data also show induction of human HO-1 in hypoxic conditions. It occurs in D407 cells, the retinal pigment epithelial cell line (431), in dermal fibroblasts (332), and in keratinocytes (185). Low oxygen tension does not affect the expression of HO-1 in either ARPE19 human retinal pigment epithelium (431) or explants of normal human chorionic villi from term placentas (25) and does not change the protein level of HO-1 in HMEC-1 (258).

HO-1 expression is induced by hypoxia in different animal cells, such as C6 rat glioma cells, bovine brain microvascular endothelial cells (BBMVECs), COS7 monkey kidney cells (211), rat vascular smooth muscle cells (289, 290), and Chinese hamster ovary cells (300). Also, anoxia (no oxygen) upregulates HO-1 expression in rat cardiac myocytes (123). However, in sheep maintained at high altitude (3,820 m), the hypoxic atmosphere does not change HO-1 levels in comparison to those in animals kept in normal conditions (210).

These variable results indicate that species and cell-type differences exist in the mechanisms of hypoxia sensing or in the response to hypoxia. Moreover, the inhibition of HO-1 observed in human tissues exposed to low oxygen tension was postulated to be an important defense strategy. First, it was hypothesized that the reduced expression of HO-1 by hypoxia may reduce energy expenditure used for heme catabolism (305). It should be remembered that the heme breakdown catalyzed by HO-1 is an energy-consuming reaction, in which at least 3 moles of oxygen and 4 moles of NADPH are required to cleave 1 mole of heme (414). Additionally, lower HO-1 expression in hypoxic conditions prevents the local accumulation of CO, iron, and bilirubin, which under certain circumstances can reveal detrimental properties. Moreover, according to Shibahara and co-workers (382), the functional evolutionary consequences of HO-1 repression by hypoxia should be taken into consideration. These authors suggested that HO-1 regulation by hypoxia can be a human defense mechanism against severe malaria caused by *Plasmodium falciparum*. HO-1 is likely to be involved in the pathogenesis of malaria, because this disease is usually associated with intravascular hemolysis, anemia, and hyperbilirubinemia (217). *Plasmodium* parasitizes erythrocytes and digests hemoglobin molecules, which results in anemia. Anemia decreases the absolute amount of oxygen transported per unit volume of blood, resulting in anemic hypoxia. Moreover, heme degradation products can be involved in the pathogenesis of cerebral malaria. As iron is essential for the proliferation of not only *Plasmodium* but also other protozoa or bacterial pathogens, they must take it up from blood or cells in the vascular wall, although they reside in hemoglobin-rich erythrocytes. In this context, the repression of HO-1 by hypoxia (as well as by heat shock, *e.g.*, during malaria fever) may be beneficial for patients with malaria (408). As suggested by Shibahara and co-workers (382–385), a repression of HO-1 and HO-2 expression under hypoxic conditions, as observed in some human cell types, may represent a defense strategy, by decreasing energy expenditure for oxidative heme breakdown and preventing local accumulation of its degradation products (see also *Polymorphism of HO-1 promoter*).

In contrast to this hypothesis, recent elegant studies in mice suggested that HO-1 can protect against cerebral malaria (330). The authors demonstrated that exogenously added CO inhibits blood–brain barrier disruption, brain microvascular congestion, and hemorrhaging, as well as suppresses neuroinflammation by the inhibition of adhesion molecule expression in the brain microvasculature and the suppression of activated $CD8^+$ T-cell sequestration in the brain. These results showing that HO-1 can be used therapeutically to suppress the pathogenesis of cerebral malaria, in contrast to a previous study (382), leave unanswered the question about the significance of HO-1 in the development of this disease.

7. Polymorphism of HO-1 promoter. The human HO-1 gene promoter is polymorphic mainly because of variation in the number of GT repeats, (GT)*n*, ranging from 11 to 40 (120). The (GT)*n* repeat length usually shows a bimodal distribution, with the median length of the short repeat being around 23 pairs and that of the long repeat being around 30 pairs in different populations studied (52, 74, 120, 374).

This purine–pyrimidine alternating sequence, possessing Z-conformation potential, can negatively affect transcription, especially as it is located between the regulatory elements and the TATA box (about −258/−198 bp) of the HO-1 gene (163) (Fig. 2). Interestingly, longer (GT)*n* sequences in this region have been associated with attenuated HO-1 transcriptional activity. Transient transfections with plasmids containing reporter genes driven by variants of the human *Hmox-1* promoter showed that the basal transcriptions levels from the short (S/S) promoter constructs (GT = 16–20) were 2.5 times higher than those from long (L/L) ones (GT = 29–38) and that only short promoters were activated in response to oxidative stress (164, 461). The association of HO-1 promoter polymorphism with cardiovascular diseases is discussed in the section entitled *Role of HO-1 in Pathologic Conditions*.

III. HO-1 Deficiency as an Indication of HO-1 Significance

The multiple protective properties of HO-1 in the cardiovascular system have been demonstrated after chemical

stimulation/inhibition or gene transfer of HO-1. However, chemical inhibitors of HO-1 (such as tin or zinc protoporphyrin) have many effects beyond altering HO enzyme activity (144, 194). Therefore, transgenic mice that are genetically deficient in HO-1 protein are a much better model to study the importance of this enzyme in the cardiovascular system. Studies on HO-1–knockout mice, as well as detailed analysis of the only described case of HO-1 human deficiency (460), demonstrated that HO-1 is a very important protein in the regulation of cardiovascular functions.

A. HO-1 knockout mice

HO-1–deficient mice were generated and described for the first time by Poss and Tonegawa (340, 341). The authors characterized HO-$1^{-/-}$ mice as an animal model of human iron-overload disorders, as several symptoms are similar to those seen in hemochromatosis patients. These include splenomegaly, tissue iron deposition with increasing age, high $CD4^+/CD8^+$ T-cell ratios, increased lipid peroxidation, fibrosis and hepatic injury, late-onset weight loss, decreased mobility, and premature mortality (340). Furthermore, mature HO-1–deficient males have a nearly 25% reduction in the size of testes as compared with similarly sized HO-1 heterozygous (HO-$1^{+/-}$) littermates (340). Hypogonadism and lack of libido are common in males affected with hereditary hemochromatosis. Further studies demonstrated that, apart from an important role in iron metabolism, HO-1 exerts many other biologic effects (*e.g.*, it is also necessary for the protection of cells from potential oxidative damage during stress) (341). HO-$1^{-/-}$ murine embryonic fibroblasts (MEFs) exposed to several oxidants, such as hemin, H_2O_2, or cadmium chloride ($CdCl_2$), showed increased free radical production (232%, 31% and 48%, respectively, over untreated cells) and reduced survival in comparison to MEFs isolated from HO-$1^{+/-}$ or wild-type embryos (341). Although HO-1–deficient mice with endotoxemia had earlier resolution of hypotension, the mortality and the incidence of end-organ damage were higher in the absence of HO-1, and they were extremely sensitive to LPS exposure, indicating that the HO-1 pathway is a crucial antiinflammatory system (341).

It should be noted that vascular endothelial and smooth muscle cells derived from HO-1 knockout mice are more sensitive to oxidized lipid-induced cell injury (68) and more susceptible to H_2O_2-induced cell death than those isolated from wild-type mice, suggesting that increased susceptibility to oxidative stress may lead to vascular cell injury in the absence of HO-1 (468).

Decreased oxygen tension is known to upregulate the HO-1 expression in rodents, and this may play an important adaptive role in protection against cardiovascular system dysfunction (228). Accordingly, HO-$1^{-/-}$ mice were also shown to have a maladaptive response to hypoxia and subsequent pulmonary hypertension (469). Exposure of HO-1–knockout mice to hypoxia (10% of oxygen) for 5–7 weeks led to greater ventricular weight than in case of wild-type mice, although the right ventricular systolic pressure was similar in both cases. Moreover, the right ventricles were more dilated in HO-1–deficient mice. After 7 weeks of hypoxia, only HO-$1^{-/-}$ animals developed right ventricular infarcts with organized mural thrombi (469). Arterial thrombosis was also accelerated in mice devoid of HO-1 after photochemical-induced vascular injury, an effect that was rescued by inhaled carbon monoxide (427).

B. Human HO-1 deficiency

The first known human case of HO-1 deficiency was described in Japan in 1999 by Yachie and co-workers (201, 460). In this HO-1–deficient child, both intravascular hemolysis and endothelial cell injury were prominent. Importantly, oxidation of hemoglobin to methemoglobin occurred in the plasma, and iron was accumulating in the low-density lipoproteins (LDLs). Iron-induced oxidative modification of lipoproteins is cytotoxic and causes endothelial damage, leading to the development of fatty streaks and fibrous plaques in the aorta. The endothelial cells of this child were susceptible to oxidative insults because of heme-mediated oxidation of LDL (186) and an associated lack of adaptive responses, suggesting that HO-1 plays a crucial role in protecting vessels from oxidative injury (reviewed in 288).

Clinicopathologic findings have shown an absence of the spleen and a significantly enlarged liver with advanced atrophy of hepatocytes. Marked amyloid deposition and many foam cells in the liver and reticuloendothelial tissue were observed in this patient. Moreover, asplenia might have contributed to the robust endothelial cell damage induced by oxidative stress because of the absence of the splenic filtering function (201). An increased level of haptoglobin in the blood additionally indicated the enhanced inflammatory reaction in the tissues. Taken together, the human case of HO-1 deficiency was much more severely affected by oxidative stress than were HO-1 knockout mice. No doubt, all these contributed to the early death of the patient.

In summary, *in vivo* studies in transgenic HO-$1^{-/-}$ mice as well as the human HO-1–deficiency case showed the important role of HO-1 activity in anatomically and functionally diverse processes and illustrate the physiologic versatility of this single enzymatic reaction. In this regard, HO-1 deficiency seems to be related to many dangerous side effects, which may implicate the impairment or loss of function of different organs and tissues. One of the most important is injury of vascular endothelium leading to cardiovascular diseases. Fortunately, apart from the only case of human HO-1 deficiency recognized so far, it seems that a much more common phenomenon in the human population is variation of HO-1 activity, depending on the length of the GT repeat in the HO-1 promoter.

IV. Protective Role of HO-1 Products

A. Carbon monoxide

Much effort was undertaken to elucidate the protective effect of HO-1 and CO in biologic systems. The study originally involved the use of exogenously administered CO gas. The recently invented CORMs (carbon-monoxide–releasing molecules, a new class of compounds that can increase the CO level) seem to be a good tool for investigating CO activities in both *in vitro* and *in vivo* systems (296).

Studies as early as in 1991 had discovered that CO may activate the soluble form of guanylyl cyclase (sGC) and be responsible for cGMP formation (134), thus working in a similar way as NO. Subsequent studies have shown its important biologic activities: modulation of vasomotor tone (116)

and involvement in neuronal transmission (42, 95) (Fig. 4). Moreover, CO can exert potent antiinflammatory effects, probably *via* the p38 mitogen-activated protein kinase (p38 MAPK) (359). It was shown to be responsible for the inhibition of proinflammatory cytokine production, including tumor necrosis factor (TNF), interleukin-1β (IL-1β), and macrophage inflammatory protein-1β (MIP-1β) in macrophages stimulated with LPS (320). In addition, upregulation of IL-10 is a further mechanism responsible for the antiinflammatory actions of CO (320). Similar results have been obtained by using CORM-2 and CORM-3, suggesting that CO carriers can be used as an effective strategy to modulate inflammation (369).

Another protective CO effect is mediated through GC/cGMP and is correlated with preventing platelet activation and aggregation (51). However, a recent article by Chlopicki *et al*. (77) suggested that CORM-3–induced platelet aggregation does not rely on GC. The authors presented supportive data showing that in the presence of an inhibitor of GC (1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one, ODQ), inhibition of collagen-induced aggregation by CORM-3 was not blocked but was potentiated (77).

CO also suppresses thrombosis and the proinflammatory response stimulated by activated platelets (77). Additionally, in macrophages, CO downregulates expression of plasminogen activator inhibitor type 1 (PAI-1), crucial for the protective effect in a model of ischemia–reperfusion (I/R) of the lung (133). In experiments performed on HO-1–deficient cells, exogenously administered CO reduced oxidized LDL (oxLDL)-elevated PAI-1 expression, again providing support for the antithrombotic effect of CO (270). Convincing data about CO-mediated protection connected with increased thrombolysis came from *in vivo* studies. It was recently shown in apolipoprotein A (ApoE)-deficient mice subjected to angioplasty, in which arterial thrombosis occurred 12 h after injury (73). Both treatment of the injured vessels with an adenovirus bearing the HO-1 gene (AdHO-1) and exposure of animals with existing arterial thrombosis to CO (250 ppm, 2 h), resulted in reduced expression of PAI-1, earlier thrombolysis, less fibrinogen deposition, decreased MIP-1 expression and macrophage infiltration, and diminished neointimal formation (73).

Apart from these activities, CO inhibits proliferation of different cell types, like T cells (328, 392) and vascular smooth muscle cells (VSMCs) (291). Inhibition of VSMC proliferation by G_0/G_1 arrest is mediated by upregulation of G_1-cyclin–dependent protein kinase inhibitor p21cip1 and activation of p38 MAPK (390). Conversely, CO prevents apoptosis in several cell types, including endothelial cells (47, 48, 320, 321), vascular smooth muscle cells (251), fibroblasts (336), osteoblasts (60), and the β-cells of the pancreas (148).

1. CO as an antiapoptotic molecule. The protective action of CO in the vascular bed is also correlated with its influ-

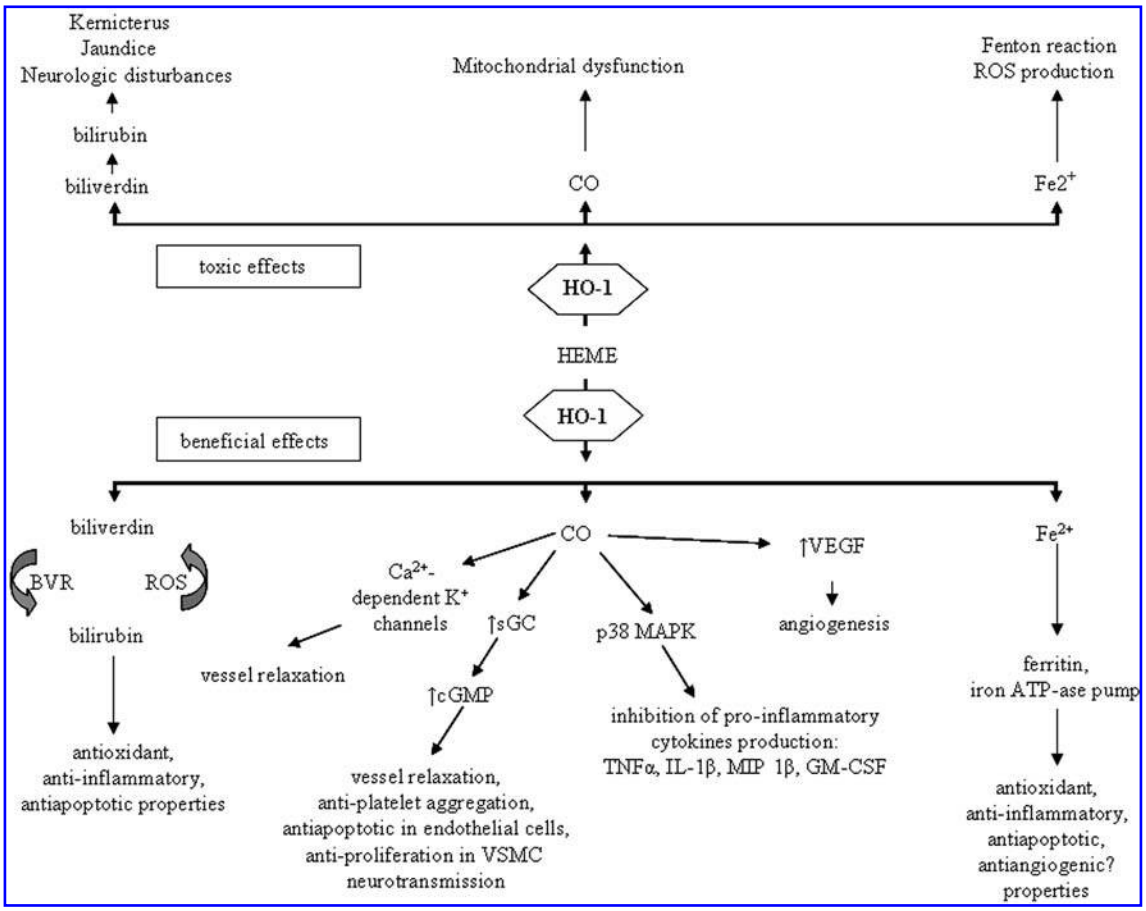

FIG. 4. The effect of HO-1 products on physiologic functions in the cardiovascular system.

ence on apoptosis of endothelial cells. Interestingly, even in the same types of cells, the effects appear to be mediated by activation of various signaling pathways (Fig. 5).

The cytoprotective effect of CO (10,000 ppm) against TNF-α–induced apoptosis in endothelial cells was diminished after treatment with SB 203580, an inhibitor of p38 MAPK or by a p38 MAPK dominant negative mutant (48), implying a role for p38 MAPK pathway. The role of a specific isoform of p38 has been investigated. In porcine aortic endothelial cells (PAECs), CO was able to modulate Fas/Fas ligand interaction and activation of executors of apoptosis: caspases 3, 8, and 9 in a p38 MAPKα–dependent way (478). In contrast, a study of murine lung endothelial cells and fibroblasts (207) highlighted the importance of the p38 MAPKβ isoform as well as heat shock factor-1 (HSF-1). Accordingly, CO was not able to protect cells from TNF-α–induced apoptosis when p38 MAPKβ was knocked down, and the CO effect was also diminished in HSF-$1^{-/-}$ (207). Thus, CO uses various p38 isoforms as mediators of the antiapoptotic effect in endothelial cells. Interestingly, p38 MAPKα and p38 MAPKβ have been shown to act in opposite ways in other cells, such as cardiac myocytes, in which p38α induces apoptosis, whereas isoform β suppresses death in the same cell type (447).

Experiments performed on a broad spectrum of endothelial cell lines [murine 2F-2B endothelial cell line, bovine aortic endothelial cells (BAECs), PAECs, and HUVECs] have also stressed the involvement of NF-κB transcription factor in the antiapoptotic effect of CO (47). Inhibition of NF-κB, by overexpression of its natural inhibitor IκBα, blocked the protective influence of HO-1/CO. However, such an effect was observed only when TNF-α–induced apoptosis was investigated. In the case of serum deprivation-induced apoptosis, even if NF-κB activity was inhibited, CO was able to protect endothelial cells (47), suggesting that different signaling pathways are involved in various models of apoptosis.

In turn, in VSMCs, cGMP but not MAPKs plays a central role in the antiapoptotic effect of CO (251). Moreover, the mechanisms of the antiapoptotic effect of CO in different cells involve regulation of the expression of both anti- and proapoptotic proteins. Expression of antiapoptotic A1, A20, c-IAP2 (47), Bcl-xl/Bag-1 (202) or Bcl-2 (478) was upregulated after CO treatment, whereas proapoptotic Bid may be downregulated (478).

Surprisingly, in contrast to many studies showing the antiapoptotic effect of CO in endothelial cells, Thom and co-workers (419) demonstrated that in bovine pulmonary artery endothelial cells (BPAECs), a low concentration of CO (100 ppm) has a proapoptotic effect. Conversely, exactly the same concentration of CO was able to inhibit apoptosis in porcine and bovine aortic endothelial cells, HUVECs, and the murine 2F-2B endothelial cell line (47). Interestingly, also in Jurkat T cells, treatment with CO (250 ppm) increased Fas/CD95-induced apoptosis, associated with the downstream activation of caspases, and the inhibition of antiapoptotic Bcl family members (391). Concomitantly, the downregulation of prosurvival ERK1/2 activity was observed (391).

In vivo models also show different effects of CO, which can be dependent on the dose of CO used. In rodent tissue, especially in brain regions, apoptosis connected with increased lipid peroxidation was observed after 1,000–3,000 ppm of CO (418). Similarly, 2,500 ppm CO caused cell loss in cortex, globus pallidus, and cerebellum of rats, and finally, CO-exposed rats showed learning and memory deficits (339). In contrast, a low CO level (50–500 ppm) protected against oxidative stress, such as hyperoxia (100% oxygen), similar to the cytoprotection observed with the elevated expression of HO-1 by chemical agents or expression vectors (319). Exogenous CO exposure attenuated lung-injury parameters such as bronchoalveolar lavage (BAL) protein and pleural effusion *in vivo*, as well as the index of inflammation (index of BAL neutrophils) (319). Inhalation of 250 ppm CO prevented I/R injury of transplanted rat lung grafts (215). In some studies, even a very low CO concentration (20 ppm)

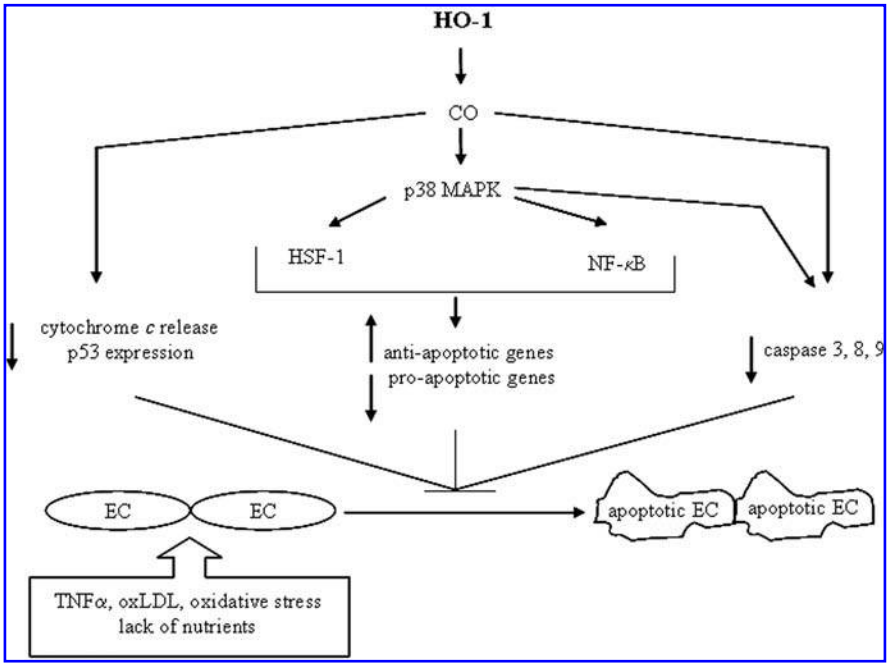

FIG. 5. Antiapoptotic action of CO in endothelial cells.

had a protective effect, as it can prevent development of chronic allograft nephropathy (309).

A majority of *in vivo* and *in vitro* studies suggest the protective effect of CO on vascular endothelium. The mechanisms are quite well characterized and involve the p38 kinase; however, the role of specific isoforms might differ in various vascular beds. Nevertheless, the reports on the potential toxic effects of CO on endothelium indicate the necessity of careful evaluation of the therapeutic effect of this gas.

B. Biliverdin and bilirubin

Biliverdin and bilirubin are widely known as bile pigments and for a long time have been regarded as waste products of heme degradation. However, recently they have been recognized as cytoprotective agents able, in many circumstances, to substitute for HO-1 activity.

Bilirubin is potentially toxic, but normally it is rapidly conjugated to glucuronic acid for urinary excretion. When the level of bilirubin exceeds 3 mg/dl, it can accumulate in the serum of neonates, causing "physiologic" jaundice, and untreated jaundice causes kernicterus (380). However, many studies have shown that this bile pigment is a physiologic neuroprotectant, reducing neural damage after various types of hypoxic insults, like stroke and neurodegenerative diseases (104, 105). Moreover, it protects lipid membranes against oxidation as efficiently as α-tocopherol and β-carotene (396). The recent description of a cycle in which biliverdin is converted to bilirubin by biliverdin reductase and bilirubin is recycled back to biliverdin on oxidation by peroxyl radicals (Fig. 1) suggests a mechanism that amplifies the antioxidant effects of the bile pigments (31). The amplification afforded by this cycle can readily explain the ability of low-nanomolar concentrations of bilirubin to overcome ~10,000-fold higher concentrations of oxidants (31).

The antioxidant properties of bilirubin have been demonstrated in a number of *in vitro* and *in vivo* studies. Bilirubin improves myocardial dysfunction after I/R (82) and protects neurons from H_2O_2-mediated cytotoxicity (105). Additionally, it possesses antiapoptotic and antiinflammatory activities. Inhibition of bilirubin production by specific siRNA against biliverdin reductase increased the ROS level and promoted apoptotic death in HeLa cells and primary neuronal cultures (31). Modulation of inflammation by bilirubin was shown in a rat model, in which it diminished LPS-induced P- and E-selectin expression in the vascular system, leading to inhibition of leukocyte rolling and adhesion *in vivo* (157).

The serum level of bilirubin can be a prognostic marker in many diseases. It was shown that slightly elevated circulatory bilirubin reduces risk of atherosclerosis and coronary artery disease (273). Another article demonstrated that a high bilirubin level was able to protect from coronary flow–reserve impairment, coronary microvascular dysfunction, and possibly coronary atherosclerosis (147). Clinical studies confirmed that having high-normal or supranormal levels of bilirubin is associated with less atherosclerotic-type disease as compared with that in individuals with low-normal levels of bilirubin (315). These results are corroborated by findings in the hyperbilirubinemic Gunn rats that exhibits mild elevations in unconjugated bilirubin and are protected against angiotensin II– and balloon injury–induced vascular injury (316, 338).

The potential therapeutic effects of bilirubin for the pharmacologic administration of bile pigments in various *in vivo* models have been suggested. The pharmacologic supplementation with bilirubin protected rats against hepatotoxicity during experimental endotoxemia (446). This action was dependent on the decrease in inducible NOS (iNOS) activity and NO production, as well as on the diminishment of proinflammatory cytokine expression (*e.g.*, TNF-α). Additionally, biliverdin/bilirubin may protect grafts from injury (128) and rejection (462). The improvement in survival of cardiac allografts was due to bilirubin inhibitory action on leukocyte infiltration and T-cell proliferation (462).

Results obtained by our group indicate that biliverdin may possess another activity. It can act as a potent inducer of proangiogenic factors (VEGF and IL-8) synthesis in human HaCaT keratinocytes both at mRNA (Fig. 6A) and protein levels (Fig. 6B) (see also 185). Administration of biliverdin remarkably increased VEGF expression, which was downregulated when specific inhibitors of ERK kinases (Fig. 6C) and Sp-1 transcription factor (Fig. 6D) were used. Interestingly, we observed a lack of the stimulatory effect of exogenously added bilirubin. It exerted rather an inhibitory effect on basal VEGF synthesis (data not shown), as well as potently diminishing H_2O_2-induced VEGF synthesis (Fig. 6E). Those results suggest that, apart from CO, biliverdin can mediate proangiogenic activities of HO-1 (see also *HO-1 and angiogenesis*). However, the effect of biliverdin may be cell-type specific, because other data indicate that it did not show any influence on VEGF expression in either rat vascular smooth muscle cells (114) or in a human endothelial cell line (unpublished data).

C. Iron

Iron, a crucial cofactor of many cellular enzymes and redox-dependent proteins, is essential for an astonishing number of biologic processes. In higher concentrations, however, it also possesses a potential to cause deleterious effects through Fenton chemistry (86, 159, 317).

HO-1 is claimed to be responsible for the regulation of the level of iron. Both in human HO-1 deficiency (460) as well as in HO-1–targeted mice (340), abnormalities in reuse of iron and tissue iron deposition have been observed. Although accumulation of iron has been detected in the liver and kidney, HO-1–deficient adult mice developed anemia associated with abnormally low serum iron levels (340). These effects might be mediated by the dysfunction of an endoplasmic reticulum–localized ATPase pump, which was found to be induced by free iron. This ATPase pump decreased the overall intracellular pool of Fe^{2+}, protected the cells from apoptosis, and, in HO-1 knockout mice, was responsible for selective tissue iron accumulation (32).

Iron can be toxic for cells and aggravate oxidative stress (*e.g.*, reactive iron liberated from heme catabolism by HO-1 was postulated to be involved in hyperoxia-mediated cytotoxicity) (400). However, free iron upregulates expression of ferritin, which possesses chelating activity toward iron and confers cytoprotection on endothelial cells in culture (28). Several studies have shown an association between the HO-1 activity and the synthesis of ferritin (435, 436). In these ex-

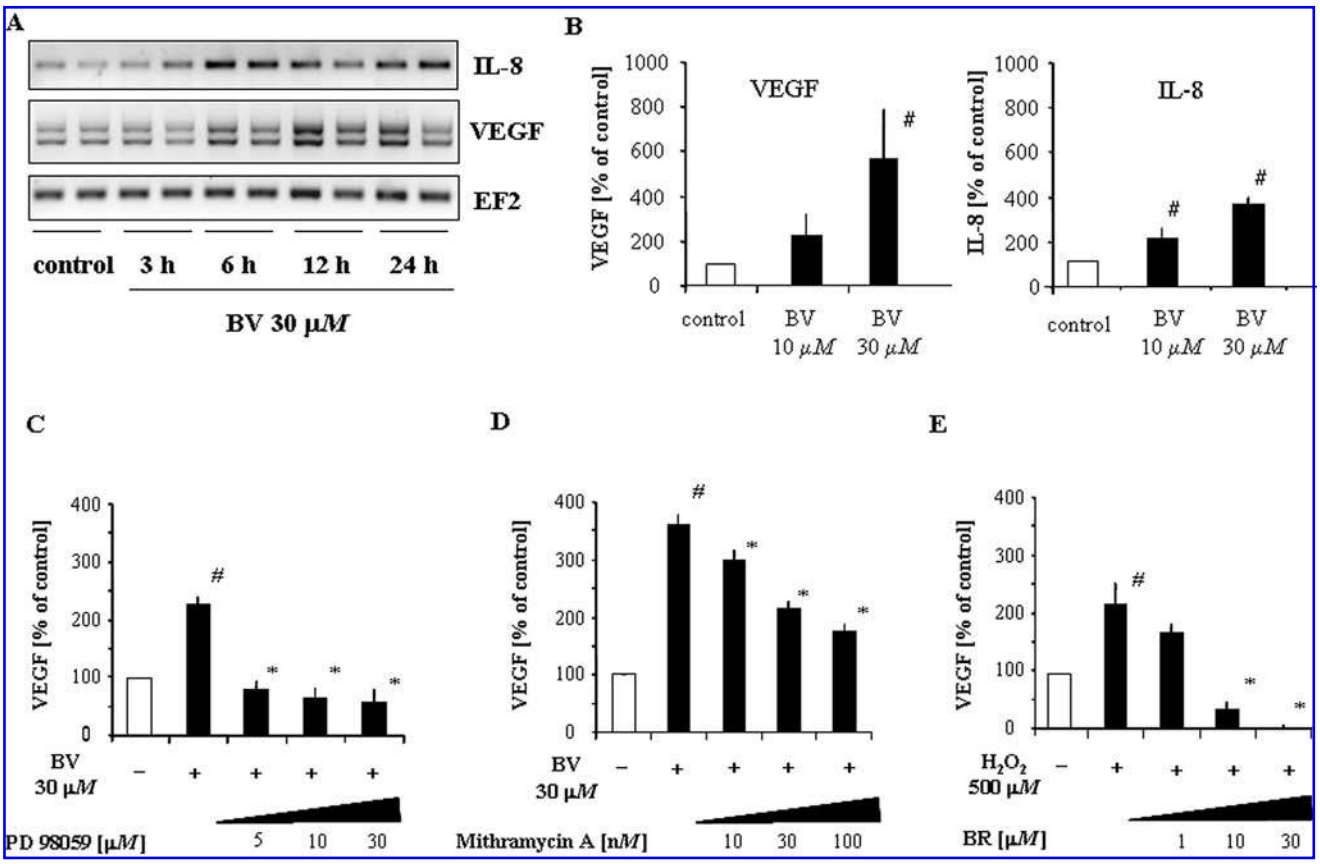

FIG. 6. Biliverdin stimulates the expression of proangiogenic factors in keratinocytes. HaCaT keratinocytes were made quiescent by serum starvation for 24 h and stimulated with 30 μM biliverdin (BV) for different time points (**A**) and with different concentrations of biliverdin (**B**). VEGF-transcript abundance was estimated by RT-PCR (**A**), and protein levels of VEGF and IL-8 were checked with ELISA (**B**). Preincubation with different concentrations of PD 98059 (5–30 μM) (**C**) or mithramycin A (10–100 nM) (**D**) attenuated biliverdin-induced VEGF synthesis, suggesting the involvement of ERK and Sp1 transcription factor. Interestingly, bilirubin showed an inhibitory effect on H_2O_2 -induced VEGF synthesis (**E**). #$p <$ 0.05 *vs.* control; *$p < 0.05$ in comparison with cells treated with biliverdin.

periments, concomitant with HO-1 induction, ferritin synthesis was upregulated 24–48 h after treatment with UVA. However, pretreating cells with HO-1 antisense oligonucleotide, SnPPIX, an inhibitor of HO activity, or with the iron-chelating agent desferrioxamine, prevented the increase in the ferritin level (435, 436).

Several studies have shown that ferritin induced by iron released from the heme moiety has a protective effect on endothelial cells. It was shown to inhibit TNF-α–induced apoptosis of endothelial cells, to protect rat liver from I/R injury, and to prevent hepatocellular damage on transplantation into recipient rats after liver engraftment (38). Accordingly, it was demonstrated that overexpression of the ferritin heavy chain has the same protective effect on livers subjected to I/R injury as does HO-1 overexpression (38). In contrast, some studies show that the potent cytoprotection provided by HO-1 induction is ferritin independent in a model of endotoxic shock in rats (127). This indicates that ferritin can substitute for the HO-1 action in only certain situations.

Both mentioned mechanisms, upregulation of ferritin and the iron ATPase pump, are regarded to contribute to the overall antioxidant effect observed after HO-1 expression in a variety of situations.

V. HO-1 and Vascular Functions

Vascular endothelium plays a crucial role in the regulation of vascular function and homeostasis (358). Under normal conditions, it constitutes an important source of substances that influence vascular tone and protect the vessel wall against inflammatory cell adhesion, thrombus formation, and vascular cell proliferation (108, 280). Pathologic conditions caused by increased oxidative stress, hyperlipidemia, and inflammation lead to endothelial dysfunction, which is characterized by reduced availability of vasodilatory and antiadhesion substances, such as NO and prostacyclin, and concomitant increases in vasoconstrictors and proadhesion factors, including endothelin-1 (ET-1), angiotensin II, and thromboxanes (280). Such remodeling of the vessel wall results in increased vascular tone, inflammatory cell and platelet adhesion, and proliferation of smooth muscle cells, which increase the occurrence of thrombosis and vascular occlusion (280).

Endogenous NO produced by nitric oxide synthase (NOS) has well-established functions as a signaling molecule in the vascular and neuronal system. Originally, it was described as endothelium-derived relaxing factor (EDRF), as it is re-

leased from endothelial cells in response to shear stress produced by blood flow and in response to activation of a variety of receptors (394). After diffusion from endothelial to vascular smooth muscle cells, NO increases intracellular cGMP concentrations through the activation of the sGC, leading to the inhibition of smooth muscle cell proliferation. NO also has antithrombogenic, antiproliferative, leukocyte-adhesion–inhibiting effects, and influences myocardial contractility. These findings established NO as a homeostatic regulator in the vasculature, the absence of which plays a role in a number of conditions and pathologic states, such as hypertension and vasospasm (286, 394). Moreover, it is well known that NO donors can activate the HO-1 gene expression and activity in a variety of tissues (130, 467).

HO-1–derived CO and NOS-derived NO share several properties, such as similar molecular masses, solubilities in water, and basal rates of production (153). Moreover, both molecules have been shown to exert comparable vasodilatory effects on the vasculature.

A. Modulation of cGMP level by CO

Although CO is toxic at elevated concentrations, low concentrations of CO can exert beneficial effects on the vasculature. It has been proposed that SMC-derived CO may regulate the vascular tone through paracrine effects on endothelial cells. CO generated by hypoxia-stimulated rat aortic SMCs caused the cGMP-dependent downregulation of the expression of mitogens, including ET-1 and platelet-derived growth factor-B (PDGF-B) in endothelial cells, thus indirectly inhibiting SMC proliferation (290).

Additionally, it played a well-established role in vasodilation. Early studies demonstrated that CO (11.5% in the inspired gas) caused vasodilation when oxygenation was normal and reduced the vasoconstriction caused by hypoxia in ventilated pig lungs (403). The mechanism of this action was suggested to arise from the binding of CO to the heme iron of cytochrome P450 (403). In isolated perfused rat hearts, the presence of CO (equilibration with 5% CO) in the perfusate significantly increased coronary flow and also reversed the vasoconstrictive effects of methoxamine. The observed effects on CO-induced vasodilation were dependent on intact endothelium or on tissue hypoxia caused by the CO (278). Finally, in a comparative study of the vasoactive effects of NO and CO in isolated rabbit aorta, exogenous CO exerted an endothelium-independent vasorelaxant response, albeit with a 1,000-fold less potency than NO under the same conditions (134).

Indeed, the vasodilatory properties of CO in the rabbit aorta are attributed to the activation of sGC and generation of cGMP by CO, although with lower efficacy than NO (134, 140, 174). This general mechanism of sGC activation has been supported by observations that exogenous or endogenous (HO-derived) CO can elevate the cGMP level in vascular (118, 290, 350) and airway (58) smooth muscle cells. In experiments on isolated porcine coronary arterial and venous ring preparations, Graser *et al*. (140) demonstrated the inhibition of CO-dependent vasodilation by methylene blue, a nonspecific inhibitor of sGC. In rabbit aortic rings, CO-dependent vasodilation was abolished by the specific sGC inhibitor ODQ, further validating the requirement for sGC in this system (174). These results confirmed the hypothesis that CO-induced relaxation is mediated by sGC.

B. The influence of CO on K^+ channel activity

Although, according to the previous observations, cGMP seems to play a major role in CO-induced vasodilation, not all experimental systems have supported those observations. Wang *et al*. (444), by using precontracted rat-tail arteries, showed that CO induced an endothelium-independent vasodilation. Moreover, either the partial inhibition of the cGMP pathway by *R*p-8-BrcGMP or the blockade of calcium-activated K (K_{Ca}) channels by charybdotoxin indicated that the response depended partially on cGMP and partially on large-conductance K_{Ca} (444). Additionally, it was found that CO hyperpolarized single SMCs isolated from rat-tail arteries by increasing outward K^+ current, which in turn inhibited voltage-gated Ca^{2+} channels, causing smooth muscle relaxation (443). 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 (232, 475). These observations highlighted a novel mechanism involving K^+ channel activity in SMCs on the vascular effect of CO.

Experiments with CORMs further validate the role for CO in vasodilation. CORM-2 exerted potent vasodilatory effects when applied to isolated rat aorta (296). The water-soluble derivatives CORM-3 and CORM-A1 produced a concentration-dependent relaxation in vessels precontracted with phenylephrine in isolated aortic rings. Inactive forms of CORMs, which do not possess the ability to release CO, did not affect the vascular tone. The vasodilation elicited by CORM-3 and CORM-A1, similar to experiments with CO gas, potentially involved both the elevation of cGMP and the activation of ATP-dependent K^+ channels (131, 297). Interestingly, inhibiting NO production or removing the endothelium significantly decreased the vasodilatation by CORM-3, suggesting that factors produced by the endothelium, such as endogenous NO, influence CORM-3 vascular activities (131).

C. Vasoconstrictive properties of CO

Despite the popular characterization of CO as a vasodilator, evidence suggests that CO may also exert a vasoconstrictive influence on vascular tone (190). Moreover, the suggested mechanism of this action involved the NO system. It has become increasingly clear that these two gases do not always work independently, but rather can modulate each other. Biochemical studies have shown that CO inhibits NO formation by binding to NO synthase (5, 452). In addition, Thorup and co-workers (420) suggested that in isolated renal resistance vessels, physiologic concentrations of CO can suppress the activity of endothelial NO synthase. In renal resistance arteries (RRAs), low concentrations (up to 100 n*M*) of CO dose-dependently increased NO release from internal stores, as measured by amperometric methods. NO release from RRAs showed dependence on L-arginine but not D-arginine, and the responses to CO were inhibited by pretreatment L-NAME, an inhibitor of NOS. High concentrations of CO, however, inhibited NO production and eNOS activity in these vessels (420).

Further studies by Johnson and co-workers (188, 189) confirmed the previous observations that 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 (188). Under these conditions, chromium mesoporphyrin (CrMP), an inhibitor of HO-dependent CO synthesis, reversed the apparent CO-dependent vasoconstriction. Therefore, strong evidence indicates that although HO-derived CO promotes vasodilation by acting directly on vascular SMC, it simultaneously exerts a vasoconstrictive effect by inhibiting formation of endothelium-derived NO (188).

Non–CO-mediated mechanisms are suggested by which HO could decrease NO production by NOS. NOS is a cytochrome P450-type hemoprotein requiring two heme molecules in its active site. Therefore, increased HO activity could directly degrade heme located in the active site of NOS or could reduce the amount of available heme for *de novo* synthesis of NOS (112, 263). Moreover iron, an end product of heme degradation by HO, could further decrease production of NOS by inhibiting its nuclear transcription (450). In addition, both HO and NOS require NADPH as a cofactor, and the subsequent reduction of biliverdin to bilirubin by biliverdin reductase also uses NADPH, which could shift competition for electrons in favor of the HO pathway (263). Indeed, induction of HO-1 by hemin negatively modulates iNOS expression and activity in an animal model of glomerulonephritis, demonstrating the potential of regulatory interactions between the two enzymes *in vivo* (93). Moreover, Ding *et al*. (102) hypothesized a complex interaction in which HO-2 inhibits NO activity by acting as an intracellular "sink" for NO. In turn, NO binding would inhibit HO-2 catalytic activity (102). These various possibilities are summarized in Fig. 7.

In conclusion, the CO-mediated effect on the vasculature seems to involve several mechanisms. The dilatory effects of CO are attributed to direct endothelium-independent effects on VSMCs, including modulation of cGMP and K^+ channel activity in VSMCs, and indirect effects on the expression of endothelium-derived vasoconstrictors and myogenic factors. Moreover, some reports suggest an accessory role for NO mobilization in CO-dependent vasodilation, whereas a vasoconstrictory effect of CO was proposed through the inhibition of enzymatic endothelium-dependent NO production. NOS-derived NO and HO-derived CO act together in a complex, dynamic, and adaptable manner, contributing to the vascular homeostasis.

VI. HO-1 and Angiogenesis

New blood vessel formation is a strictly regulated process, which starts in the early stage of embryogenesis and is continued, with limitations of occurrence, in postnatal life. It is crucial for organism development and wound healing. It also plays a role in some physiologic events in adults, such as oogenesis, the menstrual cycle, and hair growth. Two mechanisms of new blood vessel formation exist: vasculogenesis (from progenitor cells) and angiogenesis (from preexisting capillaries) (353).

Angiogenesis occurs both in embryogenesis as well as postnatally. It is based on migration and proliferation of mature, differentiated endothelial cells, which are stimulated by various factors, including VEGF (126). Increasing evidence shows the regulation of angiogenesis and vasculogenesis by HO-1 (summarized in Fig. 8) and the importance of different conditions linking HO-1 with angiogenesis (Table 3).

Our data demonstrate that HO-1 influences VEGF synthesis in VSMCs under both normoxic and hypoxic conditions (114). Inhibition of HO activity by tin protoporphyrin (SnPPIX) completely prevented cytokine- and hypoxia-induced VEGF generation. NOS inhibitors did not suppress VEGF synthesis, indicating a role for HO-1, but not NO, in hypoxia-induced VEGF expression. Stimulation of HO-1 activity by hemin enhanced VEGF production; this effect was abolished by blocking the HO pathway (114). HO-1 has also been shown to be a mediator of cross-talk between 15d-PGJ_2 and VEGF, because 15d-PGJ_2-stimulated VEGF synthesis was inhibited by SnPPIX (195). The data concerning the role of HO-1 in the regulation of VEGF expression are in line with results of experiments using HO-1 gene transfer in VSMCs and microvascular endothelial cells (114, 195). Furthermore, similar effects of HO-1 overexpression were demonstrated in *in vivo* studies. VEGF synthesis was upregulated after ad-

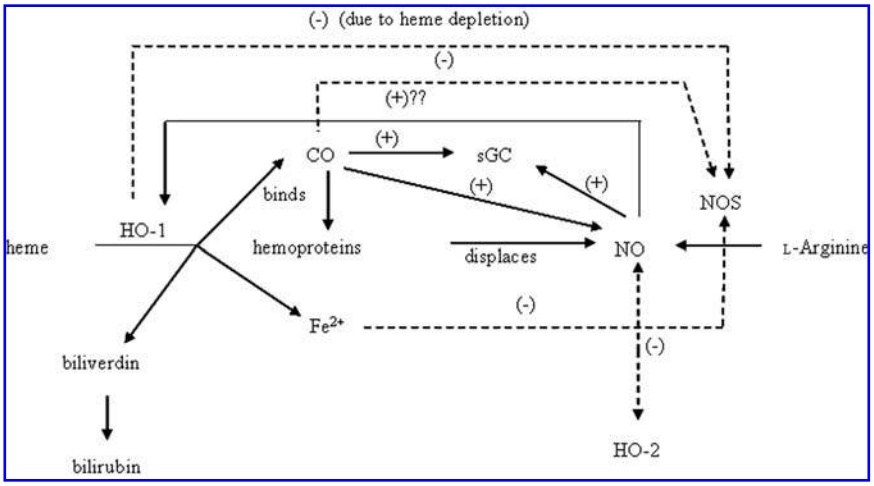

FIG. 7. Scheme of the potential regulatory interactions between the HO/CO and NOS/NO systems. Both NO and CO can activate sGC, although the effect of CO is much less potent. CO can also inhibit NOS activity.

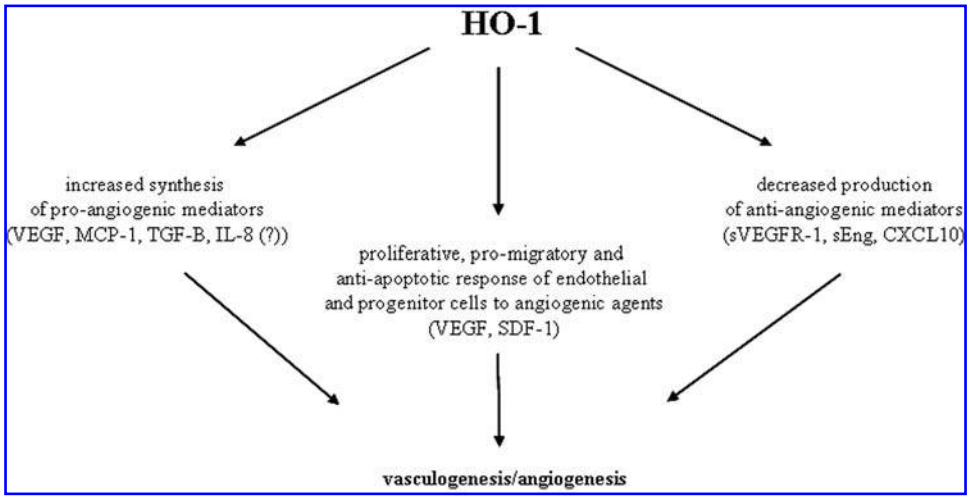

FIG. 8. Involvement of HO-1 in vasculogenesis and angiogenesis. The effect of HO-1 and/or its products can be exerted by enhancement of the synthesis of the proangiogenic mediators (*left*), decrease in the synthesis of antiangiogenic mediators (*right*), and by mediating the endothelial/progenitor cell response to stimulation with growth factors (*middle*).

enoviral-mediated HO-1 delivery both to rat placenta (219) and to rat skeletal muscle (402).

HO-1 is involved in the regulation of VEGF synthesis and also plays a crucial role in VEGF activity (Fig. 9). SnPPIX inhibited VEGF-induced proliferation and migration of HUVECs. Furthermore, tube formation by HUVECs on a basement membrane matrix (Matrigel) was shown to be HO dependent, and HO-1 overexpression led to the augmented angiogenic potential of these cells (196). In addition, VEGF was demonstrated to induce HO-1 expression *in vivo*, because the VEGF-activated angiogenic response in the chicken chorioallantoic membrane model of angiogenesis was HO-1 dependent (125). VEGF also induces HO-1 expression in endothelial cells (56, 111), enhancing *Hmox-1* promoter activity (99).

Among the different end products of HO-1 activity, CO was proposed to be involved in angiogenesis promotion, both by stimulating the production of angiogenic growth factors and by mediating VEGF and SDF-1 response in endothelial cells (99, 111, 196). CO stimulates endothelial cell proliferation (196, 242), suppresses their apoptosis (48, 388), and induces VEGF synthesis in VSMCs, macrophages, and microvascular endothelial cells (114, 195). In endothelial cells, addition of CORM or induction of HO-1 by hemin resulted in an elevation in CO production and was associated with an increase in VEGF synthesis and capillary sprouting. Interestingly, much higher levels of CO and a further increase in VEGF production were detected in cells treated with 15d-PGJ_2, a potent activator of HO-1. SnPPIX prevented the induction of CO generation and inhibited the VEGF synthesis (196). In VSMCs, 1% CO induced VEGF production (114), whereas 5% CO inhibited it (253). Thus, the effect of CO on the VEGF expression varies, depending on the cell type and the concentration of CO (for review, see 111).

It is possible that the CO effect on VEGF synthesis is similar to that exerted by hypoxia. Similar to cyanides, CO is an inhibitor of electron-transport complex IV. Cyanides have been demonstrated to increase VEGF generation in normoxia by augmenting HIF-1 stability (456). Thus, CO effects might also be HIF-1 mediated. In a recent study, stabilization of HIF-1 by CO (CORM-3) was demonstrated in macrophages, and the authors linked that activation to the enhancement of TGF-β expression in those cells. VEGF production was not determined. However, the effect of CO on HIF-1 was very

TABLE 3. CONDITIONS LINKING HO-1 WITH ANGIOGENESIS

Events	*Mechanisms*	*References*
Increased vascularization of tumors	Increased VEGF production	165, 193, 365
	Increased thymidine phosphorylase	377
	Increased thymosin β4?	449
	Unknown	399
Increased angiogenesis in rheumatoid arthritis	Increased VEGF	100
Enhanced angiogenesis by AdHO-1 delivery to rat ischemic hindlimb	Increased VEGF	402
Impaired wound healing in HO-1 knockout mice	Decreased VEGF Decreased SDF-1–induced signaling	99
Impaired retinal neovascularization in HO-1 knockout mice	Decreased SDF-1–induced signaling in endothelial progenitor cells	99
Animals without HO-1 have higher level of antiangiogenic mediators	Increased soluble Flt-1 and soluble endoglin	87

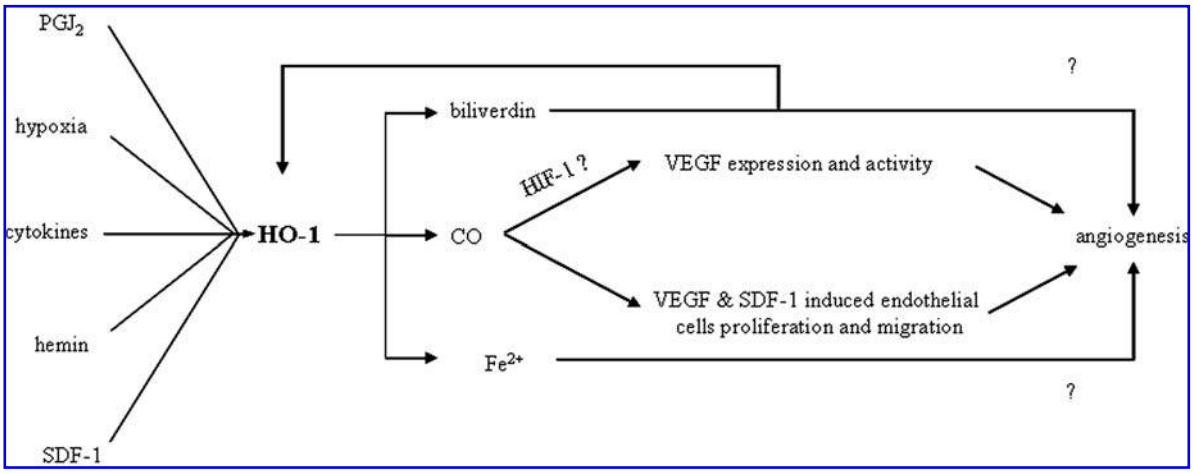

FIG. 9. CO as a regulator in HO-1–mediated angiogenesis. HO-1 is induced by various stimuli exerting proangiogenic effects. The end product responsible for these effects is CO. It stimulates VEGF expression and activity; the HIF-1 pathway is considered to be involved in this process. CO is also a downstream mediator of response of endothelial cells on stimulation with VEGF and SDF-1. The role of both biliverdin and iron ions in stimulation or inhibition of angiogenesis remains to be elucidated, although biliverdin was reported also to stimulate the synthesis of angiogenic mediators (see text for details).

short (15 min) and not potent; therefore, the biologic significance of these results is not as yet obvious (76).

Angiogenesis is regulated by several mediators. One of them is oxidative stress. It is known that ROS modulate new blood vessel formation by regulation of various angiogenic factors (271). Thus, H_2O_2 has been demonstrated to induce VEGF expression in many cell types, such as endothelial cells (79), macrophages (366), retinal pigment epithelial cells (221), and keratinocytes (376). It has also been shown to stimulate HO-1 expression in a variety of cell types (8, 203). We provided evidence that HO-1 is involved in the regulation of H_2O_2-mediated induction of VEGF synthesis (81). Treatment of fibroblasts and keratinocytes with SnPPIX resulted in downregulation of H_2O_2-induced VEGF expression. Studies in HO-1–knockout mice further corroborated these observations, showing lower basal and H_2O_2-induced production of VEGF (81). Moreover, the copper/zinc superoxide dismutase (SOD1) gene transfer resulted in enhanced intracellular generation of H_2O_2 and induction of VEGF production with a concomitant augmentation of HO-1 expression. This effect was reversed by overexpression of human catalase. We also showed that the effect of H_2O_2 is mediated by the activation of HRE as well as the Sp1 recognition site of the VEGF promoter (145).

The synthesis of another proangiogenic mediator, IL-8, can also be regulated by HO-1. Treatment of HUVECs with *S*-nitroso-penicillamine (SNAP), an NO donor, resulted in higher expression of HO-1 and elevated VEGF and IL-8 production (327). Attenuation of HO-1 expression by transfection of specific siRNA or antisense oligonucleotides diminished the SNAP-induced VEGF and IL-8 synthesis. Moreover, synthesis of IL-8 was reduced when VEGF-neutralizing antibodies were applied, whereas no attenuation of VEGF production was observed after treatment with IL-8–neutralizing antibodies. The authors proposed the existence of an NO–HO-1–VEGF–IL-8 molecular cascade in endothelial cells (327).

Conversely, our data suggest that, in contrast to VEGF, the production of IL-8 is independent of HO-1 induction in HMEC-1 cells (256). CoPPIX-induced VEGF but not IL-8 synthesis was abolished by SnPPIX, demonstrating the existence of both HO-1–dependent and independent mechanisms of proangiogenic factor synthesis. Similarly, PGJ_2-induced VEGF production was blocked in the presence of inhibitors of HO-1 activity; however, enhancement of IL-8 production was not dependent on HO-1 (195).

Recently, Datta *et al*. (92) demonstrated that, in human renal proximal tubular epithelial cells, overexpression of HO-1 induced the expression of the antiangiogenic chemokine CXCL10, along with angiogenic chemokines CXCL8 (IL-8) and CCL2 (monocyte chemotactic protein-1, MCP-1). Interestingly, the induction of CXCL10 was observed early after transfection and decreased with time. It was also dependent on the level of HO-1 expression, as transfection of higher amounts of HO-1 plasmid decreased CXCL10. A similar course of expression, dependent on the amount of HO-1 used, was observed for CCL2, whereas the expression of CXCL8 increased with time (92) .

HO-1 can also regulate the expression of antiangiogenic mediators (Fig. 8). Interestingly, in a recent study, Cudmore *et al*. (87) have demonstrated that deficiency of HO-1 resulted in the elevation of the levels of soluble endoglin (sEng) and soluble VEGFR-1, known inhibitors of angiogenesis. Accordingly, overexpression of HO-1 in endothelial cells prevented the production of antiangiogenic soluble VEGFR-1 and sEng (87). Thus, one can suggest that deficiency of HO-1 or its diminished expression can influence angiogenesis in pathologic conditions, such as limb or heart ischemia.

A. VEGF- and SDF-1–induced blood vessel formation is mediated by HO-1

Recent articles demonstrated that VEGF is able to stimulate HO-1 expression in endothelial cells (56, 111) and in the

chicken chorioallantoic membrane (CAM) (125). Bussolati *et al*. (56) provided *in vitro* and *in vivo* evidence that VEGF stimulates HO-1 synthesis in HUVECs and HMEC-1 cells and that this induction is necessary for VEGF-driven angiogenesis because SnPPIX, as well as ZnPPIX, abrogated capillary formation in a Matrigel assay *in vitro*. Similarly, VEGF-induced angiogenesis in CAM was associated with HO-1 protein expression, and ZnMP significantly attenuated this response (125). The increase in HO-1 activity and protein occurred after 24–48 h in endothelial cells (56) and after 48 h in CAM (125) exposed to VEGF. In contrast, neither fibroblast growth factor-1 (FGF-1, acidic FGF) nor FGF-2 (basic FGF) induced HO-1 expression in endothelial cells (56, 113). The mechanisms responsible for VEGF-induced HO-1 expression are not known in detail, but calcium influx and activation of protein kinase C (125), leading to augmentation of VEGF promoter activity, have been reported (99).

In another study, an increase in HO-1 mRNA and protein synthesis, associated with an increase in endothelial cell proliferation and stimulation of tube formation on Matrigel, was observed after exposure to prolactin (10 and 25 ng/ml) (268). Moreover, augmentation of endothelial tubulogenesis *in vitro* in an HO-1–dependent manner was observed after overexpression of thymidine phosphorylase or treatment with its catalytic product, 2-deoxy-D-ribose-1 phosphate and downstream 2-deoxy-D-ribose (377).

Recent elegant work by Siner *et al*. (387) provided *in vivo* evidence that VEGF exerts a protective effect against hyperoxic lung injury through induction of HO-1. Enhancement of VEGF expression in transgenic mice harboring the VEGF gene, driven by a tetracycline-regulated promoter, caused HO-1 expression and HO-1–mediated attenuation of lung cell apoptosis, lipid peroxidation, as well as enhancement of the survival of mice exposed to hyperoxia. The involvement of HO-1 in this pathway was confirmed by silencing HO-1 with a specific siRNA that abrogated the beneficial effects of VEGF (387).

Recently, HO-1 was also demonstrated to mediate the proangiogenic effects of SDF-1 (99) (Fig. 9). The experiments were done by using endothelial progenitor cells (EPCs) and aortic endothelial cells isolated from wild-type mice and from HO-1–knockout mice (HO-$1^{-/-}$). Nanomolar concentrations of SDF-1 induced HO-1 in endothelial cells through a PKC-ζ–dependent and VEGF-independent mechanism. SDF-1–induced endothelial tube formation and migration were impaired in HO-1–deficient cells. Aortic rings from HO-$1^{-/-}$ mice were unable to form capillary sprouts in response to SDF-1, and this defect was reversed by the CO donor, CORM. The functional significance of HO-1 was confirmed in Matrigel plug and wound healing *in vivo*. Absence of HO-1 was associated with impaired wound healing and weaker neovascularization in injured tissue. These findings demonstrated an important role for HO-1 in SDF-1–mediated angiogenesis and provided new avenues for therapeutic approaches in vascular repair (99).

Importantly, this study also demonstrated impairment in the function of EPCs harvested from HO-1–deficient mice, as evidenced in ischemia-induced neovascularization in the retina (99). Because EPCs have been shown to be very important in the neovascularization processes in adults, this finding may be of importance for cell-based therapeutic strategies for cardiovascular diseases.

B. Dual role of HO-1 in angiogenesis

Interestingly, the influence of HO-1 on angiogenesis depends on the underlying conditions (Fig. 10). As described earlier, Bussolati *et al*. (55, 56) showed that VEGF-induced angiogenesis is dependent on HO-1 activity, whereas in the same studies, they demonstrated that inflammation-induced blood vessel formation can be attenuated by overexpression of HO-1. During LPS-induced angiogenesis, leukocyte infiltration is followed by blood vessel formation. Induction of HO-1 in this model prevented leukocyte invasion into the Matrigel plug and subsequent angiogenesis. However, when pharmacologic inhibition of HO-1 was evaluated in non-inflammatory, VEGF-induced angiogenesis, induction of leukocyte infiltration stimulated VEGF-induced angiogenesis. Moreover, SnPPIX treatment significantly decreased angiogenesis induced by agonistic antibodies against CD40 (55).

Therefore, it was suggested that during chronic inflammation, HO-1 has two possible roles: first, it inhibits leuko-

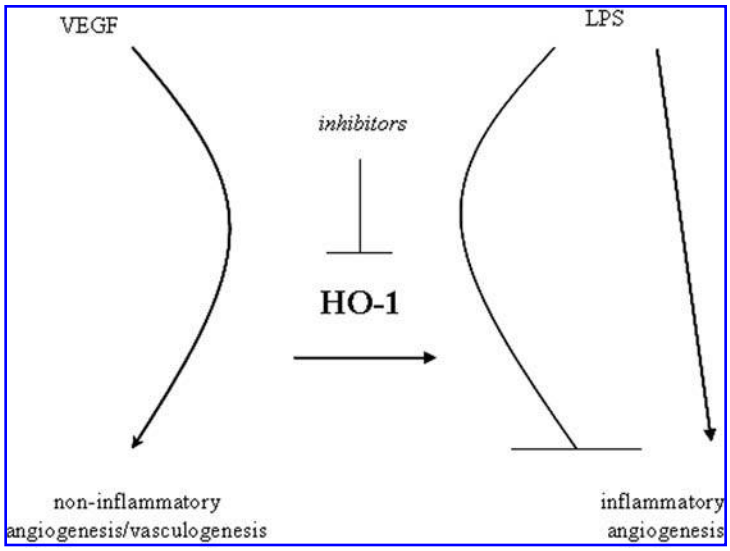

FIG. 10. Dual role of HO-1 in angiogenesis. HO-1 is proangiogenic in VEGF-driven, inflammation-independent angiogenesis (left). HO-1 blocks inflammatory angiogenesis; thus, inhibition of HO-1 can promote inflammatory angiogenesis.

cyte infiltration, and second, it promotes the VEGF-driven noninflammatory angiogenesis that facilitates tissue repair (55) (Fig. 10). Similar interactions have been reported by Angermayr *et al*. (23). In portal hypertensive rats, HO-1 simultaneously decreases stress and inflammation in the splanchnic territory as well as induces VEGF production.

These data suggest that the products of HO-1 activity act as proangiogenic factors only when HO-1 expression would occur in the environment free of inflammatory reactions. However, when the inflammation drives blood vessel formation, the influence of HO-1 on the latter process is rather inhibitory (Fig. 10). It remains to be elucidated whether this is a direct effect of HO-1 on the production of angiogenic and antiangiogenic mediators or a result of amelioration of inflammation.

VII. Role of HO-1 in Pathologic Conditions

A. HO-1 and atherosclerosis

Atherosclerosis is viewed as "the response to injury" by lipoproteins and other risk factors. Accordingly, inflammation is intimately linked to the development of atherosclerotic lesions (261, 355, 356).

In the child lacking a functional HO-1 allele, atherosclerotic changes such as fatty streaks and fibrous plaques have been detected (201, 460), accompanied by high levels of triglycerides and hyperlipidemia. Moreover, the LDLs had increased electrophoretic mobility, and other assays confirmed that they were extremely modified. These LDLs were cytotoxic to endothelial cells, mostly because of the high concentrations of lipid hydroperoxides. Preincubation of the child's LDLs with reduced glutathione/glutathione peroxidase abolished the detrimental effect of LDLs on endothelial cells (186; for a review, see 29). However, it is not known exactly whether the observed changes were a result of HO-1 deficiency or of long-term steroid treatment.

Data indicate that HO-1 might be activated throughout the entire period of atherosclerosis development. HO-1 is expressed in atherosclerotic lesions of humans and apolipoprotein (Apo) E–deficient mice (293, 442). In LDL-receptor knockout mice, HO-1 was abundantly expressed in atherosclerotic plaques after dietary cholesterol feeding (179). Conversely, oxLDL present in atherosclerotic plaque upregulates the expression of HO-1 (178). Thus, it has been suggested that HO-1 is induced as a protective gene, and its expression can ameliorate processes leading to development of atherosclerosis. Accordingly, increased HO-1 activity markedly reduces the chemotaxis of monocytes after exposure to oxLDL (178). Moreover, HO-1 overexpression reduced plaque formation, whereas inhibition of HO-1 by SnPPIX reversed the protection in such animals (179). Another support for a protective role of HO-1 against atherosclerosis was shown in Watanabe heritable hyperlipidemic rabbits (WHHL). Again, HO inhibition resulted in a marked increase of atherosclerotic lesion development in WHHL rabbits, suggesting that products of HO-1 activity can function as antiatherogenic molecules *in vivo* (180).

By using mice deficient in both HO-1 and ApoE genes, Yet *et al*. (468) demonstrated that larger and more-advanced lesions are formed in these animals than in mice lacking only ApoE (468). Also recently, Orozco *et al*. (318) showed, by using HO-1 knockout, heterozygotes and wild-type mice, that both the diminished expression and the absence of HO-1 resulted in increased lipid uptake and foam cell formation *in vitro*, which correlated with increased ROS generation and greater release of inflammatory cytokines, such as IL-6, MCP-1, and KC (a mouse equivalent of IL-8). Interestingly, in contrast to the experiments in double ApoE and HO-1 knockouts, in which the lack of HO-1 resulted in increased size of the plaque, the extent of atherosclerosis was not different in LDL-receptor–null mice that were reconstituted with bone marrow either from HO-$1^{+/+}$ or HO-$1^{-/-}$ mice (318).

Another proof for the protective role of HO-1 against atherosclerosis comes from studies with overexpression of HO-1. Juan *et al*. (198) intraventricularly delivered adenoviral vectors carrying HO-1 and observed a significant decrease in the lesion areas at the aortic root and aortic arch in both young (14 weeks old) and older (20 weeks old) Apo$E^{-/-}$ mice, compared with the animals treated with a control vector. Additionally, HO-1 gene transfer effectively reduced hemin-induced iron overload in rat aortic smooth muscle cells, and the iron overload was less in the aortic lesions of AdHO-1–treated mice (198). As elevation in iron deposition is closely associated with the progression of atherosclerotic lesions (230), and iron can increase oxidative events in the vasculature, reduction of iron overload by HO-1 can be considered a protective mechanism.

It is believed that, besides the reduction of intracellular iron accumulation, other beneficial effects are derived from the activity of HO-1 products. The inhibitory effect of HO-1 on lipid peroxidation occurs *via* biliverdin and bilirubin. These bile pigments acting as antioxidants diminished monocyte transmigration, whereas inhibition of HO by SnPPIX enhanced chemotaxis (178). CO may also contribute to the antiatherogenic effects of HO-1 in the vascular wall. The vasodilatory function of CO suppresses vasospasm, a pathologic property of vascular disease such as atherosclerosis, graft failure, and restenosis. The vasodilator properties of endothelium-derived CO might be protective in vascular injury in which NO production is inhibited or lost because of endothelial mechanical trauma. Antiatherogenic properties of CO have been demonstrated in a model of transplant atherosclerosis (419). The exposure to a relatively low concentration of CO (250 ppm) retards the development of atherosclerotic lesions after the transplantation of aortic segments of Brown Norway rats into Lewis rats (419).

In sum, HO-1 is suggested to be beneficial and to prevent atherosclerosis. However, it has been also shown that mouse of strains more sensitive to the development of atherosclerosis have higher levels of HO-1 in the blood vessels (381). Moreover, expression of HO-1 was significantly increased in the aortae of female ApoE mice at 12–34 weeks of age (*i.e*., with the progression of atherosclerosis), whereas it was not the same for other antioxidant and protective genes, such as superoxide dismutase, catalase, glutathione peroxidase, or endothelial nitric oxide synthase, which decreased with the growth of the plaque (404). Interestingly, in another recent study, Datla *et al*. (91) did not observe any basal expression of HO-1 in the aortas of 26- to 30-week-old male ApoE knockout mice. The reason for such a difference is not known, and whether the sex of animals might play a role remains to be investigated.

We demonstrated recently that overexpression of ApoE4 induces HO-1 (187). From three known ApoE isoforms (E2, E3, and E4), ApoE4 has been found to be associated with a 40–50% higher risk of cardiovascular disease (CVD) (393). In RAW264.7 macrophages stably transfected to produce equal amounts of human ApoE3 or ApoE4, the changes in both the basal and LPS-stimulated production of HO-1 were detected (187). ApoE4-macrophages demonstrated increased expression of HO-1 under baseline conditions and a stronger upregulation of HO-1 at the mRNA and protein levels after LPS application. However, because ApoE4 cells have expressed higher amounts of proinflammatory IL-1β and TNF-α, it is plausible that the induction of HO-1 by ApoE4 represents a stress-induced protective response.

In conclusion, HO-1 appears to be a marker of early inflammation in the artery wall. The data suggest also that the protective effect of HO-1 in atherosclerosis is due to the inhibition of the inflammatory response in the vascular wall. HO-1 was demonstrated to be defensive against atherosclerosis in young animals, and the clinical observations in the HO-1–deficient child indicate that it also might be protective in humans. It is doubtful, however, whether HO-1 can have therapeutic value in advanced plaques.

B. Hypertension, angiotensin II, and HO-1

Hypertension is a disruption of homeostasis in which the blood pressure level is chronically elevated. It increases the risk of heart attack, stroke, aneurysm, and renal failure.

In literature, two views of the connection of HO-1 with hypertension can be found. The increase in the HO-1 level is observed as a consequence of increasing blood pressure. Conversely, hypertension is noted to decrease (or its increase is prevented) after chemical induction of HO-1, overexpression of its gene, elevation of bilirubin, or after administration of exogenous CO.

Angiotensin II (Ang-II) is a peptide that increases blood pressure, when present continuously in the bloodstream. Ang-II, depending on the kind of tissue, can stimulate cell proliferation, hypertrophy, apoptosis, ROS production, and cause oxidative DNA damage. In many cell types, Ang-II was shown to induce HO-1. Both *in vitro* effects are prevented if HO-1 is upregulated before the administration of the peptide. Concomitantly, this prevention is not observed when HO-1 inhibitors are used. This Ang-II–HO-1 interplay was observed both *in vitro* in VSMCs (182, 422) and endothelial cells (3, 274) and *in vivo* in vessels (182, 292), hearts (129, 171), and kidneys (11, 12, 40, 94, 154, 240, 343, 434). Interesting additional evidence for the involvement of HO-1 in preventing the negative effects of Ang-II was shown in hyperbilirubinemic rats that are resistant to Ang-II–induced hypertension (338). However, in two experimental settings, HO-1 overexpression was shown either to suppress Ang-II–induced hypertrophy of myocytes (a process promoting hypertension) (171) or to have no influence on this process (129). The only two noticeable differences between these two reports were (a) the concentration of CoPPIX used for HO-1 induction, and (b) the multiplicity of infection (MOI) of viral vectors used to deliver the HO-1 gene to the cultured myocytes. Weaker stimulation inhibited Ang-II–induced hypertrophy (171) (which was suggested to be mediated by bilirubin), whereas stronger HO-1 induction had no effect on hypertrophy (129). The mechanism underlying this unexpected outcome, in which putatively a lower HO-1 level exerts a stronger effect than a higher level, remains to be elucidated.

Apart from the Ang-II–mediated induction of hypertension, spontaneously hypertensive rats (SHRs) are a common model in hypertension research. When heme was injected into such animals, their blood pressure was reduced to normal levels observed in young rats, and this effect was abolished when HO-1 inhibitors were administered before its induction (191, 308). Among the three HO-1 products, only CO was shown to be responsible for this effect (191). The blood pressure of SHRs was also observed to decrease after treatment of the animals with CO or with HO-1 substrate (322) and after administration of retroviral vectors containing the human HO-1 gene (360). These results show that the induction of HO-1 expression results in the reduction of blood pressure to normal levels. Conversely, however, hypertension itself can induce HO-1 activity (181). Wiesel *et al*. (453) performed a study on the one kidney–one clip (1K1C) model of renovascular hypertension, in which one kidney is removed and the remaining kidney undergoes arterial constriction (453), which showed that although prolonged deficiency of HO-1 does not alter basal blood pressure, HO-$1^{-/-}$ animals had more severe hypertension and cardiac hypertrophy after 1K1C injury. Additionally, in knockout animals, an increased mortality occurred within 72 h after 1K1C surgery when compared with HO-$1^{+/+}$ and HO-$1^{+/-}$ animals (453).

The connection of HO-1 and hypertension was also observed in humans. In pregnant women experiencing gestational hypertension or preeclampsia, the end-tidal CO levels were significantly lower than in healthy pregnant or nonpregnant women (34, 220), which suggests that hypertension is correlated with reduced HO-1 activity.

Hypertension is a serious complication in preeclampsia, which affects 3–8% of all pregnancies. Preeclampsia is characterized by elevation of soluble VEGFR-1 and sEng, a coreceptor for TGF-β (234). Recently, Cudmore *et al*. (87) demonstrated that adenoviral overexpression of HO-1 in endothelial cells inhibited VEGF-mediated sFlt1 release and interferon-γ and TNF-induced sEng release, whereas HO-1 inhibition potentiated soluble VEGFR-1 production in endothelial cells and placental villous explants. These studies may shed some light on the link between HO-1 and hypertension in preeclampsia.

Transplant patients are often immunosuppressed with cyclosporin, which also causes hypertension. Carvedilol can be used to reduce the blood pressure, and this effect was observed simultaneously with elevation of HO levels in the monocytes of these patients (329).

Based on data demonstrating the induction of HO-1, it was considered as a therapeutic target against hypertension. Accordingly, in an interesting recent study, Wang *et al*. (445) demonstrated that administration of hemin for 3 consecutive weeks to 12-week-old SHRs normalized systolic blood pressure, and importantly, this normalization was maintained for 9 months after discontinuation of hemin (445). Obviously, HO-1 was induced on such treatment, but the explanation for the mechanisms underlying this phenomenon is not known. One of the observed changes was a decrease in the VEGF level. This is in agreement with studies demonstrating that VEGF is an essen-

tial mediator in Ang-II–induced vascular inflammation (363, 346). As discussed in the accompanying editorial (36), this is quite an unprecedented and a remarkable finding that the applied regimen induced a reduction in blood pressure, long after treatment had ceased.

In another model of hypertension, an induction of HO-1 ameliorated renovascular hypertension (44) by interfering with the angiotensin-mediated increase in blood pressure. However, Johnson *et al*. (192) reported that HO-1 induction resulted in pro-hypertensive CO-mediated endothelial dysfunction in rats kept on a high-salt diet. HO-1 also prevented pulmonary hypertension induced by hypoxia. Agonists of HO-1 or targeted overexpression of HO-1 in the lungs of transgenic mice prevented the pulmonary inflammatory and vascular response to hypoxia (78, 282). HO-1 overexpression also reduced monocrotaline-induced pulmonary responses and the resulting right ventricular overload (139). Interestingly, pulmonary hypertension also can be prevented by rapamycin, a drug known to induce HO-1 expression (481).

Collectively, several facts indicate that a tight association exists between blood pressure and the level and activity of the HO-1 enzyme (Fig. 11). First, the enzyme level becomes elevated in animals with induced hypertension. Particularly, it is elevated by Ang-II. Second, in rats with spontaneous hypertension, chemical induction or overexpression of HO-1 reduced the blood pressure. Third, inhibition of HO-1 results in hypertension. Accordingly, the induction of HO-1 expression ameliorating hypertension is proof for the beneficial role of this enzyme in the regulation of vascular tone.

Thus, the link of HO-1 to the origin, prevention, or treatment of hypertension has been demonstrated; however, the results are still far from being conclusive.

C. HO-1 and stroke

Stroke is a clinical condition in which blood supply to the brain (or its part) is limited or severed. It can be caused by embolism, blood clotting, or by the rupture of blood vessels and consequent intracerebral hemorrhage (ICH). After central nervous system hemorrhage due to erythrocyte lysis, hemoglobin and heme degradation products may contribute to oxidative injury after hemorrhagic insults.

Although protective in many diseases, HO-1 is suggested not to be as beneficial for neurons as it is for other cell types. A lack of HO-1 or inhibition of its activity may even attenuate neuronal death. Accordingly, SnPPIX treatment can reduce brain edema induced by hemoglobin in rats (173). In porcine brains injected with blood containing HO inhibitors, less damage was observed in comparison with animals injected with blood lacking HO inhibitors (439). Moreover, chelation of iron with deferroxamine attenuated brain edema and neurologic deficits in rats (303).

Interestingly, recent data suggest that HO-1 can play opposite roles in neurons and in astrocytes. HO-1 is essential for astrocyte resistance to hemoglobin but has no protective effect in neurons (62). Astrocytes isolated from HO-1–knockout animals were more vulnerable to hemoglobin (62) and to hemin (63) than were astrocytes from wild-type animals. Additionally, HO-1 gene transfer to KO astrocytes decreased hemoglobin toxicity (62). Because of such a disparate effect of HO-1 in neurons and in astrocytes, it can be important to obtain HO-1 overexpression selectively in astrocytes. An interesting approach was performed by using the glial fibrillary acidic protein (GFAP) promoter. Ad-GFAP–HO-1 gene transfer resulted in high overexpression of HO-1 in astrocytes, but not in neurons, and was able to reduce hemin-induced cell death (37). One can speculate why astrocytes are protected by HO-1, whereas neurons are not; a possible explanation can be related to the limited ability of neurons to detoxify excess iron by increasing ferritin synthesis (459). After ICH in the rat, an increase in ferritin level was found only in glial cells (microglia and astrocytes) but not in neurons (459).

In contrast to the studies described, data also show the beneficial effects of HO-1 on neuron death. Overexpression

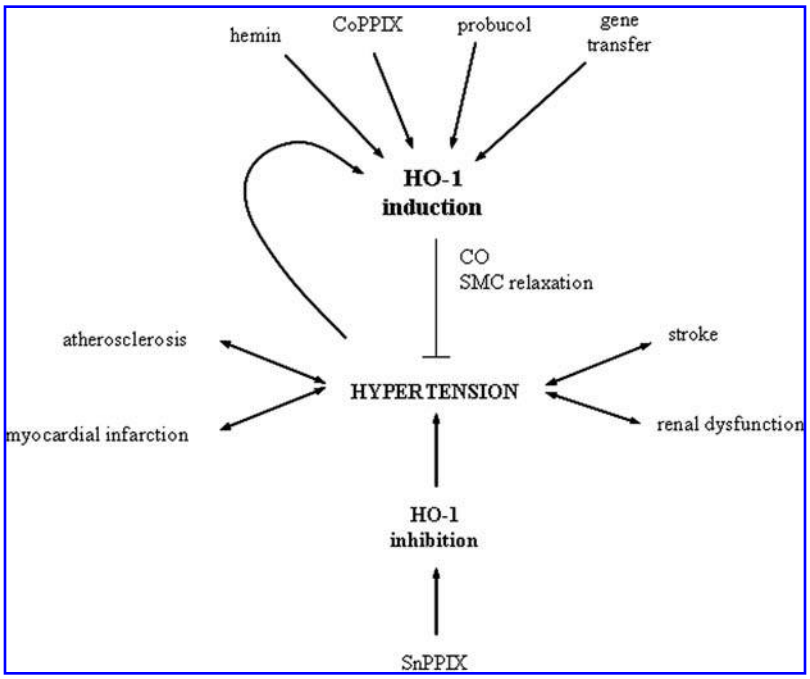

FIG. 11. Simplified scheme of the connection between HO-1 and hypertension. Induction of HO-1 can prevent the development of the hypertension, which is tightly connected with other pathologies, such as stroke, renal dysfunction, atherosclerosis, and myocardial infarction. Upregulation of HO-1 can be achieved by either chemical induction of the enzyme or introduction of its gene into the vessels. Upregulation of HO-1 in hypertension might be a physiologic mechanism developed to reduce the blood pressure. Concomitantly, inhibition of HO-1 in animal models elevated the blood pressure.

of HO-1 protects neurons from oxidative injury (67). Both glutamate- and H_2O_2-induced cell deaths were significantly decreased in cerebellar granular neurons isolated from homozygous transgenic mice that overexpressed HO-1 in comparison with neurons from wild-type animals (67). Additionally, in transgenic mice overexpressing HO-1 in the brain, middle cerebral artery occlusion (MCAO) caused less damage compared with that in wild-type mice (331). 2,2′-Dipiridyl, which is an iron chelator, was shown to induce HO-1 in rat brains and was protective in rats subjected to cerebral ischemia (96). This, however, should not be assigned only to the beneficial effects of HO-1 *per se*, but possibly also to the scavenging of deleterious iron. In a recent study, application of an antagonist of *N*-methyl-D-aspartate receptors, which limited the mortality of stroke-prone hypertensive rats, was also shown to induce HO-1 expression in the brain (135).

These conflicting results may be because HO-1 products may enhance or reduce oxidative damage, depending on concentration and environment (400). *In vitro* studies show that in contrast to limited upregulation of HO-1 (less than a fivefold increase), greater upregulation of HO-1 (especially >15-fold) is associated with significant oxidative cytotoxicity rather than with protection against cell damage (400). Therefore, it is important to remember that although HO-1 can protect the cells from damage and apoptosis, its high activity in a heme-rich environment can have very harmful effects on neurons and consequently on the brain. HO inhibitors could even be considered as drugs to be used for limiting the intracerebral bleeding–caused damage in stroke patients.

Another issue that should be taken into consideration is that HO-2 (not HO-1) might play a dominant role in neuroprotection. First, inducible HO-1 is not detectable in an unaffected brain (96), whereas constitutive HO-2 is highly present in the brain, primarily in neurons (370) but also in cultured astrocytes (370) and cerebral endothelial cells (333), and it seems to play an important role in the protection of the brain from stroke. It was shown that cultured neurons isolated from HO-2 knockout mice are more prone to oxidative damage (105). Accordingly, HO-$2^{-/-}$ animals have more injury after MCAO compared with HO-$1^{-/-}$ animals (106).

Again, data also show that the HO-2 gene deletion is cytoprotective. The inhibition of neuronal HO-2 had beneficial effects on neuron physiology both *in vitro*, in mixed neuron/astrocyte cultures (351) or in cortical neurons (354), and *in vivo*, in an experimental ICH model (347). HO-2 deletion attenuated hemin-induced ROS formation and reduced levels of oxidized proteins that resulted in reduced neuronal death in knockout cultures (351) as well as decreased hemoglobin toxicity (354). Compared with wild-type animals, HO-$2^{-/-}$ mice exert less oxidative cell injury after whole-blood injection into the mouse striatum (347).

Thus, the data obtained so far indicate that HO isoforms can play an important role in neuronal complications; however, more-detailed studies, especially with the selective inhibition or downregulation of HO-1 and HO-2, should be performed.

D. HO-1 and myocardial infarction

Ischemia/reperfusion (I/R) injury and myocardial ischemia lead to significant myocardial damage because of the formation of noxious ROS after reoxygenation of the ischemic myocardium. Current data indicate that HO-1 plays a crucial role in preventing I/R-induced cardiac dysfunction and apoptosis. First, expression of HO-1 increases in the site of I/R injury [*e.g.*, in cardiomyocytes in isolated perfused hearts (272), in hearts after renal I/R (349), and in rat lungs after I/R of the lower limbs (46)]. Further experiments showed that administration of heme or bilirubin protects hearts from ischemic damage (82, 152). Second, data from experiments with HO-1 overexpression (both chemical stimulation and gene transfer) indicate its cardioprotective effect (discussed in the section *HO-1 Gene Transfer for Therapeutic Purposes*). Studies on transgenic animals also confirmed the importance of HO-1 in ischemic heart injury. The hearts of HO-$1^{+/-}$ mice were more prone to damage caused by I/R (473), whereas when animals overexpressed the enzyme in their hearts, the organs suffered less damage (470) and cardiomyocyte apoptosis after infarction (438). A similar protective effect was observed in animals with deletion of the *Bach1* gene (466), which is a repressor of HO-1 expression.

The protective effects of HO-1 against myocardial dysfunction are probably mediated by CO. Indeed, CORM-3, a new member of CORM family, has been shown to possess cardioprotective potential (83, 149, 395). Pretreatment of cardiac cells with CORM-3 decreased the injury caused by hypoxia–reoxygenation and oxidative stress when compared with cells treated with inactive CORM. Similarly, treatment with CORM-3 during cardiac reperfusion after an ischemic event improved cardiac performance and reduced infarct size and muscle damage (83).

Gene therapy using adeno-associated virus (AAV)-mediated HO-1 gene delivery into the rat myocardium has been shown to confer sustained myocardial protection from I/R injury. In this study, HO-1 delivery to the left ventricular risk area 8 weeks in advance of myocardial infarction resulted in an ~80% reduction in infarct size. Furthermore, it was accompanied by a decrease in oxidative stress, inflammation, and interstitial fibrosis, leading to the postinfarction recovery of the risk area (281).

In another study, HO-1 protection against postinfarction heart failure was investigated. Liu *et al*. (250) studied the mortality of rats transduced with AAV–HO-1 in the anteroposterior apical region of the left ventricular wall and then subjected to 30 min of ischemia. One year after acute myocardial infarction, mortality was markedly reduced in the HO-1–treated animals compared with animals injected with LacZ. Moreover, a 62% reduction in myocardial scarring and fibrosis in HO-1–overexpressing rats has been reported (250).

E. HO-1 and vascular restenosis

Balloon angioplasty is a technique used for dilating blood vessels to increase the blood flow in the occluded arteries. This process, however, causes inflammation in vessel walls, which results in the induction of SMC proliferation, and consequently, the lumen of the artery can be even more narrowed than before the angioplasty. The phenomenon is called restenosis, and it can also occur after stent placements. It was reported that HO-1 is induced after balloon angioplasty (422). Hemin administration inhibits neointima formation after balloon angioplasty in rats (10). Both CO and biliverdin were shown to contribute to the prevention of

neointima formation (160, 316, 429). CO mediates its action by inhibition of VSMC proliferation (337, 429) and apoptosis (251), as well as abolishing inflammation (320). Bilirubin also suppresses VSMC proliferation at least in part by modulating the p38 MAPK signaling pathway (316).

Interestingly, probucol, a drug used to prevent restenosis, also induces HO-1 (97, 458). Recently, butylated hydroxyanisole, a synthetic flavonoid, was discovered to inhibit VSMC proliferation in culture and to suppress neointima formation in injured arteries of rats, an effect that was shown to be dependent on the Nrf2/HO-1 pathway (252).

Some studies demonstrated that shorter GT repeats of the HO-1 promoter leading to higher HO-1 activity were associated with a lower risk of restenosis (121, 146, 374) (see also part VII-G). This outcome was a good indicator that HO-1 inducers should be considered as drugs for the prevention of restenosis. Collectively, results show that HO-1 and its products are involved in the prevention of neointima formation.

F. HO-1 and diabetes

Diabetes is a metabolic disorder caused either by impaired insulin secretion (type 1 diabetes) or by insulin resistance (type 2 diabetes). A high blood-glucose level is a hallmark of the disease. The consequences of such lingering hyperglycemia are severe, leading to tissue damage and endothelial dysfunction. Microvascular complications are typical in the retina and may cause diabetic retinopathy and even loss of vision. The pathologic changes in small blood vessels contribute also to nephropathy and neural lesions (218). Macrovascular complications may result in myocardial injury (335) or so-called "diabetic foot syndrome," which is a chronic nonhealing wound, leading to ulcers, necrosis, and ultimately requiring amputation (255).

The upregulation of both HO-1 expression and activity is considered to play a protective role in the development of diabetic complications. Therefore, induction of HO-1 in the livers of spontaneously diabetic rats (84), and in the retina (88) or in glomerular epithelial cells (155) of streptozotocin (STZ)-induced diabetic rats can be thought of as a protective mechanism. However, the authors observed that treatment with SnPPIX reduced the number of cells damaged by oxidative stress in the hearts of STZ-injected rats (124). These seemingly paradoxic observations provide evidence of the pro-oxidant activity of HO-1 in the heart in diabetes, which could be mediated by increased iron levels. Conversely, an inhibition of HO activity was observed in aortas of STZ-induced diabetic rats, despite a lack of change in HO-1 protein level (348). Decreased expression of HO-1 mRNA was shown in human retinal pigment epithelium and in human muscles from diabetic donors. It was thus suggested that a lower expression of HO-1 may contribute to the vulnerability of the tissues to significant metabolic alterations encountered in diabetes (50, 89). Accordingly, enhancement of HO-1 can be considered as a therapeutic approach for the amelioration of diabetic complications.

In line with such a supposition, Li *et al*. (235) reported that pancreatic β cells of nonobese diabetic mice may escape autoreactive T cell–mediated destruction as a result of the CoPPIX-mediated induction of HO-1 and the associated increase in the production of CO and bilirubin. In addition, Hu *et al*. (172) demonstrated the beneficial effect of AAV-mediated HO-1 expression in nonobese diabetic (NOD) mice. HO-1 transduction reduced insulitis, characterized by the inflammation and the lymphocytic infiltration of the Langerhans islets and slowed the progression of type 1 diabetes. It downregulated the phenotypic maturity of dendritic cells and Th1 effector function. A similar effect was observed in NOD mice subjected to CO (172).

HO-1 can also attenuate hyperglycemia-induced damage to endothelial cells. Retroviral-mediated HO-1 overexpression reduced endothelial cell sloughing in STZ-induced diabetic rats (4) and glucose-induced apoptosis of human microvessel endothelial cells (3). In another study, however, HO-1 induction under hyperglycemic conditions was associated with oxidative DNA and protein damage in HUVECs (69).

HO-1 also plays a crucial role in wound healing, providing protection against oxidative stress (155, 348). In diabetes, impaired tissue vascularization contributes to poor healing of chronic wounds (35). Furthermore, a high glucose level impairs growth-factor production, including VEGF (110, 185), and HO-1 induction reverses this disadvantageous effect (185). Collectively, these findings indicate that HO-1 may have a salutary influence in the diabetic state, especially in the vasculature (Fig. 12). Possibly it may protect against development of type 1 diabetes through its antiinflammatory properties. However, the role of HO-1 in diabetic complications must be better elucidated and confirmed.

G. The role of HO-1 promoter polymorphism in cardiovascular diseases

The existence of HO-1 promoter polymorphism has been shown to be of clinical relevance. Its association with cardiovascular diseases is reviewed in Table 4. It was reported that the short (GT)*n* repeats are linked to lower plasma levels of inflammation markers (239, 373). Moreover, as demonstrated in several reports, the absence of the short allele significantly augments inflammatory reaction in the vessel wall and increases the risk of restenosis in patients undergoing percutaneous transluminal angioplasty (PTA) (121, 373, 374) and coronary stenting (74, 421).

Studies by Schillinger *et al*. (373) showed that patients with short (GT)*n* repeats (<25 GT) exhibited a significantly reduced level of inflammation in response to balloon angioplasty compared with carriers of longer (GT)*n* repeats (373). Indeed, the risk of restenosis in patients carrying the short HO-1 genotype was reduced, as was found in two independent cohorts of 96 and 381 patients undergoing peripheral PTA, with a risk reduction by 0.24 and 0.43, respectively, for carriers of the short (<25) GT repeats (121, 374).

Quantitative coronary angiography performed 6 months after stent implantation in 323 patients of the Chinese population confirmed the previous observations that the polymorphism of (GT)*n* repeats in the HO-1 gene promoter can be an independent risk factor for angiographic restenosis as well as adverse cardiac events (74). Carriers of longer (GT)*n* repeats have been shown to have a 3.74-fold increased risk of angiographic restenosis after coronary stenting (74).

These findings from different ethnic backgrounds seem to provide strong evidence for the involvement of HO-1 microsatellite polymorphisms in the pathogenesis of resteno-

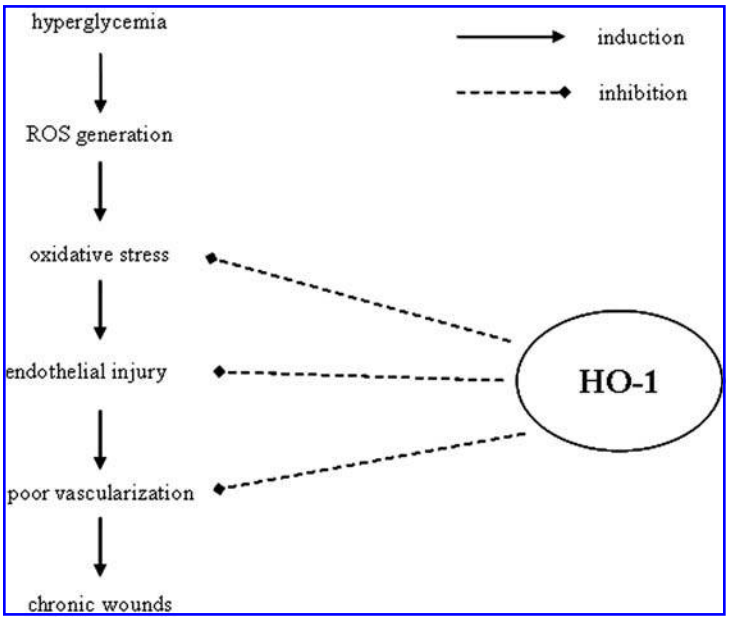

FIG. 12. The potential protective role of HO-1 in diabetic complications. Hyperglycemia induces in cells the generation of reactive oxygen species, especially superoxide anion. Endothelial cells are extremely vulnerable to hyperglycemia-induced oxidative damage. Endothelial injury may strongly influence tissue vascularity, and poor vascularization is one of the main problems in diabetic chronic wounds. HO-1 might play a protective role in such complications.

sis. However, very recently, Tiroch and co-workers (421) were not able to support a clinically relevant association of this polymorphism with restenosis and ischemic events after coronary stenting from the cohort of 1,357 German subjects with 6-month follow-up angiography. In light of these observations, the protective effect of the short HO-1 promoter on the risk of in-stent restenosis requires further investigation.

Brydun *et al*. (52) investigated the HO-1 mRNA expression in hemin-stimulated peripheral blood mononuclear cells (PBMCs) isolated from patients with coronary atherosclerosis. The level of HO-1 expression was significantly reduced in L/L genotype carriers compared with the values in the S/S genotype (52). This confirmed the previous observations that the long genotype is associated with a lowered capacity for HO-1 gene upregulation in response to oxidative stress. Moreover, the expression of HO-1 showed a strong negative correlation with the coronary score in patients, suggesting that the reduced ability to induce HO-1 may actually be involved in the mechanism of coronary atherosclerosis (52).

The presence of the long HO-1 promoter was shown to be associated with a higher extent of lipid peroxidation in serum (75), leading to the increased risk of abdominal aortic aneurysms (372) and coronary artery disease (CAD) and supporting the genetic linkage of HO-1 to defense against oxidative stress (75, 200). Chen *et al*. (75) observed that the presence of long sequences of (GT)*n* repeats (≥32 GT), resulted in a 4.7- fold increase in the incidence of CAD in 474 Chinese type II diabetic patients compared with 322 controls. Accordingly, Kaneda *et al*. (200) reported a reduced frequency of CAD in carriers of short (GT)*n* fragments (<27 GT) in 577 patients undergoing coronary angiography in the high-risk subgroups with hyperlipidemia, diabetes, and in current smokers (200). In contrast to the findings in the Asian population, Endler *et al*. (119) were unable to reproduce those

TABLE 4. STUDIES INVESTIGATING THE ROLE OF (GT) POLYMORPHISMS IN THE HO-1 GENE PROMOTER AND CARDIOVASCULAR DISEASES

Disease	*Polymorphism associated with the disease*	*No. of patients included*	*Race population*	*References*
Inflammation after balloon angioplasty	Yes	317	White	373
Abdominal aortic aneurysms	Yes	271	White	372
Restenosis after peripheral angioplasty	Yes	96	White	121
	Yes	381	White	374
Restenosis after coronary stenting	Yes	323	Asian	74
	No	1357	White	421
Myocardial infarction and stable CAD	No	649	White	119
CAD in patients with risk factors	Yes	577	Asian	200
CAD in type II diabetic patiens	Yes	796	Asian	75

results in 649 whites. Individuals with myocardial infarction ($n = 258$) or stable CAD ($n = 180$) and controls ($n = 211$) had similar genotype distributions of the (GT)*n* repeat polymorphism. Therefore, the real meaning of HO-1 genotypes in the progression of atherosclerosis, similar to that in the studies on restenosis, also remains unclear.

Increased expression of HO-1 protein in experimental transplant models correlated with reduced graft injury (151, 366, 389). Furthermore, transplant arteriosclerosis was found to be prevented by the expression of HO-1 (151). Therefore, the role of promoter polymorphism also was studied in organ grafts and predominantly in renal transplantation (26, 85, 122). Two different groups demonstrated that kidneys with the short (GT)*n* repeat genotype are less vulnerable to tissue injury, resulting in less chronic allograft nephropathy and better graft survival (26, 122). However, a recent study by Courtney *et al*. (85) showed that graft survival was not significantly affected by presence of the short allele, either in the donor or in the recipient. Similarly, Turpeinen *et al*. (430), analyzing 680 renal transplant recipients, demonstrated that another HO-1 gene polymorphism (four single-nucleotide polymorphisms and one microsatellite marker) had no significant influence on the outcome of kidney transplantation. Therefore, no unequivocal evidence exists for a protective effect of the short allele of the HO-1 promoter on graft or recipient survival in clinical renal transplantation.

Besides cardiovascular diseases, the presence of long (GT)*n* repeats has been positively associated with cigarette smoke–induced chronic obstructive pulmonary disease (COPD) (461), although again, independent studies found no such association (158). Moreover, several studies suggested that a lack of the short allele and resulting lower activity of HO-1 can be associated with a higher incidence of cancers such as oral squamous cell carcinoma (61, 248), lung adenocarcinoma (204), and gastric adenocarcinoma (254). The length of the (GT)*n* sequence in the HO-1 promoter did not influence the onset and progression of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (120, 209).

In summary, functional (GT)*n* repeat polymorphisms in the promoter region of the human HO-1 gene have been demonstrated to modulate the level of HO-1 activity in response to different stimuli, which correlates with different disease entities in various studies performed in populations of different ethnic backgrounds. Multiple investigations gave evidence that the ability of patients with certain genotypes to respond strongly in terms of upregulating HO-1 may be an important endogenous protective factor. Nevertheless, in many cases, the data are contradictory, and therefore the clinical consequences of the functional dinucleotide (GT)*n* polymorphism within the HO-1 promoter, especially in the field of cardiovascular diseases, requires further investigation and validation.

VIII. HO-1: Target of Cardiovascular Drugs

As reviewed earlier, recent studies have focused on HO-1 as an excellent target for treatment of cardiovascular diseases. Therefore, a number of drugs have been investigated for the capacity to increase HO-1 expression. Accordingly, it is supposed that the activation of HO-1 may contribute to the therapeutic benefits of statins, probucol, nonsteroidal antiinflammatory drugs, or rapamycin, used in the clinic as cholesterol-reducing agents, antiinflammatory medicines, and immunosuppressants, respectively.

A. Statins

Statins, inhibitors of 3-hydroxy 3-methyloglutaryl coenzyme A reductase, are among the most frequently prescribed drugs for treatment of cardiovascular diseases. Apart from reducing cholesterol levels, they have been shown to have both antiinflammatory and antioxidant properties (for reviews, see 109, 245). To these pleiotropic effects of statins, one can also include the modulation of HO-1 expression. However, data presented recently must be interpreted with some caution.

In two independent studies, Grosser *et al*. investigated the influence of different statins: rosuvastatin (141), lovastatin, and simvastatin (143) on HO-1 expression. In their hands, extremely high concentrations (300 μM) of rosuvastatin resulted in a modest (twofold) induction of HO-1 mRNA (141). In another study, they used also high concentrations of other statins (100–300 μM) to induce HO-1 expression (143). Thus, it must be stressed that the concentrations of statins used in those experiments do not reflect the clinical situation in patients taking statins for reducing cholesterol levels. Statins should be used up to low micromolar doses, as their plasma concentration in patients are at a nanomolar range: 0.1–0.3 μM for lovastatin (417) and 0.002–0.2 μM for atorvastatin (80). Such low, pharmacologically relevant concentrations affect the expression and activity of eNOS and Akt kinases and influence angiogenic properties of endothelial cells (257, 432).

Moreover, these experiments have been performed on the ECV304 cell line, which has been claimed to be of endothelial origin. However, recent studies have proven the nonendothelial origin of these cells, which are derived from the epithelial cells of bladder cancer (49). In this context, the effect of statins can be different on endothelial and on epithelial cells.

Our studies performed on the HMEC-1 cell line demonstrated that neither 6- nor 24-h stimulation with 0.1–1 μM atorvastatin, in both normoxic and hypoxic conditions, stimulated HO-1 mRNA and protein expression (258). Similar results were obtained in primary HUVECs (115). Other statins (pravastatin, cerivastatin, and fluvastatin) did not induce HO-1 expression in HMEC-1 but even downregulated its expression (unpublished data). These results are in line with recent studies in which the expression of HO-1 was observed in Neuro 2A cells only on exposure to very high concentrations of statins (>50 μM; lower concentrations were ineffective), which induced apoptosis of those cells (169).

Thus, it might be possible that the regulation of HO-1 expression by statins can be related to the cell type used. Lee *et al*. (231) reported a stimulatory effect of simvastatin on HO-1 expression in human and rat aortic SMCs, but not in endothelial cells and macrophages. Conversely, Chen *et al*. (65) showed that several statins induced HO-1 expression in murine RAW264.7 macrophages *via* ERK, p38 MAPK, and protein kinase G pathways (65).

Recently, Ali *et al*. (20) demonstrated that relatively low concentrations of statins (up to 5 μM) induced HO-1 in HU-

VECs and human aortic endothelial cells, providing protection against oxidative stress. The effect was demonstrated to be dependent on activation of Kruppel-like factor-2 (KLF2), a transcription factor previously shown to be activated by statins (334).

In vivo studies, in which statins have been injected into mice, demonstrated moderate induction of HO-1 expression and activity (170, 298). It remains to be established whether these effects have physiologic relevance and whether the induction of HO-1 expression is not the result of muscle injury (rhabdomyolysis) and the release of heme proteins such as myoglobin, induced by high statin concentrations.

B. Probucol

Probucol, which was earlier used as a cholesterol-reducing drug, also has antioxidant properties and inhibits atherosclerosis and restenosis (367, 368). Deng *et al*. (97) reported that this drug inhibits proliferation of VSMCs *via* activation of HO-1. However, in primary porcine aortic endothelial cells, probucol appeared to decrease HO-1 mRNA after a short (6 and 8 h) time of incubation. Data from animal studies showed that probucol induces HO-1 in balloon-injured hypercholesterolemic rabbit aortas, which was also associated with inhibition of VSMC proliferation and apoptosis (458). Noteworthy, these results were obtained only when sulfur groups of probucol were active. Accordingly, HO-1 expression was induced by probucol and its sulfur-containing metabolite, probucol dithiobisphenol, but not by probucol bisphenol, which possesses only phenol moieties (458). Moreover, in Zucker rats and in the carotid arteries of rabbits subjected to balloon injury, treatment with probucol or an analogue containing a dithioether bridge provided vascular protection, and this effect was dependent on HO-1. In contrast, vitamin E, which did not induce HO-1, was not protective in these models (458).

C. Nonsteroidal antiinflammatory drugs

Another group of compounds that are now believed to have beneficial effects on the vascular wall are nonsteroidal antiinflammatory drugs (NSAIDs), mainly aspirin and indomethacin. To elucidate the potential effect of these drugs on HO-1, Grosser *et al*. (142) used ECV304 cells as an endothelial model (see earlier). Aspirin (3 m*M*) led to an approximately twofold induction of bilirubin formation, and an even lower stimulation of CO release after 12 h was observed. However, a typical therapeutic level of aspirin *in vivo* ranges from 0.1 to 2 m*M*, whereas 5 m*M* is toxic and leads to endothelial cell apoptosis (43). At these therapeutic doses (0.3 m*M*), aspirin was able to induce HO-1 expression up to 2.6 times (142), but the effect was not exhibited by indomethacin, another member of NSAID family (142). In contrast, in primary cultures of guinea pig gastric mucosal cells, both indomethacin and aspirin were able to induce HO-1 expression (6). However, the increase in HO-1 protein was observed only after the administration of 10 and 12 m*M* aspirin, whereas lower concentrations (2–8 m*M*) even decreased HO-1 expression. An interesting finding was obtained by using 15-epi-16-(*para*-fluoro)-phenoxy-lipoxin A_4 (ATL-1), an aspirin-triggered lipoxin A_4 stable analogue, which was even more potent in inducing HO-1 expression in HUVECs than was 0.3 m*M* aspirin (306). However, the effect of ATL-1 was more pronounced in nonendothelial ECV304 cells than in HUVECs (306). Thus, the observed effects are problematic, and the nonphysiologic conditions used in some of the studies should also be taken into consideration.

D. Rapamycin

Other studies have shown that the protective effect of rapamycin, an immunosuppressive agent with antiproliferative properties against lymphocytes, vascular endothelial, and smooth muscle cells (375), can be related to the induction of HO-1 expression. Both in human pulmonary artery endothelial cells (HPAECs) and smooth muscle cells (HPASMCs), HO-1 mRNA, protein, and more important, activity level were increased in response to rapamycin (437). Also, *in vivo* studies performed in a rat monocrotaline-induced pulmonary hypertension model indicate that rapamycin acts *via* the HO-1 pathway. Zhou *et al*. (481) found that inhibition of HO-1 by using SnPPIX or a knockout of the HO-1 gene results in a loss of the protective effects of rapamycin on SMC proliferation.

E. Pentoxifylline

Pentoxifylline attenuates the cytotoxic action of TNF-α (474) and is used for the treatment of cerebrovascular disorders, occlusive arterial diseases, claudication, and septic shock (for a review, see 132). In 2003, Oh *et al*. (314) showed that pentoxifylline in a broad range of concentrations (0.1–1 m*M*) potently induced HO-1 expression and increased its activity in L929 fibroblasts. However, our experiments do not confirm such an action either in HUVEC, U937, and J744 cells or in mice injected with pentoxifylline (unpublished data).

F. Plant polyphenols

HO-1 also has been suggested to be a downstream mediator of chemopreventive agents such as resveratrol or curcumin (for review, see 344). Juan *et al*. (197) showed the biphasic action of resveratrol on HO-1 expression in human aortic smooth muscle cells (HASMCs). Resveratrol at low concentrations (1–10 μM) induces HO-1 *via* the NF-κB signaling pathway, whereas at higher concentrations ($\geq$20 μM), it suppresses IκBα phosphorylation and blocks NF-κB activity, which inhibits HO-1 induction (197). However, Das *et al*. (90) suggested that resveratrol reduces myocardial infarct size and decreases the number of apoptotic cardiomyocytes in rat hearts subjected to I/R injury through HO-1–mediated mechanisms, which are regulated by p38 MAP kinase and Akt survival signaling, but not by NF-κB activation. Another pathway involved in the HO-1–mediated activity of resveratrol depends on the Nrf2 transcription factor (64).

HO-1 can also mediate the antiinflammatory and antioxidant activities of curcumin. The upregulation of HO-1 expression after curcumin treatment has been observed in rat kidney epithelial (NRK) and porcine renal epithelial (LLC-PK1) cells (30), human renal proximal tubule cells (161), human hepatoma cells (279), and bovine aortic endothelial cells (295). Similarly, as in the case of resveratrol, induction of HO-1 by curcumin may be mediated by Nrf2 and NF-κB (30, 64, 161, 279).

G. Organic nitrates

Very recently, the concept about HO-1 acting as a modulator of organic nitrates-caused tolerance and endothelial dysfunction has been exploited (107, 451). Nitroglycerin (glyceryl trinitrate, GTN) is widely used in patients with acute myocardial infarction and in patients with chronic congestive heart failure; however, the induction of nitrate tolerance, a phenomenon of the development of therapeutic resistance to this drug, limits its effectiveness. Interestingly, it has been demonstrated that pentaerithrityl tetranitrate (PETN), contrary to other organic nitrates, does not cause tolerance. In contrast to GTN, in rats infused with PETN, no increase in ROS production in isolated heart mitochondria as well as in intact aortic vessel segments has been detected (451). HO-1 has been proposed to be responsible for the differences observed between tolerance-inducing nitrates and PETN, as both *in vitro* (312) and *in vivo* (107, 451), the remarkable induction of HO-1 has been noted after PETN but not after GTN treatment. Similarly, upregulation of HO-1 by PETN was paralleled by the increase in the expression of another cytoprotective enzyme, ferritin (107, 451).

However, further studies are necessary to confirm whether the upregulation of HO-1 is unique for PETN, as Minamiyama and co-workers (283) reported about fourfold induction of liver HO-1 expression after GTN treatment in rats. Nevertheless, it was also proven that HO-1 induction by exogenously added hemin reduced GTN-induced ROS formation and tolerance. Conversely, inhibition of HO-1 by apigenin in PETN-treated animals resulted in increased ROS formation and development of resistance to this drug (451).

Thus, the data obtained so far indicate that HO-1 induction by cardiovascular drugs is possible; however, more-detailed studies, especially with the pharmacologic concentrations of examined compounds, should be performed.

IX. HO-1 in Gene and Cell Therapy

Gene therapy with the use of viral vectors is a promising approach that can allow us not only to reconstitute missing or dysfunctional genes, but also to modify the expression of genes that are already present. Although only one report exists of a child lacking the HO-1 protein (460), it is important to remember that HO-1 expression and thus activity can vary among people because of differences in the HO-1 gene promoter sequences (discussed in *Polymorphism of HO-1 promoter*). With respect to these facts, gene therapy provides a wide spectrum of potential HO-1 gene manipulation, including the delivery of the gene under a strong promoter for stable expression, the use of highly active promoters for transient expression, and expression regulated by particular conditions, such as hypoxia. The delivery of the HO-1 gene to vascular tissues can contribute to the inhibition of the development of certain diseases as well as abrogate the severity of others (for review, see 2).

Gene transfer based on viral vectors uses most commonly one of three viruses as the vector base: adenovirus, retrovirus, or AAV. Each of these has its advantages and disadvantages. For instance, adenoviral vectors are very efficient in transducing many nondividing cell types, like endothelium *in vivo*, but, conversely, can exert a strong inflammatory response. It was shown, however, that the antiinflammatory effects of HO-1 can be exploited here, as the HO-1 gene delivery in adenoviral vectors prevented the inflammatory response and was effective even when coinjected with another adenoviral vector carrying a different gene (275).

A. Atherosclerosis

In vitro rat VSMC transduction with the HO-1 gene carried in retroviral vectors resulted in extended resistance of cells to oxidative stress, as well as slowed their proliferation rate (476). *In vivo*, delivery of the HO-1 gene in adenoviral vectors reduced inflammation, leukocyte infiltration, and VSMC proliferation, which consequently diminished neointima formation in transplanted rat aortas (45). Also in ApoE-deficient mice, HO-1 delivery in adenoviral vectors inhibited plaque formation in young animals and delayed their progression in adult animals (198).

B. Myocardial infarction

Experiments on animals showed that HO-1 is induced in hearts on I/R injury of the organ (224), and this phenomenon is mediated by ROS (272). Therefore, it was hypothesized that gene transfer of HO-1 to the heart can ameliorate detrimental effects on myocardium. Overexpression of HO-1 by AAV-mediated delivery decreased the extent of myocardial scarring, attenuated the expression of proapoptotic genes, and decreased the inflammatory reaction (281).

Few independent studies showed that transduction of rat myocardium with AAV–HO-1 vectors results in gene expression, even a long time after the vector delivery. When subsequent heart infarction was induced, HO-1 exerted many beneficial effects. The infarct size was decreased by at least 75%, proapoptotic markers levels were reduced (281), the ejection fraction was only slightly diminished and returned to normal after 3 months, fibrosis and remodeling of the ventricle wall was inhibited (249), and 1 year after the infarction, mortality was markedly reduced (250). Similar protective effects of AAV-mediated HO-1 gene transfer were observed after repeated I/R events (324).

However, one can speculate that the constitutive overexpression of human HO-1 may also cause some undesirable effects. To overcome such possible adverse effects, Tang et al. (411–413) designed a regulatable, vigilant plasmid system for HO-1 overexpression, which uses two constructs. The first plasmid contained the myosin light chain-2v (MLC-2v) promoter and a sequence encoding an oxygen-dependent degradation domain (ODD) from HIF-1α. The second plasmid contained the HO-1 gene driven by a hypoxia-responsive element. After transfection, the synthesis of HIF-1 occurred only in the cardiomyocytes, but in normal oxygen tension, HIF-1 was degraded. On infarction, HIF-1 ODD was stabilized, bound to HRE, and induced the expression of HO-1. Thus, this system, switched on by ischemia, allows transient expression of the HO-1 gene in cardiomyocytes. After the proper level of oxygenation is obtained, the system can be turned off (411–413).

The data strongly indicate that HO-1 plays an important role in the regeneration of myocordium after I/R injury or acute infarction. It is also possible that the protective effect of HO-1 gene therapy in the heart depends on the induction of angiogenesis, in a similar way as HO-1 gene transfer ameliorated hindlimb ischemia in rats (402). Therefore, it remains

to be established whether HO-1 overexpression in ischemic heart induced formation of new blood vessels.

C. Hypertension

Delivery of the HO-1 gene by retroviral vectors to 5-day-old SHRs prevented the development of hypertension in the animals (138, 360). HO-1 gene introduced to cirrhotic rats reduced vasoconstrictive responsiveness to phenylephrine in superior mesenteric artery (361). In yet another study, overexpression of HO-1 in rats introduced by retroviral vectors rendered the blood pressure more resistant to AngII (465).

D. Type I diabetes

Cultured rat and human islets infected with adenoviral vectors carrying the HO-1 gene not only were shown to be more resistant to apoptosis induced by TNF-α and cycloheximide, but also demonstrated better responsiveness in insulin production after exposure to high levels of glucose in the medium (71, 243, 244).

In another study, the IL-10 gene was introduced by AAV vectors into islets that were subsequently transplanted into NOD mice. This procedure restored the normal control of blood-sugar levels in the animals. This effect of IL-10 was suggested to be mediated by induction of antioxidative genes, including HO-1 (480). Similar effects of IL-10, delivered by using an AAV vector, were shown in a rat aortic-transplant model of allograft rejection and neoinitimal proliferation, in which the vascular protective effects were dependent on HO enzyme activity (70). Also, when HO-1 was delivered to NOD mice in adenoviral vectors (172), progression of the disease was inhibited. Therefore, HO-1 seems not only to protect islet grafts from rejection but also to contribute to the proper functioning of islet cells and inhibit their apoptosis and clearance in diabetic animals.

Recent studies have indicated that application of bone-marrow–derived progenitor cells can be beneficial in regeneration of ischemic myocardium (183, 199, 397). In numerous experimental models, it was shown that injection of bone marrow–derived endothelial progenitor cells (EPCs) into damaged tissues can stimulate revascularization (397). However, EPC function seems to be impaired in patients with vascular disorders. The number of EPCs in the blood inversely correlates with a risk of cardiovascular diseases, such as diabetes (259), hypercholesterolemia (432), and hypertension (433). Inquiry into the role of various genes in the reduced activity of EPC may provide future strategies for progenitor cell modifications leading to the improvement in their regenerative potency. One of these genes is HO-1, which may play a pivotal role in EPC function and influence the therapeutic efficiency.

The role of HO-1 in the functions of EPCs has been investigated in experimental balloon angioplasty. EPC delivery, by stimulating of regeneration of endothelium, reduced neointima formation. This effect was even stronger when the EPCs were overexpressing eNOS, but not HO-1 (216). However, although HO-1 in EPCs may not be involved in their restenosis-abolishing activity, it was proven that migration of these cells toward SDF-1 is dependent on the presence of HO-1 (99). Therefore, HO-1 may influence the therapeutic potential of EPCs. Accordingly, recent study demonstrated that inhibition of HO-1 can disrupt the natural gradient of SDF-1 expression established in ischemic conditions, in which high SDF-1 concentration in the periphery drives the migration and homing of EPCs from the bone marrow (423). An increase in Sca-1^+/Kdr$^+$ EPC in the peripheral circulation, observed after hindlimb ischemia, is reduced when HO is inhibited in mice. Consequently, the capillary density in the ischemic limbs was reduced (423). Those observations are of potential clinical relevance, indicating that future studies on EPC functions and applications should address the mechanisms that may affect HO-1 expression and activity.

Besides EPCs, which are suggested to be of hematopoietic origin, bone marrow–derived mesenchymal stem cells (MSCs) could help in the regeneration of infarcted heart muscle tissue in humans (371). After MSC-based therapy of an infarcted heart, the left ventricle ejection fraction improved in the trials, with the best outcomes by values of $\sim$ 5% (302). Although this result was statistically significant, its clinical meaning was brought into question (302). It was suggested that this beneficial role may be mediated partially by induction of HO-1 in the stem cells, as well as in cells of the surrounding myocardium (477). In an experimental setting, it was shown that when the MSCs were modified to overexpress HO-1, the improvement in regeneration of the infarcted heart tissue, inhibition of apoptosis, extended survival, and reduced remodeling of the ventricle wall were observed in comparison not only with nontreated hearts, but also in hearts treated with unmodified MSCs (413). These data strongly indicate that the role of HO-1 in regenerative cell therapy should be further investigated. Provided that experimental data continue to prove HO-1–overexpressing stem cells to be better than unmodified ones, a combination of gene therapy and cell therapy should be considered for future therapeutic strategies.

X. Novel Roles for HO-1 in Cardiovascular Disorders

HO-1 has been demonstrated to be a protein with multiple functions, playing roles of significant consequence in various processes, including angiogenesis (111), immune response (460), pregnancy (27), transplantation (463), and embryonic stem cells protection (425). The cardiovascular functions of HO-1 were, however, among the most extensively investigated, demonstrating the significant input of this enzyme to the physiology and pathology of vascular beds.

Cardiovascular diseases secondary to ischemia are currently a major medical and social problem. Therefore, methods for the effective stimulation of new blood-vessel formation, which represent a new approach to the improvement of the life quality of patients not curable with surgical revascularization treatment, are urgently needed. It is possible that such effects can be obtained by cell therapy, and EPCs are regarded as a potential therapeutic agent. In the light of recent findings (99), one can suggest that modification of EPCs with the HO-1 gene can be considered as a serious therapeutic opportunity.

Moreover, the HO-1 promoter polymorphism may be an endogenous factor modulating the HO-1 expression in response to given stimuli, and it can correlate with the occurrence of different diseases. Therefore, the significance of HO-1 promoter polymorphism in EPCs is also worthy of further consideration.

Endothelial protection could be also achieved through the induction of phase 2 detoxifying enzymes and antioxidant

enzymes. As described earlier, Nrf2 modulates stress-dependent induction of HO-1 expression (14); therefore, this transcription factor can be considered as one of the major targets in HO-1–mediated chemoprevention of cardiovascular diseases.

When discussing the protective role of HO-1 in the cardiovascular system, it is necessary also to stress the importance of BVR activity. Three different tracks of BVR function exist: a reductase that converts biliverdin to bilirubin (222), a transcription factor regulating HO-1 (9), and a kinase in the insulin-receptor/MAPK pathway (233). It was recently shown that the cardioprotective effect of HO-1 in I/R-induced myocardial injury is dependent on Akt kinase activity, and this effect is mediated, at least in part, by BVR and is mimicked by bilirubin (325). Such results show that the HO-1 pathway is linked to BVR activity, and it suggests a novel mechanism of HO-1 in myocardial infarction.

Last but not least, the intriguing question assigning new directions of HO-1 research concerns the significance of microRNAs in the regulation of HO-1. MicroRNA is a class of small RNAs playing a role in the regulation of gene expression (39). The recent discovery of the role of Mir-122 in the regulation of HO-1 expression in hepatocytes in response to hepatitis C infection (379) indicates the significance of this regulation in the HO-1 pathway as well. The potential link between microRNA, HO-1, and angiogenesis also must be considered.

HO-1 possesses many faces, and its role is much wider than heme elimination only. It is one of the most inducible genes in response to stressful conditions, and numerous experiments show that the products of HO-1 activity are involved in the maintenance of cell homeostasis. Subsequent years should provide more data elucidating the complex interactions of this elaborated antioxidant system.

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Abbreviations

AAV, adeno-associated virus; AdHO-1, adenovirus bearing HO-1 gene; Akt, protein kinase B; AngII, angiotensin II; AP-1, activator protein-1; ApoE, apolipoprotein E; ARE, antioxidant response element; ATL-1, 15-epi-16-(*para*-fluoro)-phenoxy-lipoxin A_4; BAECs, bovine aortic endothelial cells; BAL, bronchoalveolar lavage; BBMVECs, bovine brain microvascular endothelial cells; BPAECs, bovine pulmonary artery endothelial cells; BV, biliverdin; BVR, biliverdin reductase; CAD, coronary artery disease; CAECs, coronary artery endothelial cells; CAM, chicken chorioallantoic membrane; CdRE, cadmium-responsive element; cGMP, guanosine 3′,5′-monophosphate; CO, carbon monoxide; COPD, chronic obstructive pulmonary disease; CoPPIX, cobalt protoporphyrin; CORM, CO-releasing molecule; COX, cyclooxygenase; CPR, NADPH cytochrome P450 reductase; CRE, cAMP response element; CrMP, chromium mesoporphyrin; CVD, cardiovascular disease; EDRF, endothelium-derived relaxing factor; eNOS, endothelial nitric oxide synthase; EPCs, endothelial progenitor cells; ERK, extracellular signal-regulated protein kinase; ET-1, endothelin-1; FGF-1, fibroblast growth factor-1, acidic FGF; FGF-2, fibroblast growth factor-2, basic FGF; GFAP, glial fibrillary acidic protein; GST, glutathione S-transferase; (GT)*n*, GT repeats; GTN, glyceryl trinitrate (nitroglycerin); γGCS, glutamylcysteine synthetase; HAECs, human coronary aortic endothelial cells; HASMCs, human aortic smooth muscle cells; HIF-1, hypoxia-inducible factor-1; HMEC-1s, human microvascular endothelial cells; hmox-1, human oxygenase-1 gene; HNF-1, hepatocyte nuclear factor; HO-1, heme oxygenase-1; HO-$1^{-/-}$, HO-1 knockouts; HPAECs, human pulmonary artery endothelial cells; HPASMCs, human pulmonary smooth muscle cells; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; HRE, hypoxia-responsive element; HSE, heat-shock element; H_2O_2, hydrogen peroxide; HS, hypersensitive site; HSF-1, heat-shock factor-1; 4-HNE, 4-hydroxy-2-nonenal; HUVEC, human umbilical vein endothelial cell; ICH, intracerebral hemorrhage; IFN-γ, interferon γ; IL-1β, -6, -8, -10 – interleukin -1β, -6, -8, -10; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; JNK, c-Jun NH_2-terminal protein kinase; Keap1, Kelch-like ECH-associated protein-1; KLF2, Kruppel-like factor-2; 1K1C, one kidney–one clip, model of renovascular hypertension; LDL, low-density lipoprotein; LLC-PK1, porcine renal epithelial cell; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MARE, Maf recognition element, MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemotactic protein-1; MEFs, murine embryonic fibroblasts; mEH, microsomal epoxide hydrolase; MIP-1β, macrophage inflammatory protein-1; MLC-2v, myosin light chain-2v; MSCs, mesenchymal stem cells; MOI, multiplicity of infection; NF-κB, nuclear factor-κB; NO, nitric oxide; NOD, nonobese diabetic mice; NOS, nitric oxide synthase; NRE, negative regulatory element; Nrf2, nuclear factor erythroid 2–related factor; NRK, rat kidney epithelial cells; NSAIDs, nonsteroidal antiinflammatory drugs; NQO1, quinine oxidoreductase-1; ODD, oxygen-dependent degradation domain; ODQ, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one, inhibitor of GC; oxLDL, oxidized low-density lipoprotein; PAECs, porcine aortic endothelial cells; PAI-1, plasminogen activator inhibitor type 1; PBMCs, peripheral blood mononuclear cells; PDGF-B, platelet-derived growth factor-B; PETN, pentaerithrityl tetranitrate; PGH_2, prostaglandin H_2; PHDs, prolyl hydroxylases; 15d-PGJ2, 15-deoxy-D12,14-prostaglandin J2; PI3K, phosphatidylinositol 3 kinase; PKC-ζ, protein kinase C; PTA, percutaneous transluminal angioplasty; ROS, reactive oxygen species; RRAs, renal resistance arteries; SBE, smad-binding element; SDF-1, stromal cell–derived factor-1; sEng, soluble endoglin; sGC, soluble guanylyl cyclase; SHRs, spontaneously hypertensive rats; SIN-1, the 3-morpholinosydnonimine hydrochloride;

SnMPPIX, tin mesoporphyrin; SnPPIX, tin protoporphyrin; SNAP, S-nitroso-penicillamine; SOD1, copper/zinc superoxide dismutase; StRE, stress-responsive element; STZ, streptozotocin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; USF, upstream stimulatory factor; UV, ultraviolet; WHHLs, Watanabe heritable hyperlipidemic rabbits; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells.

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