Review

Heme oxygenase-1 and cardiovascular disease

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Summary. Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-controlling enzyme of heme degradation. HO-1 is up-regulated by a host of oxidative stress stimuli and has potent cytoprotective and anti-inflammatory functions via decreasing tissue levels of the prooxidant heme along with production of bilirubin and the signaling gas carbon monoxide. This review deals with recent findings that highlight the emerging significance of HO-1 in cardiovascular disease. Evidence is presented on how heme and various oxidative stress stimuli may cause endothelial cell dysfunction and how HO-1 may counteract the detrimental effects of oxidative stress in the endothelium. Recent advances in the understanding of the role of endothelial HO-1 for the regulation of the inflammatory response are summarized, including the modulation of leukocyte recruitment and transmigration through the endothelial barrier. Furthermore, experimental evidence from various cell culture and animal models is discussed which suggests an association of HO-1 with the complex sequence of events that cause atherosclerosis. In the second part of the review we present potential strategies that apply HO-1 as a therapeutic target in the treatment of cardiovascular disease. Specific inducers of HO-activity which may ultimately lead to the development of clinically relevant pharmacological applications are introduced.

Key words: Heme oxygenase-1, Bilirubin, Endothelial cells, Atherosclerosis, Inflammation

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Introduction

Heme oxygenase (HO) catalyzes the first and rate-controlling step of heme degradation (Fig. 1) (Maines, 1988). HO-1 is the inducible isozyme which is up-regulated by its substrate heme and a host of oxidative stress stimuli such as hydrogen peroxide, heavy metals, UV-light and endotoxin in different cells and tissues (Choi and Alam, 1996; Immenschuh and Ramadori, 2000). For many years the induction of HO-1 gene expression has been considered to be an adaptive autoprotective response of the cell, although the exact underlying mechanisms have not been understood in detail (Vile and Tyrrell, 1994). More recently, HO-1 has been recognized to have anti-inflammatory effects which have initially been demonstrated in an in vivo model of complement-dependent pleurisy (Willis et al., 1996). An anti-inflammatory role of HO-1 has also been observed in HO-1 knock out mice and a human case of genetic HO-1 deficiency (Poss and Tonegawa, 1997b; Yachie et al., 1999). HO-1 deficient animals exhibit chronic non-specific inflammatory changes and are highly susceptible to organ damage by the prototypical inflammatory mediator endotoxin (Poss and Tonegawa, 1997b). The anti-inflammatory role of HO-1 is currently under intense investigation and various regulatory mechanisms such as modulation of the synthesis of proinflammatory cytokines (Kapturczak et al., 2004) or that of T-cell activation by HO-1 (Otterbein et al., 2003; Brusko et al., 2005) have been discussed.

This review deals with recent findings that illustrate the significance of HO-1 in the pathogenesis and therapy of cardiovascular disease. We discuss the stress-dependent toxicity of heme in the endothelium and the emerging role of endothelial HO-1 to counteract heme-mediated oxidative stress. Mechanisms that are involved in the HO-1-dependent regulation of inflammation in EC, in particular, the potential anti-inflammatory roles of the HO products bilirubin (BR) and carbon monoxide (CO) are presented. Furthermore, we highlight specific aspects of the growing body of evidence that suggests an association of HO-1 with atherosclerosis. Potential

therapeutic strategies for the treatment of cardiovascular disease that apply the targeted regulation of HO-1 are presented.

The HO enzyme reaction

HO catalyzes the enzymatic catabolism of the tetrapyrrole heme (Fig. 1) (Tenhunen et al., 1968). Heme is a double-edged sword which on the one hand is the prosthetic group of essential hemoproteins such as hemoglobin, myoglobin, cytochrome P450s, inducible nitric oxide synthase and soluble guanylate cyclase (Wijayanti et al., 2004). These proteins have important roles for oxygen transport, drug metabolism or signal transduction. On the other hand, excess heme can be severely toxic because it has strong pro-oxidant properties if not contained by covalent or non-covalent binding to heme-binding proteins such as hemopexin (Muller-Eberhard and Fraig, 1993). Thus, the tight control of cellular heme levels is considered to be a major physiological function of the HO enzyme reaction. More recently, the significance of the HO-derived product BR as a potent antioxidant (Stocker et al., 1987) and that of CO as a signaling gas has been appreciated (Maines, 1997; Otterbein et al., 2003). Moreover, HO-1 appears to be involved in maintaining the cellular homeostasis of iron which is illustrated by findings in HO-1 knock out mice and a case of human genetic HO-1 deficiency. In either case an anemia with abnormally low iron levels and an iron-overload of kidney and liver has been reported (Poss and Tonegawa, 1997a; Yachie et al., 1999). Little, however, is known on the specific mechanisms how HO-1 may regulate cellular iron levels.

Vascular endothelium, heme and HO-1

In pathologies such as cerebral hemorrhage, sickle cell disease and malaria an excess of 'free' heme and hemoproteins causes tissue damage (Wagner et al., 2003; Wijayanti et al., 2004). During these pathological conditions the vascular endothelium is exposed to high concentrations of circulating heme and, therefore, is the first line of defense against heme-dependent oxidative damage. Balla and colleagues demonstrated in a cell culture model of EC that loading of these cells with 'free' heme causes a marked amplification of EC injury mediated by the exposure to granulocytes (Balla et al., 1991). The findings of this study were extended in a follow-up report in which heme arginate exhibited lower EC toxicity as compared to heme because the latter compound was a more potent free radical catalyst (Balla et al., 2000). A potential mechanism on how circulating heme or other hemoproteins may indirectly damage the endothelium in vivo was proposed by recent findings showing that heme-generated oxidized low-density lipoproteins (LDL) caused EC damage (Jeney et al., 2002).

Two major mechanisms that provide protection of EC against heme-dependent cellular damage have been established. First, extracellular heme-binding proteins such as the serum protein hemopexin can prevent damage to the endothelium by neutralizing the prooxidant effects of heme via non-covalent binding (Muller-Eberhard and Fraig, 1993; Wijayanti et al., 2004). Second, EC can be protected against heme-mediated damage via the up-regulation of endothelial HO activity. Such an autoprotective role of HO-1 gene induction was demonstrated in cell cultures of EC (Balla et al., 1993; Jeney et al., 2002) and was confirmed by others in human microvessel EC (Abraham et al., 1995). In the latter report overexpression of HO-1 was shown to provide specific protection against heme- and hemoglobin-mediated cell toxicity. Remarkably, enhanced HO-1 activity not only enzymatically degrades cellular levels of heme, but is also coupled to the up-regulation of ferritin to prevent iron-dependent toxicity (Nath et al., 1992).

Endothelial HO-1 and inflammation

Inflammation is a complex reaction of the innate immune system in vascularized tissues at sites of an infection, toxin exposure or cell injury. The endothelium plays an important role for the regulation of the inflammatory response, because it regulates leukocyte recruitment, in particular that of polymorphonuclear neutrophils (PMNs) (Muller, 2003; Cook-Mills and Deem, 2005). Extravasation of leukocytes through microvessel EC layers is mediated via a cytokine-mediated increase of selectin and adhesion molecule expression which, in turn, enhances the adhesion and transmigration of leukocytes to the site of an injury (Muller, 2003).

In 1999 Hayashi and colleagues hypothesized that HO-1 may be involved in the regulation of the inflammatory response in the endothelium (Hayashi et al., 1999). To prove this hypothesis the authors examined the specific effects of HO-1 on EC-leukocyte interactions by intravital microscopy in an in vivo rat model. In this model augmentation of HO activity was
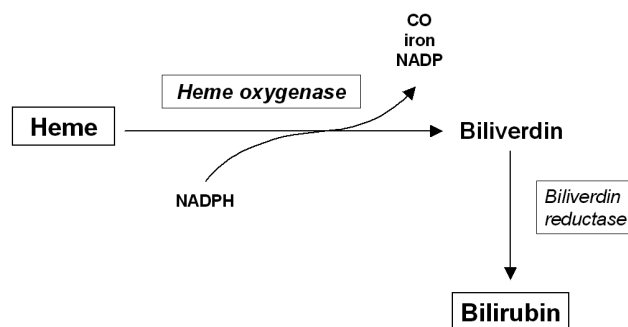

Fig. 1. The heme oxygenase enzyme reaction. Heme is enzymatically degraded to yield carbon monoxide (CO), iron and biliverdin which is converted into bilirubin in a coupled reaction.

demonstrated to down-regulate the adhesion of PMNs to microvessel EC during oxidative stress. Oxidative stress was elicited either by ischemia-reperfusion or by superfusion with hydrogen peroxide (Hayashi et al., 1999). In an independent report, it was demonstrated by immunohistochemical studies that heme lead to a marked inflammatory response in different organs of mice (Wagener et al., 2001). In this study intravenous administration of heme was shown to cause an increased influx of radioactively-labeled liposomes into pancreas, liver and spleen which occurs in parallel to an enhanced mobilization of leukocytes. The extent of heme-mediated leukocyte infiltration was potentiated by inhibition of HO enzyme activity and it was concluded that heme is a potent proinflammatory molecule regulating the permeability of the endothelial layer for PMNs and that HO activity counteracts the proinflammatory effects of heme (Wagener et al., 2001). A comprehensive review on this concept has recently been given by Wagener and associates (Wagener et al., 2003).

Similar proinflammatory effects were not only reported for heme, but also for other hemoproteins. Methemoglobin was shown to activate EC via the up-regulation of E-selectin expression in human umbilical vein EC. Here, it was shown that the effect of heme on EC is similar to that of the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) (Liu and Spolarics, 2003). A protective role of endothelial HO-1 was also demonstrated *in vivo* during the inflammatory response in a rat model. Endotoxin-dependent induction of endothelial P- and E-selectin gene expression in vasculature beds of various organs was differentially affected by HO enzyme activity. Thus, endothelial HO enzyme activity may modulate the inflammatory response via the expression of adhesion molecules (Vachharajani et al., 2000). More recently, TNFα-mediated up-regulation of E-selectin and VCAM-1 expression was shown to be inhibited by increased HO-1 activity in primary bovine aortic EC. The inhibition of adhesion molecule expression was increased either by virus-mediated HO-1 gene transfer or by inducer-dependent up-regulation of HO activity (Soares et al., 2004).

Taken together, a growing body of evidence suggests that HO-1 is involved in the modulation of the inflammatory response in the endothelium.

Endothelial HO-1 and atherosclerosis

It is generally accepted that inflammation plays a crucial role in the pathogenesis of atherosclerosis (Ross, 1999). This concept has been established by studies in which oxidized LDL were shown to up-regulate the synthesis of monocyte chemotactic protein-1 on EC which, in turn, leads to a recruitment of inflammatory cells during the early phase of atherosclerosis (Cushing et al., 1990; Rajavashisth et al., 1990). In the following, it was demonstrated that mice with genetic deficiency of monocyte chemotactic protein-1 do not develop atherosclerosis (Boring et al., 1998). Thus, HO-1 gene induction appears to be of particular importance in the early stages of atherosclerosis. Likewise, this hypothesis is supported by the fact that oxidative stress of the arterial wall due to the common risk factors diabetes, hypertension or hyperlipidemia causes EC dysfunction (Bonetti et al., 2003) also induce HO-1 gene expression (Abraham and Kappas, 2005).

Genetic evidence for a common pathway that may mediate oxidative stress, induction of HO-1 gene expression and fatty streak formation was presented by Liao et al. (1994) (Table 1). In 1997 Ishikawa and colleagues suggested an association of HO-1 with atherosclerosis by a study on co-cultures of human EC and smooth muscle cells in which it was demonstrated that HO activity can modulate chemotaxis of mononuclear cells (Ishikawa et al., 1997). Here, increased HO activity was shown to markedly reduce the chemotaxis of monocytes after exposure to oxidized LDL. Thus, the induction of HO-1 may protect against atherosclerosis via an inhibition of the inflammatory response in arterial walls. These findings were strengthened by in vivo studies in LDL-receptor knock out mice (Ishikawa et al., 2001b) and Watanabe heritable hyperlipidemic rabbits (Ishikawa et al., 2001a). In both animal models HO-1 gene expression in atherosclerotic lesions co-localized with oxidized phospholipids as determined by immunohistochemistry. The significance

Table 1. Animal and cell culture models that suggest an association of HO-1 with atherosclerosis.

ANIMAL/ CELL CULTURE MODELS	FINDINGS	REFERENCES
Inbred mice strains on atherogenic diet	HO-1 gene expression in parallel to inflammation and atherosclerotic fat streak formation	Liao et al., 1994
Cell culture of human aortic EC	Inhibition of monocyte transmigration by induction of HO-1	Ishikawa et al., 1997
Apo-E (-/-) mice	Up-regulation of HO-1 gene expression in early atherosclerotic lesions	Wang et al., 1998
Watanabe heritable hyperlipidemic rabbits	Co-localization of HO-1 with oxidized phospholipids in atherosclerotic lesions. Up-regulation of HO activity prevents lipid peroxidation	Ishikawa et al., 2001a
LDL receptor (-/-) mice	Protection against atherosclerotic lesion formation by increased HO activity. Antiatherogenic properties of HO-1 up-regulation by bilirubin	Ishikawa et al., 2001b. Kawamura et al., 2005

of HO-1 in atherosclerosis was also demonstrated by histochemical studies in atherosclerotic lesions of humans and apolipoprotein E-deficient mice (Wang et al., 1998). In this study it was shown in both human and murine samples that HO-1 was prominently expressed in EC and foam cells during the early stages of atherosclerosis (Table 1).

Despite accumulating evidence that points to a significant role of HO-1 in the complex sequence of events that cause atherosclerosis, the detailed function of this enzyme and its products is far from being fully understood. It is entirely possible that the up-regulation of HO-1 gene expression is an epiphenomenon similar to the induction of other stress-responsive genes in arterial vessel walls. Therefore, it is important to gain a better understanding on the exact role of oxidative stress in the pathogenesis of atherosclerosis (Stocker and Keaney, 2005). Comprehensive overviews on HO-1 and atherosclerosis have recently been published by others (Ishikawa, 2003; Morita, 2005).

Protective function of HO-1 and its products in cardiovascular disease

Evidence for a protective role of HO-1 in cardiovascular disease is not only provided by experimental findings in cell culture and animal models, but also by epidemiological studies in humans. Here, it was demonstrated that the long allele of the (GT)n repeat HO-1 gene promoter polymorphism which causes a lower responsiveness of HO-1 gene expression to stress stimuli is associated with diminished vascular protection from atherogenic insults (Alam et al., 2004; Exner et al., 2004). Moreover, increased levels of the HO products BR and CO have been causally linked to a higher resistance against cardiovascular disease (Ishikawa, 2003).

Biliverdin and BR

Production of the bile pigments biliverdin and BR is not only a refined defense strategy of the cell in response to oxidative stress (Foresti et al., 2004), but high-normal serum levels of BR have been reported to be inversely related to the atherogenic risk (Hopkins et al., 1996; Mayer, 2000). Since inflammation is important for the pathogenesis of atherosclerosis, the anti-atherogenic effect of BR may be due to its anti-inflammatory functions. An anti-inflammatory effect of BR in the endothelium was first demonstrated in a microvessel model in which the extent of leukocyte transmigration correlated with the concentration of HO-1-derived bilirubin (Hayashi et al., 1999). More recently, unconjugated bilirubin was also shown to block VCAM-1-dependent lymphocyte migration and to ameliorate the extent of the inflammatory response that is mediated by this adhesion molecule (Keshavan et al., 2005). Independently, others demonstrated that HO-1-derived BR directly down-regulated the proinflammatory

activation of EC and proposed that the BR-dependent inhibition of EC dysfunction may be involved in the anti-atherogenic protective mechanisms in EC (Kawamura et al., 2005).

Carbon monoxide (CO)

Besides biliverdin and BR, the HO-derived signaling gas CO was shown to modulate the inflammatory response via its interaction with EC. Morita and colleagues demonstrated that HO-1-derived CO from vascular smooth muscle cells has paracrine effects on EC under hypoxic conditions. The regulatory effects of CO were shown to be mediated via an increase of cellular cGMP levels and of endothelin-1 gene expression (Morita et al., 1995). More recently, CO was shown to suppress apoptosis in EC by activation of the mitogen-activated protein kinase p38 pathway (Brouard et al., 2000). In addition, CO appears to be a smooth muscle relaxing mediator via modulating the soluble guanylyl cyclase/cGMP signaling pathway in EC (Ryter et al., 2002).

Endothelial HO-1 as a target for cardiovascular drugs

The unique combination of tissue protective and smooth muscle relaxing properties makes HO-1 an interesting target for treatment of cardiovascular disease (Immenschuh and Ramadori, 2000; Ryter et al., 2002; Ishikawa, 2003). In addition, HO-1 seems crucial for keeping the human uterus in a relaxed state during pregnancy, and a reduced level of placental HO-1 has been connected with a higher risk for pre-eclampsia (Bainbridge and Smith, 2005). Thus, therapeutic strategies aimed at moderately increasing tissue expression of HO-1 might be beneficial in a number of disease states that are associated with, or the result of, vascular disorders, and, specifically, endothelial cell dysfunction. Until recently, however, known inducers of HO-1 such as cadmium chloride and other heavy metals, did hardly bear great promise for eventual therapeutic use in humans. More recently, a number of established as well as experimental drugs have been shown to increase endothelial HO-1 expression and may thus qualify as therapeutically useful HO-1 inducers. NO is an endogenous activator of HO-1 expression (Motterlini et al., 1996; Yee et al., 1996) and increased expression of HO-1 mediates endothelial protection by NO releasing drugs (NO donors) (Polte et al., 2000). Of all the NO donors that are currently applied in humans, the nitric acid ester pentaerythritol tetranitrate seems to be unique in its HO-1 inducing abilities (Oberle et al., 2003). Other organic nitrates such as isosorbide dinitrate failed to increase endothelial HO-1 expression under therapeutically relevant conditions, possibly due to varying kinetics and rates of NO release (Oberle et al., 2003; Daiber et al., 2004).

Cyclic GMP seems to play a major role as a

downstream signaling molecule in the events leading to HO-1 induction by NO and ensuing cytoprotection (Polte et al., 2000, 2002). This is in agreement with publications demonstrating that NO-independent activators of cGMP formation are likewise acting as HO-1 inducing agents. Atrial natriuretic peptide stimulates cyclic GMP formation by activating the particulate isozyme of guanylate cyclase and has been reported to induce HO-1 and antioxidant protection in vascular and non-vascular tissue through this mechanism (Immenschuh et al., 1998; Polte et al., 2000, 2002; Kiemer et al., 2003). It can be assumed that B-type natriuretic peptide (nesiritide), which is clinically used to treat acute heart failure, possesses HO-1-inducing properties since it activates vascular cGMP formation via pathways identical to those of atrial natriuretic peptide (Gardner, 2003; Woods, 2004).

Aspirin, known as an anti-inflammatory drug for more than 100 years and used in prevention of thrombotic events since the late 1980s, has recently turned out to be one of the long-sought 'benign' inducers of HO-1 (Grosser et al., 2003). HO-1 induction was characterized as a novel, prostaglandin-independent vasculoprotective action of aspirin, not shared by other non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (Oberle et al., 1998; Grosser et al., 2003). It is noteworthy that endothelial NO synthase and the NO/cGMP signaling system were again identified as mediators of HO-1 induction and intermediate targets of aspirin in this novel antioxidant pathway (Grosser and Schroder, 2003; Taubert et al., 2004).

Although, due to mixed study results, aspirin is currently not considered of therapeutic value in pre-eclampsia or gestational hypertension, a number of studies consistently show a protective effect of low-dose aspirin for women with risk factors for pre-eclampsia without an increase in bleeding complications, including placental abruption (Duley et al., 2001). Whether HO-1 induction might explain some of aspirin's beneficial effects under these conditions is unclear, but the functional profile of HO-1 characterized by anti-inflammatory as well as vasodilatory properties would agree with such a hypothesis (Bainbridge and Smith, 2005).

Interestingly, statins have recently emerged as inducers of HO-1 via as yet unidentified signaling routes (Grosser et al., 2004; Lee et al., 2004) confirming once more that there is some truth to the description of aspirin as being 'the poor man's statin' (Verheugt, 1998). HO-1 induction could contribute to some of the beneficial (pleiotropic) actions of statins, e.g. antioxidant activities, which cannot solely be explained by their lipid lowering properties. Although HO-1 induction by statins was reported to be a class effect, it seems to occur independently of HMG-CoA reductase blockade. In addition, activation of NO synthases, which was shown to contribute to other pleiotropic statin effects, does not seem to be involved in upregulation of HO-1 by statins (Grosser et al., 2004). Therefore, information about the

relevant HO-1 promoter sites would be important to elucidate this novel and as yet unexplored pathway and may well open up a new chapter in HO-1 research.

In summary, by use of inhibitors, antisense or knock-out models HO-1 induction was identified as a regulatory step that is of major importance for the vasculoprotective and antioxidant profile of established cardiovascular drugs as well as experimental compounds. It appears likely that the up-regulation of HO-1 in EC protects against inflammation via enzymatic degradation of the prooxidant and proinflammatory molecule heme and via the generation of its anti-inflammatory products bilirubin and CO. Therefore, a more systematic search for agents targeting HO-1 in different vascular as well as non-vascular tissues might lead to the discovery of new therapeutic avenues to treat cardiovascular disease, atherosclerosis and other inflammatory disorders.

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