

## Protective Effects of Inducible HO-1 on Oxygen Toxicity in Rat Brain Endothelial Microvessel Cells

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**Background:** Reperfusion in ischemia is believed to generate cytotoxic oxidative stress, which mediates reperfusion injury. These stress conditions can initiate lipid peroxidation and damage to proteins, as well as promote DNA strand breaks. As biliverdin and bilirubin produced by heme oxygenase isoform 1 (HO-1) have antioxidant properties, the production of both antioxidants by HO-1 may help increase the resistance of the ischemic brain to oxidative stress. In the present study, the survival effect of HO-1 was confirmed using hemin.

**Methods:** To confirm the roles of HO-1, carbon monoxide, and cyclic guanosine monophosphate further in the antioxidant effect of HO-1 and bilirubin, cells were treated with cycloheximide, desferoxamine, and zinc deuteroporphyrin IX 2,4 bis glycol, respectively.

**Results:** HO-1 itself acted as an antioxidant. Furthermore, iron, rather than carbon monoxide, was involved in the HO-1-mediated survival effect. HO-1 activity was also important in providing bilirubin as an antioxidant.

**Conclusion:** Our results suggested that HO-1 helped to increase the resistance of the ischemic brain to oxidative stress.

**Keywords:** Heme; Oxygenases; Bilirubin; Iron; Carbon monoxide

### INTRODUCTION

Heme oxygenase (HO) catalyzes the initial reaction, and subsequent rate limiting step, in heme catabolism. There are three different types of HO isoforms, HO-1, HO-2, and HO-3, although HO-3 is not catalytically active and is thought to be a pseudogene derived from HO-2 transcripts [1]. Initially, HO was thought to be only an enzyme that catalyzed the initial step in the destruction of senescent erythrocytes seen in all systemic organs. In subsequent studies, HO was also shown to undergo

adaptive regulation in response to stress. Following the discovery of this stress-related upregulation, two different isoforms of the HO enzyme, HO-1 and HO-2, were identified in 1968. Once the two different isoforms were identified, it was determined that the observed stress-related upregulation was due to a 32-kDa protein, HO-1, and that the 36-kDa isoform, HO-2, was not upregulated during times of stress.

While HO-2 is constitutively expressed, HO-1 is normally expressed at low levels in most tissues; however, it is highly inducible by a variety of stimuli including growth factors, oxi-

Received: 31 October 2013, Revised: 27 March 2014,

Accepted: 17 April 2014

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ductive stress, dietary antioxidants, or disease states [2]. HO-1 is mainly expressed in the liver and spleen, and is not expressed in the brain under normoxic conditions [3]. Indeed, HO-1 may be among the most critical cytoprotective mechanisms activated during times of cellular stress such as inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, or radiation [4], and is thought to play a key role in maintaining antioxidant/oxidant homeostasis. In particular, HO-1 is induced by hypoxia via a mechanism independent of hypoxia-inducible factors [5]. Moreover, the induction of HO-1 during conditions of cerebral oxidative stress is critical in neuroprotection [6]. Although HO-1 is an HO enzyme, it is also known as HSP32, a member of a family of proteins referred to as heat shock proteins [7,8].

The upregulation of HO-1 during times of oxidative and heat stress implied an antioxidant role. However, it was unclear whether HO-1 had inherent antioxidant properties, or whether its protective effect was a result of its catalytic products. HO uses molecular oxygen, nicotinamide adenine dinucleotide phosphate, and cytochrome P-450 reductase to catalyze the oxidative cleavage of the heme molecule, producing carbon monoxide (CO), biliverdin (subsequently converted to bilirubin by biliverdin reductase), and iron.

The ferric ion ( $\text{Fe}^{3+}$ ) is one of the products of the heme degradation reaction. Therefore, the production of free iron from the catabolism of heme could give rise to toxic hydroxyl radicals through a reaction known as the Fenton reaction, which involves hydrogen peroxide and superoxide. However, ferritin is thought to be induced to bind and sequester free iron, thus functioning as an antioxidant by preventing the production of toxic hydroxyl radicals [9].

There has also been evidence to suggest that bilirubin, another product of the heme oxygenase catalyzed reaction, can function as an antioxidant. It has been shown *in vitro* that bilirubin scavenges peroxy radicals as efficiently as  $\alpha$ -tocopherol, which is regarded as the most potent antioxidant of lipid peroxidation. Therefore, the redox cycling of bilirubin may scavenge reactive oxygen species, protecting cells from oxidative stress. Furthermore, bilirubin reacts with peroxy radicals to form biliverdin [10].

CO is another product of heme catabolism, and has been implicated as a diffusible second messenger to activate soluble guanylyl cyclase. Since the discovery of the role of nitric oxide (NO) in the central nervous system as a second messenger, research has been conducted in an attempt to elucidate a similar role for CO. Like NO, CO can bind to the iron in the heme

present in guanylyl cyclase and activate the enzyme. As was the case with NO, exogenous CO has also been shown to inhibit platelet aggregation due to the activation of soluble guanylyl cyclase [11]. The colocalization of 5-aminolevulinic acid synthase with HO-2 in the brain also indicates that the machinery for porphyrin synthesis and CO production occur together, as would be required for an endogenous second messenger [12].

The present study examined the function of HO-1 in rat brain endothelial microvessel cells (RBE4). This cell line was selected for this study because it provides an excellent model to study brain endothelial cells, which constitute the blood-brain barrier. Molecular biology methodologies were used to evaluate the functional roles of HO-1, bilirubin, and ferritin induction in cytoprotection.

## METHODS

### Cells

RBE4 cells were immortalized using the adenovirus E1A gene without oncogenic transformation. Transfection was carried out using a calcium phosphate precipitation procedure on endothelial microvessel cells. Each line was packaged with trypsin-ethylenediaminetetraacetic acid and RBE4 medium, which was composed of 500 mL of Dulbecco's Modified Eagle's Medium (DMEM; obtained from Gibco, Grand Island, NY), 55 mL of fetal bovine serum (Gibco), 11 mL of HEPES (Sigma, St. Louis, MO, USA), 146 g of L-glutamine, 167 g of geneticin (Sigma) and 55  $\mu\text{L}$  of basic fibroblast growth factor (Biomedical Technologies, Stoughton, MA, USA). Trypsin (4 mL) was added to each 10-cm<sup>2</sup> dish and the dishes were incubated for 5 minutes. Cells were washed into a 50-mL conical tube and then the dish was washed again with 4 mL of DMEM. The conical tube was then centrifuged at 1,500 rpm for 5 minutes. The supernatant was aspirated and the pellet was resuspended in RBE4 medium based on the desired dilution factor. Cell lines were diluted at concentrations of 1:3 to 1:6 with RBE4 medium.

### Viability assays

Certain cell lines were frozen in liquid nitrogen to allow for their use at a later time. Two 10-cm<sup>2</sup> dishes at 100% confluence were frozen per vial diluted with freeze medium. The freeze medium consisted of 10% dimethyl sulfoxide and 90% DMEM. Cells were trypsinized and centrifuged. Each pellet was resuspended in 500  $\mu\text{L}$  of DMEM and transferred to a

vial. Each vial was chilled on ice for 5 minutes, and then another 500  $\mu$ L of ice-cold freeze media was added to each vial. The vials were then stored at  $-20^{\circ}\text{C}$  for 1 hour prior to storage at  $-70^{\circ}\text{C}$  for 4 to 15 hours, after which vials were put into liquid nitrogen.

Cell lines were passaged on 10-cm<sup>2</sup> dishes and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) experiments were conducted on 24-well plates. The plates were coated with fibronectin to allow for the growth of cells. The experiments were comprised of three stages: induction, sensitization, and hydrogen peroxide. The induction phase was initiated at time 0 and continued for 1 hour. The sensitization phase occurred at 16 hours and was followed by the hydrogen peroxide phase, where the cell monolayers received 500  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  at 17 hours. Hydrogen peroxide was applied for 1.5 hours before the 10% MTT solution (diluted with DMEM) was placed on the cells for 3 hours. A solubilization buffer was then applied to the cells overnight. The next day, wells were measured in a spectrophotometer at 575 and 675 nm. The average of the differences between the 575 and 675 nm readings from triplicate sets was divided by the control reading. This quotient represented the percentage of cells that survived.

### Chemicals

Cells underwent oxidative stress in the peroxide phase due to the addition of 0.5 mL of 500  $\mu\text{M}$  hydrogen peroxide to each well. The induction and sensitization phases represented times during which various known compounds were introduced into the medium to test several hypotheses regarding the antioxidant role of heme oxygenase. The experiments were conducted using different concentrations of zinc deuteroporphyrin IX 2,4 bis glycol (ZnBG, Porphyrin Products Inc., Logan, UT, USA), bilirubin (Porphyrin Products Inc.), cycloheximide (Sigma), desferoxamine (Sigma), and hemin (Sigma).

The MTT cell proliferation kit I (Boehringer Mannheim, Mannheim, Germany) was used to evaluate the viability of the RBE4 cells. The MTT kit was selected to quantify cell viability because it provides a colorimetric assay that can be run expediently on a large number of cell monolayers and does not rely on cell counting. This colorimetric assay is based upon the cleavage of the tetrazolium salt, MTT, to purple formazan crystals by dehydrogenase activity in active mitochondria.

Tests were run to determine whether the variables used in the MTT assay experiments affected the cleavage of the tetrazolium salt, which could skew the data. Specific concentrations of bilirubin, hemin, ZnBG, cycloheximide, and desf-

eroxamine were placed on cells during the last 10 minutes of the sensitization phase. Nothing was placed on the cells during the induction phase. Subsequently, cells underwent the aforementioned procedure, and it was determined that none of the variables of the experiments affected the cleavage of the salt.

### Statistical analysis

All data are presented as the means  $\pm$  standard errors of the means. One-way analysis of variance was conducted and followed by Dunnett's post hoc test.

## RESULTS

### Heme oxygenase-1 promoted rat brain microvessel endothelial cell survival under oxidative stress mainly through bilirubin

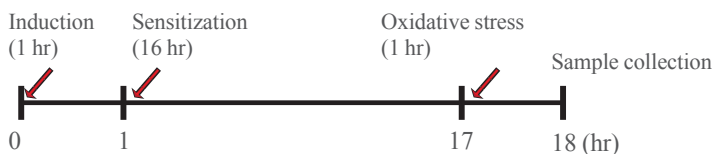
To study the role of HO activity in the protective effects observed under conditions of oxidative stress, we employed *in vitro* models with hemin, which is the HO-1 substrate for induction and sensitization under oxidative stress attributable to hydrogen peroxide (500  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) (Fig. 1A). Cell death was prevented by treatment with hemin (none vs. hemin [10  $\mu\text{M}$ ]; 11.58 vs. 36.32) (Fig. 1B). The results of the cell viability assay suggested that inducible HO-1 played an important role in the survival of RBE4 cells under oxidative stress.

### Iron was necessary for the antioxidant effects of HO-1 activity

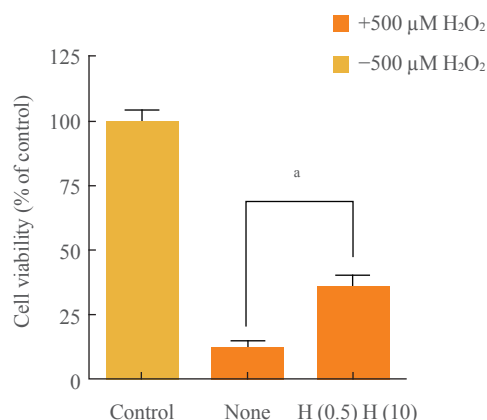
HO appears to exert most of its actions via enzymatic activity and the generation of various enzymatic byproducts that act on multiple pathways. For example, HO can promote: 1) the generation of biliverdin and its subsequent reduction to bilirubin, 2) the release of CO, and 3) the release and safe disposal of iron. These actions modulate various pathways with a high degree of overlap and/or crossover, which may help to ensure the induction of the desired biological effects [13].

To confirm the roles of HO-1, CO, and cyclic guanosine monophosphate (cGMP) in the antioxidant effects of HO-1 induced by hemin, we treated cells with cycloheximide, desferoxamine, and ZnBG, respectively, in the induction phase of experiments for 1 hour (Fig. 2A). After the induction phase, hemin was used in the sensitization phase for 16 hours. Study results revealed that the HO-1 activity induced by hemin was blocked by cycloheximide, an inhibitor of protein biosynthesis. Furthermore, cycloheximide induction completely blocked the survival effect of HO-1. Conversely, ZnBG, which is an

Experimental scheme

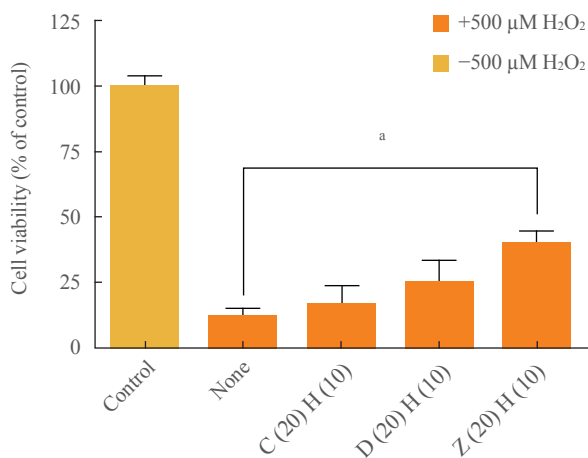


Conditions	Induction	Sensitization	Oxidative stress
None	None	None	Hydrogen peroxide (500 μM)
H (0.5) H (10)	Hemin (0.5 μM)	Hemin (10 μM)	Hydrogen peroxide (500 μM)



**Fig. 1.** Protective effects of heme under conditions of oxidative stress. (A) Experimental scheme for induction, sensitization, and oxidative stress. The “H (0.5) H (10)” condition was induction by hemin (0.5 μM) and sensitization by hemin (10 μM), while the “none” condition was no induction and no sensitization. (B) Cells untreated by H<sub>2</sub>O<sub>2</sub> were used as a positive control. Before oxidative stress was applied (500 μM H<sub>2</sub>O<sub>2</sub>), cells were induced and sensitized according to the experimental scheme. Data are mean ± SEM of three separate experiments. Analyses performed were one-way analysis of variance followed by Dunnett’s *post hoc* test. <sup>a</sup>*P* < 0.01 denote statistical significance.

Conditions	Induction	Sensitization	Oxidative stress
None	None	None	Hydrogen peroxide (500 μM)
C (20) H (10)	Cycloheximide (20 μg/mL)	Hemin (10 μM)	Hydrogen peroxide (500 μM)
D (5) H (10)	Desferoxamine (5 mM)	Hemin (10 μM)	Hydrogen peroxide (500 μM)
Z (20) H (10)	ZnBG (20 μM)	Hemin (10 μM)	Hydrogen peroxide (500 μM)

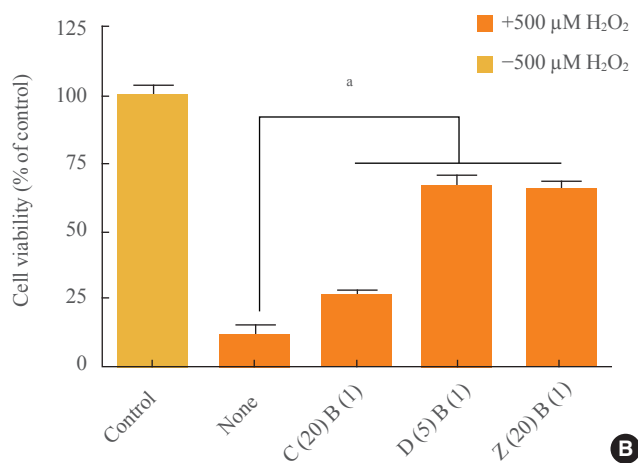


**Fig. 2.** The antioxidant effects of heme oxygenase isoform 1 activity require iron. (A) Experimental scheme for induction, sensitization, and oxidative stress. The “C (20) H (10),” “D (5) H (10),” and “Z (20) H (10)” conditions were induction by cycloheximide (20 ng/mL), desferoxamine (5 nM), and ZnBG (20 μM), respectively, with sensitization by hemin (10 μM) in each case, while the “none” condition was no induction and no sensitization. (B) Cells untreated by H<sub>2</sub>O<sub>2</sub> were used as a positive control. Before oxidative stress was applied (500 μM H<sub>2</sub>O<sub>2</sub>), cells were induced and sensitized according to the experimental scheme. Data are mean ± SEM of three separate experiments. Analyses performed were one-way analysis of variance followed by Dunnett’s *post hoc* test. <sup>a</sup>*P* < 0.01 denote statistical significance.

inhibitor of heme oxygenase induction, did not block the survival effect of HO-1 following hemin induction (cycloheximide induction vs. ZnBG induction; 16.5 vs. 39.6) (Fig. 2B). Desferoxamine, which binds specifically to iron and removes the relevant effect of iron induction, only partially blocked the survival effect of HO-1. Study results indicated that iron, not CO, was involved in the survival effects attributable to HO-1.

**HO-1 was necessary for the antioxidant effects of bilirubin**  
Bilirubin acts as a scavenger against reactive oxygen species via its own oxidation [14-16]. Indeed, bilirubin is a powerful antioxidant that can inhibit the oxidation of low-density lipoprotein and other lipids [17], scavenge oxygen radicals [18], and counteract oxidative stress in general [19]. The production of bilirubin could participate in an amplification cycle via its oxidation into biliverdin and then reduction back into bilirubin.

Conditions	Induction	Sensitization	Oxidative stress
None	None	None	Hydrogen peroxide (500 $\mu$ M)
C (20) B (1)	Cycloheximide (20 $\mu$ g/mL)	Bilirubin (1 $\mu$ M)	Hydrogen peroxide (500 $\mu$ M)
D (5) B (1)	Desferoxamine (5 $\mu$ M)	Bilirubin (1 $\mu$ M)	Hydrogen peroxide (500 $\mu$ M)
Z (20) B (1)	ZnBG (20 $\mu$ M)	Bilirubin (1 $\mu$ M)	Hydrogen peroxide (500 $\mu$ M)



**Fig. 3.** The antioxidant effects of bilirubin require heme oxygenase isoform 1 activity. (A) Experimental scheme for induction, sensitization, and oxidative stress. The “C (20) B (10),” “D (5) B (10),” and “Z (20) B (10)” conditions were induction by cycloheximide (20 ng/mL), desferoxamine (5 nM), and ZnBG (20  $\mu$ M), respectively, with sensitization by bilirubin (1  $\mu$ M) in each case, while the “none” condition was no induction and no sensitization. (B) Cells untreated by H<sub>2</sub>O<sub>2</sub> were used as a positive control. Before oxidative stress was applied (500  $\mu$ M H<sub>2</sub>O<sub>2</sub>), cells were induced and sensitized according to the experimental scheme. Data are mean  $\pm$  SEM of three separate experiments. Analyses performed were one-way analysis of variance followed by Dunnett’s *post hoc* test. <sup>a</sup>*P* < 0.001 denote statistical significance.

bin by biliverdin reductase [20].

To confirm the roles of HO-1, CO, and cGMP in the antioxidant effect of bilirubin, we used cycloheximide, desferoxamine, and ZnBG, respectively, during the induction phase of the experiments for 1 hour. After the induction phase, bilirubin was used in the sensitization phase for 16 hours (Fig. 3A). Cycloheximide induction partially blocked the survival effect of bilirubin, suggesting that HO-1 played an important role in the antioxidant effect of bilirubin. Furthermore, bilirubin did not require CO or cGMP to increase cell viability (Fig. 3B). Study results indicated that HO-1 required heme and iron to produce the observed survival effect. However, bilirubin, as an HO-1 effector for oxidative stress, did not require CO or cGMP.

## DISCUSSION

The present study examined the anti-oxidative effects of HO-1 using rat brain endothelial cells. Study results showed that the HO-1 itself acted as an antioxidant. Additionally, iron, but not CO, was demonstrated to be involved in the survival effect mediated by HO-1. The HO-1 activity was also shown to be important in providing bilirubin as an antioxidant.

Ferric ions participated in the Fenton reaction to protect cells and allowed for a comparison of the protection afforded by hemin induction. The importance of iron in inducing ferri-

tin for cytoprotection was also demonstrated when desferoxamine was added during the induction phase.

HO-1 has been shown to be a critical gene in the cellular response against pro-oxidative stimuli. When considering the cellular response as a whole, HO-1 seems to act as a hub of gene-gene interactions that modulate the triggering and activation of several protective molecular pathways.

Basal HO-1 mRNA expression levels in response to injurious stimuli appear to be more important than the degree of up-regulation or fold induction for protection against those stimuli in the vasculature. In some circumstances, the levels of HO-1 protein and HO functional activity may not be concordant, which could be due to post-translational modifications.

HO-1 has been shown to be induced inside as well as outside of areas of cerebral infarction. In particular, HO-1 has been shown to be induced in the endothelial cells within areas of infarction and in glia. More specifically, HO-1 was induced primarily in glial cells in noninfarcted areas of the cortex inside as well as outside the middle cerebral artery distribution. The cells most resistant to cerebral infarction, the endothelial cells, expressed HO-1-inducible stress genes in areas of infarction 24 hours after ischemic injuries. Because HO-1-inducible genes have heat shock elements within their promoters, it is possible that the presence of oxidative stimulus for stress related protein induction within the injured endothelial cells [21]. It

is not known whether the endothelial cells within the infarction survive, but they may do so due to the synthesis of HO-1, which could serve to protect them from further injury and therefore enable them to survive the ischemic insult [22].

HO-1 is influenced by oxidative stress in cells, as the depletion of glutathione (GSH) and ascorbic acid induce HO-1 [23]. Reperfusion in focal ischemia is believed to generate cytotoxic free radicals that mediate reperfusion injury [24]. Reactive oxygen species can initiate lipid peroxidation and damage to proteins, as well as promote DNA strand breaks. Although there are a number of pathways and enzymes in the brain that detoxify and inactivate free radicals, these antioxidants, including GSH and ascorbic acid, can be quickly overwhelmed following reperfusion injury. Because the biliverdin and bilirubin produced by HO-1 and HO-2 have antioxidant properties [4,24], the production of both antioxidants by HO-1 may help increase the resistance of the ischemic brain to oxidative stress.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGMENTS

This work was supported by the DGIST R&D Program of the ministry of science, ICT & Future Planning, Science and Technology of Korea (14-BD-0402).

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