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Upregulation of Heme Oxygenase-1 as an Adaptive Mechanism against Acrolein in RAW 264.7 Macrophages

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Abstract

Acrolein, a known toxin in cigarette smoke, is the most abundant electrophilic α, β-unsaturated aldehyde to which humans are exposed in a variety of environmental pollutants, and is also product of lipid peroxidation. Increased unsaturated aldehyde levels and reduced antioxidant status plays a major role in the pathogenesis of various diseases such as diabetes, Alzheimer's and atherosclerosis. The findings reported here show that low concentrations of acrolein induce heme oxygenase-1 (HO-1) expression in RAW 264.7 macrophages. HO-1 induction by acrolein and signal pathways was measured using reverse transcription-polymerase chain reaction, Western blot and immunofluorescence staining analyses. Inhibition of extracellular signal-regulated kinase activity significantly attenuated the induction of HO-1 protein by acrolein, while suppression of Jun N-terminal kinase and p38 activity did not affect induction of HO-1 expression. Moreover, rottlerin, an inhibitor of protein kinase δ, suppressed the upregulation of HO-1 protein production, possibly involving the interaction of NF-E2-related factor 2 (Nrf2), which has a key role as a HO-1 transcription factor. Acrolein elevated the nuclear translocation of Nrf2 in nuclear extraction. The results suggest that RAW 264.7 may protect against acrolein-mediated cellular damage via the upregulation of HO-1, which is an adaptive response to oxidative stress.

Keywords: Acrolein, HO-1, Macrophages, Adaptive response

Acrolein is a short and extremely reactive α, β-unsaturated aldehyde that is an ubiquitous environmental pollutant. Acrolein's human health concerns involve not just exogenous exposure, but also an endogenous lipid peroxidation end product[1,2]. Acrolein is formed in the incomplete combustion of organic materials, tobacco smoking, and reheated frying oils, and, physiologically, by neutrophil myeloperoxidase-mediated activity on threonine residues at sites of inflammation[3,4]. Acrolein has been reported as an end-product and initiator of lipid peroxidation, arising as a consequence of oxidative stress. A number of recent reports have described the damaging effects of acrolein in including inflammatory diseases such as chronic obstructive pulmonary disease and atherosclerosis; neurodegenerative disorders including Parkinson's disease, Alzheimer disease and diabetes; and acute airway inflammation[5-10]. Acrolein decreases glutathione (GSH) levels and alters the levels of intracellular reactive oxygen species (ROS), which preludes cell malfunction[11,12]. Indeed, acrolein-derived, ROS-mediated cell damage is an influential early factor in the pathogenesis of progression of atherosclerosis[13]. Recent reports indicate that acrolein can moderate biological reactions by a variety of downstream signaling pathways against cellular oxidative stress[14-16] and cytoprotective genes[11].

Heme oxygenase (HO) is an enzyme that catalyzes the rate-limiting reaction in the cleavage of heme catabolism, which converts heme into biliverdin, involving equimolar amounts of carbon monoxide and iron. Biliverdin is then rapidly changed to bilirubin by biliverdin reductase[17]. Three HO isoforms have been described. HO-1 is a stress-inducible isoform, and, HO-2 and HO-3 are constitutive isoforms. Among these three isoforms, HO-1 is a significant antioxidant defense enzyme that plays a cytoprotective role in oxidative stress conditions[18]. Inducible HO-1 expression is therapeutically beneficial in various pathological states such as cardiovascular disease and inflammation[19,20].

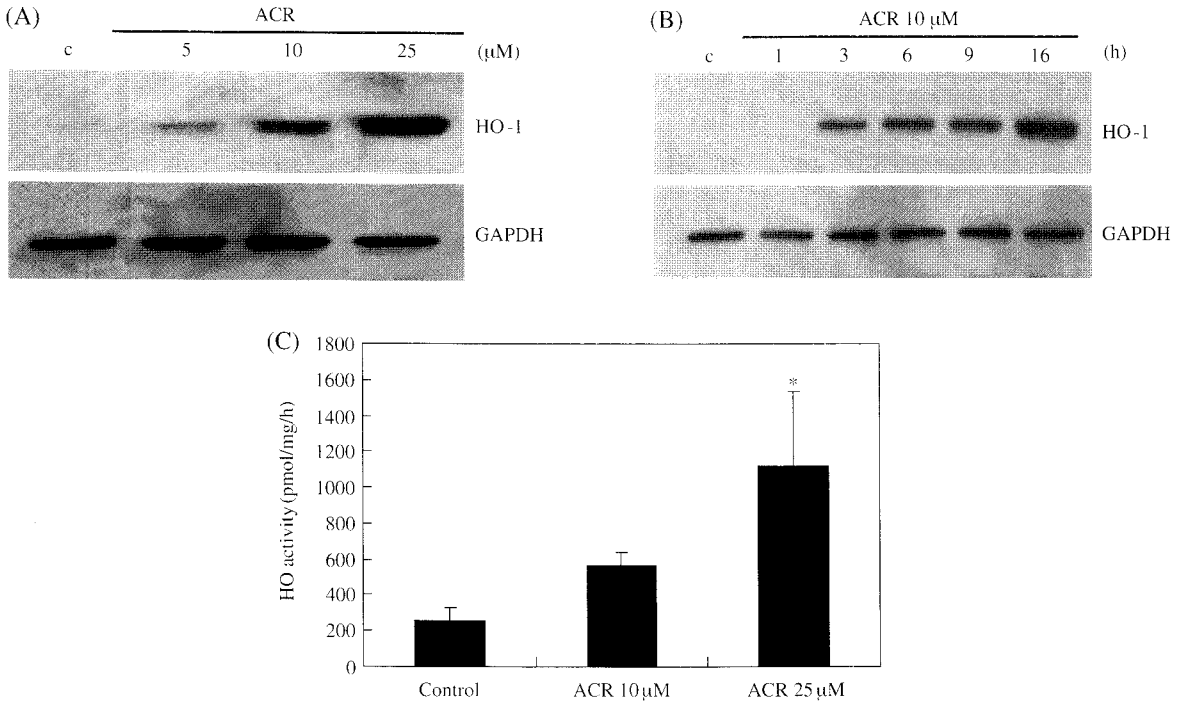

Figure 1. Increase of HO-1 expression by acorlein in RAW 264.7 cells. HO-1 expression was determined after treatment of RAW 264.7 cells with various concentrations of acrolein (A) at various times (B). The cell lysates were prepared and 40 μg samples of protein were subjected to Western blotting using anti-HO-1 antibody and anti-GAPDH antibody. (C) RAW 264.7 cells were treated with 10 μM acrolein for 16 h and analyzed for HO activity by measuring the formation of bilirubin. The data represents mean ± SD (n=3). The significant differences between the control group is indicated (*$P < 0.05$).

Moreover, HO-1 expression reduces the release of several pro-inflammatory cytokines and chemokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) in activated macrophages[21,22].

Presently, we report that acrolein induces HO-1 expression, with the subsequent signaling mechanism resulting in an adaptive response. Our findings show that acrolein increases HO-1 expression at both mRNA and protein levels, and that this induction activates protein kinase C (PKC)δ and extracellular signal-regulated kinase (ERK) 1/2. Our results also demonstrate that NF-E2-related factor-2 (Nrf2) nuclear translocation induces HO-1 expression by acrolein, indicating that the PKCδ/ERK1/2/Nrf2 pathway is very influential in the cytoprotective process.

Upregulation of HO-1 Expression by Acrolein

RAW cells were incubated with 5-25 μM of acrolein for 16 h and HO-1 protein levels were determined by Western blot. HO-1 protein levels were induced in a dose-dependent fashion (Figure 1A). Treatment with 10 μM acrolein produced a time-dependent induction of HO-1 production, with a maximum level attained after 16 h (Figure 1B). Reverse transcription-polymerase chain reaction (RT-RCR) analysis showed that the HO-1 mRNA level was highly expressed in the acrolein-treated cells, compared with control cells (Figure 2A). Induction was detected 30 min after the addition of acrolein and was maximal after 1 h (Figure 2B). Next, we tested HO-1 activity in RAW cells. As shown in Figure 1C, acrolein elevated HO-1 activity by 5-fold at 16 h.

Immunofluorescence Analysis of HO-1 Expression by Acrolein

Cells were fixed, and HO-1 expression was determined by immunofluorescence staining with an anti-HO-1 antibody, followed by fluorescence tagged secondary antibody. Immunofluorescence analysis revealed that HO-1 levels were significantly increased in RAW cells pre-incubated with 5 μM acrolein for 16 h (Figure 3, control vs. acrolein-treated cells).

Activation of ERK1/2 and PKCδ by Acrolein, and Its Relation to HO-1 Expression

To understand the signaling mechanism, we confirmed mitogen-activated protein kinase (MAPK) activation in RAW cells. Previously, it was demonstrated that activation of MAPKs is related to HO-1 expres-

sion[23,24]. To determine the signaling process by HO-1 induction, we examined the effects of PD98059 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), and SP600125 (a JNK inhibitor), and investigated the effect of rottlerin (a PKCδ inhibitor), wortmanin (a phosphatidylinositol 3-kinase (PI3K) inhibitor) to assess the upstream signaling of MAPKs on HO-1 expression. The inhibitors of the ERK1/2 and PKCδ pathways significantly reduced acrolein-induced HO-1 expression, but the other tested inhibitors had no effect (Figure 4A and 4B).

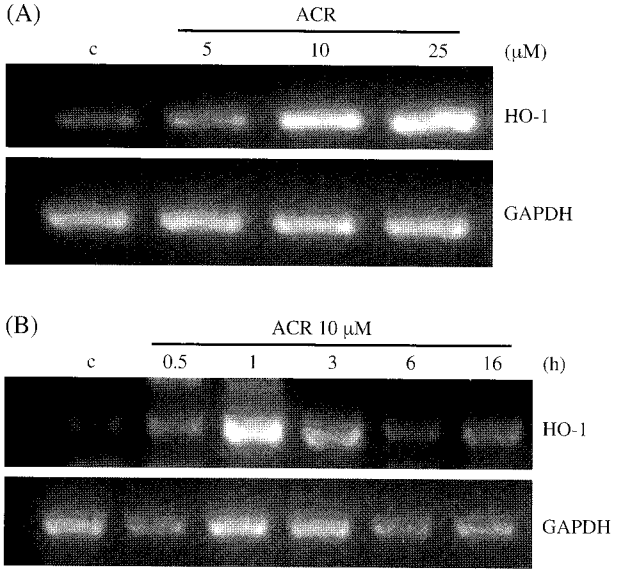

Figure 2. Acrolein-mediated increase in HO-1 mRNA expression in RAW 264.7 cells. RAW 264.7 cells were treated with acrolein at the indicated various concentrations (A) and various times (B). Total RNA was extracted, and 2 μg of the resulting RNA were analyzed by RT-PCR using HO-1 and GAPDH primers. The amplified RT-PCR product was visualized on a 1.5% agarose gel.

Nrf2 Nuclear Accumulation Is Involved in HO-1 Induction by Acrolein

Several transcription factors including activator protein-1 (AP-1), nuclear factor-kappa B (NF-kB), and Nrf2 can participate in HO-1 induction. Among them, Nrf2 participates in the regulation of HO-1 induction against oxidative damage by numerous environmental stressors[25]. To confirm that acrolein is involved with the Nrf2 transcription factor to HO-1 induction, cells were treated with 10 μM acrolein for 16 h and the nuclear fractions were examined. The accumulation of Nrf2 in the nucleus of acrolein-treated cells (Figure 5) implicated Nrf2 as being a key mediator of acrolein-induced HO-1 upregulation in RAW cells.

Discussion

HO-1 is stress-inducible protein that is expressed in most cell types. HO-1 induction protects cells from oxidative stress-mediated damage including those caused by the inflammatory response and tobacco smoke. HO-1 also has a cytoprotective effect in several pathological states of oxidative stress including atherosclerosis and inflammation[26].

In this study, we observed a significant upregulation of HO-1 in acrolein-treated RAW 264.7 macrophages. This suggests that acrolein-mediated increased production of HO-1 may be crucial in an adaptive response of macrophages to external stimuli, cigarette smoke, and oxidative stress. This is consistent with the reports that the induction of HO-1 influences the defense mechanism in stressful conditions[27,28].

Macrophages, as competent phagocytes, are critical in pro-inflammatory and immune responses. The cells are able to defend the host cell against stimuli and detrimental stress because of their phagocytic ability, which can lead to a cytoprotective mechanism against

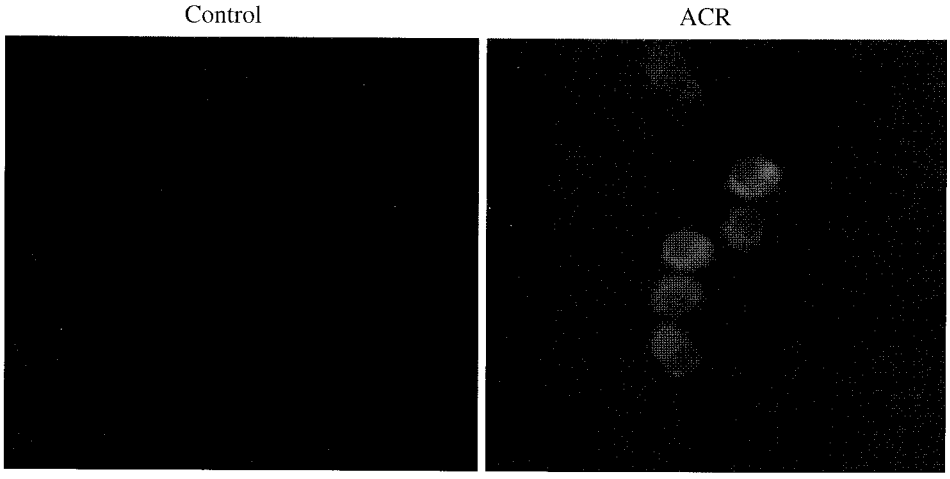

Figure. 3. Immunofluorescence staining analyses of HO-1 expression in RAW 264.7 cells. RAW 264.7 cells were pretreated with 5 μM acrolein for 16 h. Cells were fixed and HO-1 localization was determined by immunofluorescence staining with an anti-HO-1 antibody followed by fluorescence tagged secondary antibody.

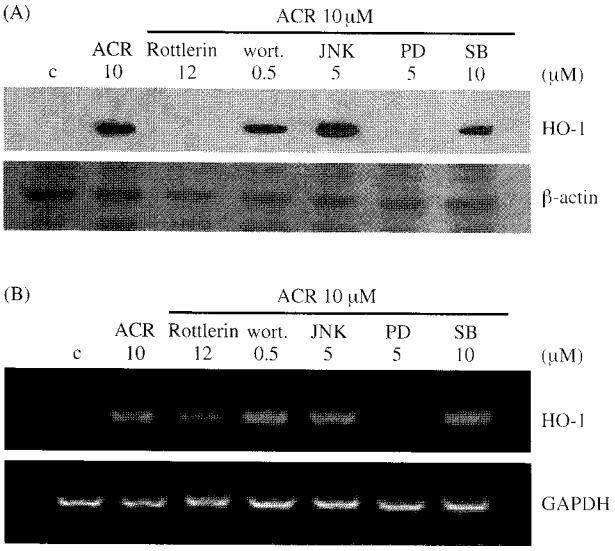

Figure. 4. PD98059 (an ERK 1/2 inhibitor) and rottlerin (a PKCδ inhibitor) inhibits acrolein-induced HO-1 expression. (A) Cells were pretreated with either inhibitor for 1 h, then incubated with 10 mM acrolein for 16 h. Whole cell lysates were prepared and subjected to Western blot analysis with antibodies against anti-HO-1 and GAPDH, as indicated. JNK (a JNK inhibitor II), SB (a p38 inhibitor), PD (an ERK inhibitor), Wort (a PI3K inhibitor) and PKCδ inhibitor were supplied at the indicated concentrations. (B) RAW 264.7 cells were pretreated with each inhibitor and incubated with 10 μM acrolein for 1 h. Total RNA was isolated and then 2 μg of the resulting RNA were amplified by RT-PCR using the HO-1 and GAPDH primers. The product was visualized on a 1.5% agarose gel.

inflammatory responses. In addition, HO-1 expression may be significant in macrophages, participating in the repair response that underlies the resolution of inflammation, leading to restoration of cell damage[29,30]. It is possible that cytoprotective function of HO-1 in macrophages involves the generation of components though the degradation of the pro-oxidant heme. HO-1 and its generated products are known to function in many processes that regulate cytoprotection, anti-inflammatory activity, cell homeostasis[17], and pro-inflammatory cytokine synthesis[22].

To elucidate the mechanism by which acrolein induces HO-1 in RAW cells, we investigated whether acrolein could activate MAPKs including ERK, JNK, and p38MAPK, leading to the enhanced expression of HO-1 in RAW cells. Because another study suggested that lipopolysaccharide (LPS)-stimulated HO-1 induction by curcumin involves PKCδ[31], we examined the role of PKCδ activation. HO-1 protein and mRNA levels were markedly inhibited 16 h after acrolein exposure with specific inhibitors of ERK 1/2 and PKCδ (Figure 4). These data suggest that the PKCδ and ERK 1/2 sig-

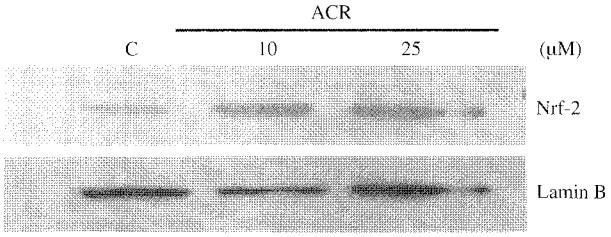

Figure 5. Acrolein increases nuclear Nrf2 protein levels for HO-1 expression in RAW 264.7 cells. RAW 264.7 cells were treated with 10 and 25 μM acrolein for 16 h. Nuclear fractions were extracted and 40 μg of total protein were detected by Western blotting using an anti-Nrf2 antibody and anti-Lamin B antibody (as a basal marker for nuclear protein).

naling pathways play an important role in acrolein-induced HO-1 expression. In addition, other studies have demonstrated that PKCδ mediates Nrf2 translocation and HO-1 transcript expression in LPS-stimulated monocytes[32]. The transcriptional mechanisms of HO-1 expression are not fully clear, but previous reports have shown that the redox-sensitive transcription factor Nrf2 is related to the induction of detoxifying/antioxidant enzyme expression, resulting in diverse electrophilic compounds and phenolic antioxidants[33-35]. The results indicate that acrolein-induced HO-1 expression requires activation of Nrf2 in RAW cells.

In conclusion, the present study shows that acrolein, which is the strongest electrophilic toxin in cigarette smoke and an end product of lipid peroxidation, induces expression of HO-1 in RAW cells through activation of the PKCδ, ERK 1/2 and Nrf2 signaling pathways. Thus, HO-1 expression by acrolein may play an important role in the adaptive response against cellular stressors in RAW 264.7 macrophages.

Material & Methods

Materials

Acrolein was obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and tissue culture reagents were obtained from WelGENE (Daegu, Korea). Trizol reagent was supplied by Invitrogen (Carlsbad, CA). TransPass R2 transfection reagent was purchased from New England Biolabs (Ipswich, MA). The HO-1 antibody was purchased from Epitomics (Burlingame, CA), and Nrf2 and LaminB antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Ab Frontier (Seoul, Korea). PD98059, SB203580, and SP600125 were purchased from Calbiochem (La Jolla,

CA). All other chemicals and reagents were of analytical grade.

Cell Culture

RAW 264.7 murine macrophages purchased from the American Type Culture Collection (Rockville, MD) were cultured in DMEM and supplemented with 10% (v/v) FBS, 50 U/mL Penicillin and 50 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% air. For all experiments, RAW cells were cultured to 60%-80% confluence.

Western Blotting

Whole cell lysate protein (40 μg) was analyzed by Western blotting. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to a nitrocellulose membrane. After blocking by incubation with 5% skim milk in Tris-buffered saline for 1 h at room temperature, the membrane was incubated using monoclonal antibody against mouse HO-1 and monoclonal antibody against mouse GAPDH. Horseradish peroxidase-conjugated anti-IgG antibodies were used as the secondary antibody to detect protein bands to which the primary antibodies bound by enhanced chemiluminescence using a WESTSAVE Up™ kit (Ab Frontier).

Isolation of RNA and RT-PCR

Total RNA was extracted from RAW cells using 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA). The isolated RNA was washed in 70% ethanol, dried, and dissolved in diethylpyrocarbonate for RNase inhibition. A total of 2 μg of RNA was quantified using a ND-100 spectrometer (NanoDrop Technologies, Wilmington, DE). After RNA preparation, cDNA was synthesized using 2 μg of total RNA. Reverse transcription was performed in steps of 5 min at 70°C, 10 min at 25°C, 60 min at 42°C, and 5 min at 95°C. For PCR, the cDNA products were used as a template and using primers for HO-1 or GAPDH as a loading control. For HO-1, annealing was applied for 35 cycles consisting of 30 s at 58°C. For GAPDH, the annealing condition was 20 cycles consisting of 30 s at 58°C. The resulting products were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide and photography under ultraviolet illumination.

Nrf-2 Nuclear Extraction

RAW cells were incubated with varying amounts of acrolein for 16 h. The cell pellets were harvested, washed using phosphate buffered saline (PBS) by centrifugation at 3,000 rpm for 5 min at 4°C, and resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with protease inhibitors. After 10 min of incubation on ice, the mixture was centrifuged at 8,000 rpm for 5 min at 4°C. The supernatant contained the cytoplasmic extract. The pellet was resuspended in solution buffer B containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitors, followed by incubation at 4°C for 30 min. Then, the mixture was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant containing the nuclear extract was collected in a new tube and stored −70°C for protein assay and Western blot analyses.

Immunofluorescence Staining

RAW cells grown in 35 mm-diameter glass bottom Microwell culture dishes (MatTek, Ashland, MA) were incubated for 16 h in presence or absence of acrolein. The cells were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature. The fixation was removed by aspiration and each sample was rinsed three times in PBS for 5 min each. After formaldehyde fixation, the cells were permeabilized by coverage with ice-cold 100% methanol for 10 min at −20°C. The reaction was blocked with 5% horse normal serum and 1% bovine serum albumin in PBS for 1 h at room temperature. The cells were incubated with a 1 : 500 dilution of anti-HO-1 antibody in PBS containing 1% bovine serum albumin overnight at 4°C. Primary antibody binding was detected with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG for 2 h at room temperature. Fluorescence microscopy was performed and the data was processed using Adobe Photoshop.

HO Activity Assay

RAW cells were harvested and centrifuged at 3,000 rpm for 3 min. The pellet was resuspended in 100 mM phosphate buffer (pH 7.4) and 2 mM $MgCl_2$ (HO activity buffer). The cells were frozen and thawed three times prior to sonication on ice. The cell lysates were centrifuged for 15 min at 4°C at 13,000 rpm and 400 μL of the supernatant was transferred to a new tube. HO enzymatic assay used a NADPH-generation system solution (200 μM) containing 100 mM PBS, 2 mM $MgCl_2$, 3 mg of rat liver cytosol, 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dyhydrogenase, and 10 μM hemin enzyme substrate plus 400 μM samples. Samples were incubated at 37°C for 60 min in the dark. Chloroform was added to terminate the reaction, and the bilirubin formed was determined by spectrophotometry measuring the difference in absorbance at 464 nm and 530 nm (ε=40

mM^{-1} cm^{-1}). The protein concentration in samples was determined using a bicinchoninic acid protein assay, and the HO activity was expressed in picomoles of bilirubin formed per milligram of protein per hour.

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