Journal of Cellular Biochemistry 100:815–823 (2007)

FAST TRACKS

Impact of Silencing HO-2 on EC-SOD and the Mitochondrial Signaling Pathway

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Abstract The contribution of heme oxygenase HO-2, the primary source of bilirubin and carbon monoxide (CO) under physiological conditions, to the regulation of vascular function has remained largely unexplored. Using siRNA HO-2, we examined the effect of suppressed levels of HO-2 on vascular antioxidant and survival proteins. In vivo HO-2 siRNA treatment decreased the basal levels of EC-SOD, pAKT proteins (serine-473 and threonine-308), without changing Akt protein expression. HO-2 siRNA treatment increased 3-nitrotyrosine (3-NT) and apoptotic signaling kinase-1 (ASK-1) ($P < 0.01$). HO activity was decreased by the use of siRNA HO-2. We extended these studies to the mitochondria, examining for the presence of HO-1 and its role in the regulation of pro- and anti-apoptotic proteins. HO activity was increased by the administration of CoPP resulting in the translocation of HO-1 into the mitochondria, mainly to the inner face of the mitochondrial inner membrane. These findings suggest that HO-2 is critical in the maintenance of heme homeostasis and also the regulation of apoptosis by controlling levels of EC-SOD, Akt, 3-NT, and ASK-1. In addition, localization of HO-1 in the mitochondrial compartment plays a critical role in mitochondria-mediated apoptosis. J. Cell. Biochem. 100: 815–823, 2007. © 2006 Wiley-Liss, Inc.

Key words: mitochondria; siRNA; apoptotic signaling kinase-1; EC-SOD; superoxide

The heme-heme oxygenase (HO) system is a regulator of endothelial cell integrity and oxidative stress. Excess heme, due to its pro-oxidant and pro-inflammatory properties, contributes to an increase in free radical formation and cell injury [Abraham et al., 1995]. Two isoforms, HO-1 and HO-2, are solely responsible for heme breakdown. The observation that HO-1 is strongly induced by its substrate, heme, and by oxidant stress in conjunction with the robust ability of HO-1 to guard against oxidative insult (for review see Abraham and Kappas, 2005; Turkseven et al., 2005) has led to an examination of the antioxidant nature of HO-1 and HO-2. Their antioxidant effects arise from the capacity to degrade heme from destabilized heme proteins and from the production of biliverdin and bilirubin, which have potent antioxidant properties [Sedlak and Snyder, 2004]. In addition, the iron released by HO is sequestered by the iron storage protein ferritin [Oberle et al., 1998]. Iron chelation has been reported to be anti-apoptotic, even in the absence of bilirubin and carbon monoxide (CO) [Berberat et al., 2003]. The ability of HO-1, or its products, to prevent cell death is attributed to its augmentation and exportation of iron-binding proteins [Ferris et al., 1999]. Further, upregulation of endogenous HO-1 and increased CO levels in endothelial cells have been shown to block apoptosis, but this effect was reversed when CO was scavenged by hemoglobin [Abraham and Kappas, 2005].

Grant sponsor: NIH; Grant numbers: DK 068134, HL55601, HL34300; Grant sponsor: Philip Morris USA, Inc.; Grant sponsor: Philip Morris International.

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Received 29 July 2006; Accepted 18 August 2006

DOI 10.1002/jcb.21138

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During apoptosis, mitochondrial membrane permeability increases and the release of pro-apoptotic factors results in an apoptotic phenotype [Green and Reed, 1998]. Mitochondria are the richest cellular source of reactive oxygen species (ROS) and play an essential role in the regulation of the key steps in apoptotic cell death by affecting energy metabolism, participating in intracellular Ca^{2+} homeostasis, regulating the activation of caspases, and releasing ROS [Liss et al., 2005]. Since ROS produced by the mitochondria are involved in apoptosis, the mitochondrial antioxidant system, including superoxide dismutase (SOD), reduced glutathione, and glutathione peroxidase, play crucial roles in regulating this process [Atzori et al., 2004]. The upregulation of HO-1 increases mitochondrial transport carriers and cytochrome oxidase via an increases in BCL-xL [Di Noia et al., 2006]. The Bcl-2 family of proteins, consisting of both anti- and pro-apoptotic proteins and serine-threonine kinase (Akt) (protein kinase B), are critical in cell death/survival pathways [Franke et al., 1997]. Akt is activated through phosphorylation at either threonine-308 or serine-473 [Alessi et al., 1996]. Activated Akt inhibits Bad, a pro-apoptotic member of the Bcl-2 family, which promotes cell death by dimerization with Bcl-2 and Bcl-XL [Yang et al., 1995].

It has been shown that increased HO activity leads to an increase in extracellular superoxide dismutase (EC-SOD) [Kruger et al., 2005; Turkseven et al., 2005]. The present report demonstrates that HO-2, the constitutive form of HO and thus the primary source of bilirubin, iron and CO under physiological conditions, regulates EC-SOD protein expression. Silencing HO-2 resulted in the induction of apoptotic signaling kinase-1 (ASK-1) and 3-NT, and a reduction in phosphorylated Akt (serine-473 and threonine-308). In addition, we demonstrate the localization of HO-1 in the mitochondria, largely on the inner face of the mitochondrial inner membrane and to a lesser extent in the matrix. We observed an increase in HO-1 in the mitochondrial in siRNA-HO-2 after CoPP treatment. The increases in HO-1 was associated with a concomitant reduction in mitochondrial release of cytochrome c into the cytosol and an enhancement of cytosolic levels of phospho-Bad, Akt, and Bcl-2 protein expression. Thus, HO-1 may have a central role in regulating the mitochondria-mediated anti-apoptotic pathways associated with mitochondrial production of ROS and the resultant induction of anti-apoptotic protein expression.

MATERIALS AND METHODS

In Vivo siRNA Transfection

RNA interference [Konnikova et al., 2003] offers great potential as a novel therapeutic strategy based on the highly specific and efficient silencing of a target gene. In the present study, three pairs of HO-2 siRNA, both scrambled and specific siRNA, were tested in cell cultures (Ambion, Inc., Austin, TX). The siRNA that were most effective in inhibiting HO-2 protein expression were selected. siRNAs (siHO-2 and siCON) were first dissolved in RNase-free water at a concentration of 80 nmol/300 μl and then mixed with 100 μl of NeoPhectin-AT (NeoPharm, Inc., Waukegan, IL). The complexes of siRNA-NeoPhectin-AT were incubated at room temperature for 20 min and diluted with 400 μl of normal saline before injection into the tail vein of 6-week-old SD rats (300 nmol/kg, once a day) for 3 days. On Day 4, rats were sacrificed for the analysis of protein expression. The chosen siRNA sequences were as follows: rat siHO-2 (sense): CAACUCUACGGCACCAGAAtt, (antisense): UUCUGGUGCCGUAGAGUUGtt and control (siCON) (sense): GACUCAGUCAGCAGAUGAUtt, (antisense): AUCAUCUGCUGACUGAGUCtt.

Animal Treatment and Tissue Preparation for HO Activity, Superoxide, and Western Blot Analysis

Animals were treated with either saline (control) or CoPP (0.5 mg/100 gr bw) by subcutaneous injection. Animals were sacrificed using pentobarbital. All experiments were approved by the Institutional Animal Care and Use Committee and conducted under NIH guidelines for the care and use of Laboratory animals. Frozen thoracic aorta and kidney segments were pulverized under liquid nitrogen and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% tergitol pH 7.5). Homogenates were centrifuged at 27,000*g* for 10 min at 4°C, supernatant was isolated, and protein levels were assayed (Bradford method). The supernatant was used for measurement of HO-1/HO-2, EC-SOD, Cu/Zn SOD, (Stressgen

Biotechnologies, Victoria, BC), ASK-1, Akt, phospho-Akt (serine-473 and threonine-308) (Cell Signaling, Danvers, MA), and 3-NT (Cayman Chemical, Ann Arbor, MI). HO activity and western blot analysis were determined as previously described [Turkseven et al., 2005].

Isolation of Mitochondrial, Cytosolic, and Microsomal Fractions

Mitochondrial, cytosolic, and microsomal fractions were isolated from renal tissues as previously described [Di Noia et al., 2006]. The fractions were used for assessing HO-1 (Stressgen Biotechnologies), Akt, Bad, phospho-Bad (ser112), Bcl-2 (Cell Signaling), VDAC-1 (Abcam, Cambridge, MA), CYT-IV (Molecular Probes, Eugene, OR), and cyt-c (BD Pharmingen, San Jose, CA) protein levels.

HO-1 Immunogold Labeling

With some modifications, HO-1 immunogold labeling was carried out according to Philimonenko et al. [2002]. The samples were immersed in 2.5% glutaraldehyde in 0.1 M TBS buffer (pH 7.4) overnight at room temperature. After washing in 0.1 M TBS, the samples were embedded in LR white resin and ultrathin sections were picked on nickel grids, which were stained by floating them on 25 μl drops of reagents on parafilm with the reaction side down. The following procedures were performed at room temperature. The grids were washed in 0.1 M TBS (pH 7.4) for 5 min, then in 0.1 M TBS containing 0.1% BSA for 30 min. After washing again in TBS, the grids were incubated in 5% normal goat serum for 30 min. After washing in TBS, the grids were then incubated in polyclonal rabbit antibody against HO-1 in a dilution 1:50 in 0.1 M TBS overnight. After washing in 0.1 M TBS (pH 7.4, 3×5 min), the grids were incubated for 1 h in a gold conjugated (15 nm) secondary antibody (goat anti-rabbit IgG) diluted 1:100 in 0.1 M TBS plus BSA. After washing in TBS (4×5 min), the grids were stained with uranyl-acetate for 5 min, washed in distilled water (3×5 min), and examined with a TEM CM10 electron microscope (Philips, Milano, Italy). For negative controls, the grids were incubated in dilution buffer instead of the primary antibody.

Statistical Analyses

The data are presented as mean ± standard error (SE) for the number of experiments. Statistical significance ($P < 0.05$) between the experimental groups was determined by the Fisher method of analysis of multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor analysis of variance (ANOVA) for multiple groups or unpaired *t*-test for two groups.

RESULTS

Silencing HO-2 Protein Expression

We have previously shown that HO-derived CO and bilirubin play an important role in cell survival and in increasing EC-SOD [Kruger et al., 2005; Turkseven et al., 2005]. To delineate the roles of HO-1 and HO-2 in EC-SOD expression in normal SD rats, we silenced constitutive HO-2 expression in vitro and in vivo using HO-2 siRNA. Endothelial cells transfected with 100 nM HO-2 siRNA had decreased HO-2 protein expression (data not shown). We evaluated the efficacy of the HO-2 siRNA in vivo by intravenous administration of HO-2 siRNA, which was effective in inhibiting HO-2 protein. Dose-response and time-course results showed that a dose of 300 nM/kg daily for 3 days was effective in significantly silencing HO-2 in various tissues, including heart, kidney, and aorta. Aortic HO-2 protein, for example, was decreased by 68% (Fig. 1A). The inhibitory effect of HO-2 produced by siRNA caused a modest, but not a significant, increase in HO-1 protein in vivo (Fig. 1B).

HO-2 siRNA Inhibits EC-SOD Protein

We tested the regulatory role of HO-2 on EC-SOD protein expression using HO-2 siRNA. Rats treated with HO-2 siRNA displayed decreased basal levels of EC-SOD (Fig. 1C). The administration of CoPP, a potent inducer of HO activity, resulted in a significant increase in EC-SOD (Fig. 1C). In contrast, 3-NT levels (a marker for peroxynitrite-mediated oxidative stress) were significantly increased by silencing HO-2 (Fig. 1D).

Differential Effect of HO-2 siRNA on Signaling Molecules

Since Akt plays a role in cell survival, we measured the levels of Akt and phospho-Akt (serine-473 and threonine-308) protein expressions in siHO-2 animals. Phospho-Akt was decreased with siRNA HO-2 treatment

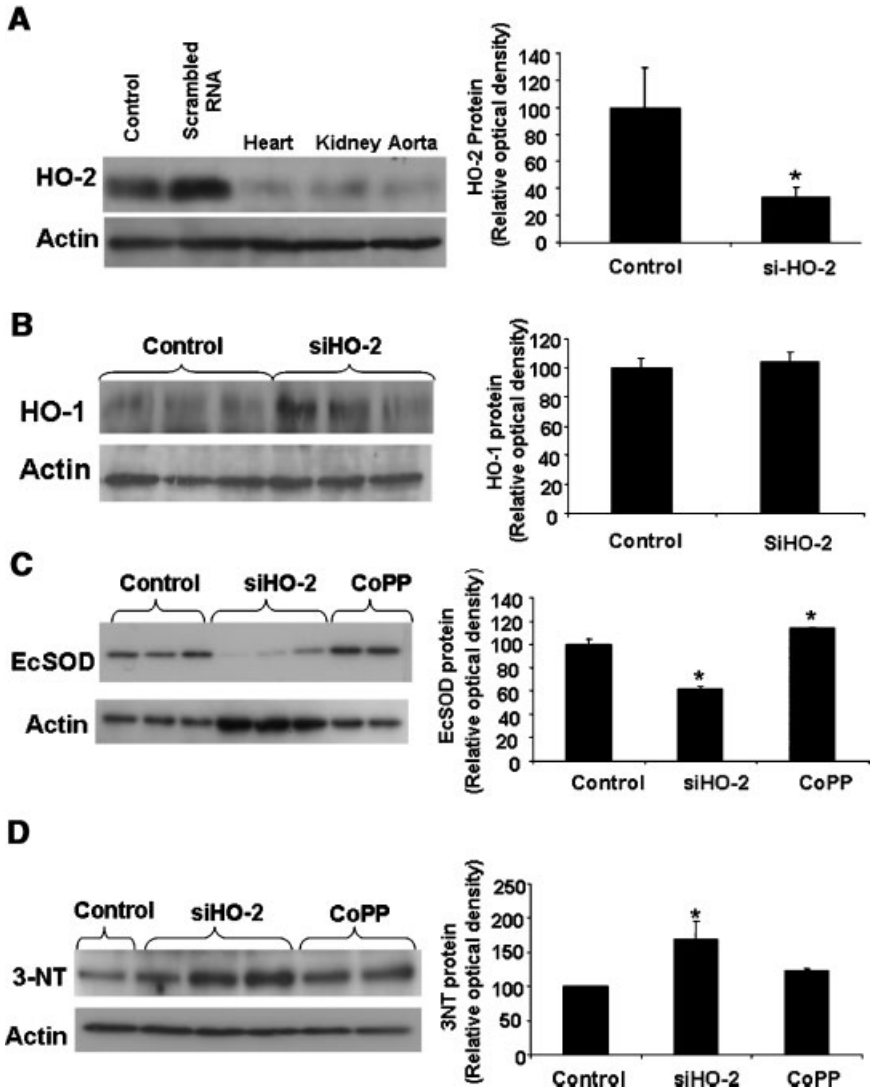

Fig. 1. Western blot analysis of **(A)** HO-2 protein expression in aorta, heart, and kidney of control rats and rats transduced with siHO-2. Quantitative densitometry evaluation of HO-2 to β-actin ratio was determined. Results are expressed as mean ± SE, n = 4; $^*P < 0.001$ versus corresponding control, **(B)** HO-1 protein expression in aorta obtained from control rats and rats treated with siHO-2. Quantitative densitometry evaluation of HO-1 to β-actin ratio was determined, **(C)** EC-SOD protein expression in aorta obtained from control rats and rats treated with siHO-2 or CoPP. Quantitative densitometry evaluation of EC-SOD and Cu/ZnSOD to β-actin ratio was determined. Results are expressed as mean ± SE, n = 4; $^*P < 0.001$ versus corresponding control, **(D)** 3-NT in the aorta of control rats and the rats treated with siHO-2 or CoPP. Results are expressed as mean ± SE, n = 4; $^*P < 0.05$ versus control rats.

(Fig. 2A). There was no change in the levels of Akt expression in siHO-2-treated rats and CoPP treatment had no effect on Akt protein expression (Fig. 2A). Since the apoptosis-regulating kinase, ASK-1, is activated by ROS, we examined ASK-1 protein expression to determine if suppressed HO-2 associated with an increase in ROS resulted in induction of ASK-1. Our results show that ASK-1 expression was significantly increased in siHO-2-treated rat aortas compared to controls (Fig. 2B). CoPP treatment had no significant effect on ASK-1 protein expression, suggesting a significant role for HO-2 in ASK-1 expression. These results demonstrate that changes in HO-2 not only influence on EC-SOD but also the expression of signaling molecules.

Detection of HO-1 in Mitochondria, Signaling Proteins, and Cytochrome c Release

Since mitochondria are the main site of ROS production and HO-1 has been shown to

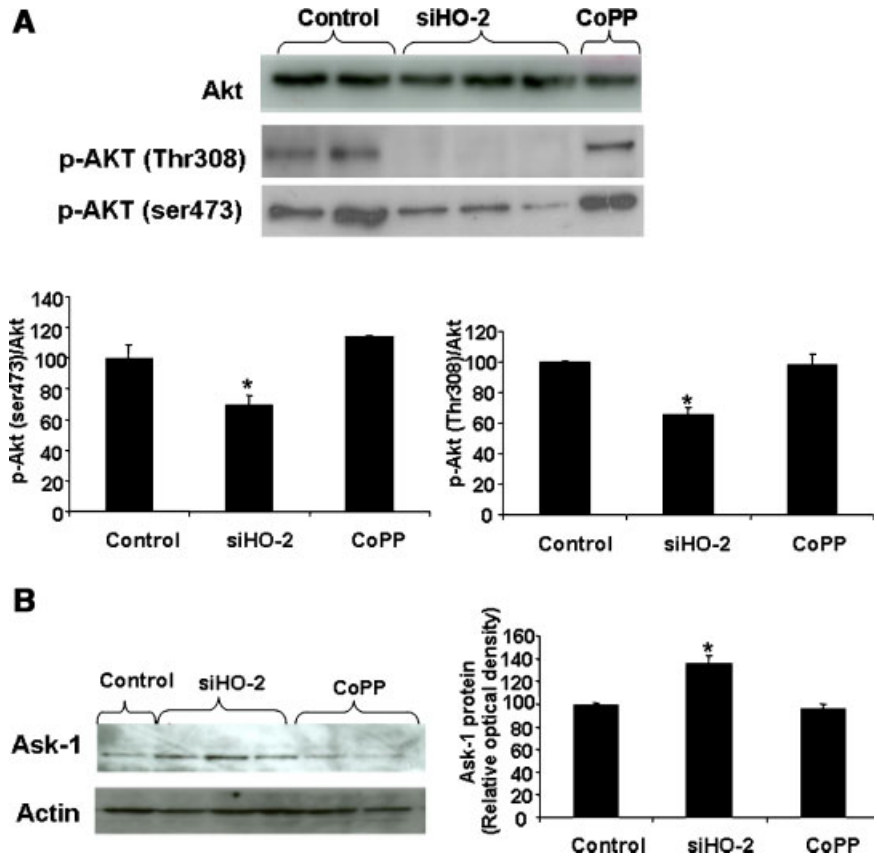

Fig. 2. Western blot analysis of **(A)** Akt, phospho-Akt (ser473), and phospho-Akt (thr308) protein expression in aorta of control rats and rats treated with siHO-2 or CoPP. Results are expressed as mean ± SE, n = 4; $^*P < 0.01$ versus control rats, **(B)** ASK-1 protein expression in the aorta of control rats and treated group. Results are expressed as mean ± SE, n = 4; $^*P < 0.01$ versus control rats.

increase mitochondrial cytochrome oxidase [Di Noia et al., 2006], we examined this organelle in renal tissue for the presence of HO-1. Western blot analysis showed that HO-1 protein is transported from the cytosol to the mitochondrial compartment under CoPP-induced conditions. HO-1 was barely detected in the mitochondria obtained from control animals; however, CoPP administration produced a robust increase in mitochondrial HO-1 (Fig. 3A). As expected, HO-1 protein expression was significantly higher in microsomes obtained from CoPP-treated rats compared to controls. We also evaluated the levels of VDAC-1, cytochrome oxidase subunit IV, and actin protein in cellular fractions, they represent mitochondrial, cytosolic, and microsomal fractions, respectively, to evaluate for possible contamination (Fig. 3A). Furthermore, HO activity was increased in both mitochondrial and microsomal fractions with CoPP treatment compared to control groups (Fig. 3B). We performed immunoelectron microscopy using goat-anti-rabbit serum conjugated with colloidal gold to measure mitochondrial HO-1 as has been previously described [Philimonenko et al., 2002]. Colloidal gold particles (10 nm diameter) were not seen in mitochondrial controls (Fig. 3C(b)). In contrast, colloidal gold particles were clearly detected in the mitochondria of kidneys in which HO-1 was upregulated by CoPP (Fig. 3C(c)). The labeling was located mainly on the inner face of the mitochondrial inner membrane and, to a lesser extent, in the matrix (Fig. 3C(c)). In addition, levels of known signaling proteins expressed in mitochondrial, cytosol, and microsomal fractions were compared. The levels of the apoptopic protein Bad were unchanged in the mitochondrial fraction obtained from CoPP-treated rats when compared to that from controls. Phospho-Bad at serine-136, the Akt phosphorylation site of the protein, as well as Bcl-2 and Akt were increased in cytosol with CoPP treatment (Fig. 4A). This

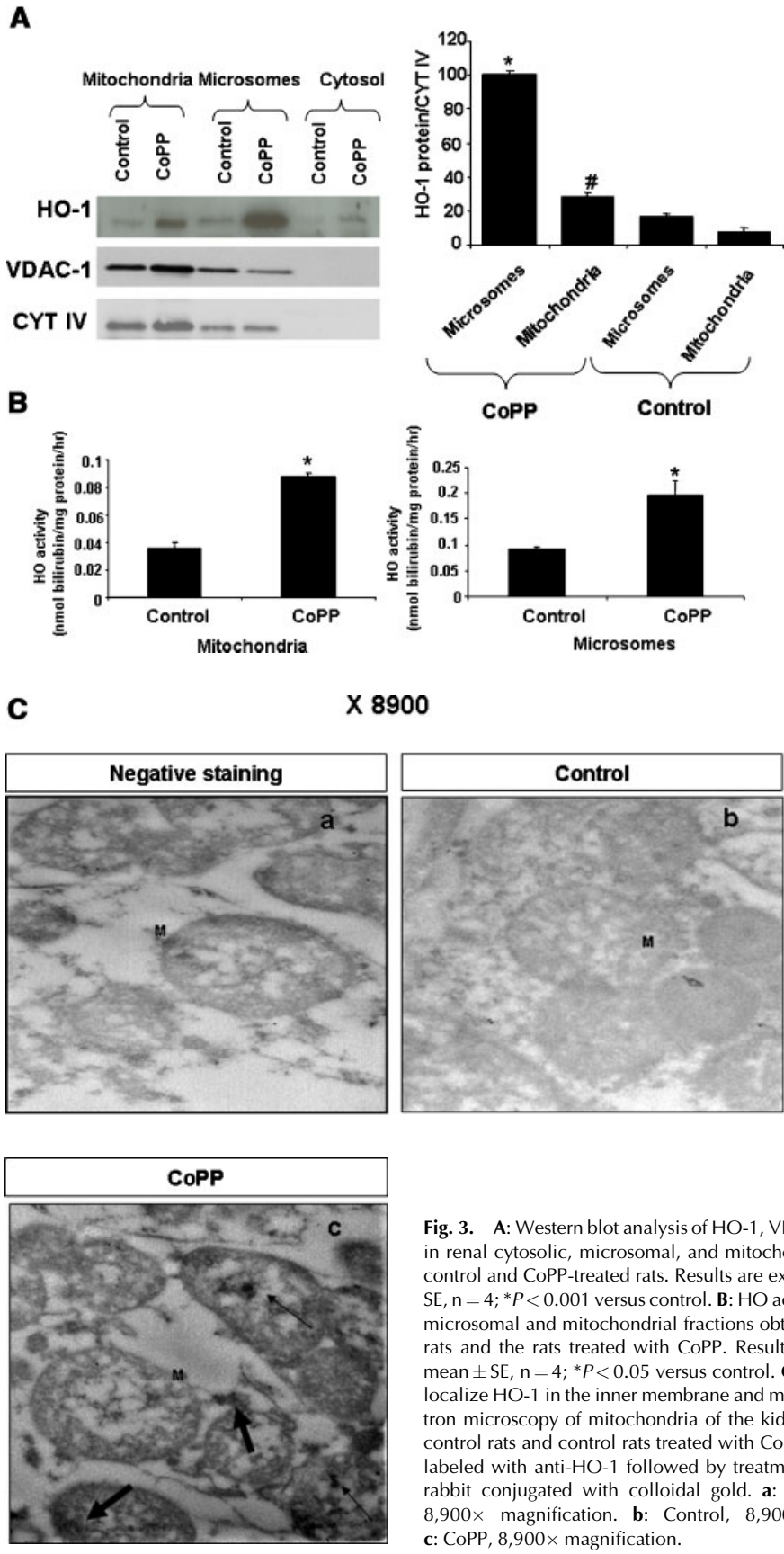

Fig. 3. **A**: Western blot analysis of HO-1, VDAC-1, and CYT IV in renal cytosolic, microsomal, and mitochondrial fractions of control and CoPP-treated rats. Results are expressed as mean ± SE, n = 4; $^*P < 0.001$ versus control. **B**: HO activity of the kidney microsomal and mitochondrial fractions obtained from control rats and the rats treated with CoPP. Results are expressed as mean ± SE, n = 4; $^*P < 0.05$ versus control. **C**: Magnification to localize HO-1 in the inner membrane and matrix. Immunoelectron microscopy of mitochondria of the kidney obtained from control rats and control rats treated with CoPP. Organelles first labeled with anti-HO-1 followed by treatment with goat-anti-rabbit conjugated with colloidal gold. **a**: Negative staining, 8,900× magnification. **b**: Control, 8,900× magnification. **c**: CoPP, 8,900× magnification.

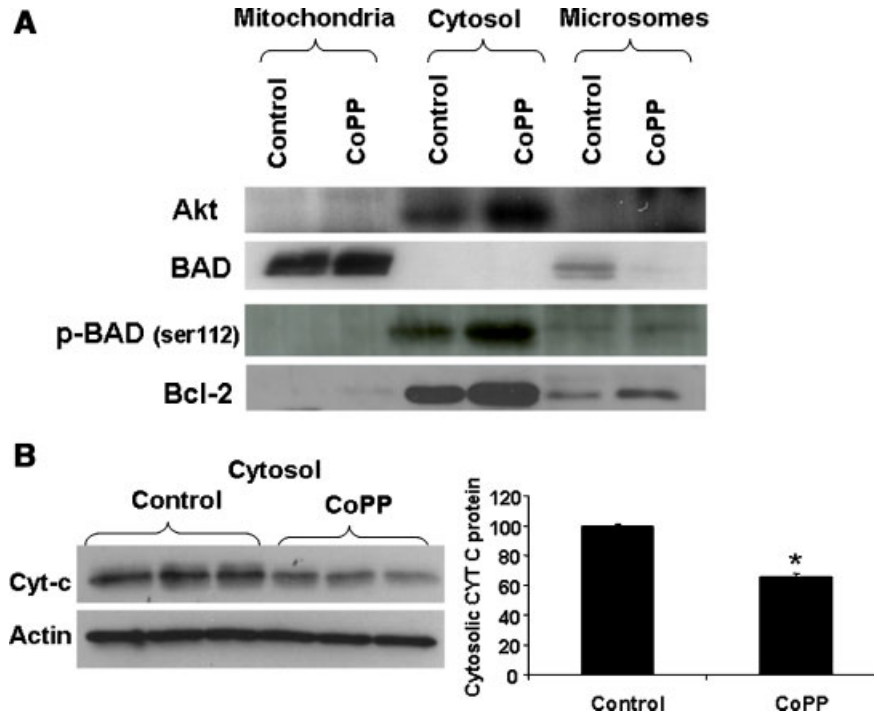

Fig. 4. Western blot analysis of **(A)** Akt, Bad, p-Bad (ser112), Bcl-2 in renal cytosolic, microsomal, and mitochondrial fractions obtained from control rats and rats treated with CoPP. **B**: Cytosolic Cyt-c in control rats and rats treated with CoPP. Results are expressed as mean ± SE, n = 4. *$P < 0.001$ versus control.

also demonstrates that the distribution, but not the organization, of these proteins responded to HO-1 induction.

As seen in Figure 4B, induction and translocation of HO-1 by CoPP result in inhibition of cytochrome c release from mitochondria into cytosol. These results suggest that HO-1 is critical in the reduction of mitochondria-mediated ROS generation and apoptosis through its presence in the mitochondria as evidenced by the decrease in cytochrome c release.

DISCUSSION

This study demonstrates that the basal levels of HO-2 participate not only in maintaining heme homeostasis but also in the cellular defense mechanisms against oxidative stress by regulating the levels of EC-SOD, Akt, 3-NT, and ASK-1, presumably by influencing the rate of heme degradation to bilirubin, CO, and iron. A number of key findings detailed in the present report substantiate this conclusion. The first critical finding was that EC-SOD protein levels were greatly diminished in rats treated with siRNA HO-2 (Fig. 1C) while levels of 3-NT were increased (Fig. 1D). Further, the levels of phospho-Akt (serine 473 and threonine 308) were decreased in siRNA HO-2-treated groups (Fig. 2A). Additionally, the decrease in HO-2 expression and HO activity produced by siRNA resulted in an increase in ASK-1 protein expression (Fig. 2B). It has been reported that ASK-1 can induce apoptosis by triggering a mitochondria-dependent pathway that includes Bid cleavage, Bax mitochondria translocation, cytochrome c release, and the subsequent activation of caspase 9 and caspase 3 [Hatai et al., 2000]. ASK-1 activity is regulated by several serine/threonine kinases. One of the critical mediators of ASK-1 activity is Akt, which phosphorylates ASK-1 at ser-83 residue to inhibit ASK-1-induced apoptosis [Kim et al., 2001]. Thus it appears HO-2 may have a critical role in the regulation of ASK-1-mediated apoptotic pathways. The anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-XL, prevent the release of apoptotic proteins from mitochondria [Wang, 2001; Cory et al., 2003]. One of the important substances released from mitochondria during apoptosis is cytochrome c, which is a component of a mitochondrial transfer chain [Liu et al., 1996]. The nucleotide

binding to the Apaf-1 cytochrome c complex triggers its oligomerization to form the apoptosome, which recruits procaspase 9 [Zou et al., 1999]. When procaspase 9 binds to apoptosome, it becomes the caspase 9 holoenzyme that is responsible for the cleavage and activation of downstream caspases [Rodriguez and Lazebnik, 1999]. The results reported here indicate that Bcl-2 transcription responded strongly to HO-1 induction, suggesting that HO-1 induction and thus increased levels of either CO and/or bilirubin are necessary to account for the prevention of apoptosis.

The BH3-only family of proteins share a sequence homology with Bcl-2 only in the BH3 domain. These proteins are normally located in various cellular compartments and only translocate to the mitochondria in response to apoptotic stimuli. Once in the mitochondria, they interact with the other Bcl-2 family members causing mitochondrial damage and the release of apoptotic proteins [Huang and Strasser, 2000]. Bad is one of the BH3-only proteins and is primarily regulated by phosphorylation and dephosphorylation [Zha et al., 1996]. Our results show that phosphorylated Bad at the serine-136 site was significantly increased in cytosol by CoPP treatment (Fig. 4A), suggesting that HO-1 can prevent Bad-regulated cell death. The link between heme-HO-1, antioxidants, and anti-apoptosis has been convincingly demonstrated [Pileggi et al., 2001; Abraham et al., 2003; Quan et al., 2004]. The balance between pro- and anti-apoptotic mechanisms is clearly related, in part, to cellular heme levels, and the anti-apoptotic and antioxidative properties of HO-1/-2, but may ultimately depend on the elimination of the substrate, heme [Abraham and Kappas, 2005]. Recently, it has been shown that the upregulation of HO-1 expression decreases heme levels and restores mitochondrial transport carriers, cytochrome oxidase, and anti-apoptotic proteins [Di Noia et al., 2006; Turkseven et al., 2006]. This may be contributing to translocation of HO-1 into the organelle with a resultant decrease in mitochondrial heme and pro-oxidants [Converso et al., 2006].

The second important finding was that HO-1, under induced conditions, is found in the mitochondria confirming that HO-1 may be translocated to the mitochondria. Although the mechanism(s) of HO-1 translocation and/or synthesis in mitochondria are not the focus of this article, these findings signify a defining a new role for HO-1 as an antioxidant in this organelle. Localization of HO-1 in the mitochondria and the cytoplasm compartments suggests that HO-1-derived CO and bilirubin may have dual roles in the anti- and pro-apoptotic pathways. Our results, demonstrating that HO-1 is localized in the mitochondrial compartment, suggest that HO-1 may also play a critical role in mitochondria-mediated anti- and pro-apoptotic pathways. This is substantiated by the inhibition of cytochrome c release after CoPP induction of HO-1 (Fig. 4B). Others have shown the potential utility of agents that affect mitochondrial metabolism as a novel mechanism to control oxidative stress [Lee et al., 2003].

In summary, the present study documents a regulatory action for HO-1/HO-2 in vascular EC-SOD and mitochondrial ASK-1 expression and in the regulation of anti-apoptotic and pro-apoptotic molecules. Given the presence of HO-1 in the mitochondria, the antioxidative, and anti-apoptotic properties of HO-1, and the central role of mitochondria in apoptosis, it is therefore reasonable to suggest that HO-1 and HO-2 act together to preserve the integrity of the mitochondria.

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