PECAM-1-Dependent Regulation and Protective Effects of Heme Oxygenase-1 in the Endothelium

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and Protective Effects of Heme Oxygenase-1
in the Endothelium

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Parts of this thesis have been submitted:


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Hendry Saragih performed experimental work, participated in research design, data analysis and writing of the paper.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AECA</td>
<td>Anti-endothelial cell antibodies</td>
</tr>
<tr>
<td>AMR</td>
<td>Antibody-mediated rejection</td>
</tr>
<tr>
<td>Anti-NS1</td>
<td>Anti against non-structural protein-1</td>
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<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>Bach1</td>
<td>BTB and CNC homolog 1</td>
</tr>
<tr>
<td>BR</td>
<td>Bilirubin</td>
</tr>
<tr>
<td>BV</td>
<td>Biliverdin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cellular adhesion molecule</td>
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<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>DHF</td>
<td>Dengue haemorrhagic fever</td>
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<tr>
<td>DSS</td>
<td>Dengue shock syndrome</td>
</tr>
<tr>
<td>DV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>GSTs</td>
<td>Glutathione-S-transferases</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HO</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular cell adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor-KB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H: quinone oxidoreductase-1</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein-1</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PPIX</td>
<td>Protoporphyrin-IX</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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1. INTRODUCTION

1.1 Inflammation

Inflammation is a complex reaction of the innate immune system in vascularized tissues, which involves leukocyte accumulation at the site of an infection, toxin exposure or cell injury (Medzhitov 2008). Inflammation leads to changes in blood vessel permeability, which promotes the coordinated delivery of blood components (plasma and leukocytes) into tissues (Majno and Joris 2004; Medzhitov 2008). Although inflammation has protective functions against infections and promotes tissue repair, it can also cause tissue damage and disease (Abbas and Lichtman 2011).

In general, acute inflammation is resolved after elimination of the triggering stimulus. However, inflammation may also persist and become chronic, if the initiating stimulus is not eliminated. In this case, the composition of infiltrating leukocytes changes from neutrophils to a mixture of mononuclear phagocytes and T cells (Medzhitov 2008). Moreover, the inflammatory stimulus changes from one sensed by pattern-recognition receptors of innate immune cells to one recognized as an antigen by activating receptors on T and B cells of the adaptive immune system. Prolonged antigenic stimulation by resistant microbes or tissue-derived autoantigens is considered to be a major cause of chronic inflammation (Pober and Sessa 2007; Nathan and Ding 2010).

1.2 Heme oxygenase (HO)

HO, which has initially been described in 1968, catalyzes the first and rate-limiting step of heme (Fe-protoporphyrin-IX (PPIX)) degradation. The HO product biliverdin (BV) (Tenhunen et al. 1968; Ryter et al. 2006;) is subsequently converted into bilirubin (BR) via NAD(P)H-dependent biliverdin reductase (Maines 1997; Ryter et al. 2006). Moreover, HO-
catalyzed heme cleavage releases iron in its ferrous form Fe(II) and gaseous carbon monoxide (CO) (Ryter et al. 2006).

**Figure 1:** The HO enzyme reaction: heme is degraded into CO, iron and BV, which is subsequently converted into BR by BV reductase.

### 1.2.1 HO isoforms

Two genetically distinct isozymes of HO are known: the inducible isoform HO-1 and the constitutive isoform HO-2 (Maines et al. 1986; Trakshel et al. 1986). HO-1, which has a molecular weight of ~32 kDa, was initially purified from rat liver and porcine spleen (Maines et al. 1977; Yoshida and Kikuchi 1978). Moreover, HO-1 has been isolated from various other species (Yoshinaga et al., 1982; Bonkovsky et al. 1990). The constitutive HO isoform, HO-2, initially identified in rat liver, spleen, brain and testes (Trakshel et al. 1989; Maines 2000), has a molecular weight of 36 kDa (Trakshel et al. 1986). Shibahara and colleagues have cloned and sequenced the cDNAs of rat and human HO-1 (Shibahara et al. 1985; Shibahara et al. 1993). Independently, Maines and colleagues have isolated cDNAs for rat and human HO-2 (Cruse and Maines 1988; Rotenberg and Maines 1990).
1.2.2 HO-1

HO-1, the inducible isoform of HO, is expressed in most cells tissues and is highly up-regulated in response to its substrate heme and multiple oxidative stress stimuli in different cells and tissues (Choi and Alam 1996). Due to its gene regulation pattern HO-1 induction has generally been considered to be an adaptive cellular response against the toxicity of prooxidant stimuli (Keyse et al. 1990; Vile et al. 1994). Moreover, HO-1 has been shown to have anti-apoptotic effects in a variety of experimental models (Brouard et al. 2000; Silva et al. 2006).

1.3 Genetic HO-1 deficiency causes proinflammatory phenotypical alterations

Studies in HO-1 knockout mice have revealed that these animals are highly sensitive to oxidative stress and exhibit signs of chronic inflammation (Poss and Tonegawa 1997a,b). Moreover, it has been demonstrated that proinflammatory cytokines are highly induced by lipopolysaccharide (LPS) (Kaputczak et al. 2004), and that adaptive immune responses are impaired in HO-1 knockout mice, respectively (George et al. 2008). More recently, it has also been demonstrated in a conditional knockout mouse model with genetic myeloid cell-specific HO-1 deficiency that these animals exhibit a defective interferon-β pathway, which causes pathological immune responses (Tzima et al. 2009). Remarkably, Yachie et al. (1999) have demonstrated in a Japanese boy with genetic HO-1 deficiency similar phenotypical alterations to those observed in HO-1 knockout mice (Fig. 2 and 3; Table 1).
**Figure 2:** Histopathology of renal glomerular in genetic HO-1 deficiency: Hematoxylin and eosin staining of renal glomeruli in a human HO-1 deficient patient showing heavy infiltration of leukocytes and glomerular capillary (black arrow) in genetic HO-1 deficiency (modified from Yachie et al. (1999) J.Clin.Invest.103, 129-135).

**Figure 3:** Histopathology of glomerular capillary in genetic HO-1 deficiency: In comparison to the normal endothelium (black arrow) in glomerular capillary (bottom panel), the endothelium of the HO-1 deficient patient (upper panel) was highly detached (arrow) and (asterisk) exhibited an unidentifiable precipitate between the detached endothelium and glomerular basement membrane (modified from Yachie et al. (1999) J.Clin.Invest.103, 129-135).

**Table 1:** Comparison of findings between human HO-1 deficiency and HO-1 targeted mouse

<table>
<thead>
<tr>
<th>Findings</th>
<th><strong>Human</strong></th>
<th><strong>Mouse</strong></th>
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</thead>
<tbody>
<tr>
<td>Intrauterine death</td>
<td>Stillbirth, abortion</td>
<td>20% birth rate</td>
</tr>
<tr>
<td>Growth failure</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anemia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Iron binding capacity</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Elevated</td>
<td>Elevated</td>
</tr>
<tr>
<td>Iron deposition</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leukocytosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Coagulation abnormality</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Endothelial injury</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

1.4 The enzymatic HO-1 reaction and inflammation

The mechanisms that mediate anti-inflammatory effects of HO-1 are incompletely understood. A growing body of experimental evidence, however, suggests that enzymatic degradation of proinflammatory free heme and production of the anti-inflammatory compounds BV/BR and CO via HO-1 appear to play major roles for these immunomodulatory functions (Soares et al. 2009).

1.4.1 Heme

Heme consists of a tetrapyrrole ring with a central iron ion. If bound to proteins, heme is the prosthetic group of various intra- and extracellular hemoproteins such as hemoglobin, myoglobin and cytochrome P450. These proteins serve essential functions in biological processes including oxygen transport and storage, mitochondrial electron transport, drug metabolism, signal transduction and regulation of gene expression (Wijayanti et al. 2004; Mense and Zhang 2006; Poulos 2014). On the other hand, if not bound to proteins, free heme is highly toxic and can cause oxidative stress. This has been shown in various cell culture and animal models (Balla et al. 1993; Belcher et al. 2010). Due to these contradictory properties of heme, enzymatic degradation of this molecule is tightly controlled (Ryter and Tyrrell 2000; Wijayanti et al. 2004). Moreover, free heme has been shown to up-regulate inducible proinflammatory adhesion molecules in cell cultures of human endothelial cells (ECs) in vitro (Wagener et al. 1997), and in mice in vivo (Wagener et al. 2001). Accordingly, a number of immune-mediated inflammatory diseases have been associated with the presence of free heme in the circulation (Andrade et al. 2010a,b; Larsen et al. 2010).
1.4.2 CO

CO is generally considered to be a toxic gas, mainly because it blocks oxygen binding to hemoglobin (Haldane 1927). However, more recently CO has been recognized to have important physiological functions, which may even have therapeutic implications (Motterlini and Otterbein 2010). For example, CO has been shown to have major signaling functions in a variety of physiological and pathophysiological situations such as neurotransmission, vasodilation, protection against apoptosis and inflammation (Kim et al. 2006). In particular, CO has been shown to induce the production of cyclic guanosine monophosphate via activating the soluble guanylyl cyclase (Morita et al. 1995) and to protect against the toxicity of LPS in vitro and in vivo through modulation of inflammatory cytokine production (Otterbein et al. 2000). Moreover, CO blocks the inducible nitric oxide synthase (iNOS) activity or production of nitric oxide (NO), but does not regulate the protein expression of iNOS (Sawle et al. 2005). Abrogation of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β production by CO is mediated by modulating mitogen-activated protein kinase (MAPKs), of which p38 is required for most of its functions. More recently, it was shown in the RAW264.7 murine macrophage cell line in vitro and also in an in vivo a mouse model for acute lung injury that CO induces the expression of peroxisome proliferator-activated receptors-gamma, which is responsible for the downstream blockage of proinflammatory genes such as early growth response protein-1 (Bilban et al. 2006). A comprehensive overview on the potential therapeutic functions of CO has been given by Motterlini and Otterbein (2010).

1.4.3 BV and BR

Similar to CO, the physiological and potentially therapeutic effects of the HO products BV and BR have only been appreciated in recent years. Initially, BR was considered a toxic waste product of the HO reaction, which is excreted in the bile. However, BR has major antioxidant
effects, comparable to that of α-tocopherol, which is an efficient antioxidant and protects
against lipid peroxidation (Stocker 2004). Hayashi et al. (1999) have shown in a microvessel
model that leukocyte transmigration was down-regulated by HO-derived BR. Moreover, it has
been demonstrated that administration of BR significantly reduced the vascular cell adhesion
molecule (VCAM)-1-dependent transmigration of leukocytes in murine EC lines (Keshavan
et al. 2005) and that BR treatment significantly blocked the hepatotoxicity after endotoxin
exposure (Wang et al. 2004). In addition, similar observations have been made in a mouse
model of endotoxemia, in which a single bolus of BR rescued the mice from endotoxemia
(Kadl et al. 2007). A review on the various effects of BR in health and disease has recently
been given by Kapitulnik and Maines (2012).

1.4.4 Iron

Iron, the third product of the HO reaction, is an important compound, which is critically
involved in various redox-dependent enzyme reactions. Similar to heme, non-protein bound
iron can cause oxidative stress in cells, and requires specific intracellular protective
mechanisms against its toxicity. Thus, HO-1 derived iron is bound by the intracellular iron
storage protein ferritin, which can then be exported from the cell (Ponka and Richardson
1997). Accordingly, it has been shown that HO-1 induction is accompanied by a parallel up-
regulation of ferritin (Balla et al. 1992; Ryter and Tyrrell 2000). Moreover, genetic HO-1
deficiency in mice (Poss and Tonegawa 1997) and humans (Yachie et al. 1999; Kawashima et
al. 2002) is associated with increased iron deposition in the liver and kidney.
1.5 Endothelial HO-1 and inflammation

Although HO-1 is expressed in all cell types, its anti-inflammatory effects appear to be critically dependent on its cell type-specific functions in ECs.

1.5.1 Endothelium and inflammation

The endothelium is made up by ECs, which are flattened cells lining the internal spaces of blood and lymphatic vessels. ECs have major physiological functions such as regulating the flow of nutrient substances and biologically active molecules. Furthermore, ECs are critically involved in the regulation of blood pressure, blood coagulation and transmigration of blood cells during the inflammatory response (Vita 2011).

The latter function is of particular importance, because the endothelium is the major barrier between the peripheral blood stream and inflamed tissues. The endothelium is equipped to respond quickly to local changes in biological needs caused by trauma or bacterial infections (Pober and Sessa 2007). During inflammation, leukocytes tether to and roll on the EC surface, then arrest and finally emigrate between ECs to the underlying tissues (Muller 2011; Noursharg and Alon 2014). In most conditions, interactions with selectins initiate and mediate tethering and rolling of leukocytes on the EC surface (Pober and Sessa 2007). There are two main components of inflammatory responses: (1) increased vascular permeability and (2) emigration, accumulation, and activation of leukocytes (Lawrence et al. 2002). The modulation of vascular permeability and the recruitment of leukocytes primarily rely on cellular adhesion molecule (CAM)-mediated intercellular communication among adjacent ECs and between ECs and leukocytes. CAM-mediated interactions allow leukocytes to migrate to the site of an inflammation, where they affect the release of inflammatory mediators (Muller 2011; Noursharg and Alon 2014). Various EC junctional molecules, such
as platelet EC adhesion molecule (PECAM)-1, junction adhesion molecules and CD99 (Dejana et al. 2008; Garrido-Urbani 2014) have been shown to be involved in these processes.

Regulation of leukocyte transmigration through the endothelium has been associated with the integration of various mechanisms including signals from clustering of apically expressed CAMs including intercellular adhesion molecule (ICAM-1) and VCAM-1, loosening of adherent junctions, recycling of PECAM-1 from the lateral border recycling compartment and regulation of paracellular migration from such molecules (Muller 2011; Muller 2013; Xiao et al. 2014).

![Figure 4: Mechanisms of leukocyte transendothelial migration to sites of infection: The multistep process of leukocyte migration through the endothelial monolayer to the site of inflammation (modified from Abbas and Lichtman (2011) Basic Immunology 3rd Ed. pp.1-30).](image)

1.5.2 Cell type-specific functions of HO-1 in ECs

Anti-inflammatory functions of HO-1 that are specifically mediated via its functions in ECs have been demonstrated in mouse models with genetic HO-1 deficiency. Major pathological
alterations of the endothelium have been observed in HO-1 knockout mice, in which ECs were more susceptible to apoptotic cell death and denudation from the extracellular matrix (True et al. 2007). Similarly, pathological alterations of the endothelium have been demonstrated in a human case of HO-1 deficiency (Yachie et al. 1999). Moreover, specific up-regulation of HO-1 in the endothelium in various in vivo models has previously been shown to protect against inflammation (Hayashi et al. 1999; Wagener et al. 2001). The mechanisms that mediate protection of the endothelium via HO-1 include inhibition of apoptosis (Brouard et al. 2000) and that of the up-regulation of inducible proinflammatory adhesion molecules (Soares et al. 1998; Soares et al. 2004). Remarkably, HO-1 has also been reported to be involved in vascular repair mechanisms by increasing the number of circulating endothelial progenitor cells derived from the bone marrow (Wu et al. 2009).

### 1.6 Regulation of HO-1 gene expression

HO-1 is induced by a large variety of stimuli including oxidative stress, cytokines, bacterial compounds and growth factors (Ryter et al. 2006). HO-1 expression is primarily regulated on the transcriptional level via the interaction of multiple regulatory elements in the HO-1 gene promoter and a complex system of transcription factors (TFs) (Alam and Cook 2007; Paine et al. 2010). Two upstream enhancer regions, E1 and E2, are of major importance for HO-1 regulation (Alam et al. 1995). Both E1 and E2 contain several copies of antioxidant response elements (AREs), which mediate the specific up-regulation of HO-1 (Nguyen et al. 2003). Interestingly, the human HO-1 gene contains a GT-microsatellite polymorphism in the proximal promoter region, which is of major biological significance for transcriptional regulation of human HO-1 and is not found in the mouse gene promoter (Yamada et al. 2000; Exner et al. 2004). Individuals carrying the HO-1 gene allele with lower number of GT repeats have been associated with higher inducibility of the HO-1 gene and seem to be protected against various diseases such as cardiovascular disorders (Yamada et al. 2000). The
TFs NF-E2 related factor 2 (Nrf2), BTB and CNC homologue I (Bach1), nuclear factor-KB (NF-KB) and activator protein (AP-1) play major roles for the regulation of inducible HO-1 gene expression.

**1.6.1 Nrf2 and Bach1**

A major regulator for HO-1 induction by oxidative stress is the TF Nrf2. Nrf2 is a master regulator of the inducible antioxidant cellular defense response and mediates the up-regulation of phase II detoxification genes such as NAD(P)H:oxidoreductase-1 (NQO1), glutathione-S-transferases (GSTs) and other inducible antioxidant and detoxification genes (Kobayashi and Yamamoto 2005; Kensler et al. 2007). In response to prooxidant stimuli Nrf2 translocates from the cytosol to the nucleus and binds to AREs in various gene promoters (Nguyen et al. 2004). Activation of Nrf2 via prooxidant stimuli is primarily controlled via the redox sensor Keap1, which forms a protein complex with Nrf2 and is regulated by various mechanisms such as ubiquitination (Ma 2013).

More recently, the transcription repressor Bach1 has been recognized to play a key role for the specific regulation of HO-1 gene expression (Ogawa et al. 2001; Igarashi and Watanabe-Matsui, 2014). Similar to Nrf2, Bach1 belongs to the cap'n'collar family of transcription factors and forms heterodimers with small Maf proteins that bind to Maf recognition elements (Motohashi et al. 2002; Igarashi and Sun 2006) such as AREs in the HO-1 promoter. Bach1 has been shown to repress HO-1 gene expression under physiological conditions and competes with Nrf2 for binding to the ARE. Specifically, high levels of intracellular heme cause Bach1 dissociation from the ARE of the HO-1 promoter, after which Nrf2 can bind to the ARE (Ogawa et al. 2001). More recently, heme has been shown to control cellular Bach1 protein levels via proteasomal degradation (Zenke-Kawasaki et al. 2007).
1.6.2 Other transcription factors

Although the Nrf2/Bach1 system plays the major role, other TFs such as NF-κB and AP-1 are also involved in HO-1 gene regulation. NF-κB which mediates the up-regulation of multiple inducible proinflammatory genes such as cytokines and adhesion molecules (Gilmore 2006), has been shown to mediate the induction of HO-1 gene expression in various in vitro and in vivo models (Naidu et al. 2008; Li et al. 2009).

Moreover, AP-1, which is composed of various members of the Jun and Fos activating TF protein family, plays a critical role in regulating transcriptional events of the stress response (Hess et al. 2004). Alam and colleagues have initially reported that AP-1 plays a critical role for the induction of murine HO-1 (Alam and Den 1992; Alam et al. 1995), which has subsequently been confirmed for the human HO-1 gene (Wright et al. 2009). Remarkably, cross-talk between Nrf2 and AP-1 has been demonstrated to be involved in the regulation of the two ARE-dependent genes NQO-1 and glutamate-cysteine ligase (Levy et al. 2009).
1.7 Objectives and goals of the thesis

1.7.1 Regulation of HO-1 via PECAM-1 in ECs

PECAM-1, also known as CD31, is a constitutively expressed 130-kDa type I transmembrane glycoprotein, which is highly abundant on ECs or platelets and to a minor extent also on mononuclear blood cells (Newman and Newman 2003; Privratsky et al. 2014). PECAM-1 has been shown to mediate EC-EC interactions via its localization at cell junctions of endothelial monolayers (Muller et al. 1993). These junctions play an important role for the integrity of the endothelium and are critically involved in the regulation of transendothelial migration of leukocytes in inflammation (Woodfin et al. 2007; Muller 2011). Moreover, PECAM-1 regulates leukocyte transmigration through adhesive interactions, activation of integrins and modulation of lateral border compartement recycling, which is important for both paracellular and transcellular leukocyte migration (Muller 2003; Muller 2011; Noursharg and Alon 2014).

More recently, PECAM-1 has also been recognized to function as a cell surface receptor, which is involved in the regulation of intracellular EC signal transduction (Newman and Newman 2003; Woodfin et al. 2007; Privratsky et al. 2014). Notably, PECAM-1 has also been shown to be part of an endothelial mechanosensory complex with VE-cadherin that transduces shear stress signals and activates the NF-KB pathway (Tzima et al. 2005). Although PECAM-1 has been implicated in a variety of endothelial signaling cascades (Newman and Newman 2003; Woodfin et al. 2007) specific target genes of this molecule are not well characterized.

Gene expression of HO-1 and PECAM-1 has previously been shown to be regulated by the proinflammatory cytokine TNF-α in human ECs. Whereas TNF-α up-regulated HO-1 (Terry et al. 1998), it down-regulated PECAM-1 gene expression in these cells (Rival et al. 1996;
Stewart et al. 1996). Therefore, we hypothesized that HO-1 gene expression might directly be linked with PECAM-1 in human ECs.

1.7.2 Regulation of HO-1 by antibodies (Abs) against Dengue non-structural (NS1) antigen in ECs

Dengue is a major arthropod-borne viral disease in tropical countries and is caused by infection with the dengue virus (DV) (Halstead 2007). Dengue infection can lead to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), both of which are potentially fatal conditions that are considered to occur as secondary infections with DV (Thein et al. 1997). Major features of DHF/DSS are vascular hemorrhage and plasma leakage, which result from structural damage of ECs (Srikiatkhachorn 2009). Although the pathogenesis of DHF is not entirely understood, interactions of cross-reactive anti-EC Abs (AECAs), that are directed against the DV NS1 antigen, and an unknown surface antigen on ECs and platelets, can cause vascular damage and thrombocytopenia in DHF (Srikiatkhachorn 2009; Tan and Alonso. 2009). Independently, Lin et al. (2002) reported that anti-NS1 Abs, which can be detected in DHF patients and interact with non-infected ECs, can cause apoptosis in these cells. Anti-NS1 Abs from mice have been shown to cross-react with human fibrinogen, platelets and ECs (Falconar 2007) and inhibit aggregation of platelets in patients with DV infection via binding to protein disulfide isomerase (PDI) (Cheng et al. 2009). To further investigate whether AECAs may play a role in EC-specific regulation of HO-1, we determined the regulatory effects of Abs against DV NS1 on HO-1 gene expression in human umbilical vein ECs (HUVECs).
1.7.3 Effect of HO-1 on human leukocyte antigen (HLA)-Ab-dependent gene expression in human ECs

AECAs play a critical role in antibody-mediated rejection (AMR), which is a major prognostic factor for long-term survival of grafts in solid organ transplantation (Singh et al. 2009). In particular the formation of Abs against HLA molecules is associated with increased rejection and graft loss in patients after heart and kidney transplantation (Zhang et al. 2005; Zhang et al. 2011). It is established that HLA Abs can cause EC injury through complement fixation (Baldwin et al. 2004), but more recently, complement-independent effects of HLA Abs have also been implicated in AMR (Valenzuela et al. 2014).

HO-1 gene expression has previously been shown to be induced by HLA class I Abs in human ECs (Narayanan et al. 2006; Iwasaki 2010). Moreover, overexpression of HO-1 has been shown to inhibit the up-regulation of inducible proinflammatory adhesion molecules in TNF-α activated ECs (Soares et al. 2004). In the current study, we hypothesized that HO-1 may specifically modulate HLA class I-Ab mediated activation of ECs.
Chapter 2  PECAM-1-Dependent Heme Oxygenase-1 Regulation via an Nrf2-Mediated Pathway in Endothelial Cells


Abstract
The antioxidant enzyme heme oxygenase-1 (HO-1), which catalyzes the first and rate-limiting step of heme degradation, has major anti-inflammatory and immunomodulatory functions via its cell type-specific effects in the endothelium. In the current study, we investigated whether the key endothelial adhesion and signaling receptor PECAM-1 (CD31) might be involved in the regulation of HO-1 gene expression in human endothelial cells (ECs). To this end PECAM-1 expression was down-regulated in human umbilical vein ECs (HUVECs) with an adenoviral vector-based knockdown approach. Knockdown of PECAM-1 markedly induced HO-1, but not the constitutive HO isoform HO-2. Nuclear translocation of the transcription factor NF-E2-related factor-2 (Nrf2), which is a master regulator of the inducible antioxidant cell response, and intracellular levels of reactive oxygen species were increased in PECAM-1-deficient HUVECs, respectively. Furthermore, PECAM-1-dependent HO-1 regulation was examined in PECAM-1 over-expressing L- and Chinese hamster ovary cells. Endogenous HO-1 gene expression and reporter gene activity of transiently transfected luciferase HO-1 promoter constructs containing Nrf2 target sequences were down-regulated in these PECAM-1 over-expressing cell lines. Finally, direct interaction of PECAM-1 with a native complex of its binding partner NB1 (CD177) and serine proteinase 3 (PR3) from human neutrophils, markedly induced HO-1 expression in HUVECs. Taken together, we demonstrate a regulatory link between HO-1 gene expression and PECAM-1 in human ECs, which might play a critical role in inflammation and may serve as a therapeutic target in cardiovascular disease.

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Chapter 3 Antibodies against Dengue Virus Nonstructural Protein-1 Induce Heme Oxygenase-1 via a Redox-Dependent Pathway in Human Endothelial Cells

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Abstract
Heme oxygenase (HO)-1, the inducible isoform of the first and rate-limiting enzyme of heme degradation, affords anti-inflammatory protection via its cell-type-specific effects in endothelial cells (ECs). In dengue hemorrhagic fever (DHF), which is the life-threatening form of dengue virus (DV) infection, endothelial interactions of cross-reactive antibodies against the DV nonstructural glycoprotein-1 (NS1) are associated with endothelial dysfunction. In this study, we investigated whether anti-NS1 antibodies might regulate HO-1 gene expression in human ECs. Serum from DHF patients with high anti-NS1 titers and a monoclonal anti-NS1 antibody upregulated HO-1 gene expression in human umbilical vein ECs, which was blocked by purified NS1 antigen. Immunoprecipitation studies showed that anti-NS1 antibodies specifically bound to the oxidoreductase protein disulfide isomerase (PDI) on ECs. Moreover, anti-NS1-mediated HO-1 induction was reduced by inhibition of PDI enzyme activity. Reactive oxygen species, which were generated by NADPH oxidase and in turn activated the phosphatidylinositol 3-kinase (PI3K)/Akt cascade, were involved in this upregulation of HO-1 gene expression. Finally, apoptosis of ECs caused by anti-NS1 antibodies was increased by pharmacological inhibition of HO-1 enzyme activity. In conclusion, HO-1 gene expression is upregulated by anti-NS1 antibodies via activation of a redoxdependent PDI/PI3K/Akt-mediated pathway in human ECs.

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Chapter 4 Heme Oxygenase-1 Modulates HLA Class I Antibody-Dependent Endothelial Cell Activation


Abstract

Background:
Antibody-mediated rejection (AMR) is a key limiting factor for long-term graft survival in heart and kidney transplantation. Activation of endothelial cells (ECs) via complement-independent effects of human leukocyte antigen class I (HLA I) antibodies (Abs) plays a major role in the pathogenesis of AMR. As the antioxidant enzyme heme oxygenase (HO)-1 is known to have cell type-specific anti-inflammatory effects in the endothelium, we investigated its role on HLA I Ab-dependent activation of human ECs.

Methods:
Regulation of inducible proinflammatory endothelial adhesion molecules and chemokines (VCAM-1, ICAM-1, IL-8 and MCP-1) by monoclonal pan- and allele-specific HLA I Abs was determined in cell cultures of primary human umbilical venous, aortic macrovascular and microvascular ECs. HO-1 was modulated by pharmacological regulators and siRNA-mediated knockdown. Adherence of THP-1 monocytes to ECs was determined by leukocyte adhesion assay.

Results:
Exposure of human macro- and microvascular EC cultures to HLA I Abs caused endothelial activation, as indicated by up-regulation of VCAM-1, ICAM-1, MCP-1 and IL-8. This up-regulation was mediated via the phosphatidylinositol-3 kinase (PI3K)/Akt and NF-κB pathways. Pharmacological induction of HO-1 with cobalt-protoporphyrin IX reduced, whereas inhibition of HO-1 with either zinc-protoporphyrin IX or siRNA-mediated knockdown increased HLA I Ab-dependent EC activation. Binding of THP-1 monocytes was enhanced in HLA I Ab-stimulated ECs. This effect was counteracted by HO-1 up-regulation.
**Conclusion:**

HLA I Ab-dependent EC activation is modulated by specific HO-1 up-regulation. Thus, targeted regulation of endothelial HO-1 may be a novel therapeutic approach for the treatment of AMR in kidney and heart transplantation.
Heme oxygenase-1 modulates HLA class I antibody-dependent endothelial cell activation

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Disclosure
The authors declare no conflicts of interest.

Running title: HO-1 modulates HLA antibody-mediated endothelial activation
FOOTNOTES

Authors’ contributions:

EZ participated in research design, performance of the research, data analysis and writing of the paper.
HS participated in research design, performance of the research data analysis and writing of the paper.
OH contributed new reagents and analysis tools.
CF, RB, GT and JUB participated in research design.
JL participated in research design and data analysis.
SI participated in research design, data analysis and writing of the paper.

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Abbreviations:
Ab, antibody; AMR, antibody-mediated rejection; CO, carbon monoxide; CoPPIX, cobalt-protoporphyrin IX; CO-RM, carbon monoxide-releasing molecule; Cox-2, cyclooxygenase-2; DSA, donor-specific antibody; EC, endothelial cell; ERK, extracellular-regulated kinase; HAEC, human aortic endothelial cell; HDMVEC, human dermal microvascular endothelial cell; HLA, human leukocyte antigen; HLA I, human leukocyte antigen class I; HO, heme oxygenase; MoAb, monoclonal antibody; PI3K, phosphatidylinositol-3 kinase; siRNA, short interfering RNA; TF, transcription factor; ZnPPIX, zinc-protoporphyrin IX.
ABSTRACT

Background:
Antibody-mediated rejection (AMR) is a key limiting factor for long-term graft survival in heart and kidney transplantation. Activation of endothelial cells (ECs) via complement-independent effects of human leukocyte antigen class I (HLA I) antibodies (Abs) plays a major role in the pathogenesis of AMR. As the antioxidant enzyme heme oxygenase (HO)-1 is known to have cell type-specific anti-inflammatory effects in the endothelium, we investigated its role on HLA I Ab-dependent activation of human ECs.

Methods:
Regulation of inducible proinflammatory endothelial adhesion molecules and chemokines (VCAM-1, ICAM-1, IL-8 and MCP-1) by monoclonal pan- and allele-specific HLA I Abs was determined in cell cultures of primary human umbilical venous, aortic macrovascular and microvascular ECs. HO-1 was modulated by pharmacological regulators and siRNA-mediated knockdown. Adherence of THP-1 monocytes to ECs was determined by leukocyte adhesion assay.

Results:
Exposure of human macro- and microvascular EC cultures to HLA I Abs caused endothelial activation, as indicated by up-regulation of VCAM-1, ICAM-1, MCP-1 and IL-8. This up-regulation was mediated via the phosphatidylinositol-3 kinase (PI3K)/Akt and NF-κB pathways. Pharmacological induction of HO-1 with cobalt-protoporphyrin IX reduced, whereas inhibition of HO-1 with either zinc-protoporphyrin IX or siRNA-mediated knockdown increased HLA I Ab-dependent EC activation. Binding of THP-1 monocytes was enhanced in HLA I Ab-stimulated ECs. This effect was counteracted by HO-1 up-regulation.

Conclusion:
HLA I Ab-dependent EC activation is modulated by specific HO-1 up-regulation. Thus, targeted regulation of endothelial HO-1 may be a novel therapeutic approach for the treatment of AMR in kidney and heart transplantation.
INTRODUCTION

Antibody (Ab)-mediated rejection (AMR) is a major limiting factor for long-term graft survival after kidney and heart transplantation (1-4). The endothelium of allografts plays a key role in the pathogenesis of AMR (5, 6), because it is targeted by donor-specific Abs (DSA), which are directed against human leukocyte antigen (HLA) and/or non-HLA molecules (7, 8). It is well established that HLA Abs can cause EC injury by complement fixation (7, 9), but more recently, complement-independent effects of HLA Abs have also been implicated in AMR (10-13). Although the underlying mechanisms of complement-independent effects of DSAs are not well understood, activation of ECs in response to HLA I Abs appears to play major role (5, 6). EC activation is critically involved in the regulation of acute and chronic inflammation (14) and is characterized by alterations of intracellular endothelial signaling, which up-regulates expression of inducible adhesion molecules and chemokines (15) modulating the coordinated recruitment of leukocytes to the site of inflammation (16, 17).

Current therapeutic regimens for AMR such as plasmapheresis and treatment with CD20 Abs (rituximab) are primarily intended to reduce levels of circulating pathogenic DSAs (4, 18, 19). The clinical success rate of these therapies, however, is limited, and alternative therapies are urgently needed. The antioxidant enzyme heme oxygenase (HO)-1, which is the inducible isoform of catalytic heme degradation (20), has previously been shown to have protective effects in the endothelium (21, 22). Moreover, overexpression of HO-1 has been shown to inhibit up-regulation of proinflammatory adhesion molecules in TNFα-activated ECs (23) and to have anti-inflammatory therapeutic potential in various cardiovascular disorders (24-27). In transplantation settings, survival of cardiac xenografts has been linked with endothelial HO-1 in a mouse-to-rat heart transplantation model (28) and genetic transfer of HO-1 into blood vessel walls has been shown to protect against allogeneic rejection of aortic vascular transplants (29). Moreover, HO-1 has been demonstrated to have beneficial effects against complement-mediated damage of HLA Abs (30). In the current study, we hypothesized that HO-1 may specifically modulate HLA I Ab-dependent activation of ECs.
It is demonstrated that HLA I Abs up-regulate inducible adhesion molecules and chemokines in human ECs and cause increased endothelial adhesion of monocytes. Both HLA I Ab-dependent effects in ECs are modulated by specific regulation of HO-1.
MATERIALS AND METHODS

Abs and chemicals

See SDC Materials and Methods.

Cell cultures and treatment of EC cultures with Abs and chemicals

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMVECs) were from PromoCell (Heidelberg, Germany) and human aortic endothelial cells (HAECs) were from Lonza (Cologne, Germany). For studies with HLA-typed cells, HUVECs with different HLA I genotypes (Donor 1 (Lot. # 696527): A*02, A*30, B*15, B*44; donor 2 (Lot. #1022301.1): A*02, A*26, B*35, B*39; donor 3 (Lot. # 1050901): A*01, A*11, B*08, B*39) were applied. Cells were used in passages 4 to 7 and were cultured in 1% gelatine-coated flasks in EC Growth Medium 2 (PromoCell) and 5% (vol/vol) fetal calf serum (Lonza). Cells were maintained until confluence at 37°C in a controlled environment of 100% humidity and 5% CO₂. For HLA I Ab simulation experiments ECs were cultured in 12-well flat bottom dishes with 2 ml EC Growth Medium 2 plus 5% fetal calf serum until confluence. After an overnight starving period in medium containing 2% fetal calf serum, cells were stimulated with 10 μg/ml of HLA I Abs or isotype control Abs. For inhibitor studies, HUVECs were treated with PI3K/Akt inhibitors wortmannin (1 μM) and LY294002 (20 μM), the NF-κB inhibitors MG132 (100 nM) and Bay 11-7082 (10 μM), the extracellular-regulated kinase (ERK) inhibitors UO126 (20 μM) and PD98059 (10 μM), the HO modulators cobalt-protoporphyrin IX (CoPPIX) and tin-protoporphyrin IX (ZnPPIX) at a final concentration of 5 μM or carbon monoxide (CO)-releasing molecule (CO-RM)-2 (25 μM) for 30 min prior to treatment with HLA I Abs.

Analysis of mRNA expression

Expression of mRNA was determined as previously described (31). Further details on the methods are given in SDC Materials and Methods.
Western blot analysis

Western blotting was performed as previously described (32). Further methodological details are given in SDC Materials and Methods.

Knockdown of HO-1 in HUVECs

HUVECs were cultured in 12-well flat bottom dishes containing 1 ml EC Growth Medium 2 supplemented with 5 % FCS and transfection was carried out with Oligofectamine™ (Life Technologies, Darmstadt, Germany) and Stealth™ RNAi (or siRNA) for HO-1 (Thermo Scientific). 150 pmol of Stealth™ RNAi were diluted in 77.5 μl of Opti-MEM I Reduced Serum Medium (Life Technologies) and were incubated for 15 min. 4 μl oligofectamine™ were mixed with 11 μl of Opti-MEM I Reduced Serum Medium for 5 min at RT. Both dilutions were combined and incubated for 15 min at RT. After removal of growth medium, cells were washed with pre-warmed Opti-MEM I medium, and 400 μl Opti-MEM I medium plus 100 μl of Stealth RNAi-Oligofectamine complexes was added. After 4 h incubation 250 μl of EC Growth Medium 2 containing 15% FCS was added to the cells without removing the transfection mixture.

Leukocyte adhesion assay

This assay was performed as previously described (33)(See also SDC Materials and Methods).

Statistical analysis

Quantitative data from Western blot analyses, real-time RT-PCR experiments and adhesion assays were analysed by two-tailed Student t test and are presented as mean ± SEM from at least three independent experiments. A p-value p<0.05 was considered as statistically significant in all analyses.
RESULTS

HLA I Abs up-regulate the expression of inducible adhesion molecules and chemokines in human ECs

The expression of inducible adhesion molecules (VCAM-1, ICAM-1) and chemokines (MCP-1, IL-8) in response to the pan-HLA I MoAb W6/32 (10 μg/ml) was determined in cell cultures of HUVECs (34-36). W6/32 markedly increased mRNA levels of VCAM-1 in a time-dependent manner with a maximum after 12 h (Figure 1A). Moreover, expression of ICAM-1, MCP-1 and IL-8 was up-regulated in response to W6/32. In contrast, the inducible proinflammatory enzyme cyclooxygenase (Cox)-2, which has previously been shown to be induced in TNFα-activated ECs (37), was down-regulated by W6/32 in HUVECs (Figure 1B).

In the following, we will apply VCAM-1 as surrogate marker for HLA I Ab-dependent EC activation. Treatment of HUVECs with a second independent pan-HLA I MoAb, G46-2.6, also up-regulated VCAM-1 expression (Figure 1C). Protein levels of VCAM-1 were up-regulated in HUVECs after treatment with W6/32 (Figure 1D).

To confirm the above findings for an allele-specific HLA I Ab, cell cultures of HLA-genotyped HUVECs were treated with the MoAb BB7.2, which is directed against a specific HLA-A2 epitope. MoAb BB7.2 up-regulated VCAM-1 mRNA expression in HUVECs from two donors with genotype HLA-A*02+ (donor 1, A*02, A*30; donor 2, A*02, A*26)(Figure 1E), but not in HUVECs from a donor with HLA-A genotype A*02- (donor 3, A*01, A*11). To determine whether HLA I Ab-mediated up-regulation of VCAM-1 would also apply to adult ECs from micro- and macrovascular blood vessels, we used cell cultures of HAECs and HDMVECs. Similar to the gene regulatory pattern in HUVECs, VCAM-1 mRNA levels were increased by W6/32 in HAECs and HDMVECs (Figure 1F). Taken together, the data indicate that HLA I Abs activate human ECs of different origins and up-regulate the expression of inducible adhesion molecules and chemokines in these cells.
HLA I Ab-dependent up-regulation of VCAM-1 is mediated via activation of PI3K/Akt and NF-κB pathways

Ligation of HLA I Abs to ECs has been shown to activate intracellular signaling cascades such as the PI3K/Akt, ERK and NF-κB pathways (5). To investigate the potential role of these cascades in HLA I Ab-dependent up-regulation of VCAM-1, we applied specific pharmacological inhibitors for pretreatment of HUVECs. The PI3K/Akt inhibitors wortmannin and LY294002 markedly decreased up-regulation of VCAM-1 mRNA levels in HUVECs by W6/32 (Figure 2A). In contrast, ERK1/2 inhibitors UO126 or PD09059 had no effect on VCAM-1 up-regulation by W6/32 (data not shown). In addition, W6/32-dependent up-regulation of VCAM-1 was blocked by pretreatment with two pharmacological inhibitors of the NF-κB pathway, MG132 and Bay 11-7082 (Figure 2B). Blocking of PI3K/Akt and NF-κB also inhibited W6/32-dependent induction of ICAM-1, MCP-1 and IL-8 (data not shown). Taken together, the PI3K/Akt and NF-κB pathways are involved in HLA I Ab-mediated up-regulation of inducible adhesion molecules and chemokines in human ECs.

Modulation of HO-1 affects HLA I Ab-dependent induction of VCAM-1 in HUVECs

HO-1 has anti-inflammatory effects via its EC-specific functions (24-26) and has previously been shown to reduce up-regulation of several adhesion molecules in TNFα-activated cell cultures of bovine ECs via inhibition of NF-κB (23, 38). To investigate whether HO-1 might also affect HLA I Ab-mediated activation of ECs, HUVECs were pretreated with a pharmacological HO inhibitor, ZnPPIX, and a HO-1 activator, CoPPIX, respectively, before exposure to W6/32. Inhibition of HO-1 enzyme activity by ZnPPIX markedly enhanced VCAM-1 mRNA induction after stimulation of HUVECs with W6/32 (Figure 3A). By contrast, CoPPIX-dependent up-regulation of HO-1 reduced VCAM-1 mRNA levels in W6/32-treated HUVECs. To confirm the role of HO-1 in HLA I Ab-mediated VCAM-1 induction, HO-1 was down-regulated in HUVECs by knockdown with siRNA against HO-1 (Figure 3B and 3C). Treatment of HO-1-depleted HUVECs with either TNFα or MoAb W6/32 enhanced relative VCAM-1 mRNA levels as compared to control cells, respectively (Figure 3D). Remarkably,
modulation of HO-1 had similar effects on W6/32-dependent expression of ICAM-1, MCP-1 and IL-8 (data not shown). The HO product CO has previously been shown to have major anti-inflammatory effects in the endothelium (39, 40). To study the effects of CO on HLA I Ab-dependent EC activation, HUVECs were exposed to a CO-releasing molecule (CORM) CORM-2 (40) prior to stimulation with W6/32. Incubation with CORM-2 markedly decreased up-regulation of VCAM-1 mRNA levels in W6/32-treated HUVECs (Figure 4) as well as that of ICAM-1, MCP-1 and IL-8 (data not shown). Taken together, the data suggest that HO-1 and its enzymatic product CO inhibit the up-regulation of inducible adhesion molecules and chemokines in HLA I antibody-activated ECs.

**HO-1 modulates increased HLA I Ab-mediated adhesion of THP-1 monocytes to HUVECs**

HLA I Abs have been shown to enhance adhesion of mononuclear cells to the endothelium in various experimental *in vivo* and *in vitro* models (41, 42). To address the question whether HLA I Ab-mediated adhesion of mononuclear cells to HUVECs might be modulated by HO-1, we performed *in vitro* leukocyte adhesion assays. HLA I Abs enhanced adhesion of THP-1 monocytes to HUVECs, which was inhibited by a VCAM-1 blocking Ab, but not by an unspecific control Ab. These findings indicate that up-regulation of VCAM-1 by W6/32 is involved in the increased recruitment of monocytes to ECs. To determine the specific effects of HO-1 modulation on monocyte adhesion to ECs, HUVECs were treated with W6/32 in the presence or absence of ZnPPiX, CoPPIX and heme, respectively. The HO inhibitor ZnPPiX increased, whereas HO-1 inducers CoPPIX and heme reduced W6/32-dependent adhesion of monocytes to HUVECs (Figure 5). The data suggest that the HLA I Ab-dependent increase monocyte adhesion to HUVECs is modulated by HO-1 in human ECs.
DISCUSSION

AMR plays a key role in graft rejection after solid organ transplantation and is a major clinical challenge due to the lack of feasible therapeutic regimens (1-4). In addition to the well-known complement-dependent effects of DSAs, complement-independent effects of such Abs, which can activate the endothelium via direct Ab-EC interactions (5, 6), have recently been shown to be involved in the pathogenesis of AMR (13). Here, we investigated, whether the inducible anti-inflammatory enzyme HO-1 can modulate HLA I Ab-dependent EC activation. The major findings of the current study are, that 1) HLA I Abs up-regulate inducible adhesion molecules and chemokines in cell cultures of primary human ECs via PI3K/Akt and NF-κB activation; 2) HO-1 specifically modulates HLA I Ab-dependent expression of these inducible proinflammatory genes; 3) HO-1 counteracts the increased HLA I Ab-dependent adhesion of monocytes to ECs.

Activation of human ECs by HLA I Abs

The current report shows, that treatment with two pan-HLA I MoAbs (W6/32, G46.6) and an allele-specific HLA MoAb (BB7.2) up-regulates proinflammatory inducible adhesion molecules and chemokines in cell cultures of primary human ECs (Figure 1). These findings agree with earlier reports, in which it has been shown that HLA I Abs activate human ECs via complement-independent effects ((41, 43-46); for reviews see (5, 42, 47)). For example, VCAM-1 has been shown to be up-regulated by HLA Abs eluted from acutely rejected renal allografts in cultured HUVECs (35). Moreover, it has been demonstrated that HLA I Abs up-regulate VEGF expression in cell cultures of HUVECs (48) or VCAM-1 and ICAM-1 in the human microvascular EC line HMEC-1 (34), respectively. Conflictingly, independent groups have also reported that VCAM-1 might not be regulated in response to HLA I Abs (46, 49). These contradictory observations could be explainable by cell type-specific differences of various ECs and/or variations of cell culture conditions in these studies. In accordance with earlier findings by others (50), we also observed that activation of the PI3K/Akt cascade is involved HLA I Ab-dependent up-regulation of inducible adhesion molecules and chemokines.
However, in contrast to a previous report \((34)\), ERK was not involved in the up-regulation of VCAM-1 by HLA I Abs in our experimental system of primary human ECs. Again, these contradictory observations may be explained by cell type-specific differences in the various cell culture models. It is also interesting to note, that in contrast to the NF-κB-regulated genes VCAM-1, ICAM-1, IL-8 and MCP-1, expression of the NF-κB-responsive gene Cox-2 \((37)\) was not affected by HLA I Abs (Figure 1B). This may suggest that additional regulatory factors and pathways are involved in HLA I Ab-dependent endothelial activation.

**HO-1 modulates HLA I Ab-dependent EC activation**

Our current findings clearly show that HLA I Ab-mediated activation of human ECs is modulated by HO-1 (Figure 3). These data correspond with previous reports on mouse and human genetic HO-1 deficiency, which revealed endothelial protective properties against prooxidant and pro-inflammatory damage for this enzyme \((21, 22)\). Independently, overexpression of HO-1 has been shown to inhibit TNFα-mediated induction of proinflammatory inducible adhesion molecules in cell cultures of primary bovine ECs \((23)\). As this HO-1 effect has been ascribed to the inhibition of NF-κB activation in a follow-up study \((38)\), such a mechanism might also be involved in HO-1-dependent inhibition of HLA I Ab-dependent EC activation in our cell culture system (Figure 3). Interestingly, HO-1 induction has previously been demonstrated to counteract the proapoptotic endothelial effects of Abs against the Dengue virus non-structural protein 1 \((32)\) and complement-dependent effects of HLA I Abs \((30)\). CO, which is one of the products of HO, is known to have major therapeutic potential in various physiological and pathophysiological settings \((40)\). As demonstrated in Figure 4, CO mediated HO-1-dependent modulation of the HLA class I Ab-mediated induction of VCAM-1 gene expression in HUVECs (Figure 4). This latter observation supports earlier studies showing that HO-1-derived CO affords protection against the rejection of mouse to rat cardiac transplants \((28, 39)\).
HO-1 as a therapeutic target for treatment of AMR

Activation of ECs is critical for the pathogenesis of various inflammatory vascular disorders including AMR (14, 51). A characteristic feature of AMR is inflammation of graft arteries as demonstrated in animal models of solid organ transplantation (52, 53). The coordinate up-regulation of inducible surface proteins such as selectins and adhesion molecules on ECs is essential for regulation of leukocyte recruitment to the site of an inflammation (16, 17). Our functional studies in a monocyte adhesion assay also confirmed that EC activation by HLA I Abs mediates binding of THP-1 monocytes to ECs (Figure 5). These findings correspond with those of recent reports by others (34, 42). In one of these reports Valenzuela and colleagues have also demonstrated a major role for P-selectin in HLA I Ab-mediated recruitment of monocytes to ECs (42).

The clinical success rate of current therapeutic regimens in AMR including treatment with CD20 Abs (rituximab) and/or plasmapheresis, which primarily aim to reduce levels of circulating serum DSAs (4, 18, 19), is poor and novel complementary therapy strategies are urgently needed. Thus, an alternative therapeutic approach in AMR could be to specifically induce anti-inflammatory protective mechanisms of the endothelium. For example, a recombinant designer protein, which binds to activated endothelium via an E-selectin-interacting domain, has been shown to counteract activation of ECs via inhibition of NF-κB (54). Similarly, targeted up-regulation of endothelial HO-1 might counteract AMR-associated inflammation in heart and kidney transplantation. As statins have previously been shown to up-regulate HO-1 in the endothelium (55-57), these widely-used pharmacological compounds might also be applicable in AMR therapy.

Conclusion:

In the current study we have shown that HLA I Ab-dependent activation of human ECs is counteracted by the anti-inflammatory enzyme HO-1. Therefore, targeting of endothelial HO-1 might be a promising strategy for the treatment of AMR in solid organ transplantation.
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FIGURE LEGENDS

FIGURE 1. Up-regulation of VCAM-1 and other proinflammatory inducible genes by HLA I Abs in cell cultures of human ECs

HUVECs (A-E), HAECs and HDMVECs (F) were cultured as described under Materials and Methods. (A) HUVECs were treated with MoAb W6/32 or isotype control for the indicated times. Cells were lysed and subjected to RT-PCR analysis. The fold induction of VCAM-1 mRNA levels is shown. (B) HUVECs were treated with W6/32 or isotype control for 18 h. Cells were lysed and subjected to RT-PCR analysis for ICAM-1, MCP-1, IL-8 and Cox-2. The fold induction of mRNA levels is shown. (C) HUVECs were treated with MoAbs W6/32 and G46-2.6 or isotype control for 18 h. Cells were lysed and subjected to RT-PCR analysis. The fold induction of VCAM-1 mRNA levels is shown. (D) HUVECs were treated with W6/32 (5 and 10 μg/ml) or isotype control for 18 h, as indicated. Cells were subjected to Western blot analysis and probed with VCAM-1 and GAPDH Abs. The fold induction of protein levels is indicated and a representative of three independent experiments is shown. (E) HLA class I-genotyped HUVECs (HLA-A*02+ or HLA-A*02−) were treated with W6/32 and an allele-specific MoAb against HLA-A2 (clone BB7.2) or isotype controls for 18 h. Cells were lysed and subjected to RT-PCR analysis. The fold induction of VCAM-1 mRNA levels is shown. (F) HUVECs, HAECs and HDMVECs were treated with W6/32 or isotype control Ab for 18 h. Cells were lysed and subjected to RT-PCR analysis. The fold induction of mRNA levels is indicated. All data are means ± SEM from three independent experiments. * p ≤ 0.05, significant differences treatment versus control.

FIGURE 2. Signaling cascades of HLA I Ab-dependent VCAM-1 up-regulation in HUVECs

(A, B) HUVECs were incubated with HLA I Ab W6/32 or isotype control for 18 h after pretreatment for 30 min with, (A) the PI3K/Akt pathway inhibitors wortmannin (1 μM) and LY294002 (20 μM), or with, (B) the NF-κB pathway inhibitors MG132 (100 nM) and Bay 11-7085 (10 μM). Cells were lysed and subjected to RT-PCR analysis. The fold induction of
mRNA levels is indicated. Data are mean ± SEM from three independent experiments. * p ≤ 0.05, significant differences treatment versus control; ** p ≤ 0.05, W6/32 versus W6/32 plus inhibitor. Wort, wortmannin; LY, LY294002; Bay, Bay 11-7085.

FIGURE 3. Pharmacological inhibition and siRNA-mediated knockdown of HO-1 reduce HLA I Ab-induced VCAM-1 expression in HUVECs

(A) HUVECs were incubated with HLA I Ab W6/32 alone and for 18 h in the presence of CoPPIX (5 μM) or ZnPPIX (5 μM), as indicated. Cells were lysed and subjected to RT-PCR analysis. The fold induction of VCAM-1 mRNA levels is shown. (B-D) HUVECs were transfected with siRNA for HO-1 or scrambled control siRNA. (B) mRNA expression was determined by RT-PCR analysis and relative levels of HO-1 mRNA are shown. (C) Protein expression was determined by Western blot analysis and probed sequentially with Abs against HO-1 and GAPDH. A representative of three independent experiments is shown. (D) Transfected HUVECs were treated with TNFα (150 ng/ml) or W6/32 for 18 h. Cells were lysed and subjected to RT-PCR analysis. The fold induction of mRNA levels is indicated. Bar graphs represent mean ± SEM from three independent experiments. * p ≤ 0.05, significant differences treatment versus control; ** p ≤ 0.05, W6/32 versus W6/32 plus CoPPIX/ ZnPPIX. Con, control.

FIGURE 4. CO-RM-2 decreases HLA class I Ab-induced VCAM-1 expression in HUVECs

Confluent HUVECs were incubated with W6/32 or isotype control for 18 h in the presence or absence of CO-RM-2 (100 μM). Cells were lysed and subjected to RT-PCR analysis. The fold induction of mRNA levels is indicated. Data are represented as mean ± SEM from three independent experiments. * p ≤ 0.05, significant differences treatment versus control; ** p ≤ 0.05, W6/32 versus W6/32 plus CO-RM-2.
FIGURE 5. HO-1 modulates HLA I Ab-dependent up-regulation of THP-1 monocyte adhesion to HUVECs

Confluent HUVECs were cultured for 24 h in the presence of MoAb W6/32 or isotype control Ab in the presence or absence of a blocking antibody against VCAM-1, a control Ab (Con Ab), ZnPPIX (5 μM), CoPPIX (5 μM) and heme (2.5 μM), as indicated. HUVECs were incubated with Cell Tracker green-labeled THP-1 monocytes for 30 min. After 5 washing steps firmly adherent monocytes were quantified with fluorescence microscopy in 15 pre-selected high-power fields by a blinded investigator. The monocyte adhesion in % of control is indicated. Data are mean ± SEM from three independent experiments. * p ≤ 0.05, significant differences treatment versus control; ** p≤ 0.05, W6/32 versus W6/32 plus anti-VCAM-1/ CoPPIX/ ZnPPIX/ heme.
Figure 2

A

Fold induction of VCAM-1 mRNA level

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B

Fold induction of VCAM-1 mRNA level

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Figure 3

A) Fold induction of VCAM-1 mRNA level.

B) Relative HO-1 mRNA level [%].

C) Western blot analysis with corresponding molecular weights.

D) Relative VCAM-1 mRNA level [%].

Legend:
- siCon: siControl
- siHO-1: siHO-1
- +: Treatment
- -: Control
SUPPLEMENTAL DIGITAL CONTENT

Abs and chemicals

The murine monoclonal pan-HLA I antibodies W6/32 and G46-2.6, both of which are directed
against different monomorphic HLA I epitopes, were from either ATCC (Manassas, VA, USA)(W6/32) and prepared as previously described (23) or BD Bioscience (San Jose, CA, USA)(G46.2.6). The murine isotype control mAb (MCA929EL) was from AbD Serotec (Oxford, UK), human HLA-A2 (clone BB7.2) and microglobulin-2 2b-57 as IgG2b isotype control were from Biolegend (London, UK). Polyclonal rabbit anti-human VCAM-1 and anti-human GAPDH for Western blot analyses were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), polyclonal rabbit anti-human HO-1 from Enzo Life Sciences (Lörrach, Germany). Secondary HRP-conjugated goat anti-rabbit IgG was from BioRad (Hercules, CA, USA), murine monoclonal VCAM-1 (51-10C9) for blocking leukocyte binding to VCAM-1 on ECs from BD Biosciences (Heidelberg, Germany) and isotype mouse IgG1 control antibody (MOPC-21) from Biolegend (London, UK). LPS (Escherichia coli serotype 0111:B4), PI3K/Akt inhibitors wortmannin and LY294002, NF-κB inhibitors, MG132 and Bay 11-7082, were from Merck Biosciences (La Jolla, CA, USA). Cobalt-protoporphyrin (CoPPIX), zinc-protoporphyrin (ZnPPIX) and heme were from Frontier Scientific (Logan, Utah, USA), CORM-2 from Sigma-Aldrich (Steinheim, Germany) and TNFα from PeproTech (Rocky Hill, NJ, USA).

Analysis of mRNA expression

RNA extraction with the RNeasy mini kit (Qiagen, Hilden, Germany), cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) were performed, as described previously (Saragih et al., 2014). Inventoried primer mixes for quantification of mRNA levels of VCAM-1, ICAM-1, MCP-1, IL-8, HO-1 and Cox-2 were from Applied Biosystems. Amplification was performed with TaqMan Gene Expression Master Mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Thermal cycling was performed at 95°C for 10 min followed by 40 cycles at
95°C for 15 s and 60°C for one min. GAPDH was used as a control for normalization of cDNA levels and the ΔΔCT method was applied for determining mRNA levels semi-quantitatively according to the manufacturer's protocol.

**Western blot analysis**

Western blotting was performed as previously described (1) with primary antibodies against VCAM-1 (1:1,000) or HO-1 (1:1,000) and secondary HRP-conjugated goat anti-rabbit Ab (1:10,000). Signals were visualized by enhanced chemiluminescence (Roth GmbH, Karlsruhe, Germany) and quantified with a Fluorchem (Alpha Innotec, San Leandro, CA, USA). Images were processed using Corel Draw Graphic Suite X5 Software (Corel Corporation, Ottawa, Canada).

**Leukocyte adhesion assay**

HUVECs were cultured in 6-well cell culture plates to confluency before incubation with W6/32 for 18 h. For blocking of VCAM-1, cells were co-incubated with 10 μg/ml mouse anti-human VCAM-1 Ab or the appropriate control Ab. Adhesion of the monocyte cell line THP-1 (ATCC, Manassas, VA) to HUVECs was determined as previously described (25) with Cell tracker green (Life Technologies). Adhering macrophages were counted by fluorescence microscopy in 15 pre-selected high-power fields by a blinded investigator. Images were acquired at RT using an Olympus IX81 microscope (Olympus, Hamburg, Germany). A QImaging Retiga EXi camera (QImaging, Surrey, BC, Canada) and QCapture Pro Software version 6.0.0.412 (QImaging) were used to capture and analyze immunofluorescence images.
Chapter 5 Discussion

HO-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation, which has antioxidant and also anti-inflammatory and immunomodulatory effects. HO-1 has been demonstrated to have beneficial protective effects in various inflammatory vascular disorders that appear to be mediated via its cell type-specific functions in the endothelium (Yachie et al. 1999; True et al. 2007). In the current thesis it is demonstrated that HO-1 gene expression is regulated by the endothelial surface adhesion receptor PECAM-1 (CD31) in a cell type-preferential manner via a pathway that involves the master redox-dependent transcriptional regulator Nrf2 in human ECs. Moreover, it is shown that specific up-regulation of endothelial HO-1 provides protection against the pathological effects of AECAs that are directed against the DV NS1 antigen and HLA class I molecules.

Role of PECAM-1 for cell type-preferential gene expression of HO-1 in ECs

PECAM-1 regulates basal HO-1 gene expression in ECs

Although HO-1 is detectable in all cells and tissues, it exhibits differential basal and regulatory expression patterns in various cell types (Maines 1997; Ryter et al. 2006; Paine et al. 2010). The findings of the current thesis indicate that the surface receptor PECAM-1, which is highly expressed on ECs and is a major endothelial differentiation marker, regulates the cell-type preferential expression of HO-1 in human ECs (Saragih et al. 2014). PECAM-1 has key functions for maintaining the integrity of the vascular endothelium (Woodfin et al. 2007; Muller 2011), because it forms homophilic interactions at cell junctions, which hold together the endothelial monolayer. Moreover, PECAM-1 plays an important role for the regulation of transendothelial leukocyte migration via heterophilic interactions between ECs and leukocytes during the inflammatory response (Muller 2003; Muller 2011; Nourshargh and Alon 2014). Besides its role for the regulation of cell-cell interactions, PECAM-1 has also been recognized to have functions for signal transduction of extracellular stimuli in ECs.
(Newman and Newman 2003: Woodfin et al 2007). The current data suggest that PECAM-1 might have a dampening effect on basal HO-1 gene expression under normal conditions in the endothelium, because targeted knockdown of PECAM-1 in ECs showed a specific up-regulation of HO-1, but did not affect expression of HO-2 or other redox-regulated inducible genes such as NQO1 and peroxiredoxin 1 (Saragih et al. 2014). Similar observations were also made in the human leukemic monocyte cell line Mono-Mac 6, which expressed lower levels of PECAM-1 in comparison to ECs and in which HO-1 was up-regulated in a dose-dependent manner via specific PECAM-1 down-regulation (Saragih et al. 2014). Similarly, overexpression of PECAM-1 in two transgenic cell lines (Chinese hamster ovary cells and L-cells; see Figures 5 and 6 in (Saragih et al. 2014)) led to a pronounced down-regulation of HO-1 gene expression. Due to the differential PECAM-1 expression in various cell types (ECs \( \sim 1-2 \times 10^6 \) PECAM-1 copies/cell; myeloid/mononuclear cells \( \sim 50,000 \) PECAM-1 copies/cell (Newman and Newman 2003)), it is conceivable that HO-1 expression is governed via PECAM-1 in an EC type-preferential manner. This conclusion might agree with an earlier report on HO-1 gene expression in various cell types of rat liver, in which hepatic sinusoidal ECs exhibited lower HO-1 levels if compared with liver tissue macrophages (Immenschuh et al. 2003). In addition, PECAM-1 might also have cell type-specific effects on intracellular signal transduction cascades, because HO-1 regulation by TNF-\( \alpha \) exhibited a counter-regulatory gene expression pattern in ECs and monocytes. Whereas TNF-\( \alpha \) markedly up-regulated HO-1 in cell cultures of human ECs ((Terry et al. 1998); see also Figure 1 in (Saragih et al. 2014)), it down-regulated HO-1 in human monocytes (Kirino et al. 2007). Notably, PECAM-1 has previously been shown to function as a negative feedback regulator for the LPS-dependent inflammatory response in mononuclear cells (Rui et al. 2007). Further studies on the underlying mechanisms that may mediate PECAM-1-dependent HO-1 gene expression in ECs are ongoing.
Role of Nrf2 for PECAM-1-dependent HO-1 regulation

PECAM-1 has been shown to affect various intracellular signaling cascades in ECs (Newman and Newman 2003; Woodfin et al. 2007; Privratsky et al. 2010) and to inhibit various extracellular stimuli that cause apoptosis (Gao et al. 2003). The findings of the present thesis suggest that PECAM-1-dependent HO-1 gene regulation is controlled via a redox-dependent pathway involving the master antioxidant transcriptional regulator Nrf2, as indicated by findings in PECAM-1-deficient HUVECs and in PECAM-1 overexpressing Chinese hamster ovary cells and L-cells (Saragih et al. 2014). As HO-1 gene expression has also been shown to be governed by other TFs such as NF-κB, AP-1 and the nuclear repressor Bach1 (Alam and Cook 2007; Paine et al. 2010), the current findings do not exclude that other transcriptional regulators might also be involved in PECAM-1-dependent HO-1 regulation. For example, PECAM-1 also modulates activation of NF-κB (Cepinskas et al. 2003), interferon-regulatory factor-3 (Ruperez et al. 2007) and signal transducer and activator of transcription-3 (Privratsky et al. 2010).

HO-1 induction via binding of PECAM-1 to a protein complex of NB1 and PR3 in ECs

PECAM-1 not only forms hemophilic interactions with other PECAM-1 molecules, but can also form heterophilic interactions with surface proteins on leukocytes (Muller 2003; Woodfin et al. 2007). In particular, binding of PECAM-1 to a protein complex with the leukocyte surface antigen NB1 (CD177) and proteinase 3 has been implicated in the regulation of neutrophil migration through the endothelial monolayer (Bayat et al. 2010; Kuckleburg et al. 2012). In the current thesis it is demonstrated that specific binding of PECAM-1 to a NB1/proteinase 3 complex up-regulated HO-1 expression in ECs. The mechanism that might be involved in HO-1 regulation by PECAM-1 interaction with NB1/proteinase 3, however, is still elusive. Up-regulation of HO-1 by PECAM-1 interaction with this protein complex might function as a potential feedback mechanism in inflammation and might counteract excessive
EC activation via the immunomodulatory effects of HO-1 and its enzymatic products (Brouard et al. 2000; Soares et al. 2004; Ryter et al. 2006). The data, which are presented in this thesis, however, are too preliminary to definitely allow such conclusion and further experimental studies are currently underway to address this question.

**HO-1 protects against pathological effects of Abs directed against endothelial antigens**

Endothelial interactions of Abs play a critical role in the pathogenesis of various diseases such as systemic lupus erythematosus or autoimmune vasculitis (van Paassen et al. 2007). Due to the beneficial effects of increased HO-1 expression in the endothelium, which has been demonstrated in various cell culture models *in vitro* and in several animal models *in vivo* (Brouard et al. 2000; Soares et al. 2004; Ryter et al. 2006; Wu et al. 2011), it was hypothesized in the current thesis that targeted modulation of endothelial HO-1 might afford protection against the pathological effects of AECAs directed against surface antigens on ECs. Therefore, the effects of AECAs, which are either directed against the DV NS1 antigen or endothelial HLA class I molecules, were investigated in cell cultures of human ECs.

**NS1 Abs and DV shock syndrome**

Dengue is a major arthropod-borne viral disease in tropical countries and is transmitted by *Aedes aegyptii* and *Aedes albopictus* mosquitoes. Dengue is caused by infection with DVs (Halstead 2007) and can lead to the life-threatening conditions DHF and DSS, both of which are characterized by vascular hemorrhage and capillary leakage (Halstead 2007; Srikiatkhacorn 2009). Although the pathogenesis of DHF is not well understood, interactions of AECAs, which are directed against the DV NS1 antigen and cross-react with an endothelial antigen, have been implicated in vascular damage in DHF (Falconar 1997; Lin et al. 2006; Srikiatkhacorn 2009; Tan et al. 2009). Moreover, anti-NS1 Abs have been shown to induce
apoptosis in human ECs (Lin et al. 2002). The findings of this thesis show that Abs against the NS1 antigen caused a marked up-regulation of HO-1 gene expression in HUVECs, which was mediated via a redox-dependent PI3K/Akt-dependent regulatory pathway (Figure 1 in (Immenschuh et al. 2013)) that involved specific interaction of anti-NS1 with PDI on human ECs. Importantly, anti-NS1 also caused apoptosis in the applied experimental system of HUVECs, which is in agreement with earlier findings by others (Lin et al. 2002) and specific up-regulation of HO-1 by cobalt-PPIX counteracted, whereas inhibition with tin-mesoporphyrin increased anti-NS1 mediated apoptosis in ECs (Figure 7 in (Immenschuh et al. 2013)). Whether such protective effects of HO-1 up-regulation against the pathological effects of anti-NS1 Abs in human ECs might also have clinical implications for potential therapies in DHF patients, remains to be established. Thus, a near term goal could be to investigate the efficiency of targeted up-regulation of endothelial HO-1 in an \textit{in vivo} animal model of DHF.

\textbf{HLA class I Abs and AMR in solid organ transplantation}

AMR is the major limiting factor for long-term graft survival after solid organ transplantation (Nankivell and Alexander 2010; Kittleson and Kobashigawa 2012; Wood and Goto 2012; Djamali et al. 2014). The endothelium of transplanted organs such as kidney and heart plays a key role in the pathogenesis of AMR (Zhang and Reed 2009; Taflin et al. 2011), because it is targeted by Abs, which are directed against HLA and/or non-HLA molecules on ECs (Colvin and Smith 2005; Sumitran-Holgersson 2008). It is well established that HLA Abs can cause EC injury by complement fixation (Colvin and Smith 2005; Wehner et al. 2007), but more recently, complement-independent effects of HLA Abs have also been implicated in AMR. Although the mechanisms of HLA Abs, which are not mediated by complement fixation, are not well understood, activation of ECs by interactions with HLA I Abs appears to play major
role in these regulatory events (Zhang and Reed 2009; Taflin et al. 2011). EC activation is critically involved in controlling the inflammatory response (Pober and Sessa 2007) and is characterized by alterations in intracellular signaling, which up-regulates expression of inducible adhesion molecules and chemokines (Xiao et al. 2014). The coordinated interplay of these complex endothelial regulation modulates recruitment of leukocytes to the site of inflammation (Muller 2011; Nourshargh and Alon 2014).

In contrast to anti-NS1 Abs, Abs against HLA class I molecules did not appreciably affect HO-1 gene expression in HUVECs (data not shown). These findings are in contradiction to previous observations by others, who have shown that HLA class I Abs induce HO-1 expression (Narayanan et al. 2004). Further studies are currently underway to resolve these conflicting observations, which might be explained by additional stimulatory factors such as low levels of endotoxin that affect activation of ECs. In accordance with previous findings the data of the current thesis indicate that HLA I Abs caused EC activation (Zilian et al., submitted). This is demonstrated by the up-regulation of VCAM-1 and a number of other inducible pro-inflammatory genes such as ICAM-1, IL-8 and MCP-1. Up-regulation of these genes was reduced by up-regulation of HO-1 via cobalt-PPIX and was increased by inhibition of HO-1 via zinc-PPIX (Zilian et al, submitted). Similarly, it has been demonstrated in an earlier report, that targeted HO-1 up-regulation counteracted TNF-α-dependent up-regulation of various inducible adhesion molecules in cell cultures of bovine ECs (Soares et al. 2004). Various molecular mechanisms that may mediate these anti-inflammatory effects of HO-1 in ECs have previously been demonstrated by others (Soares et al. 2004; Silva et al. 2006; Seldon et al. 2007; Gozzelino et al. 2010).
Potential therapeutic applications of targeted modulation of endothelial HO-1

Up-regulation of endothelial HO-1 might not only afford alternative therapeutic options in the treatment of DHF and AMR after solid organ transplantation, but may also be applicable in a number of other inflammatory vascular diseases such as atherosclerosis or autoimmune vasculitis (Immenschuh and Ramadori 2000; Paine et al. 2010; Wu et al. 2011). Therefore, compounds that up-regulate HO-1 in an EC-specific manner might have a major potential to provide therapeutic effects in such disorders. For example, pharmaceuticals that have previously been shown to induce HO-1 in the endothelium (Grosser et al. 2004; Lee et al. 2004; Calay and Mason 2014) and might be candidates for such therapeutic interventions are hydroxymethyl-glutaryl coenzyme A reductase inhibitors (statins). HO-1 induction by statins has previously been shown to exhibit pleiotropic effects including antioxidant activity. Therefore, induction of endothelial HO-1 by such compounds could be a feasible therapeutic approach for treatment of a variety of vascular inflammatory disorders. Previously, other compounds, which induce HO-1 via redox-independent pathways in ECs, have been shown to provide protection against atherosclerosis (Wu et al. 2006; Wu et al. 2012).

Conclusion and Outlook

Specific up-regulation of HO-1 in the endothelium is a promising approach for therapeutic interventions in various vascular inflammatory diseases. Therefore, a better understanding of the underlying mechanisms that govern the regulation of HO-1 in ECs and identification of further pharmacological compounds for specific induction of endothelial HO-1 might help to develop novel therapeutic strategies for the treatment of such disorders.
Chapter 6  Summary

PECAM-1-Dependent Regulation and Protective Effects of Heme Oxygenase-1 in the Endothelium

Hendry Tri Sakti Surya Gunawan Saragih

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation, which has antioxidant and anti-inflammatory effects. HO-1 affords protection against various inflammatory vascular disorders via its functions in the endothelium. In the current thesis it was hypothesized, 1) that HO-1 gene expression is regulated by the endothelial surface adhesion receptor platelet endothelial cell adhesion molecule-1 (PECAM-1)(CD31) in an endothelial cell (EC) type-preferential manner, and 2) that specific up-regulation of endothelial HO-1 provides protection against the pathological effects of anti-EC antibodies (Abs) that are directed against the dengue virus (DV) non-structural 1 (NS1) antigen and against human leukocyte antigen (HLA) class I molecules. In the first part of the thesis it is demonstrated that knockdown of PECAM-1 in ECs up-regulated HO-1, but not other stress-inducible genes. HO-1 gene regulation by PECAM-1 was mediated via a redox-dependent pathway, which involved the master antioxidant transcriptional regulator nuclear factor-erythroid 2-related factor 2 (Nrf2) as demonstrated by findings in PECAM-1-deficient human umbilical vein endothelial cells (HUVECs) and in two PECAM-1 overexpressing cell lines. Moreover, interaction of PECAM-1 with a protein complex of the leukocyte surface molecule NB1 (CD177) and the serine protease proteinase 3 markedly up-regulated HO-1 expression in ECs. In the second part of the thesis it is shown that Abs against the NS1 antigen (anti-NS1) caused a marked up-regulation of HO-1 gene expression in cell cultures of HUVECs. HO-1 induction by anti-NS1 was regulated via a redox-dependent phosphatidylinositol-3-kinase/Akt-mediated pathway involving the interaction of anti-NS1 with protein disulfide isomerase on the surface of human ECs. Anti-
NS1 caused apoptosis in cell cultures of HUVECs and up-regulation of HO-1 by cobalt-protoporphyrin IX (PPIX) counteracted, whereas inhibition of HO-1 with tin-mesoporphyrin increased anti-NS1 mediated apoptosis in ECs. Independently, it is also demonstrated that Abs against HLA class I molecules caused EC activation as indicated by the up-regulation of vascular cell adhesion molecule (VCAM)-1 and other inducible proinflammatory genes including intercellular adhesion molecule (ICAM)-1, interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 in human ECs. HLA I Ab-dependent induction of these proinflammatory genes was inhibited by up-regulation via cobalt-PPIX, and increased by blockage of HO-1 via zinc-PPIX, respectively. In conclusion, targeted up-regulation of HO-1 in the endothelium could be a promising approach for therapeutic interventions in vascular inflammatory disorders. Thus, a better understanding of the regulatory mechanisms of endothelial HO-1 will help to develop novel therapeutic strategies for the treatment of such diseases.
Chapter 7 Zusammenfassung

PECAM-1 vermittelte Regulation und Schutzeffekte von Häm Oxygenase-1 im Endothel

Hendry Tri Sakti Surya Gunawan Saragih

Häm Oxygenase (HO)-1 stellt die induzierbare Isoform des Schrittmachерenzyms des Hämabbau dar, die nicht nur antioxidative, sondern auch antientzündliche Effekte aufweist. Die HO-1 hat protektive Effekte bei entzündlichen Gefäßerkrankungen, die über ihre spezifischen Funktionen im Endothel vermittelt werden. In der vorliegenden Arbeit wurden die Hypothesen überprüft: 1) ob die Genexpression der HO-1 durch den endothelialen Oberflächenrezeptor Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)(CD31) in einer zell-präferentiellen Weise reguliert wird; 2) ob die spezifische Heraufregulation der endothelialen HO-1 gegen die pathologischen Effekte von anti-endothelialen Antikörpern (Ak), die gegen das Denguevirus (DV) non-structural-1 (NS1) Antigen und gegen humane Leukozyten Antigen (HLA) Klasse I Moleküle gerichtet sind, geschützt. Im ersten Teil der Promotionsarbeit wird gezeigt, dass durch gezielten knockdown von PECAM-1 in Endothelzellen die Genexpression der HO-1, nicht jedoch anderer stress-induzierbarer Gene hochreguliert wird. Die PECAM-1-abhängige HO-1 Genregulation wird über einen redox-abhängigen Signalweg unter Beteiligung des antioxidativen wirksamen Transkriptionsfaktors Nuclear factor erythroid 2-related factor 2 (Nrf2) vermittelt, was in PECAM-1-defizienten humanen Umbilikalvenen Endothelzellen (HUVECs) und in zwei PECAM-1 überexprimierenden Zelllinien gezeigt werden konnte. Weiterhin wurde die HO-1 Genexpression durch die Interaktion von PECAM-1 mit einem Proteinkomplex aus dem Leukozytenoberflächenmolekül NB1 (CD177) und der Serinprotease Proteinase 3 in Endothelzellen heraufreguliert. Im zweiten Teil der vorliegenden Promotionsarbeit wird gezeigt, dass Ak gegen das DV NS1 Antigen (Anti-NS1) zu einer deutlichen Heraufregulation der HO-1 Genexpression in Zellkulturen von HUVECs führte. Diese HO-1 Induktion wurde
über einen redox-abhängigen Phosphatidylinositol-3-Kinase/Akt-abhängigen Signalweg vermittelt und beinhaltete die Wechselwirkung von Anti-NS1 mit dem Enzym Proteindisulfid Isomerase auf der Oberfläche von humanen Endothelzellen. Darüberhinaus verursachte Anti-NS1 Apoptose in Zellkulturen von HUVECs. Heraufregulation der HO-1 durch Cobalt-Protoporphyrin IX (PPIX) schwächte die durch Anti-NS1 vermittelte Apoptose ab, während die gezielte Hemmung der HO-1 durch Zinn-Mesoporphyrin die Anti-NS1 abhängige Apoptose in Endothelzellen steigerte. Unabhängig hiervon konnte gezeigt werden, dass Ak gegen HLA Klasse I Moleküle zur Aktivierung von Endothelzellen führte, was anhand der Heraufregulation von Vascular cell adhesion molecule (VCAM)-1, Intercellular cell adhesion molecule (ICAM)-1, Interleukin (IL)-8 und Monocyte chemotactic protein (MCP)-1 in humanen Endothelzellen gezeigt werden konnte. Die HLA Ak-vermittelte Induktion dieser proinflamatorischen Gene wurde durch Heraufregulation der HO-1 durch Cobalt-PPIX gehemmt und durch die Blockierung der HO-1 durch Zink-PPIX verstärkt. Zusammenfassend lässt sich sagen, dass die gezielte Heraufregulation der HO-1 im Endothel eine vielversprechende Möglichkeit für therapeutische Interventionen bei entzündlichen Gefäßerkrankungen darstellt. Daher ist ein besseres Verständnis der regulatorischen Mechanismen der endothelialen HO-1 erstrebenswert, um neue therapeutische Strategien bei der Behandlung dieser Erkrankungen zu entwickeln.
Chapter 8 References


Chapter 9  Acknowledgements

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