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Elucidating the Role of Biliverdin Reductase in the Expression of Heme Oxygenase-1 as a Cytoprotective Response to Stress

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1. Introduction

Hemin is a cofactor in which an atom of iron is coordinated to the nitrogens of four pyrrole groups that make up the protoporphyrin IX ring (see figure below).
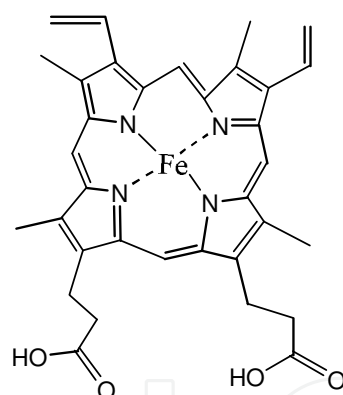

Hemin

Many types of enzymes in living systems use hemin as a prosthetic group to catalyze oxidation/reduction reactions or for the binding/transport of reactive molecules (e.g. oxygen). For instance, several cytochromes of the mitochondrial electron transport chain are "heme" enzymes as are the major drug/xenobiotic-metabolizing enzymes of the endoplasmic reticulum, the cytochromes P450 (CYP or P450). The heme group of the P450s allows these enzymes to use redox chemistry to bind molecular oxygen and cleave the O-O bond, thus forming a reactive, high-valent oxygen species that can insert oxygen into otherwise stable carbon-hydrogen bonds of drugs/xenobiotics (White and Coon, 1980). The unfavorable thermodynamics of this type of reaction has caused the P450s to be likened to "catalytic blowtorches" (Schlichting et al., 2000), and the process is essential for the elimination and clearance of many lipophilic compounds ingested from the environment. Catalase is an important protective heme enzyme that is responsible for degrading

hydrogen peroxide. Furthermore, the heme enzymes, nitric oxide synthase and cyclooxygenase, have important signaling roles in the regulation of various cellular processes such as inflammation. However, in terms of the sheer abundance in higher living systems, hemoglobin is the most important heme enzyme as it uses the cofactor to transport oxygen in blood circulation to facilitate oxidative/phosphorylation and energy generation in distal tissues. Because the heme proteins interact with reactive oxygen species (ROS), they are susceptible to ROS-mediated damage, which in turn, results in the accumulation of free or unused heme.

1.1 The toxicity of heme

The reactive nature of hemin does not come without a cost. The free (not enzyme bound) form of the cofactor has been shown *in vitro* to increase the peroxidation of lipids and the fragmentation and cross-linking of DNA and protein resulting from oxidative stress (Kumar and Bandyopadhyay, 2005;Vincent, 1989). One likely explanation for these findings can be drawn from two of the basic reactions of reactive oxygen chemistry, the Fenton reaction and the Haber-Weiss reaction. In the Fenton reaction (below), superoxide anion reduces free molecular iron.

$$O_2^- + Fe^{+3} \longrightarrow O_2 + Fe^{+2} \qquad \text{(Fenton Reaction)}$$
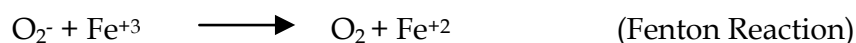
In the Haber-Weiss reaction, the reduced iron can interact with hydrogen peroxide, resulting in cleavage of the O-O bond to form hydroxyl anion and hydroxyl radical (Vincent, 1989).

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + HO^- + HO \qquad \text{(Haber-Weiss Reaction)}$$

Using the Haber-Weiss system as an analogy, it is likely that the free hemin functions in a manner similar to that of free iron as a means to produce hydroxyl radical. The hydroxyl radical has been shown to be much more destructive to proteins and DNA than both hydrogen peroxide and superoxide (Davies et al., 1987;Jackson et al., 1987).

It also has been proposed that hemin interacts with hydrogen peroxide to form a putative, hypervalent iron-oxygen species analogous to the reactive intermediate of peroxidase enzymes referred to as Compound I (Vincent, 1989).

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3}{:}O \cdot + H_2O \qquad \text{(Peroxidase-like Reaction)}$$
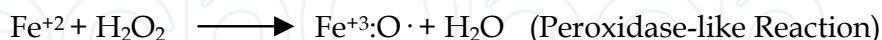
Because hemin is much more lipophilic than free iron, the oxidative stress associated with free hemin is more destructive to membrane lipids and organelles (Balla et al., 1991). In this respect, the putative iron-oxo species resulting from the reaction of hemin and hydrogen peroxide could be even more deleterious than hydroxyl radical as suggested by the fact that free radical scavengers of hydroxyl radical (e.g. dimethyl sulfoxide) did not protect lipids and proteins from damage when incubated with hemin and hydrogen peroxide (Vincent, 1989). Understandably, the propensity of hemin to damage lipid mixtures causes it to be extremely harmful to cellular membranes and organelles. Free hemin also has been shown to promote inflammatory reactions that have been associated with hepatic, renal, neuronal, and vascular injury (Kumar and Bandyopadhyay, 2005). In particular, several studies have demonstrated the contribution of hemin to the pathogenesis associated with atherosclerosis and ischemia/reperfusion (Wagener et al., 2001).

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1.2 The regulation of cellular heme levels

Because of the harmful effects of free heme accumulation, the synthesis and catabolism of heme in living systems is highly regulated. The rate-limiting enzyme for heme synthesis is α-aminolevulinic acid synthetase, and its expression and activity is highly regulated by a variety of agents and stress signals (Ponka, 1997). Heme catabolism is carried out by two enzymatic reactions. The rate-limiting step of heme catabolism is catalyzed by heme oxygenase (HO). This enzyme catalyzes a complicated, multi-step reaction that uses molecular oxygen and electrons (received from a separate redox partner, the cytochrome P450 reductase) to cleave the α-meso bridge of the protoporphyrin IX ring and form ferrous iron, CO, and biliverdin in the process (Kikuchi et al., 2005;Liu et al., 1997;Liu and Ortiz de Montellano, 2000) (see figure below).

(HO Reaction)
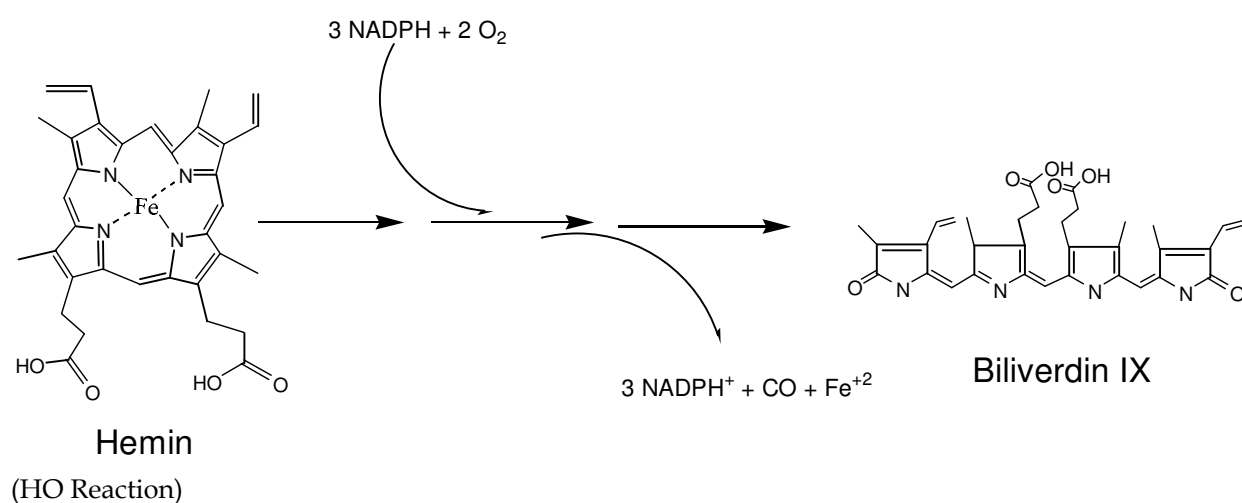
The final step of heme catabolism involves the reduction of the biliverdin formed by HO to bilirubin. This enzymatic step (below) is catalyzed by the biliverdin reductase (BVR). BVR has dual cofactor specificity as both NADH and NADPH can provide electrons to the enzyme. NADPH is the preferred cofactor under basic conditions, whereas NADH is more favorable at lower pH (< 7.0) (Noguchi et al., 1979). The activities of both HO and BVR are highly regulated to effectively coordinate heme catabolism with its synthesis under different conditions.

(BVR Reaction)
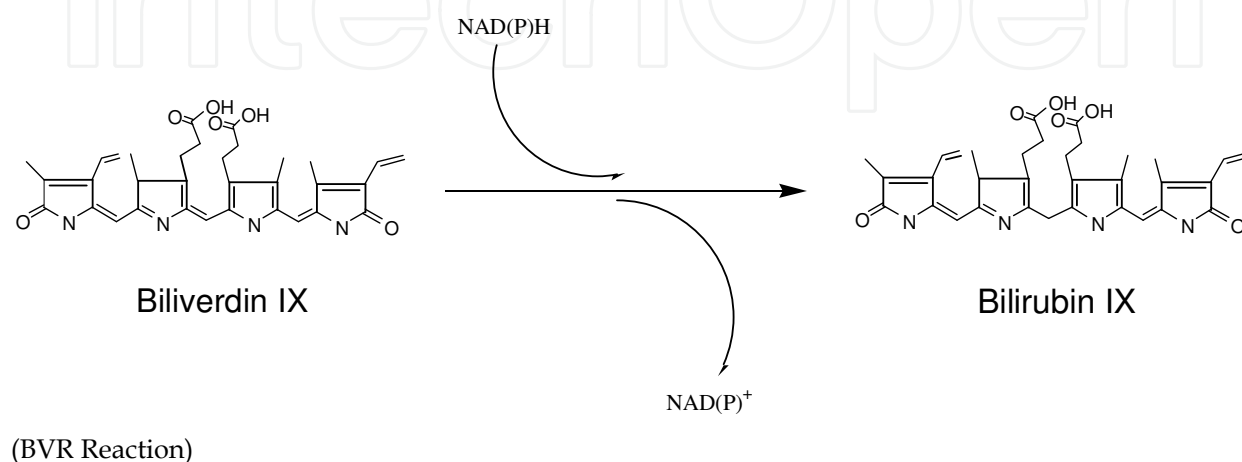
2. Cellular functions of HO-1 and HO-2

There are two isoforms of HO, known as HO-1 and HO-2 that are expressed through two different genes and are immunologically distinct (Maines et al., 1986;Maines, 1988). The two enzymes are approximately 40% homologous as both have in common a catalytic region of about 24 amino acids (Rotenberg and Maines, 1991) and a hydrophobic, C-terminal tail that serves to anchor the enzymes to the endoplasmic reticulum. HO-1, which is 33 kDa, is highly inducible by a multitude of stimuli and compounds and is constitutively expressed in liver and spleen. HO-2 is constitutively expressed in most tissues, and is highly expressed in brain, kidney, and testes. The HO-2 protein is 36 kDa as it contains additional regulatory sequences (e.g. extra heme-binding sites) which affect its activity in a tissue-specific manner and which might also be regulated by CO and NO binding (Ryter et al., 2006).

2.1 Functions of HO-2

It is generally believed that the main role of HO-2 might be to maintain homeostatic levels of heme during normal cellular metabolism. In one study, HO-2 knockout mice only displayed mild phenotypes and did not show evidence of altered iron maintenance (Poss et al., 1995). The study did show that the mice displayed ejaculation abnormalities (in males) and increased susceptibility to hyperoxic lung damage. Illustrating the importance of HO-2 to brain heme metabolism, these mice also showed dramatically reduced levels of HO activity in the brain. In addition, another study did demonstrate oxygen toxicity and iron accumulation in the lungs of HO-2 knockout mice (Dennery et al., 1998). Thus, HO-1 cannot completely compensate for the absence of HO-2 in terms of cellular function and the regulation of heme levels.

In brain, testes, and cardiovascular tissue, HO-2 activity plays a critical role in function by generating CO which functions as a tissue-specific signaling messenger that acts mainly through activation of guanylyl cyclase. In smooth muscle and endothelial tissue, CO mimics the effects of NO by causing relaxation and vasodilatation, respectively (Hangai-Hoger et al., 2007;Patel et al., 1993). CO also has anti-apoptotic and anti-inflammatory effects mediated through mitogen-activated protein kinases (MAPK) and not guanylyl cyclase (Piantadosi and Zhang, 1996). These signaling relationships will be discussed in more detail below in the chapter. CO also has been postulated to play a role in inhibiting P450 enzymes since the ferrous form of this type of heme protein forms a tight-binding complex with CO.

2.2 Functions of HO-1

Whereas HO-2 seems to be important in managing heme levels during normal cellular metabolism, HO-1 serves to maintain homeostatic levels of heme under conditions of cellular stress. Oxidative stress is associated with increased rates of heme protein damage and in turn, free heme accumulation. Early studies with HO-1 speculated on a cytoprotective role for this enzyme given the following: 1) It was identified as a 32 kDa heat shock protein (Keyse and Tyrrell, 1989) induced by a variety of stressors that included direct oxidative stress; 2) it metabolized a compound (hemin) that was known to be harmful to cells at high concentrations (Kutty and Maines, 1984); and 3) it was induced (greater than 40-fold in some instances (Wright et al., 2006)) in virtually all tissues following exposure to a variety of cellular stressors including oxidative stress, UV radiation, hyperoxia, hypoxia,

hyperthermia, heavy metals, metal porphyrins, tumor factors, insulin, endotoxin, and sulfhydryl-reactive compounds (Keyse and Tyrrell, 1989) (reviewed in (Ryter et al., 2006)). Because most of the HO-1-inducing agents also cause elevated levels of oxidative stress, it has been postulated that HO-1 induction represents an early, "sentinel-type" response by the cell to counteract the deleterious effects of oxidative stress (Otterbein et al., 2000;Poss and Tonegawa, 1997b).

3. Experimental evidence for the cytoprotective role of HO-1

3.1 In vitro evidence for the cytoprotective role of HO-1

Most of the in vitro/in vivo evidence for HO-1 playing a cytoprotective role has examined the effects of inducers and inhibitors of HO-1 when cells/animals are dosed with a stressor. These types of studies are described in the paragraphs below. More sophisticated lines of in vivo evidence using gene knockout/therapy to modulate HO-1 levels will be referred to separately. Metal porphyrins and heavy metals are often used alternately in studies to implicate a function of HO in a cellular process. Whereas in most cases, both types of compounds induce the HO-1 gene (a major exception is tin mesoporphyrin which inhibits HO-1 induction) and elevate protein expression, the metal porphyrins will often bind to the HO active site resulting in the inhibition of enzyme activity.

Both in vitro and in vivo studies have demonstrated that the elevated expression and activity of HO-1 is associated with a greater tolerance to various types of stress. It was already mentioned how an in vitro study was used to demonstrate that HO-1 was a 32 kDa heat shock protein which was induced by cellular stress and protected cells from toxicities related to these stressors (Keyse and Tyrrell, 1989). Another interesting in vitro study used HepG2 cells that were transfected to constitutively express CYP2E1 to demonstrate the protective role of HO-1 during CYP2E1-mediated metabolism and oxidative stress (Gong et al., 2004). Of the P450 enzymes, CYP2E1 is especially prone to the breakdown of its monoxygenase catalytic cycle with the concomitant release of superoxide, hydrogen peroxide, and excess water (Gorsky et al., 1984). A previous study by the same lab used these cells to show that the oxidative stress associated with CYP2E1-mediated metabolism could be cytotoxic, especially after prior cellular depletion of glutathione by treatment with L-buthionine-(S,R)-sulfoximine (Chen and Cederbaum, 1998). In the study examining the role of HO-1, the cytotoxicity associated with the CYP2E1-mediated metabolism of arachidonic acid was not observed when HO-1 expression was up-regulated by transfection of the cells with an adenovirus containing the cDNA for human HO-1 (Gong et al., 2004). Furthermore, when the cells were treated with chromium mesoporphyrin, which acts as an inhibitor of HO-1, the CYP2E1-related toxicity was potentiated. The in vitro study also implicated CO but not bilirubin in the protective effects of HO-1, probably through the CO-related inhibition of P450 activity (discussed below).

In another in vitro study, the protective effect of the flavonoid, quercetin, on the hepatotoxicity of ethanol was attributed to its induction of HO-1 in hepatocytes because the effects of quercetin were abrogated by treatment with zinc mesoporphyrin (Yao et al., 2009). Addition of free iron increased the damage caused by ethanol, whereas CO treatment protected the cells from ethanol-induced toxicity. Thus, it was thought that the protection afforded by HO-1 induction was in part caused by the inhibition of P450-mediated

activation of ethanol by CO. Another study by this group indicated that HO-1 was induced through the MAPK/Nrf2 pathways of signal transduction (Yao et al., 2007). The in vitro studies of course are critical in elucidating the signaling pathways involved in heme metabolism. These pathways are discussed more completely below. Interestingly, the second enzyme responsible for heme metabolism, BVR has a very active role in the signaling required to modulate HO-1 levels with the ever-changing levels of heme in the cell.

3.2 In vivo evidence for the cytoprotective role of HO-1

Many in vivo studies have also tested for the protective role of HO-1 after exposure to toxins. Acetominophen is a widely-used analgesic that unfortunately has a narrow therapeutic index, and overdosing results in liver failure. Cytochrome P450-mediated metabolism is responsible for the harmful effects of acetaminophen as it converts the compound to a reactive quinone-imine that alkylates cellular protein and DNA. Interestingly, cellular glutathione effectively scavenges the reactive intermediate and protects against cytotoxicity. However, when an overdose occurs, the intracellular glutathione gets depleted resulting in the destruction of critical proteins that are necessary for cell function (Gibson et al., 1996). Several studies have tested for the ability of HO-1 to protect against acetaminophen toxicity in rats. In one of these studies, acetaminophen treatment resulted in HO-1 induction. To test whether the HO-1 expression was cytoprotective, the rats were treated with hemin to induce HO-1 prior to exposure to acetaminophen. These rats were indeed protected from acetaminophen hepatotoxicity compared to animals that were not pretreated with hemin. The study also found that biliverdin pretreatment was able to protect the rats from acetaminophen-induced hepatotoxicity (Chiu et al., 2002).

HO-1 induction was also shown to be protective from liver damage caused by carbon tetrachloride (Nakahira et al., 2003) and halothane (Odaka et al., 2000). Both of these compounds can be activated to free radical species by P450-mediated metabolism. Treatment with hepatotoxic doses of these compounds resulted in the rapid accumulation of intracellular free heme which was followed by HO-1 induction. It was found that when the rats were pre-treated with hemin (to induce HO-1) before halothane administration, hepatotoxicity was not observed. Similarly, when rats were treated with tin porphyrin 1 hour before administration of carbon tetrachloride to inhibit HO-1 activity, the carbon tetrachloride-induced liver injury was exacerbated (Nakahira et al., 2003). The findings of these studies suggest that free heme accumulation, presumably derived from the destruction of P450 enzymes, may be the main source of toxicity by these compounds. Thus, HO-1 induction was proposed to be an adaptive response that was critical for recovery from the toxic insults.

Many studies have investigated the ability of HO-1 to protect against endotoxin exposure. Endotoxin is a lipopolysaccharide produced by gram negative bacteria. Tissue exposure to endotoxin results in inflammatory injury and oxidative stress (Murphy et al., 1998) (McCord, 1993). In two separate studies, HO-1 induction in rats by hemin (Wen et al., 2007) and hemoglobin (Otterbein et al., 1995) pretreatment was protective against the deleterious effects of a subsequent (otherwise lethal) dose of endotoxin. In contrast, the rats were more susceptible to endotoxin toxicity, and the protective effects of HO-1 induction were ablated when the animals were treated with metal porphyrins that inhibited the HO-1 activity.

In vivo studies also demonstrated the ability of HO-1 induction to protect against acute renal failure in rats following ischemia/reperfusion (Toda et al., 2002) and exposure to mercuric chloride (Yoneya et al., 2000). Ischemia/reperfusion involves exposing the tissue to a sequence of oxygen deprivation followed by reoxygenation. Reoxygenation is associated with high levels of oxidative stress. Thus, it is a good model to examine the protective role of HO-1. The kidney ischemia/reperfusion study used tin chloride to induce the HO-1. Tin chloride induces HO-1 in a tissue-specific manner and does not induce HO-1 in the liver but does induce it in the kidney, demonstrating the complicated regulation of the HO-1 gene (discussed below). The fundamental role of HO-1 in mediating renal protection was demonstrated by showing that treatment with tin mesoporphyrin, an inhibitor of HO-1, did not prevent renal injury in the rats (Toda et al., 1995).

3.3 Gene knockout/therapy evidence for the cytoprotective role of HO-1

Over the last 10-15 years, novel research studies and interesting clinical findings have confirmed the cytoprotective role for HO-1. One of the seminal studies to demonstrate the protective role of HO-1 examined embryonic fibroblasts from HO-1 knockout mice and compared their attributes to those from normal wild-type animals (Poss and Tonegawa, 1997b). The cells from the knockout mice produced higher levels of ROS and also were less resistant to toxicity caused by hydrogen peroxide, paraquat, heavy metals, and heme exposure. The effects of HO-1 in the protection from free hemin exposure were quite dramatic offering 50% survival at a hemin concentration (200 µM) that was completely toxic to the cells from knockout mice. Another study from the same group, also compared the response of wild-type and HO-1 knockout mice to an intraperitoneal injection of endotoxin (Poss and Tonegawa, 1997a). Because the adult HO-1 knockout mice had a variety of health issues including anemia, iron-overloading, and chronic inflammation, younger mice (6 to 9 weeks) that did not display these phenotypes were used to study the effects of endotoxin. In terms of survival, the knockout mice were significantly more sensitive to endotoxin treatment and demonstrated higher levels of hepatic injury including increased serum liver enzyme levels and liver vacuolization. Interestingly, the hepatic injury seemed to be spatially and temporally related to iron loading malfunctions in both Kupffer cells and hepatocytes. Iron also accumulated in renal proximal cortical tubules.

Gene therapy studies to upregulate HO-1 have also been instrumental in proving that HO-1 is protective against cellular stress. In a study to demonstrate the role of HO-1 in vascular protection, a retroviral vector was used to transfect the human HO-1 gene into rat lung microvessel endothelium (Yang et al., 1999). Cells transfected with the retrovirus had over a 2-fold increase in HO-1 expression and activity. Furthermore, cGMP levels (probably regulated by CO activation of guanyly cyclase was almost 3-fold higher. These endothelial cells were significantly more resistant than untransfected cells to toxicity resulting from hydrogen peroxide and heme exposure. This protection was abolished upon treatment with stannic mesoporphyrin indicating the role of HO-1.

In another gene therapy study to investigate the ability of HO-1 to protect against the exposure of endotoxin in lung, an adenovirus encoding HO-1 was directly inoculated into rat trachea (Inoue et al., 2001). As a result, HO-1 was upregulated in both airway epithelium

and alveolar macrophages. This therapy was found to be as effective as HO-1 induction by hemin pretreatment in preventing the inflammatory reaction caused by aerosolized endotoxin exposure. Furthermore, the protection conferred by increased HO-1 expression seemed to be related to higher endogenous levels of Interleukin-10 production by the macrophages.

Gene therapy was also used to compare the oxidative stress resistance of cerebellar granular neurons isolated from wild type and transgenic, homozygous mice that were engineered to overexpress HO-1 (Chen et al., 2000) The transgenic mice overexpressing HO-1 generated lower levels of ROS and were more resistant to oxidative stress resulting from either glutamate or hydrogen peroxide treatment.

3.4 Clinical evidence for the cytoprotective role of HO-1

Finally, in a tragic clinical example, the protective role of HO-1 was profoundly demonstrated by an individual who did not have a functional HO-1 gene (Kawashima et al., 2002). The six-year old male patient presented with growth retardation, anemia, elevated levels of ferritin and heme in serum, low serum bilirubin, intravascular hemolysis, and hyperlipidemia. In contrast to the HO-1 knockout mice showing toxicity from iron overloading, the endothelial tissue of the human patient was more severely affected causing a spectrum of cardiovascular maladies. A lymphoblastoid-derived cell line from this patient was also extremely sensitive to hemin-induced oxidative stress (Yachie et al., 1999).

3.5 Therapeutic potential of HO-1 modulation

On the basis of these scientific and clinical findings, the role of HO-1 in maintaining homeostasis and protecting against cellular stress is now well established. In conjunction, the enzyme has been shown to be protective in various types of disease/injury models including the following: 1) inflammation (sepsis, atherosclerosis), 2) lung injury (pulmonary fibrosis, ventilator-induced injury), 3) cardiovascular injury/disease (myocardial infarction, hypertension), 4) ischemia/reperfusion, and 5) organ transplantation/rejection. There are now several excellent reviews that discuss the pharmacologic potential of HO-1 induction (Abraham and Kappas, 2008;Mancuso and Barone, 2009;Ryter et al., 2006). Unfortunately, this type of therapy is not straightforward as it has been observed that over-expression of HO-1 can be harmful from the accumulation of reactive iron (Suttner and Dennery, 1999) and the bilirubin that results from HO-1-mediated heme catabolism (Claireaux et al., 1953). Thus, HO-1 expression in this type of therapeutic treatment would need to be highly regulated to prevent over-expression of the enzyme.

4. Mechanisms of cellular protection by HO-1

Originally, it was thought that the sole mechanism by which HO-1 protected cells was through the catabolism of free heme and the elimination of its prooxidant activities (discussed at the beginning of the chapter). Ironically, it was originally thought that the other products of the HO-1 reaction were useless (or even toxic) by-products. Now, it is known that CO and biliverdin play multiple roles in protection and that there are actually several mechanisms by which HO-1 performs its cytoprotective functions. It is very likely that there are more mechanisms yet to be identified.

4.1 Protective roles of CO: Cell signaling mediated by CO

The most definitive proof for the protective effects of CO has been derived from studies using CO-releasing molecules (Motterlini et al., 2003) as a surrogate for HO-1-derived CO. As indicated above, the CO formed by HO-1 has been shown to activate guanylyl cyclase to mediate the relaxation and dilation responses of smooth muscle and vascular endothelial cells, respectively (Cardell et al., 1998;Christodoulides et al., 1995). In vascular tissue, CO has also been shown to stimulate relaxation of endothelial smooth muscle cells by activation of calcium-dependent potassium channels (Williams et al., 2004) via a poorly-understood mechanism that does not involve guanylyl cyclase. CO has been shown to serve as a partial agonist to nitric oxide synthetase (NOS) and thus, may down-regulate the level of NOS-dependent signaling (Hangai-Hoger et al., 2007;Ishikawa et al., 2005).

Interestingly, it also was reported that cGMP-dependent signaling was able to induce HO-1 through the cAMP responsive element in its promoter by a mechanism that was not elucidated (Immenschuh et al., 1998a). Logically, these cGMP-related effects may be initiated by HO-1-generated CO. Of course, the cAMP promoter element also allows HO-1 to be induced directly through cAMP-dependent signaling and activation of protein kinase A (Immenschuh et al., 1998b).

Many of the details about other types of signaling involving CO are poorly understood as it appears to be both cell type- and stressor-specific (Song et al., 2003a){Song, 2003 2563 /id}. Most studies have implicated the ability of CO (and CO-releasing molecules) to activate the P38 MAPK pathway in carrying out its anti-apoptotic and anti-inflammatory effects (Brouard et al., 2000;Dérijard et al., 1995;Keum et al., 2006;Otterbein et al., 2000).

The MAPK pathways have been shown to regulate processes such as inflammation, differentiation, tumor promotion, proliferation, apoptosis, stress response, and ion channels (reviewed in (Shen et al., 2005;Wada and Penninger, 2004)). Two of the three arms of the MAPK pathway (JNK and p38) have been implicated in the cellular stress response. Downstream activation of the MAPK pathway, and specifically the JNK arm, leads to the dimerization and DNA binding of the stress response factors, c-Jun and c-fos. CO dramatically inhibited JNK MAPK signaling in murine macrophages exposed to endotoxin which resulted in lower production of inflammatory cytokine, IL-6 (Morse et al., 2003). Furthermore, c-Jun activation has been linked to cellular proliferation (Yoshioka et al., 1995) so this would explain part of the role of CO in mediating proliferation/transformation.

The p38 arm of the MAPK kinase activates ATF-2 which competes with c-fos for binding to c-Jun. This heterodimer binds with greater affinity to the HO-1 promoter than the c-fos/c-Jun heterodimer (Kravets et al., 2004). Furthermore, ATF-2 dimers can bind and directly activate the cyclic AMP responsive element (CRE) in the promoter region of HO-1 (Lee et al., 2002). ATF-2 activation may play a role in the activation of transcription factor, NFκB (Kaszubska et al., 1993). NFκB plays an essential role in the response to both apoptotic and inflammatory stimuli and regulates the expression of cytokines, growth factors, and cell cycle effector proteins (reviewed in (Bonizzi and Karin, 2004;Shen et al., 2005)). ATF-2 involvement in the activation of NFκB could explain the anti-apoptotic/anti-inflammatory affects of CO (see below for more details). In support of the idea that ATF-2 and c-Jun oppose one another in the protective gene expression associated with HO-1 induction, c-Jun has been shown to inhibit activation of NFκB (Tan et al., 2009).

The ERK 1/2 MAPK regulates cellular growth and differentiation. Stimulation of the pathway has been shown to be protective against apoptosis (Wada and Penninger, 2004). However, overstimulation of ERK appears to be the major mechanism by which some oncogenes transforms cells (e.g. Ha-Ras (Hibi et al., 1993)). In one study of human airway smooth muscle cells, CO-mediated affects on guanylyl cyclase led to inhibition of ERK1/2 MAPK (Song et al., 2003b). Thus, this effect of CO on ERK 1/2 MAPK would serve to prevent overstimulation of this pathway and in turn, the uncontrolled proliferation of cells. ERK has been shown to phosphorylate the inhibitory protein of NFκB and thus facilitate activation of the transcription factor (Chun et al., 2003).

It has also been postulated that CO can activate transcription factors indirectly by mitochondrial-driven ROS production (Piantadosi, 2008). More specifically, it is known that CO is a potent inhibitor of the complex III-mediated terminal step of oxidative phosphorylation. The inhibition of this step of the mitochondrial electron transport chain results in excess ROS production which in turn, reacts with critical thiol groups of the phosphatases that turn off activated transcription factors. In this regard, CO-mediated, mitochondrial ROS production has been implicated in the prolonged activation of the phosphoinositide-3-kinase (PI3 kinase)/Akt pathway (Piantadosi, 2008;Pischke et al., 2005). Numerous studies have implicated this pathway in the protective effects of plant-derived antioxidants (that include induction of HO-1) by ultimately leading to the activation of the transcription factors, Nrf2 (Martin et al., 2004;Park et al., 2011;Pugazhenthi et al., 2007). Nrf2 is a member of the Cap-N-Collar/basic leucine zipper family of transcription factor that responds directly and indirectly to oxidative stress to mediate cytoprotective gene transcription through the antioxidant response elements (ARE) of gene promoters (reviewed in (Itoh et al., 1997;Itoh et al., 2003;Kwak et al., 2004)). Thus, CO-mediated activation of PI3 kinase/Akt provides anti-oxidative protection.

4.2 Regulation of important enzymes by HO-1

It was also discussed how the CO released from HO-1 can inhibit various enzyme activities. The ability of the molecule to inhibit cyclooxygenase may provide an anti-inflammatory effect by preventing the synthesis of inflammatory prostaglandins from arachidonic acid. CO also inhibits cytochromes P450. This action could be cytoprotective because unproductive P450-mediated metabolism results in the release of hydrogen peroxide and/or superoxide from the P450 active site. This activity is an unavoidable consequence of metabolism by these enzymes, and the amount of ROS produced in this manner is dependent on the substrate being metabolized and the specific type of P450 carrying out the reaction (Gorsky et al., 1984;Gruenke et al., 1995;Reed and Hollenberg, 2003). Furthermore, it has been reported that P450-mediated metabolism can generate destructive hydroxyl radicals under certain circumstances (Paller and Jacob, 1994;Terelius and Ingelman-Sundberg, 1988). The oxidative stress generated by P450-mediated metabolism is significant as it has been estimated that the rate of ROS formation by the endoplasmic reticulum can be as much as 30% of that by mitochondria during oxidative phosphorylation (Zangar et al., 2004).

In an idea originally proposed at the turn of the century, HO-1 may provide cytoprotection by indirectly inhibiting P450 activity (and its associated production of ROS) through its competition with P450 for binding to the P450 reductase (Emerson and LeVine, 2000). Both P450s and HO-1 obtain electrons needed for their respective reactions by binding to the P450

reductase. One of the odd aspects of the stoichiometry of these enzymes in the endoplasmic reticulum is that the amount of P450 enzymes far outnumber the amount of P450 reductase (with estimates as high as 25 P450s for every P450 reductase (Peterson et al., 1976). Thus, P450-mediated metabolism in the liver endoplasmic reticulum is extremely limited by the amount of available P450 reductase. Although the level of HO-1 in unstressed liver is very low, it can be induced to an amount that is comparable to that of P450s (Reed et al., 2011). Therefore, it seems likely that the induction of HO-1 would attenuate the rate of P450-mediated metabolism by limiting the ability of P450 to interact with P450 reductase. A preliminary investigation from our lab has provided support for this effect of HO-1 induction (Reed et al., 2011). Furthermore, recent studies in which cells were protected from oxidative stress by the transfection and induction of a mutant HO-1 which was not able to catalyze heme degradation also provides support for this type of indirect mechanism of cytoprotection (Lin et al., 2007;Lin et al., 2008). However as discussed below, the results with the shortened, inactive mutant could be explained if the mutant serves as a heme-carrier to shuttle heme to the nucleus in order to directly modulate gene transcription.

Although far from proven, HO-1 also might actually interact with P450 enzymes to accelerate the degradation of the P450s. Several studies, including a few that were cited above (Nakahira et al., 2003;Odaka et al., 2000), have postulated that HO-1 induction following a stress event coincides with a rapid accumulation of free heme which presumably originates from damaged P450 enzymes. Evidence also was derived by observing a dramatic increase in the rate of degradation of labeled heme from P450 enzymes after HO-1 was induced by either hemin or endotoxin treatment (Bissell and Hammaker, 1976). More specifically, the data suggested that HO-1 increased the degradation of the P450 and not just the catabolism of the heme released from the P450. Again, it is not possible to ascertain whether the damaged P450 releases the heme or the HO-1 binds to the P450 to scavenge and catabolize its heme group. More direct evidence of this putative effect of HO-1 was reported in a study finding that the incubation of purified P450s with either HO-1 and HO-2 caused the heme of the P450 enzymes to be degraded to biliverdin in essentially a 1 to 1 ratio (Kutty et al., 1988). The results also were consistent with the interaction of HO-1 and P450 causing one P450 (two were studied in the publication) to degrade to an inactive form.

Surprisingly, the research supporting the idea that HO-1 facilitates degradation of P450s is decades old but has not been followed up on and confirmed. One reason for this is the fact that the full-length HO-1 is very unstable and susceptible to truncation that generates an inactive, soluble form (28 kDa). The C-terminal part of the protein that is cleaved causes the HO-1 to interact with membrane lipids, and its removal alters the manner by which the enzyme interacts with potential membrane binding partners (Huber, III et al., 2009;Huber, III and Backes, 2007). Most in vitro studies of HO-1 have expressed and purified a modified, but active, 30 kDa form of the enzyme that lacks the C-terminal membrane-binding sequence and is soluble as a result. Our lab has recently modified the amino acid sequence of full-length HO-1 to remove a thrombin cleavage site in the C-terminal tale of HO-1 (Huber, III and Backes, 2007). This mutant is full-length and binds to lipid vesicles. The full-length HO-1 mutant also binds much tighter to the P450 reductase and has much higher catalytic efficiency than the active, soluble form of the enzyme (Huber, III et al., 2009). Thus, studies with this mutant will finally enable researchers to understand the enzymatic capability of HO-1 with respect to those of the other potential binding partners in the endoplasmic reticulum. The putative interaction of HO-1 with P450 may allow for very

efficient inhibition of the P450 by the HO-1-generated CO, providing a cytoprotective role by effectively removing the P450 as a contributor to cellular, oxidative stress.

4.3 Protective role of biliverdin/bilirubin

Originally biliverdin and the bilirubin formed by the BVR-catalyzed reduction of biliverdin were thought to be cellular waste products. However, it is apparent that both compounds have antioxidant properties and elicit various cytoprotective effects. Early studies implicated the antioxidant effects of these compounds by showing that they reacted with enzymatically generated superoxide in vitro (Galliani et al., 1985;Robertson, Jr. and Fridovich, 1982). Subsequent studies showed bilirubin to be a more potent antioxidant than α-tocopherol with respect to scavenging lipid peroxides (Neuzil and Stocker, 1993). In fact, both biliverdin and bilirubin were found to interact synergistically with vitamin E to prevent lipid peroxidation by an azo compound (Stocker and Peterhans, 1989). The fact that both of these bile pigments are lipophilic, especially bilirubin, makes them typically more effective than water soluble antioxidants in preventing the damage of membranes and organelles. Bilirubin bound to albumin was also shown to be an effective antioxidant in plasma by protecting the oxidation of low density lipoproteins (Stocker et al., 1987). In addition to its ability to scavenge ROS, bilirubin also inhibits the superoxide-generating NADPH oxidase (Kwak et al., 1991).

Evidence for cytoprotection mediated by the bile pigments comes from several studies. Bilirubin was shown to protect both neuronal cultures (Dore et al., 1999) and HeLa cells (Baranano et al., 2002) from hydrogen peroxide-induced toxicity. Furthermore, when cellular bilirubin was depleted by incubation of the cells with short antisense RNA to BVR, preventing the expression of BVR and in turn, its catalyzed conversion of biliverdin to bilirubin, intracellular levels of ROS increased and promoted apoptotic death of neuronal and HeLa cells (Baranano et al., 2002). It was found that the effects of bilirubin depletion had a greater pro-oxidant effect than depletion of cellular glutathione. In another study, pretreatment of cultured endothelial cells with bilirubin also protected cultured endothelial cells from pro-inflammatory responses after challenge by oxidized LDL and TNF-α (Kawamura et al., 2005). The level of protection of the endothelial cells was comparable to that achieved by preinduction of HO-1 with hemin. Interestingly, CO treatment of the cells did not protect them from these responses.

Bilirubin and biliverdin have also been shown to be protective in animal studies. Injection of bilirubin into rats prevented glutathione depletion following administration of cadmium chloride (Ossola and Tomaro, 1995). The two bile pigments also have been shown to be effective in various models of ischemia/reperfusion injury (Clark et al., 2000;Fondevila et al., 2004). Biliverdin treatment was as effective as hemin-mediated HO-1 induction in protecting rats from acetaminophen toxicity (Chiu et al., 2002). Bilirubin treatment also protected rats challenged with endotoxin by preventing an inflammatory response in the animals (Wang et al., 2004). Biliverdin and bilirubin also react with reactive nitrogen species such as nitric oxide and peroxynitrite. Thus, the compounds can attenuate NO signaling, and this was believed to be the cause of the anti-inflammatory effect in the endotoxin study (Wang et al., 2004). Another anti-nitrosative effect of HO-1 recently discovered is the finding that increased HO-1 expression was associated with induction of endothelial cell superoxide dismutase (Kruger et al., 2005). This, in turn, would lower the amount of superoxide available to react with NO to form peroxynitrite.

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Biliverdin also inhibits activation of NF-κB in HEK293 cells (Gibbs and Maines, 2007). The effect was observed to be specific for biliverdin and not bilirubin. In fact, overexpression of BVR, which converts the biliverdin to bilirubin, overcame the biliverdin-mediated inhibition of NF-κB. Thus, part of the anti-inflammatory effect of biliverdin may be caused by preventing activation of NF-κB. Biliverdin has also been shown to be a potent inhibitor of c-Jun N-terminal kinase and AP-1 pathway (Tang et al., 2007), and this effect has been associated with pro-inflammatory and pro-apoptotic responses. Bilirubin has been shown to modulate ERK1/2 signaling pathways (Taillé et al., 2003). Furthermore, it has now been shown that both biliverdin and bilirubin activate the aryl hydrocarbon receptor to induce expression of a spectrum of genes including CYP1A1 (Phelan et al., 1998). At this point, it is not fully appreciated how these cell-signaling effects mediated by biliverdin and bilirubin relate to cytoprotection.

In summary, the effects of biliverdin and bilirubin are complex and are poorly understood. Protection by these compounds seems to derive from antioxidant properties of the compounds as well as anti-nitrosative effects from scavenging NO. However, many more mechanisms may be involved to explain their effects. In fact, the effects of these compounds on cell signaling pathways are only beginning to be elucidated. It should also be mentioned that the balance between cytoprotection and toxicity is delicate in the case of bilirubin. High concentrations of this compound are neurotoxic and pro-oxidative (Claireaux et al., 1953; Stocker and Ames, 1987) adding to the complexity and importance of cellular heme regulation.

4.4 Ferrous iron release: The participation of ferritin and HO-1

You can put a rose on a herring, but it will still stink and be red. The same analogy can be used when trying to argue the protective "benefits" of HO-1-mediated ferrous iron production. Free ferrous iron will be a "smelly, red herring" with regards to oxidative/reductive homeostasis in the cell. As mentioned at the beginning of the chapter, the metal is prone to generating highly destructive hydroxyl radicals. Thus, it is a powerful pro-oxidant which would cause it to potentiate oxidative stress. In fact, a study which over-expressed HO-1 through a tetracycline-inducible vector found that the mutant cells were much more prone to deleterious iron-overload (Suttner and Dennery, 1999). Thus, HO-1 expression had a negative effect on cell survival in this instance. Presumably, HO-1 over-expression also could have a negative health impact from the potential build-up of bilirubin levels, as described above.

On the other hand, the metal is essential for the synthesis of heme and in turn, for all of the functions carried out by the heme proteins. Thus, HO-1 does provide a way for the iron in free heme or heme attached to damaged enzymes (which may act as even more potent pro-oxidants than free iron) to be recycled for future heme synthesis in the cell, so its activity does have a net positive effect on cellular health. Furthermore, heme induction also activates expression of the iron-storage protein, ferritin (Eisenstein et al., 1991). Thus, during heme-related stress, both ferritin and HO-1 are coordinately regulated (Tsuji et al., 2000). Ferritin is an iron storage protein that allows for the controlled release of iron to coincide with the metabolic needs of the cell (Ponka, 1997).

Interestingly, it has been suggested that there are *at least* two types of HO-1 inducing agents, heme-dependent and heme-independent (Bauer and Bauer, 2002). Subsequently, it was shown that heme-independent HO-1 induction did not necessarily induce ferritin (Sheftel et al., 2007). However, HO-1 will only produce excess free iron when there is an abundant supply of heme, so the ferritin will be induced in the cell when it is needed. In the study cited above (Suttner and Dennery, 1999) indicating iron over-load in cells overexpressing HO-1, the cells were transfected with an expression vector. Thus, hemin was not involved in the induction of HO-1 and consequently, ferritin was not induced enough to protect against iron overload from catalytically active HO-1.

Although it has not been proven definitively, it has been speculated that the location of HO-1 in the endoplasmic reticulum may facilitate the migration of free iron to the extracellular space and in turn, help maintain iron blood levels (Poss and Tonegawa, 1997a). Whether or not this putative role of HO-1 exists, the activity of the enzyme and the co-ordinate regulation of ferritin when HO-1 is induced through its cognate promoter give the cell a protective way to recycle iron and manage its levels in the cell.

5. Mechanisms of cellular protection by BVR

5.1 BVR-catalyzed redox cycle with lipid peroxides

The anti-oxidant effects and other positive health benefits of bilirubin, that are attributable to catalysis by BVR, are obviously important means of BVR-mediated cytoprotection. The antioxidant effects of this compound were discussed above, but the reason for its potency involves metabolism by BVR. In a study examining the cytoprotective effects of bilirubin in neuronal cells, it was found that as little as 10 nM bilirubin (physiologic levels) protected against 10,000-fold higher concentrations of hydrogen peroxide (Baranano et al., 2002). From these results, it was postulated that BVR participates in a redox cycle with lipid peroxides in which bilirubin is oxidized by the lipid peroxides to biliverdin which, in turn, is reduced by BVR to reform bilirubin (Figure 1). Thus, BVR may have an important role in extending the antioxidant potency of bilirubin. It should be noted that a recent study concluded that the cytoprotective role of this redox cycle was limited as BVR overexpression and inhibition of BVR expression with antisense RNA did not seem to influence hydrogen peroxide-mediated cytotoxicity (Maghzal et al., 2009).
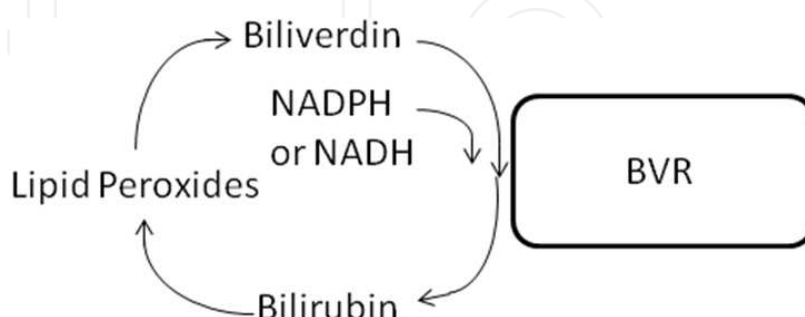

Fig. 1. BVR-catalyzed redox cycle with lipid peroxides. See text for details

5.2 BVR-mediated modulation of cell signaling

BVR may be the most versatile protein known. Research over the last decade has revealed new functions for the enzyme that have broadened its role in the cytoprotective response to

cellular stress signals. It is now known that BVR also functions as a dual-specific kinase of serine/threonine and tyrosine residues in proteins, and in this capacity, BVR affects the signaling and cellular responses to a variety of stimuli (Reviewed in (Kapitulnik and Maines, 2009)). Kinases capable of phosphorylating both threonine and tyrosine residues have been identified as those regulating upstream events in signal transduction pathways (Pawson and Scott, 2005). The discovery of this function of BVR was preceded by finding that its ability to metabolize biliverdin was dependent on protein phosphorylation and that the enzyme could catalyze autophosphorylation of this residue (Salim et al., 2001).

Subsequently, it was shown that BVR is regulated by insulin/insulin growth factor stimulation through receptor-mediated tyrosine phosphorylation (Lerner-Marmarosh et al., 2005). BVR binding to this receptor competes with insulin receptor substrates (IRS) 1 and 2 for binding to the receptor. BVR phosphorylates serine residues of the IRS which attenuates their affinity for the insulin receptor kinase, essentially inactivating them. Phosphorylated BVR can activate two protein kinase C proteins, βII and ζ, which are involved in cross-talk between the upstream components of the MAPK and phosphatidylinositol 3-kinase pathways, respectively. Protein kinase C βII also can activate BVR which partly contributes to the activation of BVR by stress signals (Maines et al., 2007). The activation of protein kinase C βII by BVR leads to activation of all three arms of the MAPK signaling. Thus, all of the effects of CO caused by its activation of the P38 MAPK (discussed above) also apply to the activation of this pathway by BVR.

BVR-mediated signaling appears to play a critical role in the recruiting transcription factor, NFκB to the HO-1 promoter (Gibbs and Maines, 2007). Furthermore, NFκB has been shown to be activated by protein kinase Cζ which in turn, is directly activated by BVR (Lerner-Marmarosh et al., 2007). As described in detail below, the involvement of NFκB appears to be important in mediating the anti-apoptotic, anti-inflammatory, and anti-proliferative effects associated with expression of HO-1.

BVR has the ability to form protein complexes with itself and other proteins, and serves to shuttle activated transcription factors to the nucleus. The ability of BVR to function as a dual cofactor enzyme with different pH optima expands its range of function in the cell (Kapitulnik and Maines, 2009). In addition to the activation of the ERK MAPK pathway by BVR through protein kinase C βII, BVR has been shown to play a critical role in shuttling the activated ERK to the nucleus to influence gene transcription (Lerner-Marmarosh et al., 2008).

Interestingly, BVR also binds to NFκB (Gibbs and Maines, 2007). This is intriguing because the HO-1 promoter does not have a prototypical response element for NFκB, and it has been conjectured that it must be recruited to the promoter by other transcription factors (Alam and Cook, 2007). Thus, through this interaction, BVR also may play a role in allowing NFκB to influence HO-1 gene transcription.

In another transport capacity, BVR also complexes with heme and shuttles it to the nucleus where the heme can bind to regulatory elements that influence gene transcription. In fact, heme-mediated gene induction has been shown to be dependent on BVR in renal cells (Tudor et al., 2008). Interestingly, although HO-1 is most typically observed in the endoplasmic reticulum, instances of it being located in other parts of the cell including the plasma membrane (where it localizes to caveolae rafts (Kim et al., 2004)), mitochondria

(Converso et al., 2006), and even as a shortened, soluble component in the nucleus have been reported (Lin et al., 2007). The significance of these findings has yet to be elucidated, but they may indicate that HO-1 also can transport heme to the nucleus (discussed below) and may be involved in targeting BVR to different organelles to mediate signaling or the biliverdin/BVR redox cycle to scavenge ROS from the mitochondria (discussed above).

5.3 BVR-mediated modulation of gene transcription through DNA binding

Although the kinase-dependent activity described above ultimately results in the modulation of gene transcription, BVR also has the capacity to bind to DNA and directly influence gene expression through a basic leucine zipper-binding motif. BVR binds to AP-1/CRE sites in DNA which are typically bound by c-Jun/fos heterodimers to mediate the stress response (Ahmad et al., 2002). In this capacity, BVR binds as homo- and hetero-dimers to recruit or block the binding of other transcription factors and thus, BVR can enhance or inhibit gene expression directly. At this point, BVR-mediated gene transcription has been shown to induce ATF-2 (Kravets et al., 2004). ATF-2 has also been shown to be capable of binding to and activating NFκB (Kaszubska et al., 1993).

Thus, with regards to the role of BVR in recruiting NFκB to the HO-1 promoter, BVR can facilitate its binding directly or through the upregulation and activation of ATF-2. However, it is likely that NFκB would bind to the promoter in different 5'-flanking regions when bound to BVR and ATF-2. Most of the specific effects of BVR-DNA binding on gene expression have not been elucidated. But the enzyme has been shown to translocate to the nucleus of HeLa cells (Tudor et al., 2008) after hemin induction and in rat kidney after exposure of animals to nephrotoxins (Maines et al., 2001). In fact, HeLa, heme-mediated induction of HO-1 expression was shown to be dependent on BVR expression (Tudor et al., 2008).

6. Assessing the dependence of HO-1-mediated cytoprotection on BVR

Given the diverse abilities of BVR to regulate cellular processes, it has been postulated that the HO-1 cytoprotective response is completely dependent on BVR. The hypothesis proposes that the increased expression of HO-1 caused by the wide variety of inducing agents is mediated by the signaling and DNA binding activities of BVR. Furthermore, in a study examining the catalytic steps of the HO-1 reaction, it was determined that binding of BVR and HO-1 was required to increase the rate of release of biliverdin from the HO-1 active site. The study found that in the absence of BVR, biliverdin release was the rate-limiting step of the HO-1 reaction, and its presence dramatically increased turnover by HO-1. In showing that even the catalytic activity of the enzyme was dependent on BVR, the study supported the idea that the HO-1 cytoprotective response was totally dependent on BVR. However, finding that HO-1 activity was dependent on BVR was counter-intuitive because the level of HO-1 can be induced many-fold by cellular exposure to stress signals (Wright et al., 2006). In contrast, the expression level of BVR is typically unchanged after stress (although it may be inducible in kidney (Maines et al., 2001)). Thus, it would seem to be unproductive for the cell to induce HO-1 and have its activity be limited by the supply of BVR. Such a situation also would allow little opportunity for biliverdin to accumulate in cells. Yet, studies show that biliverdin has completely different cytoprotective roles than bilirubin (see above). Thus, the enzymatic data showing that BVR regulated HO-1 enzymatic activity were baffling.

More recent enzymatic studies measuring steady state metabolism by HO-1 showed that BVR had no effect on the rate of HO-1-mediated catalysis (Reed et al., 2010). In fact, this study showed that the rates of HO-1-mediated heme catabolism measured by the formation of both biliverdin and a ferrozene:ferrous iron complex in the absence of BVR were slightly higher than that measured by bilirubin formation in the presence of BVR. The earlier results finding that HO-1 activity was dependent on BVR were attributed to the unusual conditions required to monitor the individual catalytic steps (namely anaerobic with limiting concentrations of NADPH).

The question still remains whether the HO-1-related gene response following exposure to stress signals is completely dependent on BVR. The HO-1 promoter contains a variety of responsive elements that includes two AP-1 sites, a CRE, a Maf recognition domain (binding partner to Nrf2), and a partial site for NFκB binding (Alam and Cook, 2007). Understanding how these responsive elements recruit transcription factors to influence gene transcription is complicated by the fact that many of the response elements overlap and thus, may inhibit or enhance DNA binding by factors to adjacent elements. The transcription factors that activate the HO-1 promoter will vary with the types of stress to which the cell is exposed (reviewed in (Alam and Cook, 2007). Furthermore, studies have shown that the signaling pathways activated by the same stress signals also can vary in different cell types (Ryter et al., 2006). As a result, the transcription factors mediating a specific cytoprotective response can vary in different cell types. Thus, a type of HO-1-mediated cytoprotection may depend on BVR in some cell types but not others. Because of these considerations, the discussion pertaining to the dependence of HO-1 expression on BVR will be a generalization based on the typical roles of the implicated transcription factors in cellular processes.

6.1 Anti-oxidant protection

With this perspective in mind, the various protective roles of cell signaling and gene transcription associated with HO-1 induction are mediated largely through activation of either Nrf2 or NFκB. Certainly one of the first evolutionary roles needed for survival of a cell would have been to develop a defense system for the oxidative stress associated with the activity of heme enzymes. Heme oxygenase is the only enzyme that uses its heme cofactor as a substrate. Furthermore, as discussed below, HO-1 can be induced directly by heme interaction with the HO-1 gene repressor Bach-1. Therefore, it is the only known eukaryotic enzyme that has a substrate that regulates a transcription factor needed for its expression. This scenario would suggest that heme oxygenase represents a very old link in the evolutionary chain. By the same line of reasoning used above to rationalize the evolutionary role of HO-1 function, Nrf2/anti-oxidant response must represent an early evolutionary adaptation to allow living organisms to survive oxidative stress. Interestingly, Bach-1 is an effective repressor of the HO-1 gene because it blocks Nrf2 from binding to the HO-1 promoter. Because heme metabolism is intimately connected to reductive/oxidative homeostasis in the cell, it seems plausible that Nrf2 can mediate HO-1 expression independently from BVR as a response (initially at least) to oxidative stress.

One caveat to the hypothesis that the anti-oxidative response is independent of BVR is dictated by whether or not the shuttling of heme to the nucleus by BVR is the only mechanism by which this can occur (discussed above). As alluded to above, Bach 1 blocks the binding of Nrf2 to the antioxidant response element when Bach 1 is not bound to heme

(Sun et al., 2002). In support of the idea that an anti-oxidant response can be mediated directly by Nrf2 without involvement of BVR, HO-1 was found to be constitutively expressed in Bach 1 knockout mice (Sun et al., 2002). Thus, it is proposed that nuclear heme localization and functional Nrf2 are the essential components of the initial gene response to oxidative stress. In some instances, BVR has been shown to be critical for heme translocation to the nucleus. However, the results are mixed and may be cell type-specific. For instance, the same laboratory has shown that inhibition of BVR expression with small interference (antisense) RNA blocked the hemin-mediated induction of HO-1 cells in HeLa cells (Tudor et al., 2008) but had no effect in COS cells (Ahmad et al., 2002).

Recent studies showing that shortened HO-1 also translocates to the nucleus may serve as another mechanism by which hemin is transported to the nucleus to directly influence gene transcription (Lin et al., 2007). This would allow the shuttling role of BVR to be bypassed in some cells and would explain results showing that increased expression of a catalytically inactive mutant was also able to up-regulate HO-1 expression (Lin et al., 2008). Another way that cells could possibly bypass BVR-mediated heme shuttling can be postulated from the results of a study showing that heme bound to and stabilized Nrf2 (Alam et al., 2003). This led to activation of Nrf2 and the heme-responsive element in the HO-1 promoter. Thus, Nrf2 could also transport heme to the nucleus to influence gene transcription during oxidative stress. Therefore, it does seem necessary to require BVR to transport heme to the nucleus for most cell types.

There also are several general experimental findings that support this inutitive argument. First, Nrf2 is believed to be the primary transcription factor activated directly by oxidative stress, and its activation is associated with induction of phase II and antioxidant enzymes (Bellezza et al., 2010;Ishii et al., 2002;Shen et al., 2005). A multitude of studies have implicated Nrf2 in the induction of HO-1 during oxidative stress (Alam et al., 2003;Gong and Cederbaum, 2006a;Gong and Cederbaum, 2006b;Sun Jang et al., 2009). Finally, in two studies looking at the effects of oxidative stress (by hydrogen peroxide treatment), it was found that cell viability was only marginally affected (Baranano et al., 2002) or not affected at all (Maghzal et al., 2009) by silencing BVR (with interference RNA). The latter study also showed that BVR induction and over-expression also did not provide protection to the cells exposed to hydrogen peroxide. Thus, it may not be a coincidence that studies have not yet implicated a connection between BVR actions and Nrf2. In fact, the binding site for Nrf2 in the HO-1 promoter overlaps with one of the AP-1 sites to which BVR can bind (Alam and Cook, 2007). Differences between antioxidant response elements and AP-1 binding sites have been distinguished previously (Yoshioka et al., 1995). Thus, it appears Nrf2 binding and AP-1 binding by BVR might be somewhat competitive with Nrf2 binding occurring initially after oxidative stress and BVR replacing the transcription factor from the antioxidant response element after prolonged oxidative stress.

In addition, to the demonstration of constitutive HO-1 expression in Bach-1 knockout mice, there appears to be a unique aspect of the HO-1 promoter which allows Nrf2 to initiate transcription without recruitment of other transcription factors. It has been found that the chromatin remodeling protein, BRG1, interacts with Nrf2 to form a Z-DNA structure which permits access for RNA polymerase II to initiate transcription of the HO-1 gene (Zhang et al., 2006). This interaction between BRG-1 and Nrf2 was exclusive to the HO-1 gene (but not other Nrf2–regulated genes) by virtue of a series of TG repeats that are present in the HO-1

promoter. In fact, genetic polymorphisms affecting this region have been shown to correspond to susceptibility to oxidative stress-related diseases (Ishii et al., 2000).

Thus, the right panel of Figure 2 is drawn to indicate the BVR-independent activation of HO-1 gene transcription after exposure to oxidative stress. ROS can activate Nrf2 directly by oxidizing Keap-1 which is responsible for binding Nrf2 in the cytoplasm and facilitating its degradation (Itoh et al., 2003). Oxidation of critical cysteine residues in Keap-1 releases Nrf2, allowing it to translocate to the nucleus and activate gene transcription (reviewed in (Kwak et al., 2004)). In addition, CO inhibits mitochondrial electron transport resulting in ROS production which inhibit phosphatases needed to inactivate PI3K. Prolonged activation of PI3K results in stimulation of Akt which can phosphorylate Nrf2 (Piantadosi, 2008). Phosphorylation causes Keap-1 to release and activate Nrf2. ROS can also lead to activation of JNK MAPK. It has been shown that JNK target, c-Jun, can bind to Nrf2 (Shen et al., 2005). Thus, c-Jun/fos dimers binding to the AP-1 site facilitate the recruitment of Nrf2 to bind to the overlapping Maf-recognition element and induce HO-1 transcription. Previous binding by the c-Jun/fos dimer may help facilitate the binding of Nrf2 if Nrf2 can exchange with fos for binding to c-Jun. BVR is shown in this panel to serve as a heme transporter to the nucleus. That putative role is also indicated for the shortened form of HO-1 that has been identified in the nucleus (Lin et al., 2007) and for Nrf2. One additional role that BVR could play in the prolonged anti-oxidant response is to modulate the initial response by increasing or decreasing the anti-oxidant response depending on the needs of the cell.

6.2 Anti-apoptotic/proliferation protection

For the reasons below, it is postulated that BVR plays an integral role in the signaling responsible for anti-proliferative, anti-inflammatory, and anti-apoptotic responses by facilitating the interaction of NFκB with the HO-1 promoter and by modulating the activity of the transcription factor. As mentioned above, NFκB regulates the expression of cytokines, growth factors, and cell cycle effector proteins (Du et al., 1993;Hayden and Ghosh, 2011;Peng et al., 1995). As a result, the factor regulates important physiological processes such as immune response and apoptosis, and overstimulation or dysregulation of this factor is associated with inflammation, transformation, and proliferation of cells (Bubici et al., 2006). Thus, the cytoprotective role of HO-1 induction for processes such as inflammation, apoptosis, and proliferation would logically involve mechanisms affecting NFκB function. Scientific studies have shown that NFκB and Nrf2 are oppositely and variably regulated by different types of cellular stress (Bellezza et al., 2010). Nrf2 is typically activated by low or moderate levels oxidative stress, whereas NFκB is turned on by inflammatory signals or very high levels of oxidative stress. Thus, with regards to the HO-1 promoter and various cytoprotective responses mediated by enzyme induction, stress signals will specifically alter the relative levels of cellular transcription factors and in turn, will determine whether Nrf2 or NFκB binds to activate transcription of the HO-1 gene. The JNK MAPK target, c-Jun has been shown to bind to Nrf2 (Shen et al., 2005), and inhibit NFκB (Tan et al., 2009). However, the P38 MAPK target, ATF-2 has been shown to bind to NFκB (Kaszubska et al., 1993). The model below proposes that the relative levels of c-Jun and ATF-2 play a major role in different cytoprotective responses mediated by HO-1 induction.

One essential role that BVR may play in modulating cytoprotective gene expression associated with HO-1 induction is the activation and recruitment of NFκB to the HO-1

promoter (Gibbs and Maines, 2007). It is proposed that this function is the main determinant in mediating the anti-apoptotic response associated with HO-1 induction. NFκB activation has been associated with prevention of apoptosis following treatment with cytokines and tumor promoters (Papa et al., 2006;Sen et al., 1996). Biliverdin inhibits activation of NFκB (Gibbs and Maines, 2007), and BVR reverses this affect by metabolizing biliverdin to bilirubin and by promoting PKCζ-mediated phosphorylation and activation of the transcription factor (Lerner-Marmarosh et al., 2007).

The importance of BVR in mediating the apoptotic response has been demonstrated repeatedly. When BVR was over-expressed in HEK 293 and MCF-7 cells, the cells were arrested in G_1/G_o stage (Gibbs and Maines, 2007). Furthermore, over-expression of BVR also protected cells from NFκB-mediated proliferation after stimulation with TNF-α (Gibbs et al., 2010). These findings are consistent with a number of studies that have used various cell types (HEK293 (Miralem et al., 2005), HeLa (Ahmad et al., 2002), cardiomyocytes (Pachori et al., 2007) and renal epithelial cells (Young et al., 2009)) to show that the inhibition of BVR expression using interference RNA resulted in apoptosis after the cells were challenged with arsenite, hydrogen peroxide, hypoxia/reoxygenation, and angiotensin II, respectively. Thus, repression of BVR expression consistently leads to apoptosis after cells are challenged with various types of stressors. Most importantly, with regards to protection from apoptosis, NFκB has been shown to be a necessary factor for the activation of the tumor suppressor protein, P53 (Ryan et al., 2000). Consistent with the role of BVR in preventing apoptosis, bilirubin also has been shown to have anti-apoptotic effects in a variety of studies (Bulmer et al., 2008;Kim et al., 2006;Parfenova et al., 2006). In addition, activation of ERK MAP kinases (mediated by BVR (Lerner-Marmarosh et al., 2008)) has been shown to favor anti-apoptotic responses (Wada and Penninger, 2004) which would also be consistent with the anti-apoptotic role of BVR. For these reasons, it seems probable that the BVR-mediated recruitment and activation of NFκB are essential for anti-apoptotic HO-1 induction.

It is speculated that BVR also plays another important role in the anti-apoptotic signal. Studies have shown that BVR induces expression of P38 MAPK target, ATF-2 (Kravets et al., 2004). ATF-2 is constitutively expressed and not induced by environmental stimuli (unlike c-Jun) (Angel and Karin, 1991;Herdegen and Leah, 1998). Furthermore, it can form mixed dimers with c-Jun, and this hetero-dimer binds with much tighter affinity to AP-1 sites than c-Jun/fos dimers (Benbrook and Jones, 1990). Furthermore, ATF-2 dimerizes with itself and binds to the CRE response element instead of the AP-1 stress response element. Most importantly with respect to anti-proliferative, anti-inflammatory, and anti-apoptotic cytoprotection, ATF-2 also has been shown to dimerize with NFκB (Kaszubska et al., 1993). It is proposed in the mechanism below that these functions of ATF-2 along with the participation of BVR mediate HO-1-related cytoprotection from apoptotic, inflammatory, and hyper-proliferative stimuli.

The left panel of Figure 2 shows cell signaling cascades that may mediate the general anti-apoptotic HO-1 response. It seems likely that apoptotic/anti-apoptotic specificity involves a change in gene expression mediated by c-Jun/fos dimerization to one mediated by ATF-2 homo-dimerization. The critical events in this transition are BVR-mediated activation of NFκB (by direct phosphorylation and metabolism of biliverdin (BV in figure) to bilirubin (BR) and amplification of P38 MAPK relative to the c-Jun arm of MAPK). As mentioned above, biliverdin has been shown to be an inhibitor of NFκB (Gibbs and Maines, 2007). The

amplification of P38 MAPK relative to JNK and ERK MAPKs would be consistent with the effects of CO as the molecule activates P38 but inhibits JNK/ERK MAPKs (summarized above). In addition, biliverdin has been shown to be a potent inhibitor of JNK MAPK (Tang et al., 2007). Furthermore, c-Jun activation has been linked to cellular proliferation (Yoshioka et al., 1995) so switching from c-Jun-driven to ATF-2-driven transcription would be protective against proliferation/transformation. To show attenuation of the JNK MAPK pathway, the JNK arm of MAPK is shown as a dashed arrow in the figure panel to show that its activation is attenuated relative to that of P38 kinase. Because BVR has been shown to activate ERK MAPK (Lerner-Marmarosh et al., 2008), the arrow from ERK is a mixed dash/dot symbol to show moderate activation. Activation of ERK MAPK has been shown to facilitate anti-apoptotic responses (Wada and Penninger, 2004), and this might be related to the ability of ERK proteins to catalyze phosphorylation of the NFκB-inhibitory protein that keeps NFκB in the cytosol (for review of NFκB activation see (Shen et al., 2005)). As described above, signaling and DNA transcription mediated by BVR also lead to activation and increased expression of the P38 target, ATF-2, so this is another factor that increases the relative activation and concentration of ATF-2 (note the arrow from BVR to ATF-2 in the panel). Furthermore, BVR activates PKCβII (the latter can also activate BVR so a double headed arrow connects the two kinases in the panel) which serves to activate all three arms of MAPK signaling.

As ATF-2 concentrations and its level of activation increases relative to c-Jun and fos, c-Jun/fos hetero-dimerization would be replaced with c-Jun/ATF-2 dimerization at the AP-1 site. Because a P38-mediated pathway leading to activation of Nrf2 has been reported as a cytoprotective response in a cell line derived from human bronchial epithelial cells that were exposed to CeO2 nanoparticles, it is possible that the c-Jun/ATF-2 dimer serves as a more potent transcription factor in the recruitment of Nrf2 to the HO-1 promoter (Eom and Choi, 2009). Further increases in the concentration of ATF-2 would favor homo-dimerization of the transcription factor at the CRE site instead of the AP-1 site. ATF-2 has been shown to bind to NFκB (Kaszubska et al., 1993), and it has been shown that P38-mediated phosphorylation of Nrf2 promotes its association with the inhibitory protein, Keap1 (Keum et al., 2006). Both of these aspects of P38 pathway activation would favor activation of NFκB over Nrf2. Thus, it is proposed that the ATF-2 dimerization is the key signal that recruits NFκB to bind to the HO-1 promoter to induce expression of the gene. As proposed in the anti-oxidant response with the c-Jun/fos dimer facilitating recruitment of Nrf2 to the promoter, the ATF-2 dimer would allow NFκB to bind to the promoter as it exchanges with one of the ATF-2 units of the dimer. Another consistent aspect of the transition from the binding of c-Jun to that of ATF-2 in the recruitment of NFκB is the finding that c-Jun has been shown to inhibit NFκB activation (Tan et al., 2009).Thus, in the left panel of figure 2, the role of ATF-2 is represented by having its arrow point towards that for NFκB in the nucleus. Consistent with studies showing that Nrf2 and NFκB are co-regulated in opposite directions in response to stress signals (Bellezza et al., 2010), binding by NFκB is proposed to displace Nrf2 from the HO-1 promoter. The change in binding to the CRE site also may be critical in this regard because the AP-1 site overlaps more with the Nrf2 binding site than the CRE site. Thus, there would be less competition between Nrf2 and NFκB for binding to the HO-1 promoter. Because BVR can bind to both CRE and NFκB (Gibbs and Maines, 2007;Kravets et al., 2004), it may also serve to recruit NFκB to bind near the CRE.

In addition to activating NFκB, BVR also binds to the transcription factor in a manner that modulates its activity to favor protective gene expression relative to harmful pro-apoptotic,

pro-inflammatory and pro-proliferative targets (Gibbs et al., 2010). Consistent with the possibility that BVR regulates the activity of NFκB, TNF-α-mediated stimulation caused NFκB to act as a repressor of BVR expression which demonstrates that BVR is competitive with the inflammation process mediated by TNF-α through activation of NFκB. Because of the BVR-mediated modulation of NFκB, the arrow from NFκB is drawn as a dash in the panel.

6.3 Anti-inflammatory protection

It appears that the anti-inflammatory effects associated with HO-1 induction (middle panel of figure 2) might be mediated by both BVR-dependent and BVR-independent processes. ROS formation (produced by immune cells) is a big component of inflammation. Thus, it seems unlikely that the anti-inflammatory response is totally regulated by BVR (middle panel of figure 2). Thus, for the reasons given in the preceding paragraph, Nrf2 will be activated independently of BVR, probably as an initial response to inflammatory stimuli. Another implication of the role of Nrf2 in the HO-1-related anti-inflammatory response has come from studies with plant-derived, phenolic diterpenes that elicit both anti-oxidant and anti-inflammatory responses. Not coincidentally, these compounds also mediate HO-1 induction through Nrf2 following stimulation of phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling (Martin et al., 2004;Pugazhenthi et al., 2007). The Akt protein kinase activated by PI3K is distinct from the PKCζ that is known to be activated by BVR (Lerner-Marmarosh et al., 2007). As described above, CO has been implicated in mediating signaling through Akt (Piantadosi and Zhang, 1996). Thus, this pathway of Nrf2 activation appears to be related directly to the catalytic activity of HO-1. These mechanisms activating Nrf2 contribute to the anti-inflammatory effects associated with HO-1 induction.

An appealing hypothesis, that seems consistent with research findings, proposes that BVR-independent processes activate Nrf2 at the early stages of inflammation, whereas BVR-mediated MAPK and NFκB activation play critical roles in the cellular response at later stages of inflammation. In support of this idea, NFκB signaling typically opposes Nrf2 mediated signaling as a later event in response to many stress events (Bellezza et al., 2010). The putative ability of BVR to bind and modulate the activity of NFκB is important in the latter response because agonist binding to immune receptors cause potent activation of NFκB. It is important to emphasize that this modulating role is not the only way BVR would protect cells from NFκB-mediated stress. By replacing NFκB with Nrf2 at the HO-1 promoter, BVR would be diverting NFκB from promoting harmful gene expression while freeing up Nrf2 to activate protective gene expression. The anti-inflammatory effects of CO generated by HO-1 activity also would act independently of BVR (described above). A recent study demonstrated that HO-1 activity (as opposed to merely BVR-related cell signaling and DNA binding) was essential for the anti-inflammatory effects following treatment with endotoxin (Tamion et al., 2006). The anti-inflammatory response demonstrated by the treated animals was explained by both the inhibition of tumor necrosis factor-α production and the elevation of interleukin-10. Because the anti-inflammatory effects required catalytic activity by the induced HO-1 (activity was inhibited by treatment with tin mesoporphyrin), it can be assumed that either BVR-mediated signaling/DNA-binding was dependent on HO-1 activity or the effects were caused by bilirubin/CO. Either premise dictates that BVR effects occurred secondary to those mediated by HO-1 activity. For these reasons, it seems that the anti-inflammatory response is pleiotropic and depends on both BVR-dependent and BVR-independent signaling and gene transcription.

In the middle panel, the anti-inflammatory signaling that results in the later-staged recruitment of NFκB to the HO-1 promoter is represented. The scheme for Nrf2 activation preceding NFκB activation is shown in the far right panel. In the anti-inflammatory response, CO formed by HO-1 inhibits the JNK and ERK MAPK pathways. The CO-mediated inhibition of c-Jun protects against uncontrolled proliferation. However, BVR modulates ERK to protect against apoptosis through activation of NFκB. Initially, c-Jun inhibits NFκB activation which is shown as the red block line. Eventually, ERK activity and ATF-2 dimerization at the HO-1 promoter will favor gene transcription mediated by NFκB. BVR also modulates NFκB activity to favor protective gene expression, so the arrow from NFκB is dashed. In addition, as mentioned above, diversion of NFκB to stimulate the protective induction of HO-1 limits its ability to stimulate inflammatory gene expression and allows more Nrf2 to activate transcription of other anti-oxidant genes.
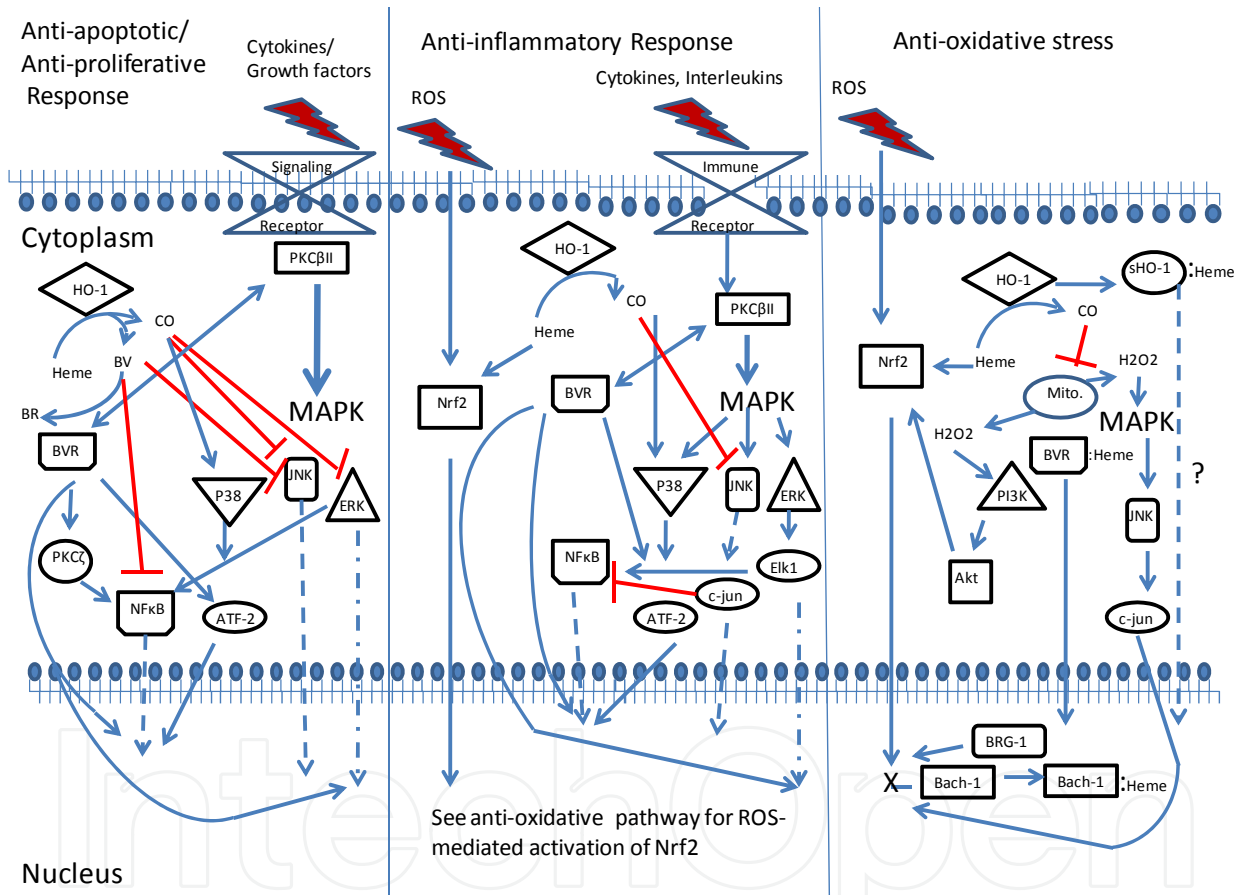

Fig. 2. Signal transduction regulation of the HO-1 protective response to cellular stress signals. The schematic diagram shows the putative signaling pathways responsible for gene induction/repression following different types of cellular stress (apoptotic signals, inflammatory stimuli, and oxidative stress). Arrows ending in the cytoplasm point at downstream kinases or transcription factors activated by the stimuli at the base of the arrow. Arrows ending in the nuclei represent the binding of transcription factors to gene promoters to affect expression and mediate the cytoprotective responses. Double-headed arrows represent kinase reactions that can occur in both directions (see text for details). Dashed arrows indicate attenuated activation, and arrows directed to the same point either have a role in binding together at the promoter or modulating the activity of one another.

7. Conclusions

Hemin is an essential cofactor for heme proteins that carry out a multitude of vital oxidative and oxygen transport-related functions in the cell. Unfortunately, hemin is also reactive and extremely harmful to cells when accumulated in the free form. A highly regulated system has evolved to control the levels of cellular heme. HO-1 and BVR catalyze the steps involved in heme catabolism. Interestingly, the enzymes are also involved in a host of cytoprotective functions mediating anti-oxidant, anti-apoptotic, anti-proliferative, and anti-inflammatory responses that have been proven to be therapeutic in many clinical disease models. HO-1 directly mediates most anti-oxidant effects through the following mechanisms: 1) the removal of heme in coordination with the up-regulation of the iron storage protein, ferritin; 2) the production of a lipophilic, anti-oxidant, biliverdin; 3) the regulation of both cell signaling and gene expression; and 4) the regulation of the cytochrome P450 system. Gene transcription mediated by Nrf2 largely mediates the cellular response to oxidative stress. BVR contributes to the antioxidant response by catalyzing a redox cycle that involves the BVR-mediated conversion of the potent antioxidant, bilirubin. In addition to catalyzing the second step of heme catabolism, BVR also acts as an upstream activator of MAPK and phosphatidylinositol-3-kinase pathway; directly binds to DNA; and participates in the transactivation of AP-1 sites in the HO-1 promoter. The anti-apoptotic effects associated with HO-1 induction are most often caused by BVR-mediated cell signaling and DNA-binding that leads to NFκB activation, but signaling effects related to HO-1-catalyzed CO production work in concert with the effects of BVR to protect against inflammation. Much remains to be learned about the specifics of cytoprotection via BVR-mediated signaling and gene transcription in addition to the roles of biliverdin and bilirubin in altering gene transcription. Similarly, because most in vitro studies of the enzymology of HO-1 have used a shortened mutant that does not bind to membrane or interact with membrane binding partners in the same manner as full length HO-1, almost nothing is known about how interactions between HO-1, BVR, and cytochrome P450 reductase are regulated to influence cell signaling, gene expression, the metabolism by HO-1, and oxidative stress related to P450-mediated, metabolism.

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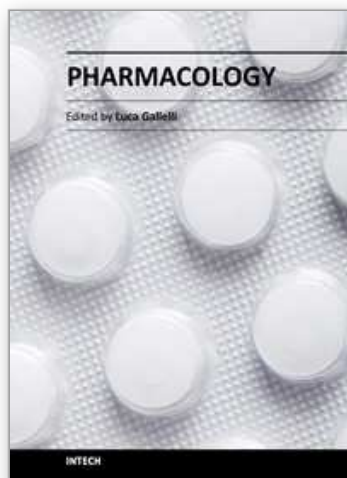
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The history of pharmacology travels together to history of scientific method and the latest frontiers of pharmacology open a new world in the search of drugs. New technologies and continuing progress in the field of pharmacology has also changed radically the way of designing a new drug. In fact, modern drug discovery is based on deep knowledge of the disease and of both cellular and molecular mechanisms involved in its development. The purpose of this book was to give a new idea from the beginning of the pharmacology, starting from pharmacodynamic and reaching the new field of pharmacogenetic and ethnopharmacology.

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