ADAPTIVE RESPONSES TO TISSUE INJURY: ROLE OF HEME OXYGENASE-1

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ABSTRACT

Tissue injury may result as a consequence of a physical, chemical, or biological insult. Such injury recruits an adaptive response to restore homeostasis and protect against further injury. One of the most prompt protective and adaptive responses by all tissues is the robust activation of the highly inducible, anti-inflammatory, anti-oxidant, and anti-apoptotic protein, heme oxygenase-1 (HO-1). HO-1, a microsomal enzyme, catalyzes the breakdown of pro-oxidant heme, which is released from heme proteins to equimolar quantities of iron, carbon monoxide, and biliverdin. Biliverdin is converted to bilirubin by biliverdin reductase. The beneficial effects of HO-1 expression are not merely due to heme degradation but are also attributed to the cytoprotective properties of the byproducts of the reaction. Manipulation of this enzymatic system in a myriad of disease models has provided substantial evidence to support its role as a cytoprotective enzyme and is therefore an emerging therapeutic molecule.

INTRODUCTION

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme, which catalyzes the oxidative degradation of heme to equimolar quantities of carbon monoxide (CO), iron, and biliverdin (Figure 1). Biliverdin is consequently reduced to bilirubin by biliverdin reductase (1). Heme is derived from a number of heme proteins including hemoglobin, myoglobin, cytochromes, and enzymes such as nitric oxide synthase, myeloperoxidase, catalase and others that are present ubiquitously in all cells. Heme oxygenase primarily exists as two isoforms. HO-1, the inducible isoform is encoded on chromosome 22 (22q12) in the human genome and translates to a 32-KDa protein. On the other hand, HO-2 is the constitutive isoform encoded on chromosome 16 (16q12) and translates to a 36-kD protein. Although HO-1 and HO-2

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Potential Conflicts of Interest: None disclosed.

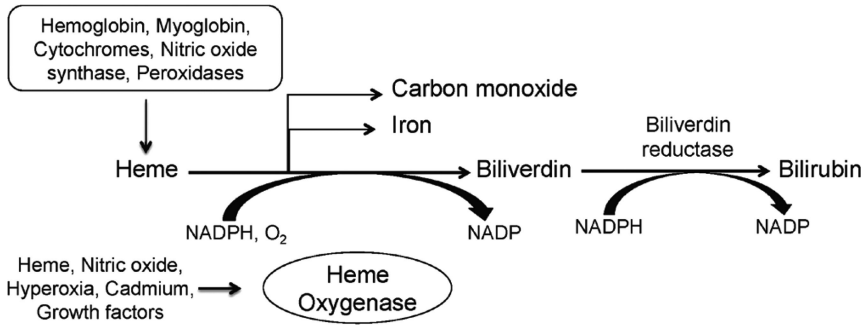

FIG. 1. Enzymatic reaction catalyzed by heme oxygenase-1.

share 40% homology, catalyze the same reaction, and have similar cofactor requirements (NADPH, O_2), they substantially differ with respect to regulation and expression pattern. HO-2 is constitutively expressed in the testes, brain, and endothelium; however, HO-1 is expressed in all cells at low levels but is highly inducible by a wide variety of stimuli, including its own substrate heme (2). HO-2 is thought to regulate normal physiological functions, whereas HO-1 plays a protective role in modulating tissue responses to injury in several diseases including acute kidney injury, atherosclerosis, sepsis, organ transplant rejection, and others (Table 1).

HO-1 Deficiency in Humans and Mice

Heme oxygenase was first described by Tenhunen et al. in 1968 as an important regulator of heme turnover (3). They showed that this enzyme was capable of oxidative degradation of heme to bilirubin. After this observation, several researchers aimed at identifying the regulation and expression of this enzyme in different tissues. Seminal work by Nath et al. in 1992 provided the first demonstration that HO-1 was rapidly induced in the kidney *in vivo* during rhabdomyolysis and that this induction served as an important protective response against the inflicted heme-mediated injury (4). This study sparked an enormous interest in HO research that is reflected by an exponential increase in the number of articles published concerning this enzyme (Figure 2).

Another breakthrough in the field was the generation of HO-1 knockout (HO-$1^{-/-}$) mice by Poss and Tonegawa in 1997. These mice exhibited a decreased birth rate, growth retardation, microcytic hypochromic anemia, hematuria, proteinuria, kidney and liver iron deposition, along with a chronic inflammatory phenotype consisting of hepatosplenomegaly, lymphadenopathy, hepatic periportal inflammation, leukocytosis, and glomerulonephritis (5). The absence of

TABLE 1
Protective Role of HO-1 Expression in Diseases

Organ	Disease	Reference
Brain	Cerebrovascular accident	(36, 37)
Eye	Corneal inflammation	(38)
	Uveitis	(39)
	Ocular hypertension	(40)
Ear	Noise-induced hearing loss	(41)
	Drug-induced ototoxicity	(42)
Lung	Hyperoxic injury	(43)
	Pulmonary hypertension	(44)
	Pulmonary Fibrosis	(45)
Heart	Cardiac transplant rejection	(46)
	Ischemic heart disease	(47)
Gastrointestinal	Inflammatory bowel disease	(48)
Liver	Ischemia/reperfusion injury	(49)
	Drug-induced hepatotoxicity	(50)
	Transplantation	(51)
Vasculature	Transplant arteriosclerosis	(52)
	Atherosclerosis	(53)
	Hypertension	(54)
Bone marrow	Transplantation	(55)
Kidney	Acute kidney injury	
	Rhabdomyolysis	(4)
	Ischemia/reperfusion	(56, 57)
	Cisplatin nephrotoxicity	(58, 59)
	Contrast induced toxicity	(60)
	Mercuric chloride induced toxicity	(61)
	Glomerulonephritis	(62)
	Nephrotoxic nephritis	(63)
	Chronic renal allograft rejection	(64)
	Polycystic kidney disease	(65, 66)

HO-1 in a patient with phenotypic features similar to the HO-1–deficient mouse was described in 1999 by Yachie et al. (6). This patient, a 6-year-old boy, exhibited hematuria, proteinuria, hyperlipidemia, and hypobilirubinemia, and displayed progressive tubulointerstitial injury, iron deposition, inflammatory cell infiltrate, and vasculopathy in the kidney. The patient died at age 6 and was found to have high levels of oxidatively modified LDL in plasma and extensive fatty streaks and fibrous plaques in the aorta. A second patient with HO-1 deficiency was recently reported with a similar phenotype (7). The indisputable similarity between the mouse model and human HO-1 deficiency propelled research in this field to enable a comprehensive evaluation of the role of HO-1 in physiological and pathophysiological states.

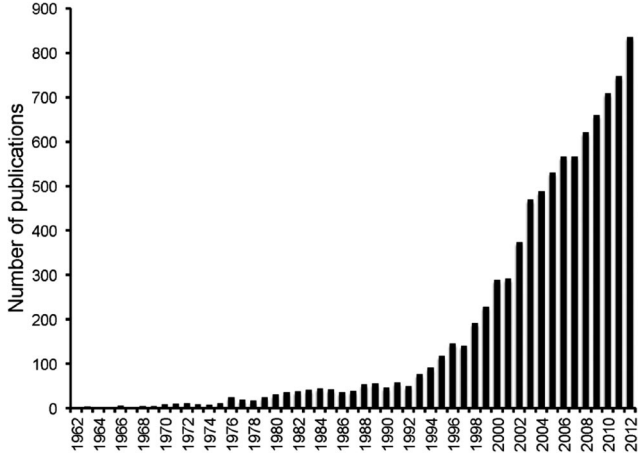

FIG. 2. Number of publications from PubMed using the search term "heme oxygenase."

Humanized HO-1 Transgenic Mice

Previous studies have shown major differences between the molecular regulation of the human HO-1 gene compared to the rodent HO-1 genes, although the function of the protein and enzymatic reaction is the same in both humans and rodents (8). To enable investigation of the human HO-1 gene *in vivo*, Kim et al. developed "humanized" HO-1 transgenic mice using an 87-kb bacterial artificial chromosome containing the entire HO-1 gene bred with HO-1 knockout mice (9). These humanized mice were able to rescue the phenotype of the HO-1–deficient mice described above and showed inducibility via transcriptional activation after injury. These mice offer a new tool to facilitate further translational studies to explore the therapeutic application of HO-1 in human diseases.

Modes of Protection

Although compelling evidence supports a role for HO-1 in cellular protection and survival, the mechanism(s) underlying such protection is attributable to multiple reasons. First, HO-1–mediated protection clearly relates to degradation of its substrate, heme, that has prooxidant effects (10, 11). Second, the by-products of the HO reaction also contribute to the protective response. CO, previously regarded as a toxic air pollutant and referred to as a "silent killer," exerts cytoprotective effects through its potent anti-inflammatory, anti-apoptotic, and vasodilatory properties (12). CO also inhibits platelet aggregation and has bactericidal effects (13). Similarly, the bile pigments released from the HO reaction, biliverdin and bilirubin, are potent peroxy

radical scavengers and can inhibit complement activation (14, 15). On the other hand, iron that is released from the reaction is capable of amplifying oxidative stress through reactive oxygen species generation. However, iron is safely sequestered by ferritin, an intracellular iron repository protein that is co-induced with HO-1 (16). Therefore, HO-1 not only removes toxic oxidant moities, but also protects the cells by providing anti-oxidant and anti-inflammatory molecules (17). Finally, the activation of HO-1 is also associated with upregulation of the cell cycle regulatory protein, p21, the latter mediating some of the cellular protective effects of HO-1 (18).

Clinical Implications

Given the significance of HO-1 expression during injury, research in the past decade has focused on targeting this enzymatic system as a therapeutic stratagem against diseases (reviewed in (19–24)). One approach that has gained considerable interest is the utilization of mesenchymal stem cells (MSC) to treat clinical conditions. These cells are multi-potent stem cells that have anti-inflammatory, angiogenic, and immunomodulatory properties that enable rapid repair and regeneration of damaged tissue (25). In fact, there are multiple ongoing clinical trials pertaining to various organ systems including the heart and kidney designed to evaluate the efficacy of MSC treatment during injury.

We have previously shown that MSCs lacking HO-1 expression have impaired paracrine effects and therapeutic potential (26). HO-1–deficient MSCs have reduced expression and secretion of important pro-angiogenic and growth factors such as stromal cell-derived factor-1, vascular endothelial growth factor–A, and hepatocyte growth factor. Furthermore, we showed that although conditioned media from HO-$1^{+/+}$ MSCs provided significant structural and functional protection against cisplatin-induced acute kidney injury, conditioned medium from HO-1–deficient MSCs was not effective (Figure 3). These results indicate the important role of HO-1 in MSC-mediated protection and highlight the importance of screening MSC donors for HO-1 expression to increase their efficacy and therapeutic potential. This is particularly relevant because human HO-1 gene expression is regulated by the number of GT repeats in the proximal HO-1 promoter. Length polymorphisms in this GT repeat region correlate with levels of HO-1 expression and associates with several diseases (27). For example, shorter GT repeats (<25) account for higher HO-1 expression and are associated with better outcomes in clinical settings such as emphysema, vascular restenosis in coronary arteries, and hemodialysis-related arteriovenous fistulae, and in the setting of renal transplanta-

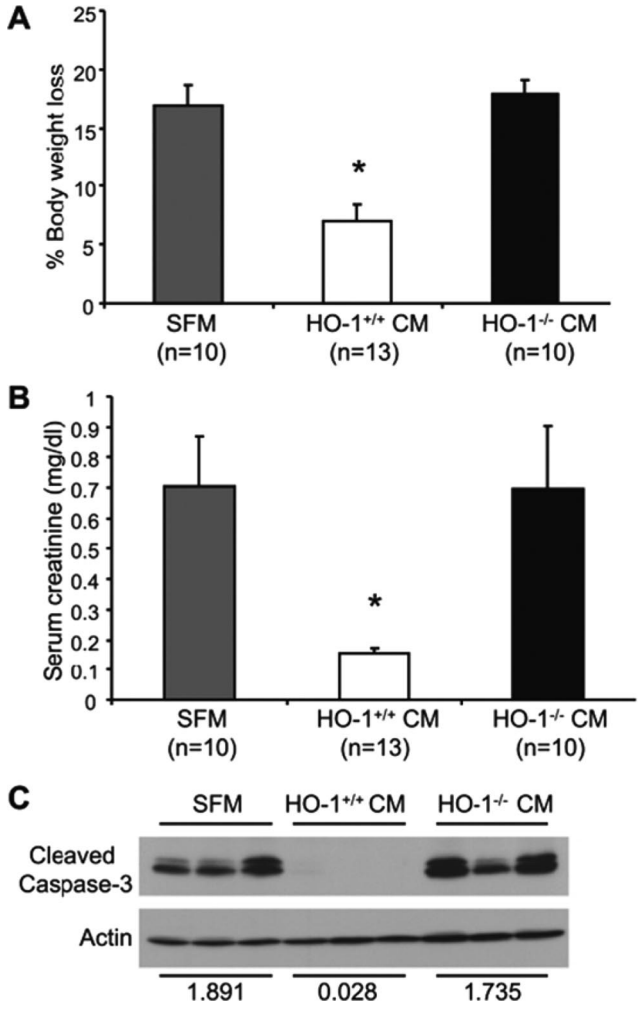

FIG. 3. Conditioned media (CM) from HO-$1^{+/+}$ mesenchymal stem cells (MSC) protects against cisplatin nephrotoxicity**. (A)** The amount of body weight loss following cisplatin administration was calculated and expressed as a percentage of the original weight. **(B)** Serum creatinine levels were measured and expressed as mg/dl. **(C)** Western blot analysis of cleaved caspase 3 expression in kidneys. Densitometric analysis of the bands on the caspase-3 and actin blots is indicated. Data are expressed as means ± SE and *$P < 0.05$. Reproduced with permission from Zarjou et al. (26).

tion. In contrast, long GT repeats (>25) are associated with lower levels of HO-1 expression and worse outcomes in these disorders.

Although these studies mainly focused on the induction of HO-1, recent work has directed attention to CO and CO-releasing molecules

as a protective strategy against injury. Neto et al. showed in a model of transplant-induced renal ischemia reperfusion injury that low-dose CO inhalation led to increased protection and graft survival (28). Currently, there are several ongoing clinical trials that are aimed at CO-based therapies of clinical conditions such as pulmonary hypertension and fibrosis and renal transplantation.

Another area with important clinical implications stems from the observations that several protective agents that exert pleotropic effects such as neutrophil gelatinase-associated lipocalin (NGAL), statins, erythropoietin, α-melanocyte–stimulating hormone, and interleukin-10 — all require HO-1, at least in part, for their beneficial properties (29–34). For example, the protective effects of exogenous NGAL are lost in acute kidney injury when HO activity is inhibited (29). IL-10 is protective in sepsis and prevents transplant vascular rejection; however, these effects are lost when HO activity is blocked (35).

In summary, the biological implications of HO-1 expression are beyond just heme degradation and encompass regulation of cellular function through modulation of a myriad of pathways. Importantly, HO-1 expression is protective in tissue injury and is a common downstream mediator and therapeutic target for drugs and other small molecules. Furthermore, these protective effects are mediated via one or more of the byproducts of the HO reaction. Therefore, modulation of the HO-1 enzyme can determine the fate of cells during injury and targeting the heme oxygenase system is an emerging therapeutic intervention for diseases.

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DISCUSSION

Sacher, Cincinnati: Thank you very much, very nice talk. I am intrigued about the Gilbert's relationship because, in fact, I think it's well-known that in Gilbert's syndrome individuals who, in fact, are starved, indirect bilirubin goes up and this is a protective functional response to stress.

Agarwal, Birmingham: I don't know if anyone has looked into that in particular but there have been large population studies since the initial work from the Czech Republic by Vitek and colleagues that unconjugated levels of bilirubin are protective, but when they've looked at obstructive jaundice, for example, it's mainly the conjugated fraction. That, in fact, is more toxic. For example, kids with neonatal hyperbilirubinemia from prematurity can get kernicterus. I don't think anybody has specifically studied the starvation response in terms of the higher level of bilirubin, but it is certainly worth exploring.

Quesenberry, Providence: Great talk, very exciting. There is some work we are collaborating with Giovanni Camussi in Torino who has looked at the mesenchymal stem cell healing of kidney injury from cisplatinum, glycerol, and ischemia reperfusion and his data offer an alternative potential and that's the transfer, probably, of microRNA in vesicles into damaged cells. Your comments?

Agarwal, Birmingham: That's a great question. There is elegant work from Camussi and others that, including endothelial progenitor cells or mesenchymal stem cells, when injected actually release microvesicles. These microvesicles are loaded with multiple factors including microRNA, DNA, and other particles that may be really getting transferred to the injured cell enhancing repair. We have preliminary data suggesting a defect in the HO-1–deficient mesenchymal stem cells in terms of secreting an adequate number of these microvesicles.

Quesenberry, Providence: I am just going to say that you are offering an alternative to his observations and actually since there's a common fund grant application coming out, that could be very helpful.

Agarwal, Birmingham: Thank you.

Lippman, Miami: I really enjoyed this talk though I'm not sure I completely follow. Perhaps you could say a bit more of the exact mechanism, what's known about how carbon monoxide actually has these beneficial effects, and whether or not in the knockout mouse you can ameliorate the effects by providing low doses of carbon monoxide.

Agarwal, Birmingham: We have not really done any of the CO experiments ourselves, but extensive work has been done by Augustine Choi, Leo Otterbein, Fritz Bach, and others where they showed that concentrations in the order of 250 parts per million (ppm) of CO afforded protection. A lot of skepticism was involved because for every molecule of heme that's degraded, equimolar amounts of CO are generated and you can rarely achieve 250 ppm concentrations of CO from endogenous breakdown of heme per se. Initially it was thought that CO was activating p38 MAP kinase and hence eliciting

the protective effect, but now there is very nice data from several people where actually CO causes a preconditioning effect. It essentially poisons the mitochondrial cytochrome c-oxidase and stuns the cell and when the cell gets challenged with another insult it is ready to defend itself and this is, you know, a preconditioning phenomenon that has been well-known for over decades in different organ systems. It is believed that the heat shock proteins such as Hsp27 and Hsp90 are also involved with such a protective mechanism.

Lippman, Miami: So, would hypoxia be equivalent in that sort of suggestion to carbon monoxide?

Agarwal, Birmingham: Yes, so essentially CO is causing hypoxia to the cell. It's a hypoxic preconditioning and we know that CO binds to hemoglobin very avidly and, you know, that's how patients essentially die from CO toxicity. These results are just recently emerging that there are other alternate mechanisms.

Hochberg, Baltimore: I may have missed it if you mentioned it, but did you mention any agents which are able to induce the heme oxygenase enzyme?

Agarwal, Birmingham: There are multiple inducers of the heme oxygenase pathway. In fact, we've all heard about a drug that was just withdrawn yesterday, bardoxolone methyl, which is a triterpenoid, very active inducer of heme oxygenase. Actually a proposed mechanism of action was through this pathway because it activates the transcription factor Nrf2. There are several other transcription factors involved. There are also drugs like statins, erythropoietin, IL-10, alpha-MSH, a lot of these agents have pleotropic effects, which are believed to work through the HO-1 pathway. For example, in HO-1–deficient animals or when HO activity is pharmacologically inhibited, these agents lose their protective effect. So, it could be a downstream mediator and several molecules that activate this enzyme system have been described.