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Gene Regulation of Heme Oxygenase-1 as a Therapeutic Target

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ABSTRACT. Heme oxygenase (HO)-1 is the inducible isoform of the rate-limiting enzyme of heme degradation. HO regulates the cellular content of the pro-oxidant heme and produces catabolites with physiological functions. HO-1 is induced by a host of oxidative stress stimuli, and the activation of *HO-1* gene expression is considered to be an adaptive cellular response to survive exposure to environmental stresses. Since overexpression of the *HO-1* gene is also protective against the deleterious effects of experimental injuries, the specific induction of HO-1 by 'non-stressful' stimuli, eg. stimuli that are not associated with oxidative stress, such as adenosine 3′,5′-cyclic monophosphate or cyclic guanosine 3′,5′-monophosphate, may have important clinical implications. This review summarizes recent advances in the understanding of regulatory mechanisms of *HO-1* gene expression, in particular the role of various redox-dependent and redox-independent signaling pathways. Models of experimental injuries are highlighted in which specific overexpression of the *HO-1* gene either by targeted gene transfer or by pharmacological modulation has been demonstrated to provide therapeutic effects. BIOCHEM PHARMACOL **60**;8:1121–1128, 2000. © 2000 Elsevier Science Inc.

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HO† catalyzes the rate-controlling step of heme degradation (Fig. 1) [1]. Two genetically distinct isozymes, HO-1 and HO-2, are known which share about 43% amino acid and nucleotide sequence similarity [2, 3]. A recently identified third isozyme of HO, HO-3, exhibits only low enzyme activity and may be involved in heme binding or heme sensing [4]. HO-1 and HO-2 have a different tissue-specific gene expression pattern, and in contrast to the constitutive isozyme HO-2, HO-1 is strongly induced by its substrate heme and by numerous stress stimuli such as UV light, heavy metals, lipopolysaccharide, heat shock, and hyperoxia [5]. The exact functional role of HO-1 induction in response to oxidative stress is not understood. However, as HO-1 provides cytoprotection in various cell culture and animal models, *HO-1* gene activation is considered to be an adaptive cellular defense mechanism [6, 7]. Overexpression of the *HO-1* gene has been shown to attenuate the toxic effects of heme and hemoproteins in transfected coronary endothelial cells [7] and to protect pulmonary epithelial cells against hyperoxia [6]. The important physiological function of HO-1 has been confirmed by observations in HO-1 knockout mice [8, 9]. Cultured fibroblasts from these animals are highly susceptible to heme- or hydrogen peroxide-mediated toxicity [9]. In addition, exposure of HO-1-deficient mice to endotoxin results in increased hepatocellular necrosis and in higher mortality from endotoxic shock as compared to control animals [9]. HO-1 knockout mice also develop an iron deficiency anemia along with hepatic iron overload, causing chronic inflammation of the liver [8]. The findings in HO-1-deficient mice were essentially confirmed in human HO-1 deficiency [10]. Since the specific induction of heat shock proteins by pharmacological stimuli has significant clinical implications [11, 12], targeted induction of *HO-1* gene expression by 'non-stressful' stimuli may serve as a novel approach to therapeutic intervention.

FUNCTIONAL SIGNIFICANCE OF HO ENZYME REACTION AND ITS PRODUCTS

The HO enzyme reaction (Fig. 1) is physiologically significant because HO degrades the pro-oxidant heme and produces equimolar amounts of catabolites, which serve regulatory and protective functions [13].

Regulation of Cellular Heme Homeostasis

Heme is a double-edged sword. When heme is covalently or non-covalently linked to specific proteins such as catalase or cytochromes, it is essential for the reversible binding of

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† *Abbreviations:* AP-1, activator Protein-1; cAMP, adenosine 3′, 5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PK, protein kinase; PP, protein phosphatase; RE, regulatory element; ROS, reactive oxygen species; TF, transcription factor; and TPA, 12-*o*-tetradecanoylphorbol-13-acetate.

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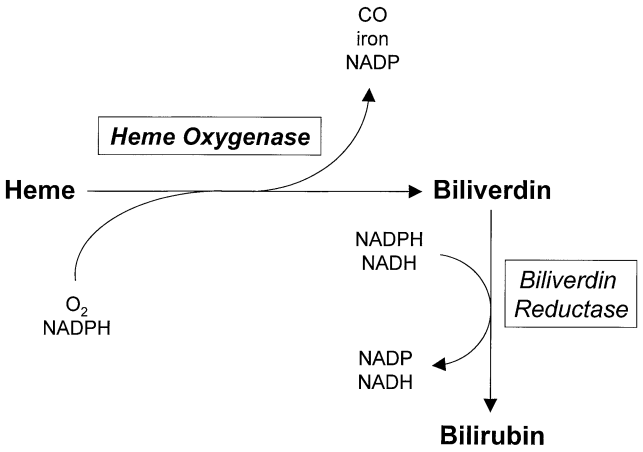

FIG. 1. The heme oxygenase enzyme reaction. Scheme of catalytic conversion of heme into bilirubin, carbon monoxide (CO), and iron.

oxygen or the transport of electrons [14]. On the other hand, heme may catalyze the production of cytotoxic ROS when it reacts with molecular oxygen which, in turn, causes DNA damage, lipid peroxidation, and protein denaturation [15]. Therefore, the 'regulatory heme pool' of the cell needs to be tightly controlled by heme synthesis and degradation. Besides HO as the rate-limiting step of heme degradation, major players in keeping the cellular 'heme pool' in check are the heme synthetic enzyme δ-aminolevulinate synthase and heme-binding proteins such as heme-binding protein/liver fatty acid-binding protein [16], heme-binding protein 23 [17] or p22 heme-binding protein [18], which neutralize the toxic effects of heme by non-covalent binding [19]. The importance of a strictly controlled cellular heme pool is illustrated by the regulatory functions of heme for the synthesis of iNOS, which catalyzes the enzymatic production of NO. The availability of sufficient heme is essential for the formation of functional iNOS dimers and catalytic activity [20]. Thus, HO-1-mediated heme degradation appears to be a negative feedback regulation for iNOS-derived NO production [3, 21]. In addition, heme of cytochrome P450 isozymes is a good substrate of HO enzyme activity in liver, and HO has been shown to specifically degrade various cytochrome P450 isozymes [22].

Bilirubin, a Cytoprotective Antioxidant

The HO product biliverdin is rapidly converted into bilirubin by biliverdin reductase in mammalian cells (Fig. 1). It has been shown that the bile pigment bilirubin has potent antioxidant properties [23]. These observations have recently been corroborated by Dore *et al.*, who demonstrated that HO-derived bilirubin provides cellular protection to neuronal cells [24].

Carbon Monoxide (CO), a Signaling Gas

HO-derived CO has been recognized to be an important cellular messenger with various physiological functions (for review see [25]). The signaling functions of CO resemble that of the signaling gas NO. In contrast to NO, however, which forms peroxynitrite with superoxide, CO does not form radicals. As to the liver, CO is involved in the regulation of hepatobiliary functions such as cytochrome P450-dependent biotransformation and, in addition, HO-1-derived CO has been shown to protect the hepatic microcirculation under stress conditions [25]. Furthermore, CO appears to play a prominent regulatory role for the tone of the cardiovascular system by promoting vasodilatation [26].

Iron

Enzymatic degradation of heme by HO produces iron (Fig. 1). Iron is an essential cofactor of numerous cellular enzymes and redox-dependent proteins, although excess iron is cytotoxic via the production of ROS by Fenton chemistry. It has been demonstrated that synthesis of the iron carrier ferritin is up-regulated depending on increased HO-1 activity during the cellular stress response of skin fibroblasts, thereby preventing iron-mediated cytotoxicity [27]. The notion that *HO-1* gene expression is involved in the physiological regulation of iron homeostasis is also supported by findings that HO-1-deficiency simultaneously leads to iron deficiency anemia and hepatic iron overload [8]. More recently, it has been demonstrated that cytoprotection of cell cultures by HO-1 may be attributed to augmented cellular iron efflux [28].

SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF *HO-1* GENE EXPRESSION

Redox Signaling

It has been reported in a large number of studies that *HO-1* gene expression is induced by stimuli that increase the cellular production of ROS, including heme, heavy metals, UV light, hydrogen peroxide, and lipopolysaccharide, or by stimuli that deplete cellular glutathione stores, including buthionine sulfoximine, sodium arsenite, and iodoacetamide [5, 29]. Furthermore, it has been shown that scavengers of ROS, such as *N*-acetyl cysteine, inhibit or reduce the magnitude of HO-1 induction by oxidative stress [30]. These findings indicate that an increase in intracellular ROS plays a crucial role in the regulation of *HO-1* gene expression. Although there are still significant gaps in the understanding of the exact mechanisms of redox signaling, in particular the intracellular targets of ROS [31], changes in the cellular redox state may modify the activity of specific regulatory protein kinases and protein phosphatases which affect the regulation of gene expression [31]. Although redox signaling appears to play a major role in HO-1 regulation, various redox-independent pathways are also involved in *HO-1* gene regulation [32].

Mitogen-Activated Protein Kinases

MAPKs are a family of serine–threonine protein kinases that are activated by a variety of extracellular stimuli and are assumed to play a crucial role in the signal transduction of cellular stress and in the regulation of cell proliferation and differentiation [33]. HO-1 is induced by numerous stimuli that are also known to enhance the activity of MAPKs. Therefore, it is not surprising that activation of MAPKs is involved in signaling pathways that induce *HO-1* gene expression. It has been shown that c-jun N-terminal kinase mediates the induction of *HO-1* gene expression by the glutathione depletor phorone in rat liver [34]. Moreover, it has recently been demonstrated that MAPKs extracellular signal-regulated kinases 1 and 2 as well as p38 are involved in the sodium arsenite-dependent induction of *HO-1* gene transcription in hepatoma cells [35].

Protein Kinase C

PKC represents a family of related serine–threonine kinases that play an important role in cellular responses mediated by diacylglycerol and phorbol esters such as TPA. The tumor promoter TPA induces *HO-1* gene expression in various cell culture systems [36–38]. TPA-dependent *HO-1* gene activation is attenuated by specific inhibitors of PKC, but not by free radical scavengers in fibroblasts, indicating that HO-1 induction by TPA is not mediated by the generation of ROS [37]. Furthermore, in this study the authors show that HO-1 is induced by heme and heavy metals independent of PKC activation [37]. It has also been shown that TPA-dependent HO-1 induction in fibroblasts and macrophages is mediated by distinct *cis*-acting REs of the human and mouse *HO-1* genes, suggesting that at least two distinct cell-specific signaling pathways are involved in this regulatory mechanism [36, 38]. Terry *et al.* have recently reported that activation of PKC is involved in the induction of *HO-1* gene expression by tumor necrosis factor-α and interleukin-1β in human endothelial cells [39].

cAMP Dependent Protein Kinase A (Protein Kinase A)

Intracellular levels of the second messenger cAMP are elevated by a large number of hormones and extracellular stimuli, resulting in the activation of PKA. Bakken and colleagues have demonstrated that treatment of rats with glucagon and Bt_2cAMP induces hepatic HO enzyme activity *in vivo* [40]. Others have previously shown that the same compounds inhibit basal and cobalt chloride-induced HO-1 enzyme activity in cultured chicken embryo hepatocytes [41]. In contrast to these latter findings, Bt_2cAMP has recently been shown to induce *HO-1* gene expression in primary cultures of rat hepatocytes [42], as well as in cultured vascular smooth muscle cells, via activation of the PKA signaling pathway [43]. The PKA-dependent induction of HO-1 appears to be cell type-specific and is mediated via a cAMP response element/ AP-1 site of the *HO-1* gene.*

cGMP-Dependent Protein Kinase G (Protein Kinase G)

An increase in intracellular NO either by NO-releasing agents or via the induction of iNOS by lipopolysaccharide or cytokines up-regulates *HO-1* gene expression in a number of cell lines [44–49]. cGMP is known to be the second messenger of at least the short-term actions of NO, which activates the soluble guanylate cyclase. However, there are contradictory observations on the regulatory role of cGMP for *HO-1* gene expression. While several authors have reported that cGMP has no effect on *HO-1* gene expression in different cell cultures [44, 46, 47], we and others have shown that HO-1 is induced by cGMP in primary rat hepatocyte cultures and in bovine pulmonary artery endothelial cells [49, 50]. Moreover, it has been demonstrated that NO-dependent induction of the *HO-1* gene is mediated via activation of soluble guanylate cyclase [49]. Similar to PKA-dependent induction of HO-1, cGMP-dependent induction of this gene appears to be cell type-specific for hepatocytes and endothelial cells.

Protein Phosphatases

The balance between specific PKs and PPs is critical for the control of cellular homeostasis, and the important role of PPs in the regulation of gene expression has been demonstrated in recent studies [51]. We have shown that okadaic acid, which is a specific inhibitor of the serine–threonine PPs 1 and 2A, induces *HO-1* gene expression and that this induction is mediated via a cAMP response element/ AP-1 site that is also responsible for cAMP- and cGMP dependent HO-1 induction [52]. Specific phosphatases also appear to be involved in the transcriptional regulation of *HO-1* gene expression by Δ^{12}-prostaglandin J_2 [53, 54]. These findings suggest that a complex interplay of upstream activators and the inhibition by specific phosphatases may significantly affect the magnitude of *HO-1* gene activation by stress stimuli and that this needs to be addressed in more detail.

Signaling Pathways Suppressing HO-1 *Gene Expression*

In contrast to the host of stimuli that induce *HO-1* gene expression, much less is known about the mechanisms that suppress *HO-1* gene expression. Ishizaka and Griendling have demonstrated that the *HO-1* gene is down-regulated by angiotensin II in vascular smooth muscle cells [55]. This down-regulation is apparently mediated by an increase in intracellular calcium levels [55]. More recently, the group of Shibahara has shown that interferon-γ suppresses *HO-1* gene expression in glioblastoma cells [56]. In addition, it

* Immenschuh S and Kietzmann T, unpublished results.

has been shown in vascular smooth muscle cells and in rat liver tissue macrophages that lipopolysaccharide-dependent induction of HO-1 is inhibited by the cytokines transforming growth factor-β_1 and interleukin-10 [57, 58].

REGULATORY ELEMENTS OF THE *HO-1* GENE PROMOTER

Stimulation of the *HO-1* gene by most stimuli is primarily controlled at the transcriptional level, which is governed by REs localized in the promoter 5′-flanking region of the *HO-1* gene. Thus, identification and characterization of inducer-dependent REs provides important information as to the mechanisms of *HO-1* gene regulation. The sequence and organization of the human, rat, mouse, and chicken *HO-1* genes are known, and the regulation of the promoter 5′-flanking regions has been analyzed by functional studies in transiently and stably transfected cell cultures and by studies on DNA–protein interaction. Findings on REs and the putative TFs that may regulate transcriptional *HO-1* gene expression are summarized in recent reviews [59, 60]. While considerable information is available on inducible REs of the *HO-1* gene, much less is known about the TFs that actually mediate this induction. Due to space limitations, only recent findings in regard to the stress-dependent induction of the *HO-1* gene are highlighted in what follows.

A prominent role for *HO-1* gene regulation by oxidative stress has been ascribed to various stress REs localized at distinct proximal and distal sites of the HO-1 promoter 5′-flanking region [37, 38, 61]. A DNA sequence containing an AP-1 consensus site that was initially shown to mediate stress responsiveness by interaction with AP-1 proteins [38, 61] has recently been shown to be regulated by the TF Nrf2 [62]. Nrf2 belongs to the family of Cap'n'Collar basic leucine zipper proteins and has previously been shown to be essential for gene induction mediated by the antioxidant response element [63]. The activity of Nrf2 is functional in a wide-ranging metabolic response to oxidative stress and constitutes a cellular sensor for oxidative stress by a nuclear shuttling mechanism with the cytosolic regulator protein Keap1 [64]. The important role of Nrf2 in the stress-dependent induction of HO-1 is confirmed by the finding that HO-1 is not inducible in Nrf2 null mice [65]. Other AP-1-binding sites of the *HO-1* gene promoter have been shown to be involved in the transcriptional induction of HO-1 by MAPKs, cyclic nucleotides, and the serine–threonine phosphatase inhibitor okadaic acid [35, 50, 52].

HO-1 GENE EXPRESSION IN EXPERIMENTAL DISEASES AND SPECIFIC *HO-1* ACTIVATION AS A THERAPEUTIC TARGET

HO-1 is induced in a number of experimental injuries and diseases of various organs, including carrageenin-induced pleurisy [66], congestive heart failure [67], kidney reperfusion injury [68], caerulein-induced pancreatitis [69], carbon tetrachloride-induced liver injury [70], corneal inflammation [71], neurodegenerative disease [72], or ischemic stroke [73]. It has been demonstrated that activation of the endogenous *HO-1* gene may be protective against the deleterious effects of stress-mediated injury in various animal models. Nath *et al.* have shown *in vivo* that the induction of HO-1 by an infusion of hemoglobin prior to an experimentally induced rhabdomyolysis provides protection against kidney failure and reduces mortality in rats, whereas specific inhibition of HO enzyme activity by tin-protoporphyrin IX decreases the protection by hemoglobin [74]. Similarly, a carrageenin-induced complement-dependent pleurisy is attenuated by induction of HO-1 enzyme activity, while inhibition of HO enzyme activity potentiates this inflammatory response [66]. Others have recently shown that HO-1 induction attenuates the experimentally induced inflammation of rabbit cornea [71]. These observations in different organs along with the findings that HO-1-deficient mice are more susceptible to the deleterious effects of endotoxin [9] or hypoxia [75] provide strong evidence that the induction of HO-1 has therapeutic implications in diseases associated with oxidative stress, although it has also been shown that *HO-1* gene activation is not protective under certain experimental circumstances [76, 77].

Organ Protection by Targeted Gene Transfer of HO-1

In a recent study, the effect of increased *HO-1* gene expression in neuronal cells of transgenic mice during ischemic stroke was investigated [73]. The authors show that specific overexpression of the *HO-1* gene under the control of a neuron-specific enolase promoter amelioriates the deleterious effects of ischemic injury during the acute phase of ischemia in brain. Furthermore, in conduction of initial experiments showing that hemoglobin-dependent induction of HO-1 protects against lung injury [78], Otterbein *et al.* recently investigated the therapeutic potential of targeted *HO-1* gene transfer. This study not only shows the feasibility of specific gene transfer into rat lung with a recombinant adenovirus vector containing the HO-1 cDNA, but also demonstrates that overexpression of HO-1 in lung leads to marked reduction of inflammatory injury caused by hyperoxia [79]. Amersi *et al.* have shown that targeted *HO-1* gene transfer into liver with an adenovirus vector protects against ischemia/reperfusion injury [80]. A different strategy, in which *HO-1* gene expression is increased via gene transfer of immunomodulatory peptides, has been applied by DeBruyne *et al.* in a heart allograft model. These authors demonstrate that plasmid-mediated gene transfer of immunosuppressive peptides into heart allografts increases the survival rate of transplanted organs coincidental with enhanced HO-1 activity [81]. However, since gene therapy approaches are still hampered by numerous technical problems which must be resolved before they may benefit patients, specific induction of HO-1

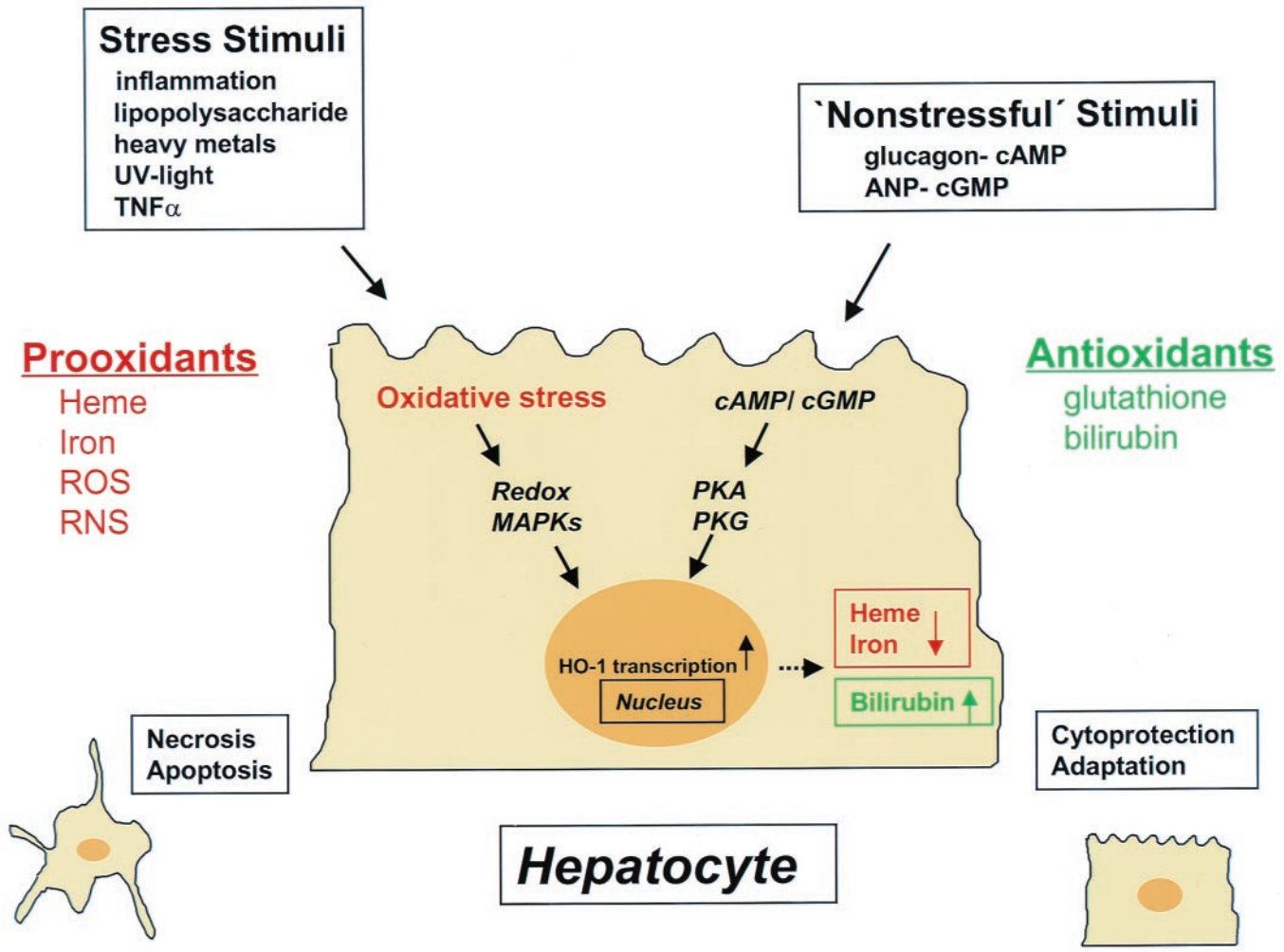

FIG. 2. Signaling pathways of hepatic heme oxygenase-1 gene regulation. *HO-1* gene transcription is induced by various stress stimuli and by 'non-stressful' stimuli, eg. stimuli not associated with oxidant stress. The induction of the *HO-1* gene decreases the cellular content of the pro-oxidants heme and iron and increases the cellular content of the antioxidant bilirubin. PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; ANP, atrial natriuretic peptide; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-α.

expression by pharmacological modulation may be a more promising strategy for the near future.

Pharmacological Modulation of HO-1 Gene Expression as a Therapeutic Target

Significant therapeutic potential of modulation of *HO-1* gene induction has been implicated in various immune functions which are important in transplantation medicine. It has been shown in a mouse-to-rat transplantation model that heart xenograft survival is functionally associated with *HO-1* gene expression [82]. Rapid induction of the *HO-1* gene by cobra venom factor and cyclosporine A in endothelial and smooth muscle cells of cardiac xenografts prior to transplantation significantly prolongs the long-term survival rate of these organs [82]. Up-regulation of *HO-1* gene expression by an anti-CD40L monoclonal antibody also protects mouse allografts against transplant rejection and development of transplant arteriosclerosis [83]. Similar observations have been made in a liver isograft transplantation model in which fatty livers of genetically obese Zucker rats were transplanted into normal Zucker rats. Pretreatment of donor animals with the HO-1 inducer cobalt-protoporphyrin IX prior to organ explantation significantly improved the viability and survival rate of steatotic livers after transplantation [80]. These latter findings may not only constitute a strategy to improve the overall success of liver transplantation, but may also help to develop new treatments to increase the supply of usable donor livers. Finally, the induction of *HO-1* gene expression by cobalt-protoporphyrin IX has been shown to protect against acute graft-versus-host disease in a mouse model [84].

What compounds may be applicable for pharmacological modulation of *HO-1* gene expression? It is conceivable that an ideal inducer of HO-1 activity for therapeutic intervention is a 'non-stressful' compound that activates HO-1 in a specific target organ or tissue (Fig. 2). The cyclic nucleotide cGMP and atrial natriuretic peptide as the receptor agonist

that increases intracellular levels of cGMP may be such compounds, since both have been shown to specifically induce *HO-1* gene expression in hepatocyte [50] and endothelial cell cultures [49]. The cGMP/ atrial natriuretic peptide-dependent induction of *HO-1* gene expression has also been shown to provide cytoprotection to endothelial cells against tumor necrosis factor-α-mediated cell toxicity in a specific manner [49, 85]. Therefore, it is conceivable that cGMP/atrial natriuretic peptide-dependent induction of HO-1 in hepatocytes may also provide liver protection during ischemia–reperfusion injury. This assumption could be in agreement with observations showing that treatment with cGMP/atrial natriuretic peptide provides cytoprotection to hepatocytes against oxidant stress in a model of rat liver ischemia–reperfusion injury [86]. Another candidate that may turn out to be applicable for pharmacological modulation of *HO-1* gene expression is Δ^{12}-prostaglandin J_2 [53].

CONCLUSIONS

The induction of HO-1 by various forms of oxidative stress stimuli has been implicated in a number of injuries and diseases such as ischemia–reperfusion injury or inflammation. Since overexpression of the *HO-1* gene provides cytoprotection against oxidative stress, the specific activation of *HO-1* gene expression by pharmacological modulation may represent a novel target for therapeutic intervention.

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