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Heme oxygenase, inflammation, and fibrosis: the good, the bad, and the ugly?

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Upon injury, prolonged inflammation and oxidative stress may cause pathological wound healing and fibrosis, leading to formation of excessive scar tissue. Fibrogenesis can occur in most organs and tissues and may ultimately lead to organ dysfunction and failure. The underlying mechanisms of pathological wound healing still remain unclear, and are considered to be multifactorial, but so far, no efficient anti-fibrotic therapies exist. Extra- and intracellular levels of free heme may be increased in a variety of pathological conditions due to release from hemoproteins. Free heme possesses pro-inflammatory and oxidative properties, and may act as a danger signal. Effects of free heme may be counteracted by heme-binding proteins or by heme degradation. Heme is degraded by heme oxygenase (HO) that exists as two isoforms: inducible HO-1 and constitutively expressed HO-2. HO generates the effector molecules biliverdin/bilirubin, carbon monoxide, and free iron/ferritin. HO deficiency in mouse and man leads to exaggerated inflammation following mild insults, and accumulating epidemiological and preclinical studies support the widely recognized notion of the cytoprotective, anti-oxidative, and anti-inflammatory effects of the activity of the HO system and its effector molecules. In this review, we address the potential effects of targeted HO-1 induction or administration of HO-effector molecules as therapeutic targets in fibrotic conditions to counteract inflammatory and oxidative insults. This is exemplified by various clinically relevant conditions, such as hypertrophic scarring, chronic inflammatory liver disease, chronic pancreatitis, and chronic graft rejection in transplantation.

Keywords: heme oxygenase, heme, HO-effector molecules, fibrosis, therapy

INTRODUCTION

Tissue injury can occur after physical, chemical, or infectious insults, such as burns, trauma, and toxins. Wound healing is a dynamic and complex process involving the coordinated action of different cell types. The quality of the wound healing process is dependent on different factors, including the severity of the insult and the inflammatory and redox status of the wound. Pathological wound healing can lead to two extreme cases: chronic, non-healing wounds or fibrosis, which ultimately may lead to excessive scarring. Both types of complications related to pathological wound healing have major impact on patient quality of life in terms of aesthetical and functional problems.

Numerous clinical and experimental observations have demonstrated that prolonged inflammation and oxidative stress may cause pathological wound healing and the development of fibrosis. However, the underlying mechanisms still remain largely unclear. Inflammation is a complex response of the innate immune system in vascularized tissues initiated to protect the organism against invading pathogens as well as to restore tissue homeostasis at the wound site.

WOUND HEALING AND FIBROSIS – WHEN GOOD TURNS BAD

The primary goal of tissue repair is to restore tissue integrity and homeostasis. Hereto, wounding initiates a complex cascade

of events to stop blood loss, to eliminate invading pathogens, and, ultimately, to promote tissue integrity and homeostasis (Chettibi et al., 1999; Hunt et al., 2000).

Wound healing occurs in three distinct, but overlapping phases: (1) inflammation, (2) regeneration, and (3) remodeling, and involves a well-coordinated sequence of cellular responses.

The *inflammatory phase* is preceded by blood clot formation and coagulation to stop blood loss and to reduce pathogen invasion. This fibrin-containing clot also serves as an early provisional extracellular matrix (ECM) to provide structural support for cellular attachment and proliferation. Coagulation also triggers the production of pro-inflammatory agents, and activation of the complement system. This leads to increased blood vessel permeability, chemokine expression, vascular adhesion molecule expression, and recruitment of immune cells. At the wound site, granulocytes and macrophages are pivotal for the innate immune system-mediated elimination of invading pathogens through the generation of reactive oxygen species (ROS; Fialkow et al., 2007), and the production of pro-inflammatory chemokines and cytokines (Ryan et al., 2004).

The temporal and spatial pattern of inflammation resolution is crucial for proper wound healing and is characterized by reduced levels of pro-inflammatory adhesion molecules and cytokines and a decreasing number of inflammatory cells at the site of injury.

This is followed by the initiation of the *regenerative phase* that mediates re-epithelialization, neovascularization, and wound closure. In particular, fibroblasts produce and deposit ECM components to substitute the provisional matrix. Also, recruited keratinocytes and endothelial cells are crucial to this process.

When the wound area is fully re-epithelialized, *remodeling* takes over from regeneration. Fibroblasts differentiate into myofibroblasts causing wound contraction and ECM reorganization by synthesis and deposition of ECM components and by providing matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which ultimately leads to wound closure. At the end of this phase, myofibroblasts present in the granulation tissue disappear by apoptosis, producing a rather acellular scar. It is important to realize that scar formation is a natural part of the wound healing process, as normal scar tissue, which mainly consists of connective tissue, represents a stable restoration of the skin (for a recent review, see Guo and Dipietro, 2010).

Prolonged inflammatory conditions accompanied by oxidative stress may interfere with the normal wound healing process, leading to an extended presence of myofibroblasts and excessive scar formation, a process known as fibrosis. Fibrosis is not only restricted to dermal wound healing, but also occurs in palatal tissue, lungs, heart, liver, intestine, and joints, and causes major medical problems ranging from disfigurement to progressive disability and even death.

Despite the fact that disorders that are associated with fibrosis account for up to 45% of deaths in the developed world, no successful anti-fibrotic therapies exist (Wynn, 2008).

Extensive research has shown that the heme–heme oxygenase (HO) system is closely involved in the regulation of various (patho)physiological processes, in particular in cellular adaptation to oxidative stress, and the anti-inflammatory response.

In this review, we address the role of heme, HO-1 and HO-effector molecules in inflammation and fibrosis with an emphasis on hypertrophic scarring, chronic inflammatory liver disease, chronic pancreatitis, and transplantation complications.

INFLAMMATION AND SCARRING – WHEN BAD TURNS UGLY

A prolonged inflammatory phase is considered a major cause of fibrosis and excessive scar formation. A clear link between inflammation and fibrosis has been revealed through embryonic studies. Mammalian embryos heal without scar formation, if wound healing occurs without influx of inflammatory cells (Ashcroft et al., 1999), indicating that inflammatory cell recruitment to the wound site is a prerequisite for scar formation. Wound healing studies in different knockout and transgenic mouse models (Fathke et al., 2004; Duffield et al., 2005; Oakley et al., 2005; Tabibiazar et al., 2006; Saito et al., 2008; Goren et al., 2009), athymic mice (Gawronska-Kozak et al., 2006), and *in vivo* antisense knockdown studies (Mori et al., 2006, 2008; Mathew et al., 2007) further support this notion. Also, an overwhelming amount of data have shown that inflammatory cells emigrating from blood, such as granulocytes, macrophages, and helper T cells, provide signals that promote granulation and fibrosis, including ROS, ECM deposition, and chemokines and cytokines (reviewed in Martin and Leibovich, 2005).

Clinical data demonstrate elevated inflammatory profiles in patients with pathological scarring (Harty et al., 2003; Martin and Leibovich, 2005). Also, chronic wounds demonstrate impaired resolution of the inflammatory phase, and thus remain in a chronic inflammatory state (Loots et al., 1998). Time is a critical factor for abnormal scar formation, as long-lasting wound healing significantly increases the risk of hypertrophic or excessive scarring (Deitch et al., 1983).

The continuous presence of inflammatory mediators results in increased secretion of growth factors, extending the proliferative phase via the prolonged presence of myofibroblasts (Singer and Clark, 1999). This in turn causes excessive deposition of ECM components, exaggerating the outcome of the proliferative phase and contributing to hypertrophic scarring or fibrosis (Aarabi et al., 2007). Thus, the inflammatory cell-derived ROS generated during the early inflammatory phase must be tightly controlled by cellular anti-oxidative and anti-inflammatory systems, as impaired ROS clearance exacerbates wound healing (Frantz et al., 1993; Steiling et al., 1999).

The unifying hallmark of fibrotic disorders is abnormal and excessive deposition of ECM components. As exemplified in the following sections, the broad range of affected organs, the progressive and often irreversible nature of fibrosis, and the large number of affected patients combined with ineffective treatment poses a challenge to the development of novel therapeutic approaches toward limiting fibrosis.

HYPERTROPHIC SCARRING

The skin is the organ with the largest surface area in the body, functioning as a barrier against the external milieu, and is important in protecting the body against pathogens and dehydration. Other functions include temperature regulation, tactile sensation, and vitamin D synthesis.

Upon cutaneous injury, dermal wound healing is crucial for restoration of the damaged skin barrier. In most cases, wound healing will result in the formation of a visible scar. However, prolonged inflammation and high levels of oxidative stress (e.g., due to infections) may result in excessive deposition of predominantly collagen I (Sidgwick and Bayat, 2012), leading to fibrosis and excessive scarring that in skin is manifested as a hypertrophic or a keloid scar. Both keloids and hypertrophic scars are characterized by excessive collagen deposition and are discolored with a rough, stiff appearance, but whereas hypertrophic scars are confined to the original wound area, keloids overgrow these boundaries. Hypertrophic scars may occur in persons of any age and at any site, whereas keloids develop with higher risk in certain ethnic groups (Brissett and Sherris, 2001). Almost all abnormal scars are associated with prolonged inflammation caused by foreign body reaction, bacterial infections, tattoos, burns, injections, bites, cuts, vaccination, trauma, surgery, or infections. Importantly, immunological alterations have been reported in hypertrophic scars, including abnormal growth factor and cytokine levels (van der Veer et al., 2009).

Often patients report itching and pain as major issues besides the aesthetical and functional problems (Bock et al., 2006). These complications may require surgical corrections, which may then lead to further scarring complications.

FIBROSIS IN CHRONIC INFLAMMATORY LIVER DISEASE AND CHRONIC PANCREATITIS

Liver and pancreas are two major abdominal organs which have specific exocrine and endocrine functions within the gastrointestinal tract and in control of the body's metabolic homeostasis.

The liver is not only a major regulator of systemic metabolism but also has key functions in detoxification of endogenous and exogenous toxins. Hepatic fibrosis is mainly caused by chronic hepatitis B and C, alcoholic liver disease, and fatty liver disease/non-alcoholic steatohepatitis (extensively reviewed in Hernandez-Gea and Friedman, 2011). Albeit from different origins, these disorders have similar early pro-inflammatory features, which can progress into hepatic fibrosis and ultimately into end stage liver cirrhosis. Hepatic fibrosis can be regarded as a result of the hepatic wound healing response to repeated injury. If hepatic injury sustains, failure of liver regeneration occurs and results in fibrosis, in which activated hepatic stellate cells (HSCs) play a key role via excessive production of ECM components. More specifically, HSC activation is the result of a complex sequence of events such as secretion of pro-inflammatory cytokines by other hepatic cells such as liver tissue macrophages (Kupffer cells). Ultimately, hepatic fibrosis and cirrhosis lead to a replacement of healthy hepatic tissue with scars and regenerative nodules, causing loss of liver function.

The pancreas is a major gland organ and has crucial exocrine functions in the gastrointestinal digestive tract. In addition, it is a major endocrine organ and produces the glucose-regulating peptide hormones insulin and glucagon. Chronic pancreatitis is a progressive inflammatory disease (Witt et al., 2007), which is most commonly caused by chronic alcoholism or is of idiopathic origin. Similar to the liver, pancreatic stellate cells (PSCs) play a key role in pancreatic fibrogenesis (Omary et al., 2007). Normally, PSCs regulate the synthesis and degradation of ECM proteins and, thereby, the maintenance of the healthy tissue architecture. Upon pancreatic injury, PSCs differentiate into an activated phenotype which is secreting excessive amounts of ECM components and leads to the formation of fibrotic tissue (Omary et al., 2007). Persistent inflammation of the pancreas causes permanent structural deterioration with tissue fibrosis and ductal obstruction, and subsequently irreversible, declined exocrine and endocrine function (pancreatic insufficiency).

FIBROSIS ASSOCIATED WITH GRAFT REJECTION

During transplantation, cells, tissues, or organs are transferred between organisms from one individual to another or from one site to another in the same individual to correct the loss or dysfunction of an organ (e.g., kidney, liver, skin). A major problem in transplantation biology is the immunological barrier between donor and recipient, which may cause graft rejection. Based on clinical and pathological criteria, graft rejection has been classified into three major forms: hyperacute, acute, and chronic graft rejection (Azimzadeh et al., 2011). Hyperacute rejection starts within minutes of transplantation and exhibits thrombosis of graft vessels and ischemic necrosis due to circulating recipient antibodies, and organ failure occurs hours after transplantation. Moreover, acute graft rejection generally occurs within days or weeks after organ transplantation and leads to graft failure within the first

year post-transplantation. This form of rejection has been primarily linked to a T cell-mediated reaction of graft destruction. Finally, chronic transplant rejection (CTR) is less well defined and results from multifactorial, pathological events, involving both immunological and non-immunological factors. Immune cells such as T cells and macrophages, chemokines, pro-inflammatory cytokines, and allo-antibodies have all been linked to the initiation and progression of the rejection process (Cornell et al., 2008; Ashoor and Najafian, 2012). Organs subjected to CTR generally display fibrotic scarring and vascular damage due to transplant vasculopathy (TV), which has similar features to atherosclerosis (Mitchell, 2009; Racusen and Regele, 2010).

Together, impaired resolution of inflammation and/or elevated levels of ROS may facilitate fibrosis and scar formation. It is now commonly accepted that the heme-HO system is a key player in the control of the inflammation and oxidative stress (Keyse and Tyrrell, 1989; Willis et al., 1996; Rushworth and O'Connell, 2004; Pae et al., 2008).

THE HEME-HO SYSTEM

The microsomal enzyme heme oxygenase (HO) catalyzes the oxidative degradation of free heme, and generates carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin (Tenhunen et al., 1968; Maines, 1988). Two genetically distinct HO isoforms exist: an inducible form, HO-1, and the constitutively expressed HO-2 (Immenschuh and Ramadori, 2000). Accumulating data demonstrate that the HO enzymes execute anti-inflammatory, anti-apoptotic, and anti-proliferative functions through the effector molecules generated by heme catabolism (reviewed in Willis et al., 1996; Abraham and Kappas, 2008). Biliverdin is almost instantaneously converted into bilirubin by biliverdin reductase, and the free iron is rapidly scavenged by co-induced ferritin (Wagener et al., 2003a). CO, Fe^{2+} /ferritin, and biliverdin/bilirubin affect different biological processes (Abraham and Kappas, 2008), including resolution of inflammation (Willoughby et al., 2000); however, the executed effects depend on the generated amounts and the microenvironment (Wagener et al., 2003b).

HEME AS A MOLECULAR SWITCH

Heme (iron protoporphyrin IX) is composed of an iron atom conjugated to a porphyrin group. Heme is synthesized by every mammalian cell, and enables in physiological concentrations a wide range of essential biological functions, by acting as the prosthetic group for hemoproteins (hemoglobin, cyclooxygenases, peroxidases) and cellular signaling (reviewed in Wagener et al., 2003b).

However, upon injury, free heme is released from hemoproteins, causing severe tissue damage (Nath et al., 1995; Balla et al., 2000; Ryter and Tyrrell, 2000; Jeney et al., 2002), predominantly by generating ROS through the Fenton reaction (Halliwell and Gutteridge, 1984) and oxidative modifications of proteins (Nath et al., 1995; Jeney et al., 2002). Also, local accumulation of high levels of free heme, e.g., in blood clots, or atherosclerotic lesions (Hasan and Schafer, 2008), overwhelms cellular detoxification systems by prolonged oxidative stress, which may cause ROS-dependent oxidation of lipids, proteins, and DNA, subsequently damaging cells and tissues (Balla et al., 1991, 1993; Jeney et al., 2002).

Furthermore, accumulating evidence shows that heme besides being a pro-oxidant in high concentration also possesses pro-inflammatory properties, because heme increases the expression of vascular adhesion molecules ICAM-1, VCAM-1, and E-selectin (Wagener et al., 1997, 1999, 2001a; Tolosano et al., 2002; Belcher et al., 2006), upregulates vascular permeability (Wagener et al., 2001b), promotes leukocyte recruitment (Wagener et al., 2001b; Porto et al., 2007), and induces the release of pro-inflammatory cytokines (Natarajan et al., 2007; Cosgrove et al., 2011; Hao et al., 2011). Administration of large amounts of heme has been demonstrated to not only result in oxidative and inflammatory stress (Wagener et al., 2001a,b; Tolosano et al., 2002), but also to exacerbate different disease settings (Pamplona et al., 2007; Seixas et al., 2009; Larsen et al., 2010). Together, this heme-induced injury has been associated with pathological manifestations of different conditions, e.g., malaria (Pamplona et al., 2007). Interestingly, increased heme levels have also been associated with fibrosis formation (Tolosano et al., 2002; Kovtunovych et al., 2010).

Furthermore, independent studies have supported a role for heme in wound healing. In moderate concentrations, heme stimulates vasoconstriction, pro-coagulation, complement activation, platelet aggregation, and cell differentiation (Nakajima et al., 1999). Also, low concentrations of free heme down-regulates the levels of pro-inflammatory cytokines, contributing to the resolution of inflammation, likely by inducing HO-1 expression (Ma et al., 2007; Cambos et al., 2010; Cambos and Scorza, 2011).

Consequently, heme can be considered as a molecular switch, since different concentrations of free heme generate different cellular responses (Wagener et al., 2003a). In small amounts, it provides essential cellular functions and cytoprotection via HO-1 induction, whereas in high concentrations, free heme can cause severe tissue injury. It is thus of importance to control the levels of free heme at sites of injury.

THE HEME–HO SYSTEM AND INFLAMMATION – FRIENDS OR FOE?

The dose-dependency of the effects mediated by free heme underscores the importance of proper control of the cellular levels hereof by the HO system.

High levels of free heme in the vascular system have been suggested to be involved in the initiation and progression of atherosclerosis, a chronic inflammatory vascular disease with fibrotic plaque pathology (Hasan and Schafer, 2008). Heme-induced oxidative stress induces expression of immediate early gene early growth response (Egr)-1 protein (Hasan and Schafer, 2008) that has been directly linked to vascular pathologies (McCaffrey et al., 2000; Blaschke et al., 2004). Importantly, this induction is inhibited by CO (Hasan and Schafer, 2008), underscoring the importance of the heme–HO feedback loop as a mechanism to counteract pathological levels of free heme. Heme also triggers vascular pro-inflammatory processes by promoting foam cell formation through oxidative modification of low-density lipoprotein (LDL) and apolipoprotein B100, which are major risk factors for the development of atherosclerosis (Tsimikas and Miller, 2011).

In contrast, free heme is an inducer of the expression of stress-sensitive genes, including HO-1, ferritin, Hsp70 as well as chemokines, and adhesion molecules (Theodorakis et al., 1989; Wagener et al., 1997; Kanakiriya et al., 2003; Iwasaki et al., 2006).

Also, the local release of large amounts of free heme upon injury induces inflammatory processes, suggesting that heme acts as a danger signal (Wagener et al., 2003a; Figueiredo et al., 2007).

Furthermore, the induction of HO-1 by increased levels of heme at the site of injury also functions as a feedback system by improving the anti-inflammatory response, as HO activity counteracts a diverse range of cellular stresses (Abraham et al., 1988).

Recently, we and others have demonstrated a clear link between HO activity and diverse (pathological) cellular processes, as HO activity has been shown to be cytoprotective and anti-apoptotic as well as to reduce oxidative stress and inflammation in a multitude of different cellular and rodent models (Willis et al., 1996; Soares et al., 1998; Brouard et al., 2000; Rucker et al., 2001; Wagener et al., 2003a, 2010; Ryter and Choi, 2009; Gozzelino et al., 2010). These effects are mediated through the actions of the effector molecules generated by HO activity (Abraham and Kappas, 2008), as biliverdin and bilirubin possess strong anti-oxidant properties and CO is implicated in different signaling cascades and vasodilatation (Siow et al., 1999).

Moreover, overexpression of HO-1 counteracts the cytotoxic, pro-oxidative and pro-inflammatory effects caused by heme via down-regulating inflammatory adhesion molecules and abrogating tissue influx of leukocytes (Wagener et al., 1999, 2001b, 2003b; Rucker et al., 2001). On the contrary, inhibition of HO activity intensifies the heme-mediated oxidative and inflammatory injury *in vitro* and *in vivo* (Hayashi et al., 1999; Vachharajani et al., 2000; Rucker et al., 2001; Takahashi et al., 2007, 2009; **Figure 1**).

This is further exemplified by studies in HO-1 knockout mice and a patient with genetic HO-1 deficiency, in which an increased expression of vascular adhesion molecules and more severe inflammation was observed (Yachie et al., 1999; Kawashima et al., 2002; Wagener et al., 2003a; Kartikasari et al., 2009; Ferenbach et al., 2010; Radhakrishnan et al., 2011). Recent studies also

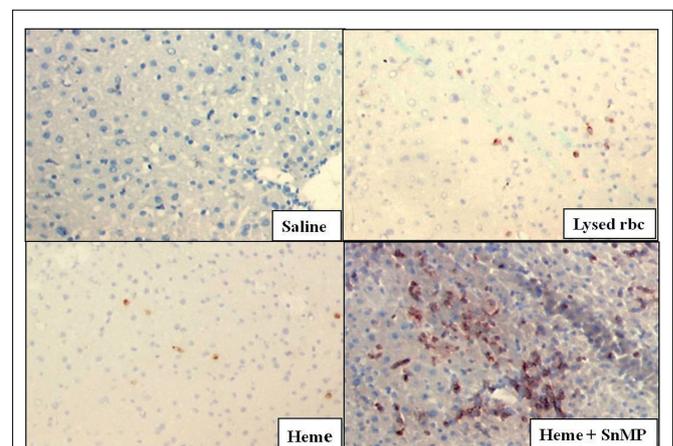


FIGURE 1 | One hour after administration of heme (750 μ M) or lysed red blood cells (rbc) to mouse livers, influx of leukocytes was evident by immunohistological staining. After saline administration, no leukocytes were detected. Importantly, inhibition of HO activity before heme exposure (24 h pretreatment, SnMP) exacerbated leukocyte influx (for more details, see Wagener et al., 2001b).

suggest that HO-2 is important for the resolution of inflammation, as HO-2 deficient mice demonstrate slower corneal epithelial wound healing and an amplified inflammatory response (Bellner et al., 2008, 2009, 2011; Halilovic et al., 2011; Marrazzo et al., 2011).

In contrast, transgenic HO-1 mice are better protected from oxidative stress, inflammation, and vascular dysfunction as well as fibrotic tissue formation post-infarction (Wang et al., 2010).

Also, clinical studies have suggested that swift activation of HO-1 gene expression might be important to cope with cellular stresses. Several HO-1 gene promoter polymorphisms have been discovered, determining the levels of HO-1 induction in humans (Exner et al., 2004). The clinical relevance of the HO-1 promoter variability has been demonstrated in independent studies and higher HO-1 induction has been associated with lower incidence for cardiovascular and inflammatory diseases (Wagener et al., 2008; Grochot-Przeczek et al., 2012).

Thus the degradation of the pro-inflammatory and pro-oxidative heme molecules by HO, the signaling actions of CO, the anti-oxidant properties of bilirubin combined with the scavenging of the pro-oxidant free iron by ferritin may all contribute to the anti-inflammatory effects of the HO system.

By degrading heme, the HO system efficiently converts the pro-oxidative and pro-inflammatory heme molecule into anti-inflammatory and anti-oxidant molecules, thereby contributing to improved wound healing. This suggests that the heme–HO system executes regulatory functions in a diverse range of cellular processes with a direct influence on a timely execution of the inflammatory phase, including resolution of inflammation, apoptosis, and proliferation. Differential protection against oxidative and pro-inflammatory insults may also explain clinical differences in fibrogenesis.

PHARMACOLOGICAL REGULATION OF HO-1 AND ITS EFFECTOR MOLECULES AS THERAPEUTIC TARGETS IN INFLAMMATION AND FIBROSIS

A rapidly growing body of experimental evidence shows that specific overexpression of HO-1 has protective effects in inflammation and fibrosis (Bauer et al., 2008). Accordingly, pharmacological induction of HO-1 or administration of its effector molecules may be a promising therapeutic approach for the treatment of inflammatory disorders associated with fibrosis in various organs. However, to apply HO-1 for specific therapeutic interventions a number of important issues have to be taken into consideration.

ANTI-INFLAMMATORY EFFECTS OF HO-1 ARE MEDIATED IN A CELL TYPE-SPECIFIC MANNER

HO-1 gene expression is up-regulated in a wide range of cell types and tissues. The specific anti-inflammatory effects of HO-1, however, seem to be dependent on its coordinate up-regulation in a cell type- and cell-context-specific manner. Notably, the regulation of HO-1 expression in mononuclear/myeloid and in endothelial cells appears to be of major importance for the immunomodulatory and anti-inflammatory functions of this enzyme.

The key immunomodulatory role of HO-1 in mononuclear cells has been shown in conditional knockout mice, in which the HO-1 gene has been specifically deleted in myeloid cells. In this report, various pathological immune reactions in experimental

infectious and autoimmune conditions have been demonstrated *in vivo* (Tzima et al., 2009). HO-1 has also been demonstrated to play a major role in the function of dendritic cells, which are a bone marrow-derived myeloid cell population involved in mediating adaptive immune responses (Remy et al., 2009; Park et al., 2010). Moreover, HO-1 appears to be of major significance for modulating inflammatory responses in endothelial cells. This has been shown in the initial reports on HO-1 knockout mice (Poss and Tonegawa, 1997) and in the first human case of genetic HO-1 deficiency (Yachie et al., 1999). In either case, the endothelium revealed specific pathological alterations, which have been ascribed to an increased endothelial sensitivity to oxidative stress. Accordingly, it was reported that genetic HO-1 deficiency exhibited phenotypic alterations of the vascular endothelium, causing accelerated formation of arterial thrombosis (True et al., 2007).

REGULATION OF HO-1 GENE EXPRESSION

To apply HO-1 induction by specific pharmacological compounds for therapeutic interventions, it is not only important to understand the physiological functions of HO-1 and its products.

It is also important to understand the specific regulatory pathways that up-regulate HO-1. Although primarily known to be induced by oxidative stress, HO-1 is also up-regulated by a variety of stress-independent stimuli including various well-known pharmacologic compounds (Immenschuh and Ramadori, 2000; Li et al., 2007). Regulation of HO-1 is mainly, but not exclusively governed on the transcriptional level. Accordingly, a large variety of transcription factors has been shown to be involved in HO-1 induction. In particular, redox-dependent transcription factors such as Nrf2, which is a master regulator of the anti-oxidant response, activator protein-1 (AP-1) and nuclear factor (NF)- κ B, but also other nuclear factors such as Egr-1, upstream regulatory factor-2 (USF-2), and specificity protein (SP)-1 have been shown to mediate HO-1 induction (Ryter et al., 2006; Paine et al., 2010). A key role in governing HO-1 expression has also been recognized for the transcriptional repressor Bach1. Bach1 is a heme-binding protein, the activity of which is primarily regulated via intracellular levels of free heme (Ogawa et al., 2001; Igarashi and Sun, 2006). As both, Bach1 and Nrf2, interact with the anti-oxidant response element (ARE), which is the major *cis*-acting regulatory DNA element in the HO-1 gene promoter, the interplay of these two nuclear factors appears to be of major importance for inducible HO-1 gene expression. Whether and how Bach1 mediates the specific regulation of HO-1 if compared to other ARE-dependent stress response genes such as (NADPH):oxidoreductase-1 (NQO1) or glutathione-S-transferase (GST)-1 is a subject for currently ongoing investigations (Dhakshinamoorthy et al., 2005; MacLeod et al., 2009; Okada et al., 2010). The complexity of HO-1 gene regulation is further illustrated by the array of signaling cascades including protein kinase Cs (PKCs) and mitogen-activated protein kinases (MAPKs) that are involved in the regulation of this gene (for a review, see Paine et al., 2010). In addition, some of these pathways such as p38 MAPK appear to have contradictory cell-specific regulatory effects on HO-1 gene expression (Naidu et al., 2009) indicating that HO-1 is governed by a highly complex interplay of various signaling modules. Macrophage-specific up-regulation of HO-1 gene expression in inflammation has been

demonstrated in various experimental models (Paine et al., 2010). As an example, HO-1 in liver tissue macrophages (Kupffer cells) is up-regulated in response to the cytokine adiponectin via an IL-10 dependent pathway (Mandal et al., 2010). Moreover, macrophage-specific induction of HO-1 by various toll-like receptors (TLR) ligands, including lipopolysaccharides (LPS), requires activation of the Tec kinase Bruton's tyrosine kinase (Vijayan et al., 2011).

TARGETED PHARMACOLOGICAL HO-1 INDUCTION AS THERAPY FOR INFLAMMATION AND FIBROSIS

Numerous compounds have been shown to provide therapeutic effects via HO-1 induction. However, rather than giving an exhaustive list of compounds that may up-regulate HO-1 for therapeutic purposes in inflammatory disorders, we refer to more specific overviews on this topic (Li et al., 2007; Abraham and Kappas, 2008; Vijayan et al., 2010).

Briefly, cobalt-protoporphyrin (CoPPiX), which has been extensively applied in various experimental models to induce HO-1, does not seem an ideal compound for targeted therapeutic HO-1 up-regulation due to possible toxicity. Interestingly, systemic administration of heme (e.g., heme arginate) to healthy individuals has been shown to increase HO-1 levels in serum (Doberer et al., 2010), and has been used in the clinics for years in the treatment of porphyria (Tenhunen and Mustajoki, 1998; Ma et al., 2011). More research is warranted to explore the potential induction of systemic HO activity in the human setting by heme arginate.

Natural products such as quercetin or theaflavin (Loke et al., 2008, 2010) are less toxic and may be more appropriate for potential therapeutic applications in inflammatory disorders. Interestingly, Loke et al. (2010) recently demonstrated increased expression of endothelial HO-1 in aortic lesions and subsequently attenuation of aortic lesion formation in quercetin-fed ApoE knockout mice, underscoring the potential of targeted HO-1 induction in clinical therapies.

Curcumin is another naturally occurring compound with a broad range of pharmacological activities, including anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-diabetic, and anti-viral effects (Motterlini et al., 2000; McNally et al., 2007), mediating cellular protection against ROS (Barzegar and Moosavi-Movahedi, 2011; Wang et al., 2012; Yin et al., 2012) by up-regulating HO-1 through Nrf2 (Balogun et al., 2003). Recent studies have shown beneficial effects of curcumin in a liver injury model (Cerny et al., 2011), and curcumin treatment reduced radiation-induced lung fibrosis (Lee et al., 2010).

Moreover, macrophage-specific (Wijayanti et al., 2005) HO-1 induction by defined compounds such as 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and endothelial cell-specific HO-1 induction by statins (Grosser et al., 2004; Lee et al., 2004), might be useful for such therapeutic applications.

Another key issue that needs attention when applying HO-1 induction for therapeutic applications deals with the question at what stage of the inflammatory phase HO-1 should be induced to afford its salutary protective effects. Importantly, HO-1 induction after the onset of an inflammatory disorder or in full-blown inflammation seems to be not effective in certain circumstances. This has been shown in an experimental model of dextran-sulfate-

induced colitis, in which HO-1 failed to have anti-inflammatory effects if induced after the onset of this disease (Paul et al., 2005) or in an experimental model of acute pancreatitis (Nakamichi et al., 2005). Therefore, the time point of targeted HO-1 induction for therapeutic applications needs further attention. Alternatively, administration of HO-effector molecules may mediate more direct anti-inflammatory effects.

EXPOSURE TO HO-EFFECTOR MOLECULES CO AND BILIRUBIN: POSSIBLE AMELIORATING EFFECTS ON INFLAMMATION AND FIBROSIS

A wide array of preclinical and epidemiological evidence suggests that the protective properties of HO can also be mediated via its effector molecules bilirubin, CO, and co-induced ferritin. In inflammatory and fibrotic models both CO and bilirubin demonstrated significant protection (recently reviewed in Grochot-Przeczek et al., 2012).

CO exerts its cytoprotective effects through different mechanisms, including anti-inflammatory (Neto et al., 2006; Chora et al., 2007), vasodilatory (Sammur et al., 1998), anti-coagulative (Chlopicki et al., 2006), anti-apoptotic (Wang et al., 2007), and anti-fibrotic pathways (Neto et al., 2006). CO deliverance is achieved through inhalation (Mayr et al., 2005; Moore et al., 2005), CO saturated physiological solutions (Nakao et al., 2006), and CO-releasing molecules (CO-RMs; Motterlini et al., 2002).

Interestingly, there are recent attempts to translate the promising findings with CO inhalation and administration of CO-RMs toward the human setting (Motterlini and Otterbein, 2010). The CO-RMs have been demonstrated to provide cytoprotective effects in different *in vitro* (Clark et al., 2003; Sandouka et al., 2005; Sawle et al., 2005; Srisook et al., 2006; Zobi et al., 2010) and *in vivo* (Vera et al., 2005; Sandouka et al., 2006; Zhou et al., 2009; Wei et al., 2010) models, which urge for translation toward the clinic.

However, the potential safety issues with these compounds must be thoroughly addressed. CO-RMs are complexes of heavy metal, like nickel, cobalt, iron, or ruthenium surrounded by carbonyl groups that are released as CO under the appropriate conditions (Motterlini et al., 2002, 2003). Ruthenium-based CO-RMs have recently been suggested to generate cytotoxic, CO-depleted by-products *in vitro* (Winburn et al., 2012). However, the development of water-soluble CO-RMs (Clark et al., 2003; Foresti et al., 2004), the use of other transition metals, e.g., manganese, in the chemical structure of CO-RMs (Motterlini et al., 2002; Crook et al., 2011), and even the use of CO-RMs devoid of transition metals (Motterlini et al., 2005) may promote the development of clinically safe CO-RMs due to less confounding effects.

Bilirubin is another generated HO-effector molecule with promising clinical applications. Besides being a powerful anti-oxidant (Stocker et al., 1987), several studies have shown that biliverdin/bilirubin administration has cytoprotective effects during ischemia-reperfusion injury and graft rejection after transplantation (Clark et al., 2000; Fondevila et al., 2004). Also, bilirubin exerts immunomodulatory and anti-inflammatory effects (Willis et al., 1996) by down-regulating expression of inflammatory cytokines and adhesion molecules (Nakao et al., 2004), as well as by reducing immune cell infiltration (Hayashi et al., 1999; Nakao et al., 2004).

Individuals with Gilbert's syndrome have a polymorphism in the UGT1A1 promoter and are protected against cardiovascular complications (Schwertner and Vitek, 2008). This polymorphism results in slower glucuronidation and therefore diminished excretion of bilirubin, leading to elevated bilirubin levels. Unfortunately, bilirubin is not available for human use. We and others postulated that experimentally induced mild hyperbilirubinemia would mimic the positive effects seen in Gilbert's syndrome (McCarty, 2007; Dekker et al., 2011). In a double-blind, placebo-controlled crossover design, we demonstrated that elevating bilirubin levels using the HIV protease inhibitor atazanavir indeed ameliorates vascular function in type 2 diabetes mellitus patients (Dekker et al., 2011). In addition, the redox status in these patients was improved following induction of mild hyperbilirubinemia (Dekker et al., 2011). This demonstrates for the first time the potential beneficial power of bilirubin as a novel mechanism in humans. We expect that these clinically significant protective effects of HO-effector molecules in humans strongly encourage more translational research toward the protective properties of HO and its effector molecules in inflammatory and fibrotic conditions.

Finally, three examples of inflammatory disorders, which are linked to organ fibrosis and in which HO-1 and its effector molecules may be of therapeutic use, are discussed in more detail.

The heme–HO system in hypertrophic scarring

Except for fast healing wounds, such as shallow scratches, most wounds will result in visible scar formation, which is a natural part of the wound healing process. However, in some individuals – depending on geographic and ethnographic distribution as well as the character of the injury – excessive deposition of ECM occurs, resulting in the formation of a raised, discolored hypertrophic scar.

The involvement of the HO system is evident from knockout mouse models being devoid of HO-1 or HO-2, respectively. Both models demonstrate delayed wound healing due to poor response toward inflammatory and oxidative insults (Braun et al., 2002; Seta et al., 2006; Deshane et al., 2007; Bellner et al., 2008, 2009, 2011; Patil et al., 2008; Kovtunovych et al., 2010; Halilovic et al., 2011). Also, pharmacological inhibition of HO activity has a negative impact on dermal wound healing (Grochot-Przeczek et al., 2009).

Also in dermal wound healing, the duality of heme is evident. Local accumulation of free heme liberated by spontaneous degradation of hemoglobin at the wound site may promote free radical formation and oxidative damage (Balla et al., 1991, 2000; Jeney et al., 2002). We have postulated that this injury-derived heme may be the trigger that initiates the inflammatory and the innate immunity response (Wagener et al., 2003a). To rapidly cope with oxidative stress, dermal fibroblasts are capable to rapidly induce HO-1 expression as a feedback response, and keratinocytes contain high levels of HO-2 (Applegate et al., 1995). When HO-1 levels are increased at the wound site resolution of inflammation will take place and the wound healing cascade can enter the next phase.

Pre-induction of HO by daily heme administration (30 mg/kg/day i.p.) in a mouse excisional wound healing model enhances wound contraction by increasing cellular proliferation and collagen synthesis (Ahanger et al., 2010). HO-1 induction also resulted in reduced transcription of pro-inflammatory cytokines, e.g., TNF α , and an up-regulated transcription of the

anti-inflammatory cytokine interleukin-10 (Ahanger et al., 2010). This demonstrates that low levels of heme may promote wound healing via induction of HO-1, whereas high levels of heme may prolong oxidative and inflammatory stress and result in a pro-fibrotic environment. Indeed, up-regulation of HO-1 expression in keratinocytes in transgenic mice bearing the HO-1 gene under the control of keratin 14 promoter resulted in improved neovascularization and accelerated wound healing (Grochot-Przeczek et al., 2009). Also, adenoviral mediated HO-1 gene delivery significantly improved dermal wound healing (Grochot-Przeczek et al., 2009).

Together, these data suggest that increased levels of HO-1 may improve wound healing and reduce dermal scarring.

Also, (myo)fibroblast apoptosis is essential in normal and hypertrophic scarring, as fibroblasts in pathological wound healing remain in the wound area and deposit excessive amounts of ECM components, contributing to fibrogenesis. At low concentrations, curcumin has been shown to induce HO expression, whereas HO-1 expression and activity was negligible at high curcumin doses (Scharstuhl et al., 2009). Despite this, HO-1 expression has been demonstrated to protect against fibroblast apoptosis induced by high concentrations of curcumin through the actions of effector molecules biliverdin/bilirubin (Scharstuhl et al., 2009). Importantly, pre-induction of HO-1 with low doses of curcumin also protected fibroblasts against curcumin-induced apoptosis (Scharstuhl et al., 2009). This suggest that curcumin in high doses may affect pathological scar formation through affecting fibroblast apoptosis, while HO-1 and its effector molecules can fine-tune this response.

The heme–HO system as therapeutic target in hepatic and pancreatic fibrosis

The current therapeutic options in specific treatment of both hepatic (Popov and Schuppan, 2009) as well as pancreatic fibrosis (Braganza et al., 2011) are only limited and novel therapeutic options are urgently needed. Interestingly, independent groups have demonstrated that specific overexpression of HO-1 had an inhibitory effect on chronic viral hepatitis (Zhu et al., 2008; Hou et al., 2010) as one of the major causes of hepatic fibrosis. Exposure to high levels of heme has been demonstrated to result in inflammatory leukocyte influx and fibrosis into the liver (Wagener et al., 2001b; Tolosano et al., 2002). Moreover, in a more recent study pharmacological HO-1 induction via CoPPiX has been shown to prevent liver fibrosis in a Mdr2 knockout mice (Barikbin et al., 2012). Importantly, in this report up-regulation of HO-1 not only reduced activation of HSCs, but also reversed established hepatic fibrosis in this mouse model.

Heme exposure has also shown to strongly promote inflammation in the pancreas, as illustrated by increased vascular permeabilization (Wagener et al., 2001b). HO-1 might also serve as a therapeutic target in pancreatic fibrosis. Schwer et al. (2008) have shown that up-regulation of HO-1 by curcumin inhibited PSC proliferation, which plays a crucial role in the progression of pancreatic fibrosis. These findings have been extended in a more recent report, in which PSC proliferation was inhibited via activation of the p38 MAPK/HO-1 pathway (Schwer et al., 2010). Moreover, these authors also indicated that the protective effect on PSC may be mediated via HO-1-dependent up-regulation of the cell cycle inhibitor p21/CIP-1 (Schwer et al., 2010).

The heme–HO system in CTR

A late but severe clinical complication after solid organ transplantation is CTR, which is currently considered the major limiting factor for long-term graft survival in particular in kidney and heart transplantation (Mitchell, 2009). A hallmark of CTR is TV, also termed graft vascular disease or allograft arteriopathy, in which the success rate of current therapeutic regimens including treatment with immunosuppressive drugs is very poor (Cornell et al., 2008). Pathologic features of TV include diffuse concentric intimal hyperplasia and adventitial sclerosis due to proliferation of various cell types of the vascular wall.

Survival of cardiac xenografts has been shown to be critically dependent on the expression of endothelial HO-1 (Soares et al., 1998). Accordingly, targeted overexpression of HO-1 via a gene therapy approach has been demonstrated to protect against TV and to cause a prolonged survival of chronic allogenic rejection of aortic vascular transplants in a rat model (Chauveau et al., 2002). Additionally, HO-1 activity has been shown to reduce atherosclerosis (Bouche et al., 2002; Ferran, 2006; Du et al., 2007), which could further contribute to a higher transplantation success rate.

Although the underlying mechanisms of how HO-1 mediates its protective effects are not well understood, one major beneficial effect of HO-1 may be its anti-proliferative effects in the vasculature (Valenzuela and Reed, 2011), which has previously also been shown in an experimental angioplasty rat model (Duckers et al., 2001). In corroboration, an increasing number of studies have proven HO-1 activity to be important for transplant graft survival (Ollinger and Pratschke, 2010), as HO-1 expression induction improves recipient immune tolerance (see reviews of Soares and Bach, 2007; Blancou et al., 2011) and donor graft survival after ischemia/reperfusion injury (for recent reviews, see Ferenbach et al., 2010; Sass et al., 2012).

The beneficial effects of HO-1 activity have been linked to the induction level of HO-1, as HO-1 gene promoter polymorphisms affect transplantation outcome (Immenschuh and Ramadori, 2000; Baan et al., 2004; Geuken et al., 2005; Holweg et al., 2005; Courtney et al., 2007; Gerbitz et al., 2008). However, it is not only the direct effect of HO-1 on limiting heme levels in injury sites that mediates the beneficial outcome of HO-1 activity. Also, the cytoprotective and anti-inflammatory effects of the end products generated by heme degradation, CO and biliverdin/bilirubin, have proven pivotal in improving the success of graft transplantation (Soares et al., 1998; Immenschuh and Ramadori, 2000; Kato et al., 2003; Nakao et al., 2004; Lee et al., 2007; Brugger et al., 2010).

The implication of heme-mediated regulation of the expression of the transcription factor and inflammatory mediator Egr-1

in atherosclerosis may have a significant impact on the success rate of graft survival after transplantation, as atherosclerosis is one of the causative agents for graft rejection and transplantation failure. Interestingly, HO-1 effects have been associated with its functional interaction with the cyclin-dependent kinase inhibitor p21/CIP-1, which belongs to the Cip/Kip protein family of cell proliferation inhibitors (Duckers et al., 2001). In addition, the function of p21/CIP-1 has been demonstrated to up-regulate the cellular anti-oxidant response via direct binding to the master HO-1 gene transcriptional regulator Nrf2 (Chen et al., 2009). These findings are in keeping with a recent report showing a major regulatory role for p21/CIP-1 in endothelial cell proliferation in atherosclerosis (Obikane et al., 2010), and may suggest that the interplay of p21/CIP-1 with HO-1 and/or Nrf2 is involved in the pathogenesis of TV.

Together, this exemplifies that the HO system is of major interest for the development of future therapeutic approaches that take advantage of pharmacological induction of HO-1 in endothelial cells. As a precedent, it has recently been demonstrated in a rodent atherosclerosis model that HO-1 induction by the pharmacological compound probucol had salutary effects via inhibition of vascular smooth muscle cell proliferation (Wu et al., 2006). Also, the importance of transcriptional regulation of HO-1 further underscores the clinical significance of the HO-1 promoter polymorphisms on therapy success, and the need for personalized medicine in the future.

FUTURE PERSPECTIVES

In this review we showed examples clearly demonstrating that excess levels of heme may promote pro-oxidant, pro-inflammatory, and subsequently fibrotic processes, in which the HO system may have counter-regulatory effects. These effects may occur via degradation of pro-inflammatory free heme and by the cytoprotective and anti-inflammatory effects of the HO-effector molecules CO and biliverdin/bilirubin. Promising (pre)clinical data support the utilization of HO and its effector molecules as novel therapeutic targets to reduce tissue inflammation, oxidative stress, and fibrosis. However, in order to translate this toward the clinical setting, safe and potent inducers of HO-1 are needed.

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