

Cellular iron depletion weakens induction of heme oxygenase-1 by cadmium

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Abstract:

Heme oxygenase-1 is an inducible cytoprotective gene, although its induction by environmental factors is not completely understood. This study aimed to ascertain if specific nutritive factors or related compounds influence heme oxygenase-1 expression. In HCT-116 cells, cadmium increased heme oxygenase-1 enzymatic activity. This effect of cadmium was weaker in cells made iron-deficient with the iron chelator, desferrioxamine, which was associated with repression of heme oxygenase-1 protein and mRNA expression. The repression by desferrioxamine of cadmium-induced heme oxygenase-1 upregulation was reversed upon iron replenishment of the cells. Additionally, it was found that thiol antioxidants inhibited the heme oxygenase-1 upregulation caused by cadmium and also by ethacrynic acid, which each decreased intracellular glutathione as did buthionine sulfoxamine. Interestingly, cadmium and ethacrynic acid increased nuclear translocation of Nrf2 and subsequent heme oxygenase-1 expression, but buthionine sulfoxamine did not. Furthermore, NADPH oxidase inhibitors (diphenyleneiodonium and apocynin, and a superoxide scavenger (Tiron) inhibited cadmium-induced upregulation of heme oxygenase-1. Diphenyleneiodonium was the most potent and inhibited NADPH-cytochrome P450 reductase as well, whereas apocynin and Tiron did not. It is concluded that adequate amounts of iron, which at the atomic level can serve as the pivotal element of heme in NADPH oxidase, must be present in cells to permit what appears to be thiol redox-sensitive, NADPH oxidase-dependent upregulation of heme oxygenase-1. Thus, these findings are significant because they suggest that cells without adequate iron would be unable to fully express the stress gene, heme oxygenase-1, when confronted with the toxic metal, cadmium.

Keywords:

Cadmium, Glutathione, Heme oxygenase-1, Iron, NADPH oxidase

Article:

1. INTRODUCTION

Cells in living organisms are inevitably confronted with noxious components originating from the external environment. To cope with the situation, cells express cytoprotective genes, such as heme oxygenase-1 (HO-1). Some inducers of HO-1 include strong oxidants (Li et al., 2006), UV radiation (Tyrrell, 2004), chemical carcinogens (Chin et al., 2003), and heavy metals (Takeda et al., 1994; Elbekai and El-Kadi, 2007). The HO-1 gene codes for a microsomal enzyme that catalyzes the degradation of heme to three main products (Kikuchi et al., 2005). The first product is biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Both biliverdin and bilirubin are antioxidants (Stocker, 2004). However, recent research suggests that HO-1 can protect cells against oxidative stress independent of generating the heme-derived antioxidants (Sheftel et al., 2007). The second product is ferrous iron (Fe^{++}), which apparently induces biosynthesis of ferritin that can bind Fe^{++} . This binding negates potential Fe^{++} -mediated formation of reactive oxygen species (ROS) (Galaris and Pantopoulos, 2008). The third product is carbon monoxide, which can have anti-inflammatory effects (Sawle et al., 2005; Ning et al., 2005) as a result of influencing cell signaling events (Ning et al., 2005).

Certain metal elements have contrasting biological significance and effects on HO-1. For example, iron and cadmium are normally regarded as a vital micronutrient commonly deficient in humans (Zimmermann and Hurrell, 2007) and as a toxic environmental pollutant (Bertin and Averbeck, 2006), respectively. At certain concentrations (less than 100 μM), iron (i.e., Fe^{++} ions) has not been demonstrated by anyone to significantly induce HO-1 in cells, whereas cadmium (i.e., Cd^{++} ions) stimulates ROS production (Chen et al., 2008) and HO-1 gene activation (Takeda et al., 1994; Elbekai and El-Kadi, 2007). It is unclear whether these two effects of cadmium require iron, specifically in adequate amounts as a nutritive cellular component, in order to happen. Iron is directly and indirectly involved in the transformation of molecular oxygen to ROS that can influence cellular processes. The involvement of free or elemental iron in the formation of ROS that can in turn mediate signaling events in cells is well known (Galaris and Pantopoulos, 2008). On the other hand, iron bound as the pivotal transition metal of heme molecules in NADPH oxidase (NOX) permits this enzyme system to work. More specifically, during enzyme catalysis, iron facilitates electron transfer from NADPH to molecular oxygen to produce superoxide (Zhu and Silverman, 2008). Non-phagocytic isoforms of NOX are known to exist and can generate a source of ROS to act as mediators of signal transduction (Park et al., 2004). Hence, working in a rather inconspicuous and intricate manner, iron facilitates the production of signaling oxidants by NOX, whose enzymatic activity is known to be induced by cadmium (Souza et al., 2009). It is not known if iron-containing NOX is involved in cadmium-induced upregulation of redox-sensitive HO-1.

In light of the above gaps in knowledge, we aimed to identify some nutritive factors or related components capable of influencing HO-1 upregulation as caused by cadmium in HCT-116 cells. Just recently, it was reported that desferrioxamine (DFO), which depletes cells of iron, abrogated induction of GRP78 and GADD153 stress gene expression caused by the anti-malaria and anti-cancer drug, artemisinin, in HCT-116 cells (Lu et al., 2010). Also, DFO induced activation of the transcription factor, HIF-1 α , in HCT-116 cells (Erez et al., 2003). These two studies suggest that the iron status of HCT-116 cells influences gene expression. For the first time, we principally report that cellular iron influences HO-1 induction.

2. MATERIALS AND METHODS

2.1. Materials

HCT-116 human colon adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). All reagents were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Norcross, GA), unless otherwise stated. Goat HO-1 polyclonal antibody (C-20), rabbit Nrf2 polyclonal antibody (H-300), rabbit NOX-1 polyclonal antibody (H-75), rabbit anti-goat IgG-HRP, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse β -actin monoclonal antibody (AC-15) was obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture, treatment of cells, and assessment of cell viability

HCT-116 cells were propagated in McCoy's 5A medium supplemented with 100 ml/L fetal bovine serum, 0.54 (mol/L amphotericin B, 100,000 units/L penicillin, and 100 mg/L streptomycin. Upon reaching 70–80% confluency, cell samples (i.e., cells grown in petri dishes, microplates, or chamber slides) were exposed to 10–50 μM cadmium (i.e., cadmium chloride) for 2–6 h depending on the experiment. Other cell samples were just heat-shocked by incubating them at 43 $^{\circ}\text{C}$ for 1 h and allowing them to recover at 37 $^{\circ}\text{C}$ for 4 h. Also, some cell samples were exposed to 50 μM ethacrynic acid or 50–100 μM buthionine sulfoxamine for 2–16 h depending on the experiment. In conjunction, cell samples were pretreated with two iron chelators, i.e., 10–100 μM desferrioxamine (DFO) or 21,21- dipyridyl, for 16–24 h before exposing the cell samples to cadmium. Moreover, cell samples were pre-treated with the iron chelators, and then treated with 50–100 μM ferrous sulfate (FeSO_4), copper sulfate (CuSO_4), or zinc sulfate (ZnSO_4) for 3 h before exposure to cadmium. In other experiments, cell samples were pretreated with 0.05–20 μM diphenyleneiodonium, 0.5–2 μM rotenone, 5–20 mM Tiron, or 0.5–4 mM apocynin for 0.5–1 h, or pretreated with 10 mM glutathione or N-acetylcysteine for 2 h, before exposing the cell samples to cadmium.

The viability of the cells was evaluated with MitoTracker Red CMXRos from Invitrogen (Carlsbad, CA). This molecular probe fluoresces when it undergoes oxidation and sequestration by mitochondria but only in actively respiring cells. Cell death was assessed by staining nuclei with 41,6-diamidino-2-phenylindole and looking for any chromatin condensation/fragmentation, as previously described (Powolny et al., 2001).

2.3. Determination of HO-1 and HSP70 mRNA expression

Total RNA was isolated from cells using an RNeasy Mini kit (Qiagen Inc., Valencia, CA). The expression levels of HO-1 mRNA were determined by multiplex relative RT-PCR analysis of total RNA using a Qiagen OneStep RT-PCR kit and gene-specific primers. The primer sequences for HO-1 (NIH, NCBI, UniSTS: 92045, RH27826) were: forward, TGTGGCAGCTGTCTCAAACCTCCA; reverse, TTGAG-GCTGAGCCAGGAACAGAGT. A β -actin/competimer pair (Ambion Inc., Austin, TX) was used as the internal standard to normalize the data. The RT-PCR conditions were 30 min at 50 °C followed by 15 min at 95°C (RT), then 0.5 min at 95 °C, 0.5 min at 60 °C, and 1 min at 72 °C (PCR) for 25 cycles. The resulting cDNA products were separated by 2% agarose gel electrophoresis with ethidium bromide staining. For the target gene (HO-1), the PCR product size was 175 bp. For the internal control (β -actin), the PCR product size was 294 bp. The primer sequences for HSP70 and RT-PCR conditions have been previously reported (Pan et al., 2004).

2.4. Determination of HO-1 protein expression

Harvested cells were sonicated in lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and placed in ice for 30 min. The lysed samples were centrifuged at 16,000 x g for 20 min at 4 °C. Sample aliquots were electrophoresed using 4-12% Bis-Tris NuPAGE mini-gels (Invitrogen, Carlsbad, CA) before electroblotting to nitrocellulose membrane. After blocking with 5% skim milk powder in TBST (20mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4), the blocked membrane was incubated overnight at 4 °C with goat HO-1 polyclonal antibody followed by anti-goat IgG-HRP. A SuperSignal WestFemto Maximum Sensitivity Kit (Pierce, Rockford, IL) was used to generate the chemiluminescence signal that was captured (Kodak 440 CF image station). Afterwards, the membrane was stripped and reprobed with mouse β -actin monoclonal antibody and anti-mouse IgG-HRP for imaging analysis as before.

2.5. Assay of heme oxygenase and NADPH-cytochrome P450 reductase activities

The heme oxygenase assay was essentially performed as described (Polte et al., 2000). Cells were washed with PBS and pelleted by centrifugation. The cell pellets were then frozen at -80 °C. Upon thawing and sonication of the pellets in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM MgCl₂ and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), the cell lysates were centrifuged at 18,000 x g for 20 min. The supernatant (700 μ g protein) was added to a reaction mixture containing 1 mM NADPH, 2 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 25 μ M hemin, and rat liver cytosol (2 mg protein) in a total volume of 1 ml. After incubation at 37 °C for 1 h in the dark, 1 ml chloroform was added to the samples, which were then vortexed. The bottom chloroform phase containing the product, bilirubin, was analyzed spectrophotometrically (difference in absorbance between 464 and 530nm). Using an extinction coefficient of 40 mM⁻¹ cm⁻¹, heme oxygenase activity was calculated and expressed as pmol/h/mg protein.

For the assay of NADPH-cytochrome P450 reductase activity (Zhukov and Ingelman-Sundberg, 1999), cell lysates were prepared and centrifuged as above but using 0.3 M potassium phosphate buffer (pH 7.6). The supernatant (50 μ g protein) was added to a buffer-based reaction mixture containing 50 μ M NADPH and 50 μ M cytochrome c in a total volume of 1 ml. The increase in absorbance at 550 nm was monitored for 10 min. Using an extinction coefficient of 21 mM⁻¹ cm⁻¹, enzyme activity was calculated and expressed as μ mol/min/mg protein.

2.6. Assessment of intracellular glutathione

Intracellular concentrations of reduced glutathione (GSH) were determined spectrophotometrically using Ellman's reagent (Mazza et al., 2003). Additionally, visual assessment of GSH in whole cells was performed by

fluorescence microscopy. The cells in microplates were washed with Hanks buffered saline solution (HBSS), before incubation with 20 μ M monochlorobimane (Molecular Probes Inc., Eugene, OR) for 30 min at 37°C. After washing with HBSS, the cell samples were then microscopically examined (UV filter).

2.7. Evaluation of Nrf2 expression

Nrf2 expression was evaluated three ways. First, determination of Nrf2 protein expression in whole cell lysates was performed by Western-immunoblotting analysis as described above, but using rabbit Nrf2 polyclonal primary antibody and goat anti-rabbit IgGHRP secondary antibody. Secondly, determination of the activated form of Nrf2 in nuclear extracts was performed by initially preparing nuclear extracts using a Nuclear Extract Kit (Active Motif, Carlsbad, CA), and then, spectrophotometrically measuring the amount of activated Nrf2 in aliquots of the nuclear extracts using a TransAM Nrf2 kit (Active Motif, Carlsbad, CA). Thirdly, for the visual assessment of Nrf2 expression by immunocytofluorescence microscopy, the cells in LabTek chamber slides were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 3% bovine serum albumin, the cells were incubated overnight with rabbit Nrf2 polyclonal antibody followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes Inc., Eugene OR). Counterstaining was with 4',6-diamidino-2-phenylindole to visualize nuclei. After extensive washing, the slides were viewed (FITC and UV filters).

3. RESULTS

3.1. Cadmium differentially affects stress gene expression

It was of initial interest to partially characterize stress gene expression in HCT-116 cells exposed to two different stressors. Two highly recognizable stress genes are HO-1 (HSP32) and HSP70. Cadmium increased expression of HO-1 mRNA (Fig. 1A), but not HSP70 mRNA (Fig. 1B). Heat shock increased expression of HSP70 mRNA (Fig. 1C) but not HO-1 mRNA (Fig. 1D). Additionally, cadmium in a concentration-dependent manner increased the expression of HO-1 mRNA and also protein (Fig. 1E).

3.2. Iron chelators weaken induction of HO-1

Standard culture media provide cells with adequate or normal amounts of iron. Since iron is essential for metabolism, depleting iron in cells would be expected to impair biochemical reactions or related processes. To determine if adequate iron is required in HCT-116 cells for full induction of HO-1, intracellular iron was depleted by pre-treating the cells with DFO (iron chelator) for 24 h, and then, the iron-deficient cells were exposed to cadmium. Such 24-h pretreatment with DFO did not result in morphological features of apoptosis, such as membrane blebbing/apoptotic body formation (Fig. 2A, a) and chromatin condensation/fragmentation

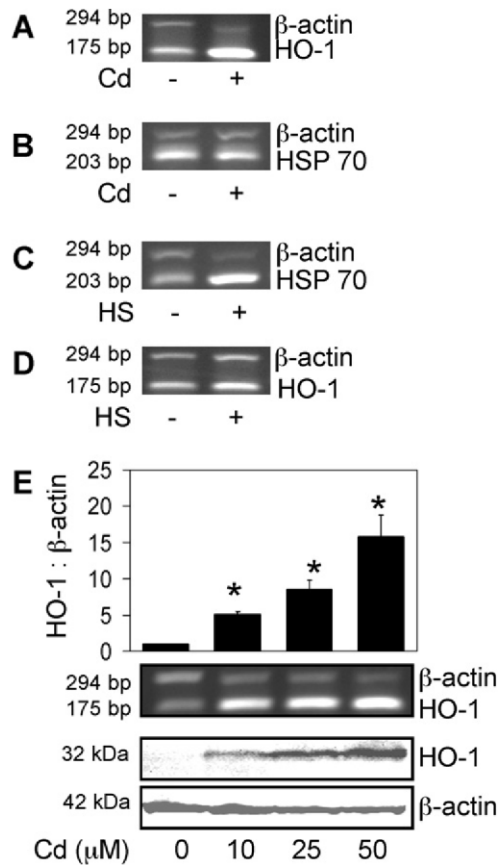


Fig. 1. Differential effects of two stress agents, i.e., cadmium (Cd) and heat shock (HS), on expression of HO-1 and HSP70 in HCT-116 cells. The cells were exposed to 50 μM Cd for 5 h (A and B), or heat shocked for 1 h at 42 °C followed by recovery at 37 °C for 4 h (C and D). Multiplex relative RT-PCR analysis was performed to determine mRNA expression of the target genes, HO-1 (A and D) and HSP70 (B and C), and internal control gene, β-actin. In further examining the effect of Cd on HO-1 (E), cells were exposed to different concentrations of Cd for 5 h before performing RT-PCR and densitometry (bar graph with gel image immediately below), or for 6 h before performing Western-blotting analysis (bottom coupled images). The results are representative of 3 different experiments. * $P < 0.05$ versus 0 μM Cd (E).

(Fig. 2A, b) that were seen at 48 h. Moreover, mitochondria of 24- h DFO-pretreated cells were found to be active based on their ability to concentrate and oxidize a standard molecular probe (to a fluorescent product) as well as mitochondria of control cells (Fig. 2A, c).

In control cells, cadmium induced heme oxygenase activity (Fig. 2B). But, such enzyme induction was weakened in cells made iron-deficient with DFO. Hence, DFO blunted the induction of heme oxygenase activity apparently at the level of HO-1 protein and mRNA expression (Fig. 2C), as both were suppressed in the iron-deficient cells. Similar results were obtained when the DFO was removed from the cells (that is, the DFO-containing cell culture medium taken off and replaced with serum-free medium) and then exposed to cadmium (data not shown). Furthermore, a structurally different iron chelator (2',2'-dipyridyl) blunted the upregulation of HO-1 mRNA and protein caused by cadmium (Fig. 2D).

3.3. Iron replenishment reverses the weakening of cadmium-induced HO-1 upregulation caused by DFO

Using FeSO₄, replenishing iron in the iron-deficient cells restored the ability of the cells to fully express HO-1 mRNA and protein when they were exposed to cadmium (Fig. 3A). Consequently, full induction of heme oxygenase activity by cadmium was

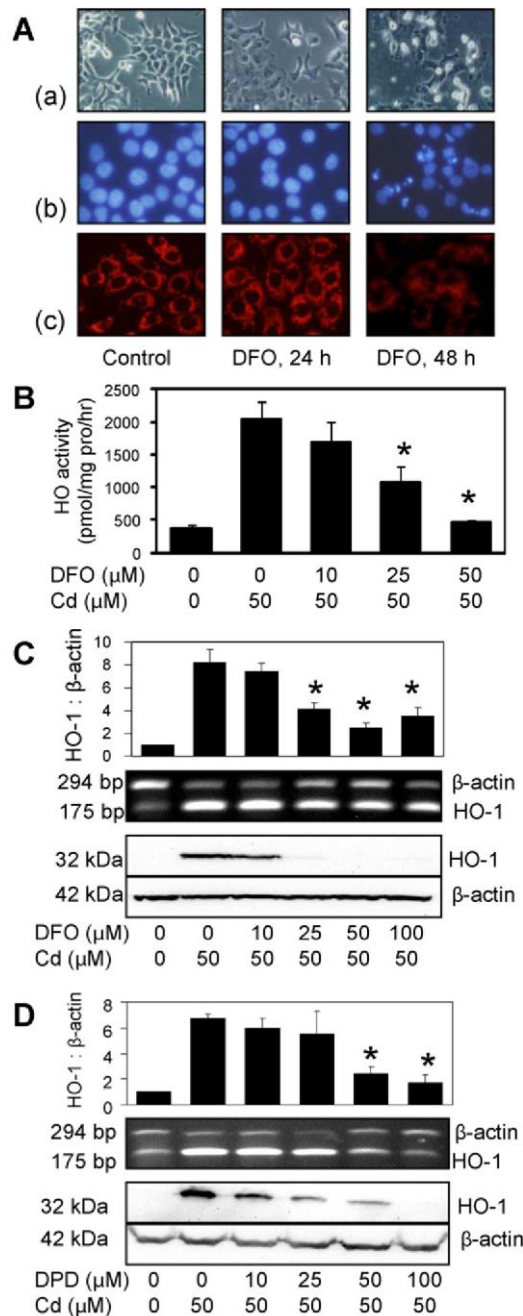


Fig. 2. Cadmium-induced HO-1 upregulation is suppressed in cells made iron-deficient with iron chelators. In the first panel (capital A), HCT-116 cells were incubated with 100 μM desferrioxamine (DFO) for 24 h or 48 h. Then, the morphology (a), fluorescently stained nuclei (b) and mitochondria (c) were imaged. In the three other panels, cells were pretreated with different concentrations of DFO or 2',2'-dipyridyl (DPD) for 24 h and then exposed to cadmium (Cd) for 5–6 h. Heme oxygenase activity was measured spectrophotometrically (B). Expression of HO-1 mRNA and protein (C and D) were determined by RT-PCR/densitometry (bar graphs with gel images immediately below) and Western-blotting analysis (bottom coupled images). The results are representative of 3 different experiments. * $P < 0.05$ versus 0 μM DFO/50 μM Cd (B and C) and 0 μM DPD/50 μM Cd (D).

restored in the iron-replenished cells (Fig. 3B). When HCT-116 cells were made iron-deficient with 2',2'-dipyridyl and then treated with FeSO_4 before subsequent exposure to cadmium, the iron replenishment similarly restored the ability of the cells to fully express HO-1 (data not shown).

To determine if the effects of DFO were specific to iron, the “rescue” capability of other divalent metals (copper and zinc) was

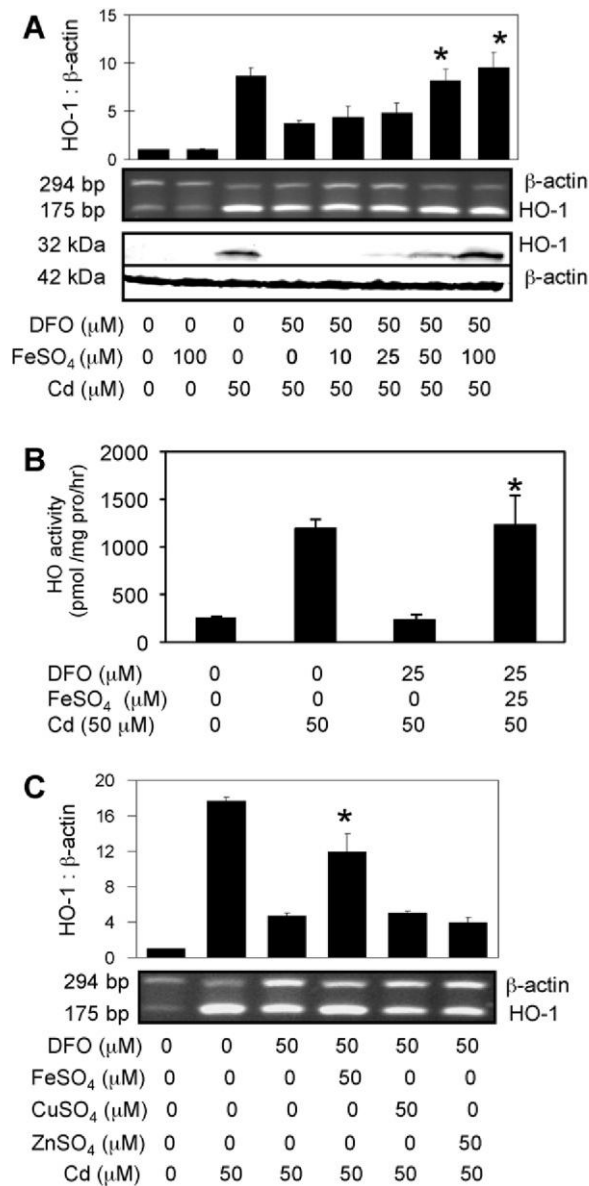


Fig. 3. Suppression by iron chelators of cadmium-induced HO-1 upregulation is overcome by replenishment with iron, but not zinc or copper. HCT-116 cells were pretreated with desferrioxamine (DFO) for 24 h, and then treated with iron (ferrous) sulfate, copper sulfate, or zinc sulfate for 3 h before being exposed to cadmium (Cd) for 5–6 h. Expression of HO-1 mRNA and protein (A and C) were determined by RT-PCR/densitometry (bar graphs with gel images immediately below) and Western-blotting analysis (bottom coupled images). Heme oxygenase activity was assayed spectrophotometrically (B). The results are representative of 3 different experiments. * $P < 0.05$ versus 50 μ M DFO/50 μ M Cd (A and C) and 25 μ M DFO/50 μ M Cd (B).

evaluated (Fig. 3C). CuSO₄ and ZnSO₄ were each ineffective. Only rescue of DFO-pretreated cells with FeSO₄ was able to largely restore the upregulation of HO-1 expression caused by cadmium. Thus, DFO appears to suppress cadmium-induced HO-1 upregulation by creating a negative balance of iron, rather than of zinc or copper, in the cells.

3.4. Significance of glutathione in cadmium-induced upregulation of HO-1 expression

Cadmium binds to glutathione (Leverrier et al., 2007), and also induces formation of ROS (Chen et al., 2008) that can oxidize glutathione. This would result in a lowering of the intracellu-

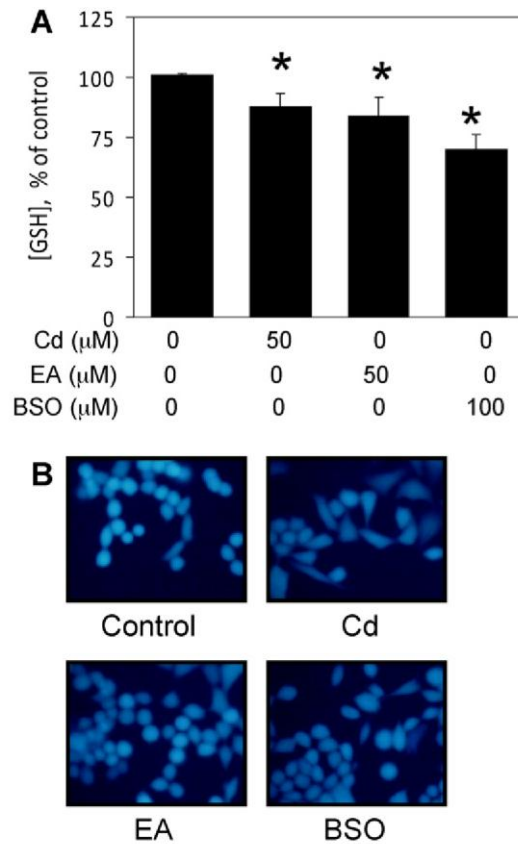


Fig. 4. Effects of cadmium (Cd), ethacrynic acid (EA), and buthionine sulfoximine (BSO) on intracellular glutathione. HCT-116 cells were incubated with Cd, EA, and BSO for 2 h, before assessment of glutathione by spectrophotometry (A) and fluorescence microscopy (B).

lar GSH:GSSG ratio, which can influence redox-sensitive gene expression (Tanaka et al., 1997). Hence, to determine if glutathione is implicated in the upregulation of HO-1 caused by cadmium, the intracellular concentration of the reduced form of glutathione was initially ascertained in HCT-116 cells exposed to cadmium. For comparison, however, cells were also exposed to ethacrynic acid, which is known to react covalently with glutathione, and to buthionine sulfoximine which is known to inhibit glutathione biosynthesis. Based on a biochemical assay, glutathione was decreased by exposing the cells to cadmium, ethacrynic acid, and buthionine sulfoximine (Fig. 4A). This finding was confirmed by microscopic examination of the cells post-labeled with monochlorobimane (Fig. 4B), which emits blue fluorescence upon reacting with the reduced form of intracellular glutathione. Cells exposed to cadmium, ethacrynic acid, and buthionine sulfoximine showed less fluorescence intensity, and therefore lower glutathione levels, compared to control cells.

In comparing the effects of ethacrynic acid and buthionine sulfoximine with cadmium on HO-1 expression (Fig. 5A–C), some similarities and differences can be seen. Ethacrynic acid had a similar effect as cadmium in that ethacrynic acid increased HO-1 mRNA and protein expression (Fig. 5B). Buthionine sulfoximine had a dissimilar effect as cadmium and ethacrynic acid in that buthionine sulfoximine at the concentrations tested did not increase HO-1 mRNA expression even with prolonged exposure time (Fig. 5C). Hence, these findings would imply that merely decreasing glutathione by inhibiting its biosynthesis with buthionine sulfoximine, in contrast to decreasing glutathione by binding or conjugating it with cadmium or ethacrynic acid, does not result in HO-1 mRNA upregulation.

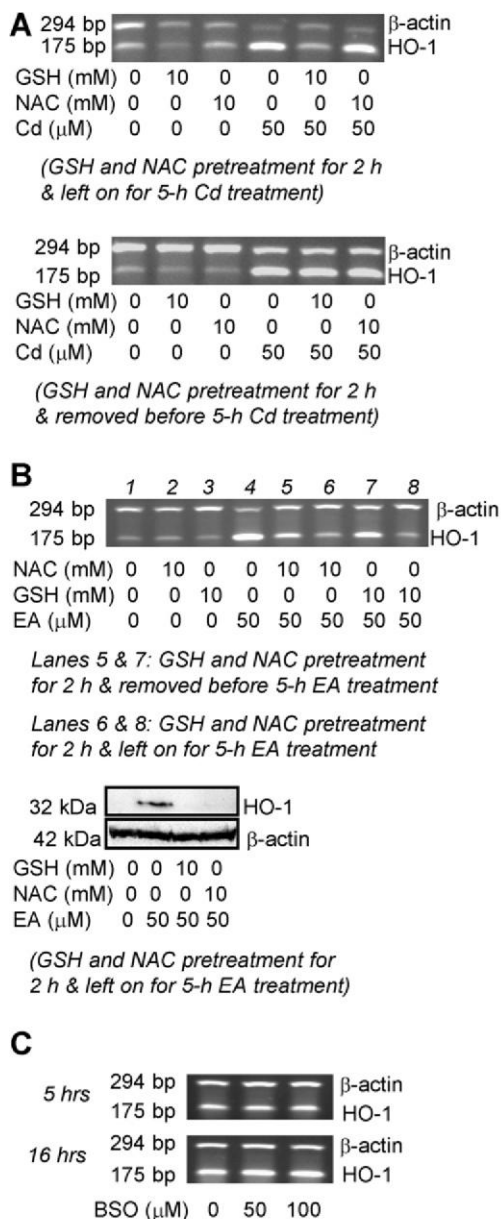


Fig. 5. Effects of modulators of intracellular glutathione on HO-1 expression. HCT-116 cells were incubated (for the times indicated above) with cadmium (Cd) (A) and ethacrynic acid (EA) (B), after being pre-treated (for the times indicated above) with reduced glutathione (GSH) or N-acetylcysteine (NAC). Cells were also incubated with buthionine sulfoximine (BSO) (C) without any pretreatment. Afterwards, HO-1 expression was assessed. The results are representative of 3 different experiments

To better clarify any involvement of glutathione in influencing cadmium-induced and ethacrynic acid-induced HO-1 upregulation, the effects of exogenously-added reduced glutathione (GSH), and also N-acetylcysteine (NAC), on upregulation of HO-1 caused by cadmium (Fig. 5A) and ethacrynic acid (Fig. 5B) were also examined. When cells were pretreated with GSH or NAC for 2 h and these two thiol antioxidants left on the cells for the 5-h cadmium exposure, GSH, but not NAC, inhibited cadmium-induced HO-1 mRNA upregulation (Fig. 5A). As can be further seen, when GSH and NAC were removed after the 2-h pretreatment before the subsequent 5-h cadmium exposure, neither GSH nor NAC inhibited cadmium-induced HO-1 mRNA upregulation. With respect to HO-1 upregulation caused by ethacrynic acid, both GSH and NAC had some inhibitory effects at the mRNA and protein levels (Fig. 5B), particularly when these thiol antioxidants were present during

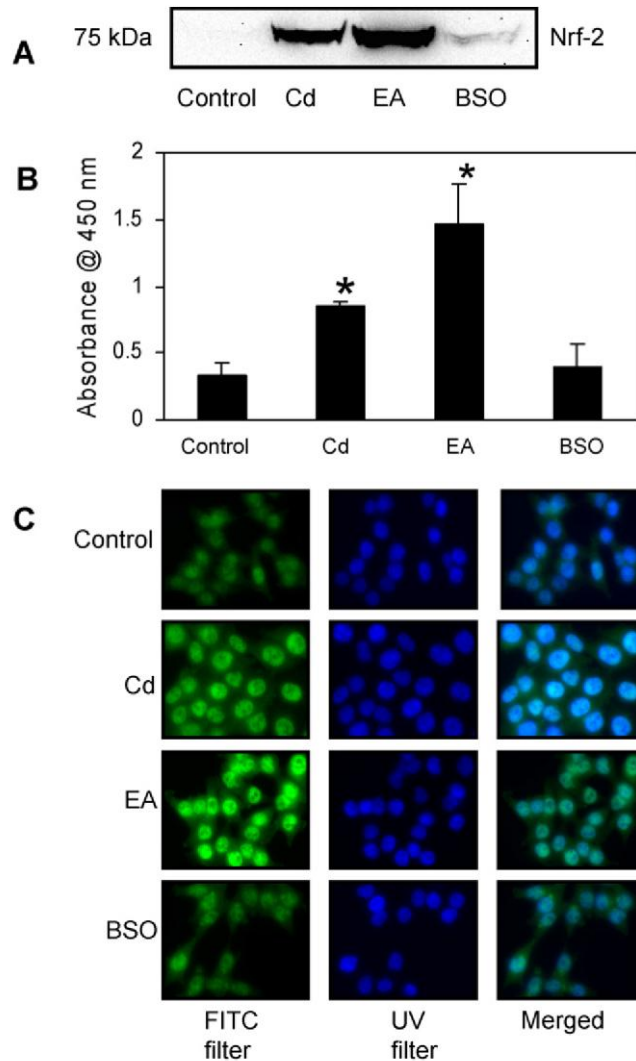


Fig. 6. Cadmium (Cd) and ethacrynic acid (EA), but not buthionine sulfoximine (BSO), increase nuclear expression of Nrf2. HCT-116 cells were exposed to 50 μ M Cd, EA, or BSO for 4 h. Nuclear extracts were prepared and analyzed for Nrf2 expression by Western-blotting (A), TransAM Nrf2 immunoassay (B), and immunocytofluorescence microscopy (C) as detailed in Section 2. The results are representative of 3 different experiments. * $P < 0.05$ versus control (B).

the entire incubation period of 7 h. Taken together, these findings would suggest that GSH and NAC work partly by reacting with some of the cadmium and ethacrynic acid molecules in the extracellular compartment (that is, in the cell culture medium), thereby reducing the amount of cadmium and ethacrynic acid available for entry into the cells.

3.5. Effect of cadmium, ethacrynic acid, and buthionine sulfoximine on Nrf2 expression and localization

To help explain why buthionine sulfoximine, in contrast to cadmium and ethacrynic acid, failed to upregulate HO-1 mRNA expression (Fig. 5C), the effects of cadmium, ethacrynic acid, and buthionine sulfoximine on the expression of one of the primary transcription factors known to activate the HO-1 gene, namely, Nrf2, were examined next. As shown by the Western-blotting results in Fig. 6A, both cadmium and ethacrynic acid increased Nrf2 protein expression in nuclear extracts prepared from the cell samples, whereas buthionine sulfoximine had only a slight effect. These findings were essentially confirmed by analysis of the nuclear extracts for Nrf2 binding to a consensus base sequence

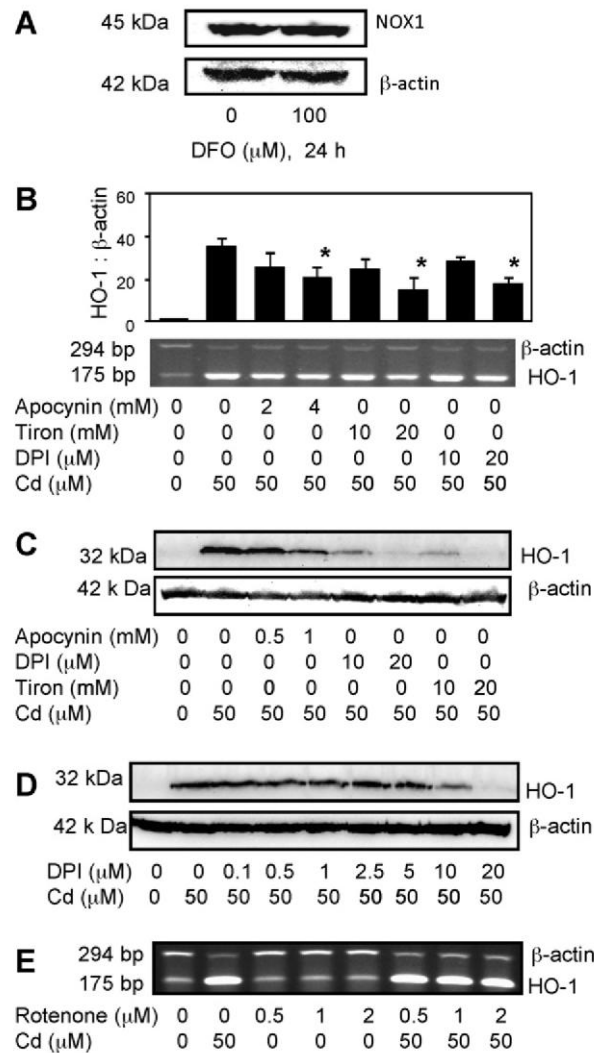


Fig. 7. Expression of NOX-1 in HCT-116 cells and attenuation of cadmium-induced upregulation of HO-1 by the NOX inhibitors, diphenyleneiodonium (DPI) and apocynin, or the superoxide scavenger, Tiron. Western-blotting analysis was performed on HCT-116 cells using anti-NOX1 antibody (A). Additionally, cells were pre-treated with DPI, apocynin, or Tiron for 0.5 h, and with rotenone for 1 h before being exposed to cadmium (Cd) for 5–6 h. Then, HO-1 mRNA (B,E) and protein (C,D) were determined. The results are representative of 3 different experiments. * $P < 0.05$ versus 50 μ M Cd (B).

reflecting the stress regulatory element of the HO-1 gene (Fig. 6B). Additionally, as shown by the images generated by immunocytofluorescence microscopy (Fig. 6C), both cadmium and ethacrynic acid increased the translocation of Nrf2 from cytosol into nuclei (as indicated by the more intense green fluorescence in nuclei counter-stained with DAPI to emit blue fluorescence). In contrast, buthionine sulfoxamine did not increase nuclear translocation of Nrf2.

3.6. Effects of NOX inhibitors, superoxide scavenger, and rotenone on cadmium-induced upregulation of HO-1

To enzymatically function, the catalytic subunit of nonphagocytic NOX depends on heme-iron to facilitate electron transfer to molecular oxygen (Zhu and Silverman, 2008), eventually generating signaling oxidants. HCT-116 cells were found to express the NOX1 isoform, based on immunoreactivity with a NOX1 antibody, although the expression of NOX-1 protein was not affected in cells made iron-deficient with DFO (Fig. 7A). Nevertheless, to deter-

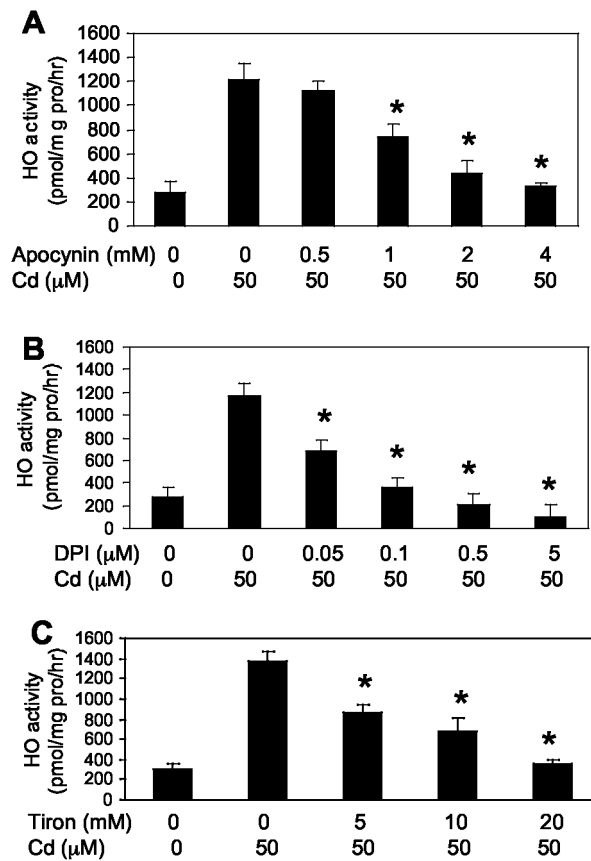


Fig. 8. Induction of heme oxygenase (HO) activity caused by cadmium is attenuated by the NOX inhibitors, diphenyleneiodonium (DPI) and apocynin, or the superoxide scavenger, Tiron. HCT-116 cells were pre-treated with apocynin (A) and DPI (B), or Tiron (C) for 0.5 h, before being exposed to cadmium (Cd) for 6 h. Then, HO enzyme activity was assayed spectrophotometrically. The results are representative of 3 different experiments. * $P < 0.05$ versus 50 μM Cd (A–C).

mine if NOX or superoxide is involved in cadmium-induced HO-1 upregulation, the effects of two NOX inhibitors (diphenyleneiodonium and apocynin) and a superoxide scavenger (Tiron) on HO-1 upregulation caused by cadmium was assessed. To some extent, all three of these test agents variably inhibited cadmium-induced upregulation of HO-1 mRNA (Fig. 7B) and protein (Fig. 7C and D). When HCT-116 cells were pre-treated with rotenone, an inhibitor of mitochondrial electron transfer, before subsequent exposure to cadmium, induction of HO-1 mRNA expression by cadmium was not affected (Fig. 7E). More notable and compelling, the induction of heme oxygenase activity caused by cadmium was inhibited by apocynin (Fig. 8A), diphenyleneiodonium (Fig. 8B), and Tiron (Fig. 8C), but diphenyleneiodonium had the greatest inhibitory effect. Thus, the effects of apocynin, diphenyleneiodonium, and Tiron in inhibiting cadmium-mediated induction of heme oxygenase activity are consistent with their inhibitory effects on HO-1 protein expression.

3.7. Effects of NOX inhibitors or superoxide scavenger on NADPH-cytochrome P450 reductase activity in cadmium-treated cells

During the catalytic cycle, heme oxygenase works in tandem with NADPH-cytochrome P450 reductase. NADPH binds to the active site of NADPH-cytochrome P450 reductase, and electrons from NADPH are eventually transferred via FAD/FMN to the heme (having ferric ion) that is bound to HO-1 as the main substrate. To determine if diphenyleneiodonium, apocynin, and Tiron might

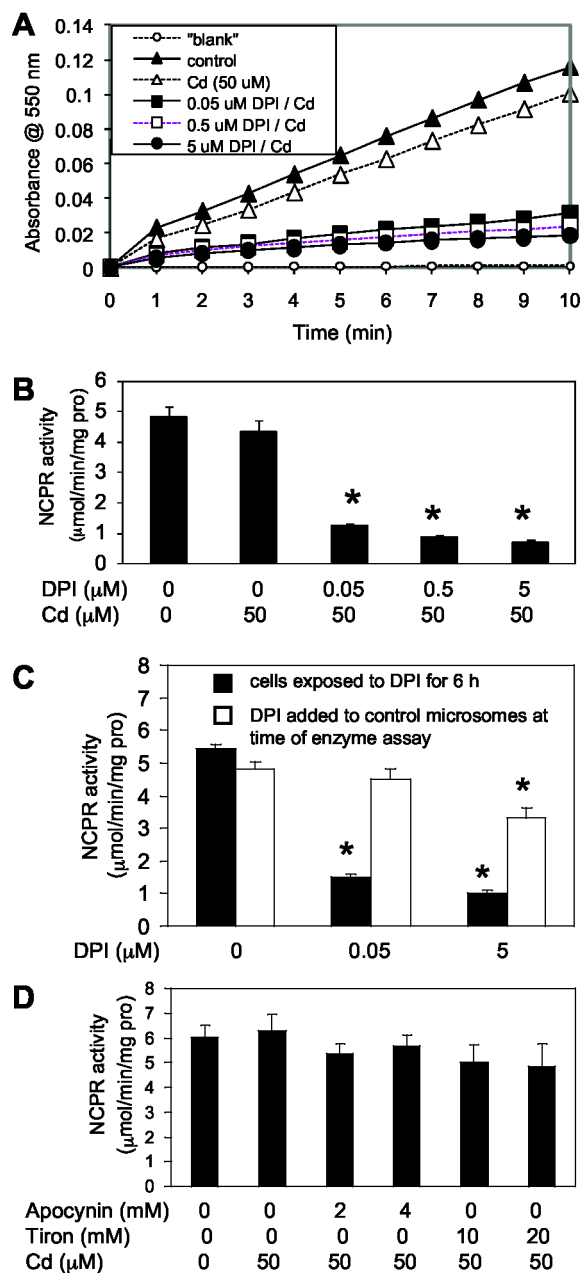


Fig. 9. Inhibition by diphenyleneiodonium (DPI), but not by apocynin or Tiron, of NADPH-cytochrome P450 reductase (NCPR) activity in cadmium-treated HCT-116 cells. The cells were pre-treated with DPI, apocynin, or Tiron for 0.5h, and then exposed to cadmium (Cd) for 6 h. NCPR activity was measured spectrophotometrically using microsomal preparations. The results are representative of 4 different experiments. * $P < 0.05$ versus 50 μ M Cd (B) and respective control (C).

influence NADPH-cytochrome P450 reductase in inhibiting the induction of heme oxygenase activity as caused by cadmium, the effects of diphenyleneiodonium, apocynin, and Tiron on NADPH-cytochrome P450 reductase activity in microsomal membrane preparations from HCT-116 cells exposed to cadmium was examined. Diphenyleneiodonium inhibited NADPH-cytochrome P450 reductase activity (Fig. 9A–C), whereas apocynin and Tiron at much higher concentrations than diphenyleneiodonium had no appreciable effects (Fig. 9D). The inhibitory effect of diphenyleneiodonium on NADPH-cytochrome P450 reductase is understandable since this enzyme belongs to the large family of flavoenzymes, which are known to be inhibited by diphenyleneiodonium (O'Donnell et al., 1993). Thus, the greater potency of diphenyleneiodonium in inhibiting cadmium-mediated induction of heme oxygenase activity could be due to diphenyleneiodonium's dual action in attenuating cadmium-induced upregulation of HO-1 protein expression and inhibiting the activity of NADPH-cytochrome P450 reductase.

4. Discussion

HO-1 contributes to the maintenance of good health, based on seminal studies (Yachie et al., 1999; Kawashima et al., 2002) showing that human HO-1 deficiency increases susceptibility to oxidative stress and promotes inflammation. It is widely recognized that persistent oxidative stress and inflammation are conducive to the development of several chronic degenerative diseases, such as cancer, atherosclerosis, and Alzheimer's disease. Therefore, it is desirable for cells in the body to have the full ability to express HO-1, but especially during adversity such as exposure to toxic environmental agents. Under these challenging circumstances, any weakening of the ability of cells to express HO-1 can be viewed as undesirable. As such, the significance of the current work becomes apparent.

Increased expression of HO-1 as caused by cadmium is likely part of a defensive tactic by cells to protect themselves against cadmium toxicity. Cadmium induces ROS and a state of oxidative stress (Chen et al., 2008), which can result in DNA damage and carcinogenesis (Bertin and Averbeck, 2006). Hence, considering the adverse effects of cadmium, it is critical for cells to be able to optimally express HO-1 upon cadmium exposure in order to help safeguard themselves the greatest. As such, it would be enlightening to have knowledge of any key factors, such as nutritive constituents present intracellularly, that could potentially influence the ability of cells to optimally express HO-1 upon cadmium exposure. It is well established that adequately nourished cells, which would be achieved when cells are cultured in media containing adequate amounts of all vital nutrients, have the ability to robustly express HO-1 upon exposure to cadmium, as exemplified by two previous studies (Takeda et al., 1994; Elbekai and El-Kadi, 2007). However, it is entirely unknown if cells can optimally express HO-1 when the cells are deficient in a single micronutrient and exposed to cadmium to induce HO-1. A central finding in the present study is that DFO, which is generally regarded as an iron chelator, weakened HO-1 upregulation caused by cadmium, suggesting that adequate amounts of iron are needed in cells to achieve maximum HO-1 expression upon induction. The effect of DFO was specific to iron, based on the observation that replenishment or "rescue" of the DFO-pretreated cells with iron significantly restored the ability of the cells to fully express HO-1 upon cadmium exposure, whereas replenishment of these cells with copper and zinc was ineffective in providing such restoration.

How adequate amounts of iron enable cells to fully express HO-1 upon induction by cadmium exposure is not completely clear. But, the generation of ROS to function as signaling oxidants appears paramount considering that the HO-1 gene, which contains a stress response element (Alam and Cook, 2003), is activated when cells are exposed to stress-inducing agents (Li et al., 2006; Tyrrell, 2004; Chin et al., 2003; Takeda et al., 1994; Elbekai and El-Kadi, 2007) that typically induce ROS generation. A potential source of such ROS may come from the reaction catalyzed by non-phagocytic isoforms of NOX that are expressed in different tissues (Takeya and Sumimoto, 2006). Cells of colonic origin are known to abundantly express the NOX-1 isoform (Fukuyama et al., 2005), and Western analysis revealed that the HCT-116 cells used in the current study express NOX-1. Enzyme catalysis by NOX produces superoxide, which has been proposed to act in its native form as a signaling oxidant (Buetler et al., 2004). On the other hand, a downstream product of superoxide, namely, hydrogen peroxide, is the more recognizable signaling oxidant (Stone and Yang, 2006). It has been reported that NOX1 is involved in ultimately producing a source of hydrogen peroxide for growth factor-mediated cell signaling (Park et al., 2004). In any event, superoxide and/or hydrogen peroxide produced enzymatically by NOX may initiate a redox-sensitive molecular pathway leading to HO-1 gene activation.

Regarding a possible connection between NOX and the cadmium-induced upregulation of HO-1 in the present study, an involvement of NOX in the signaling events is suggested by the finding that NOX inhibitors (diphenyleneiodonium and apocynin) and a superoxide scavenger (Tiron) inhibited such HO-1 upregulation. Therefore, because the iron atoms in heme of NOX facilitate transfer of electrons from NADPH to molecular oxygen in producing superoxide (Zhu and Silverman, 2008), it is conceivable that adequate iron is needed in cells to provide and sustain the iron atoms in the heme molecules necessary for catalytic operation of NOX, and hence, generation of a signaling oxidant that would mediate a cascade of events leading to HO-1 upregulation.

As such, without adequate iron in cells to furnish the iron atoms in heme of NOX, cells would be unable to strongly express HO-1 when exposed to cadmium. In other words, the iron-deficient cells would only be able to weakly express HO-1 when exposed to cadmium due to the expected impairment of NOX, and hence, lower production of ROS needed to act as signaling oxidant. There is recent evidence that iron is required to enable cadmium to induce ROS formation (Liu et al., 2009).

Another source of ROS that could possibly be responsible for triggering cadmium-induced HO-1 upregulation is the mitochondrial respiratory system, whose electron transferers (cytochromes) require iron atoms to function. It has been reported that cadmium stimulated ROS production in isolated mitochondria, which was suggested to be attributed to inhibition by cadmium of mitochondrial electron transfer resulting in increased formation of superoxide (Wang et al., 2004). Thus, mitochondrial ROS might act as the signaling oxidant to trigger the metabolic pathway leading to HO-1 gene activation caused by cadmium. Using electron transport inhibitors such as rotenone, it has been concluded that mitochondria-derived ROS can be involved in signal transduction cascades (Kulisz et al., 2002). However, in the present study, it was found that rotenone neither induced HO-1 upregulation nor inhibited cadmium-induced HO-1 upregulation, suggesting that mitochondrial ROS are not involved in cadmium-induced HO-1 upregulation.

In addition to iron, glutathione was also investigated as a possible factor influencing the ability of HCT-116 cells to express HO-1 upon exposing them to cadmium. Cadmium can bind to the free sulfhydryl groups of thiol antioxidants, especially glutathione (Leverrier et al., 2007), thereby rendering the thiol antioxidants incapable of scavenging ROS. By diminishing the overall ROS scavenging capacity in this way when incubated with cells, cadmium induces ROS formation (Stohs and Bagchi, 1995) by apparently permitting the accumulation of ROS in cells or through other indirect means. Being a redox-inactive metal, cadmium is not known to react in a Fenton reaction-like manner to directly generate ROS, in contrast to the standard redox-active metal, iron (Winterbourn, 1995). Thus, as the present work confirms, cadmium, but also ethacrynic acid that conjugates with glutathione (Ploemen et al., 1994), each decreased glutathione in HCT-116 cells. This lowering of glutathione was associated with HO-1 expression being upregulated, which on first thought would imply that cadmium and ethacrynic acid may induce HO-1 upregulation through a thiol redox-sensitive molecular pathway. However, this notion is not entirely supported by the results from the experiments with GSH and NAC. Although the HO-1 upregulation caused by ethacrynic acid was inhibited by pre-treating the cells with GSH or NAC, the HO-1 upregulation caused by cadmium was not unequivocally inhibited with GSH or NAC.

On the other hand, merely lowering intracellular glutathione per se does not appear to be sufficient to result in HO-1 upregulation. Decreasing intracellular glutathione in HCT-116 cells by inhibiting glutathione biosynthesis with buthionine sulfoxamine did not upregulate HO-1 expression. Consistent with this lack of an effect of buthionine sulfoxamine on HO-1 and in contrast to the effects of cadmium and ethacrynic acid on Nrf2, buthionine sulfoxamine did not increase expression and nuclear translocation of Nrf2. This basic leucine zipper transcription factor is responsible for largely activating stress response element-containing genes, such as HO-1 (Alam et al., 1999; Alam and Cook, 2003). In trying to explain why cadmium and ethacrynic acid upregulated HO-1 expression but buthionine sulfoxamine did not, the chemical reactivity of these three compounds needs to be considered. Cadmium and ethacrynic acid react with the thiol functional group, whereas buthionine sulfoxamine is not known to have such reactivity. Thus, when incubated with cells, cadmium and ethacrynic acid probably react with some sulfhydryl (cysteine-containing) proteins. As such, it is possible that cadmium and ethacrynic acid, but not buthionine sulfoxamine, may react with the thiol functional group of multiple cysteine residues in the sulfhydryl protein, Keap1, which is believed to serve as a molecular sensor for specific xenobiotics that induce cytoprotective gene expression (Dinkova-Kostova et al., 2002). In the absence of the xenobiotics, Keap1 immobilizes Nrf2 by binding to it in the cytosol. In the presence of the xenobiotics, the thiol functional group of key cysteinyl residues of Keap1 is covalently or oxidatively modified. As a result, Nrf2 becomes unbound from Keap1 for translocation into the nucleus to eventually transcriptionally activate responsive genes such as HO-1.

In summarizing the key findings, the ability of HCT-116 cells to express HO-1 when exposed to cadmium is influenced by the iron balance, but also thiol antioxidant status, in the cells. Upon exposure to cadmium, iron-sufficient cells strongly express HO-1, whereas iron-deficient cells weakly express HO-1. Cadmium-induced HO-1 upregulation seems to be mediated in part by NOX. Because iron is the pivotal element needed by NOX for production of signaling oxidant, it is logical to think that cells without adequate iron would be unable to optimally express redox-sensitive HO-1 when they are exposed to cadmium. Other cell lines, including primary cells, need to be used as experimental models to replicate and expand the current findings before definitive conclusions can be attained regarding the potential impact of iron on cadmium-induced upregulation of HO-1.

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