ABSTRACT:

Recently, we demonstrated the ability of heavy metals, particularly Hg\(^{2+}\), Pb\(^{2+}\), and Cu\(^{2+}\), to differentially modulate in Hepa 1c1c7 cells the expression of the phase II xenobiotic metabolizing enzymes NAD(P)H:quinone oxidoreductase 1 (Nqo1) and glutathione S-transferase subunit Ya (Gst ya) genes, yet the mechanisms involved remain unknown. To investigate the molecular mechanisms involved in the regulation of Nqo1 and Gst ya genes by heavy metals, Hepa 1c1c7 cells were treated with Hg\(^{2+}\), Pb\(^{2+}\), or Cu\(^{2+}\) in the presence and absence of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent inducer of Nqo1, Gst ya, and Cyp1a1 genes. Analysis of the time-dependent effects of heavy metals revealed that Hg\(^{2+}\) and Pb\(^{2+}\) increased whereas Cu\(^{2+}\) inhibited the constitutive and inducible expression of Nqo1 and Gst ya mRNAs in a time-dependent manner. The RNA synthesis inhibitor actinomycin D significantly inhibited the Nqo1 and Gst ya mRNA induction in response to metals, indicating a requirement of de novo RNA synthesis. The protein synthesis inhibitor cycloheximide significantly inhibited metal-mediated induction of Nqo1 and Gst ya mRNAs, which coincided with a decrease in the nuclear factor erythroid 2-related factor (Nrf2) protein expression, implying the requirement of Nrf2 for the induction of these genes. Furthermore, inhibition of Nrf2 protein degradation by carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132), a 26S proteasome inhibitor, significantly reversed the cycloheximide-mediated inhibition of Nqo1 and Gst ya mRNAs, which coincided with an increase in the expression of Nrf2, confirming that a transcriptional mechanism is involved. Nqo1 and Gst ya mRNA and protein decay experiments revealed lack of post-transcriptional and post-translational mechanisms. This is the first demonstration that heavy metals regulate the expression of Nqo1 and Gst ya genes through a transcriptional mechanism.

Chronic exposure of humans to toxic xenobiotics and environmental contaminants, such as polycyclic aromatic hydrocarbons and heavy metals, has multiple biological consequences, including effects on the xenobiotic metabolizing enzymes (XMEs) (Aitio et al., 1978; Korashy and El-Kadi, 2004). Several studies have demonstrated the association between the inhibition of NQO1 and GST Ya activities and increased risk of carcinogenesis (Smith et al., 2001; Iskander and Jaiswal, 2005; Saldivar et al., 2005). NQO1 is a cytosolic flavoprotein that is expressed constitutively in a wide range of mammalian tissues and cell lines. NQO1 catalyzes the two-electron reduction of several environmental contaminants, electrophilic and endogenous compounds (Chen and Kunsch, 2004; Pinaire et al., 2004). GST Ya belongs to a family of XMEs that catalyzes the conjugation of electrophilic compounds with glutathione, which in turn is enzymatically degraded to mercapturates and excreted (Lamb and Franklin, 2002; Hayes et al., 2005).

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ABBREVIATIONS: XME, xenobiotic metabolizing enzyme; Nqo1, NAD(P)H:quinone oxidoreductase 1; Gst ya, glutathione S-transferase subunit Ya; XRE, xenobiotic responsive element; ARE, antioxidant responsive element; AhR, aryl hydrocarbon receptor; Nrf2, nuclear factor erythroid 2-related factor 2; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TEMED, N,N,N',N'-tetramethylethylenediamine; Act-D, actinomycin D; CHX, cycloheximide; MG-132, carbobenzoxy-L-leucyl-L-leucyl-leucinal; PBS, phosphate-buffered saline; DCPII, 2,6-dichlorophenolindophenol; CHP, cumene hydroperoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.
Nrf2 dissociates from Keap1 protein and then translocates to the nucleus, where it dimerizes with a small Maf protein. The Nrf2-Maf complex then binds to the ARE consensus sequence located in the promoter region of NQO1 and GST Ya genes, resulting in the induction of the transcription process (Chen and Kunsch, 2004; Jaiswal, 2004; Nioi and Hayes, 2004).

The XRE- and ARE-driven regulation of NQO1 and GST Ya genes was generally thought to function independently. However, the proximity of the two sequence sites suggests a possible cross-talk and functional overlap. Recent reports suggest that bifunctional inducers, which activate both XRE and ARE signaling pathways, such as 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD), require direct cross-talk between the XRE- and ARE-mediated pathways for the induction of NQO1. Furthermore, it has been reported that the induction of NQO1 by ARE inducers requires the presence of AhR, suggesting a more direct cross-talk between the XRE- and ARE-mediated pathways (Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005).

Recently, we have demonstrated that heavy metals such as mercury (Hg²⁺), lead (Pb²⁺), and copper (Cu²⁺) differentially regulate the expression of Nqo1 and Gst ya genes at the constitutive and inducible levels in Hepa 1c1c7 cells (Korashy and El-Kadi, 2004). However, it is still not clear which molecular steps are targeted by heavy metals to modulate the expression of these genes. Therefore, the objectives of the current study were to investigate the molecular mechanisms involved in the regulation of Nqo1 and Gst ya genes expressions by heavy metals at both the transcriptional and post-transcriptional levels. To perform that, we used single but different concentrations from each metal, which were 5, 25, and 10 μM for Hg²⁺, Pb²⁺, and Cu²⁺, respectively. The concentrations of metals used in the current study were chosen after determining the ability of a range of concentrations to modulate the Nqo1 and Gst ya gene expressions without significantly affecting Hepa 1c1c7 cell viability (Korashy and El-Kadi, 2004). In addition, these concentrations caused submaximal effect on Nqo1 and Gst Ya genes investigated in the current study (Korashy and El-Kadi, 2004). We provided here the first demonstration that heavy metals regulate the expression of Nqo1 and Gst ya genes through a transcriptional mechanism. In addition, the transcription factor Nrf2 is required for the induction of these genes by heavy metals.

Materials and Methods

Materials. 2,6-Dichlorophenolindophenol, cumene hydroperoxide, cycloheximide, glutathione reductase, lead nitrate, mercuric chloride, reduced glutathione, lead nitrate, mercuric chloride, reduced glutathione (GAPDH) cDNA probes, consecutively. The intensities of the Nqo1 and Gst ya enzyme activities.

Determinations of Nqo1 Activity. Nqo1 activity was determined by the continuous spectrophotometric assay to quantitate the reduction of its substrate, 2,6-dichlorophenolindophenol (DCPIP) as described previously (Korashy and El-Kadi, 2004). Briefly, 10 μg of cell homogenate protein was incubated with 1 ml of the assay buffer [40 μM DCPIP, 0.2 mM NADPH, 5 μM flavin-adenine dinucleotide, 25 mM Tris-HCl, pH 7.8, 0.1% (v/v) Tween 20, and 0.023% bovine serum albumin]. The rate of DCPIP reduction was monitored for 1.5 min at 600 nm with an extinction coefficient (ε) of 2.1 mM⁻¹ cm⁻¹. The Nqo1 activity was calculated as the decrease in absorbance per minute per milligram of total protein of the sample.

Determinations of Gst ya Activity. Spectrophotometric assay for Gst ya catalytic activity using cumene hydroperoxide (CHP), as a substrate, was carried out as described previously (Korashy and El-Kadi, 2004). Briefly, cell homogenate protein (0.2 mg) was incubated with 0.5 mM CHP, 1 mM reduced glutathione, 0.1 mM NADPH, and 0.3 units of glutathione reductase. The rate of NADPH disappearance was monitored for 1.5 min at 340 nm with an ε of 6.2 mM⁻¹ cm⁻¹. The Gst ya activity was calculated as the decrease in absorbance per minute per mg of total protein of the sample.

RNA Extraction and Western Blot Analysis. After incubation with the test compounds for the indicated time periods, total RNA was isolated from the cells using TRizol reagent, according to manufacturer’s instructions (Invitrogen). Western blot analysis was performed as described previously (Sambrook et al., 1989; Korashy and El-Kadi, 2004). Briefly, total RNA (20 μg) was electrophoresed on a formaldehyde-agarose denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [32P]-labeled cDNA probe specific for mouse Nqo1. The nylon membrane blots were subsequently stripped and rehybridized with Gst ya and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes, consecutively. The intensities of the Nqo1 and Gst ya mRNAs were quantified, relative to the signals obtained for GAPDH mRNA, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/].

The mouse Nqo1 cDNA probe was prepared as described previously (Chen et al., 1994; Korashy and El-Kadi, 2004). The cDNA probes for mouse Gst ya and GAPDH mRNA were generously provided by Dr. David Eaton (University of Washington, Seattle, WA) and Dr. John R. Bend (University of Western Ontario, London, ON, Canada), respectively.

Protein Extraction and Western Blot Analysis. After incubation with the test compounds for the indicated time periods, Hepa 1c1c7 cells were collected in lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μM protease inhibitor cocktail]. The total cellular proteins were prepared by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10
min, followed by centrifugation at 12,000g for 10 min at 4°C. Western blot analysis was performed using a previously described method (Sambrook et al., 1989; Korashy and El-Kadi, 2004). Briefly, for Nrf2 immunodetection, 75 μg of protein from each treatment group were separated on 10% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked overnight at 4°C, followed by incubation with primary antibody against Nrf2 for 3 h at room temperature, and then 1 h incubation with a peroxidase-conjugated anti-rabbit secondary antibody at room temperature. For Nqo1 and Gst ya immunodetection, 200 μg of protein from each treatment group were separated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked overnight at 4°C, followed by incubation with rabbit anti-human polyclonal primary antibody against Nqo1 overnight at 4°C, and then 2 h incubation with a peroxidase-conjugated anti-rabbit secondary antibody at room temperature. Nqo1 blots were subsequently stripped in a solution containing 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50°C and then reprobed with goat anti-rat primary antibody against Gst ya as described above. The bands were visualized using enhanced chemiluminescence method according to the manufacturer’s instructions (GE Healthcare). The intensities of the Nqo1, Gst ya, and Nfr2 proteins were quantified, relative to the signals obtained for β-actin, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij].

**Determination of Nqo1 and Gst ya mRNA Half-Lives.** Act-D chase experiments were performed to determine the effect of heavy metals on the half-lives of constitutive and inducible Nqo1 and Gst ya mRNAs. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then preincubated for 12 h with either vehicle (double deionized water) or 1 nM TCDD to induce the mRNA. Thereafter, the media were removed, and the cells were washed three times with PBS before they were incubated in fresh serum-free media containing 5 μg/ml Act-D, a RNA synthesis inhibitor, to block RNA synthesis. Thirty minutes later, 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ was added. Total RNA was isolated at 0, 1, 3, 6, 12, and 24 h after the addition of Act-D followed by Northern blot quantification analysis. The half-lives of both constitutive and inducible Nqo1 and Gst ya mRNAs, in the presence and absence of heavy metals, were calculated from the slope of the semilogarithmically transformed best-fit line. The decay curves were analyzed individually using linear regression of mRNA amount, expressed as percentage of mRNA remaining versus time. The half-lives obtained from three separate experiments were then used to calculate the mean half-life (mean ± S.E.M.).

**Determination of Nqo1 and Gst ya Protein Half-Lives.** The effect of heavy metals on the constitutive and inducible Nqo1 and Gst ya protein half-lives were determined using CHX chase experiment. Briefly, Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then preincubated for 24 h with either vehicle or 1 nM TCDD to induce the Nqo1 and Gst ya proteins. Thereafter, the media were decanted and the cells were washed three times with PBS, before they were incubated in fresh serum-free media containing 10 μg/ml CHX, a protein synthesis inhibitor, to block further protein synthesis. Thirty minutes later, 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ was added, and total cellular protein was extracted at 0, 1, 3, 6, 12, and 24 h after the addition of CHX followed by Nqo1 and Gst ya immunodetection. The half-lives of both constitutive and inducible Nqo1 and Gst ya proteins, in the presence and absence of heavy metals, were calculated as described above.

**Statistical Analysis.** All results are presented as mean ± S.E.M. The comparison of the results from the various experimental groups with their corresponding controls was carried out by a one-way analysis of variance followed by Student-Newman-Kuels test to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when \( p < 0.05 \).
Results

Time-Dependent Effects of Heavy Metals on Constitutive and TCDD-Inducible Expression of Nqo1 and Gst ya mRNAs. To better understand the effect of heavy metals on the kinetics of Nqo1 and Gst ya mRNAs compared with TCDD, the constitutive and inducible expression of Nqo1 and Gst ya mRNAs were measured at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence of 1 nM TCDD for the time point indicated. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [32P]-labeled cDNA probe specific for mouse Nqo1. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for Gst ya and GAPDH, which was used as a loading control. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± S.E.M., n = 3) expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. +, p < 0.05 compared with control (t = 0); and *, p < 0.05 compared with TCDD.

FIG. 2. Time-dependent effects of heavy metals on the inducible expression of (A) Nqo1 and (B) Gst ya mRNAs. Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence of 1 nM TCDD for the time point indicated. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [32P]-labeled cDNA probe specific for mouse Nqo1. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for Gst ya and GAPDH, which was used as a loading control. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± S.E.M., n = 3) expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. +, p < 0.05 compared with control (t = 0); and *, p < 0.05 compared with TCDD.

At the constitutive level, Fig. 1, A and B, shows that Hg^{2+} and Pb^{2+} significantly increased the Nqo1 and Gst ya mRNA transcripts in a time-dependent manner. Hg^{2+} treatment caused a maximal induction of the Nqo1 (70%) and Gst ya (250%) between 6 and 12 h. With Pb^{2+}, a 1.5-fold induction of Nqo1 and Gst ya mRNAs occurred earlier at 6 h, followed by a 70% (Nqo1) and 40% (Gst ya) drop of the maximal mRNA levels at 24 h. In contrast, Cu^{2+} caused a time-dependent decrease in the Nqo1 and Gst ya mRNA levels (Fig. 1, A and B).

At the inducible level, cotreatment of the cells with TCDD and either Hg^{2+} or Pb^{2+} further increased the TCDD-mediated induction of Nqo1 and Gst ya mRNAs in a time-dependent manner, whereas cotreatment with TCDD and Cu^{2+} inhibited the induction (Fig. 2, A and B). A densitometric scan of the autoradiogram indicates that Hg^{2+}, in the presence of TCDD, significantly increased the steady-state Nqo1 and Gst ya mRNA levels by 1- and 2-fold, respectively. Yet, in the presence of Cu^{2+}, the steady-state mRNA levels of Nqo1 and Gst ya were decreased by approximately 50 and 25%, respectively. Taken together, the changes in the steady state of Nqo1 and Gst ya mRNA levels by heavy metals reflect an alteration in the rate of synthesis and/or degradation of these genes.
Effects of Heavy Metals on the TCDD Concentration-Dependent Induction of Nqo1 and Gst ya mRNAs and Activities. To further examine the effect of heavy metals on the kinetics of Nqo1 and Gst ya mRNAs and activities induced by different concentrations of TCDD, Hepa 1c1c7 cells were treated with 5 μM Hg²⁺, 25 μM Pb²⁺, or 10 μM Cu²⁺ in the presence and absence of increasing concentrations of TCDD (0.1, 1, or 10 nM) for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [³²P]-labeled cDNA probe specific for mouse Nqo1. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for Gst ya and GAPDH, which was used as a loading control. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± S.E.M., n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels.

Inhibition of Heavy Metal-Mediated Induction of Nqo1 and Gst ya mRNAs by a RNA Synthesis Inhibitor. To investigate whether heavy metals are capable of increasing the de novo Nqo1 or Gst ya mRNA synthesis, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg²⁺, 25 μM Pb²⁺, or 10 μM Cu²⁺ in the presence and absence of 5 μg/ml Act-D, a RNA synthesis inhibitor. Total RNA was then isolated and quantified by Northern blot analysis. If metals increase the amount of Nqo1 or Gst ya mRNA through increasing their de novo RNA synthesis, under these circumstances, we would expect to observe a decrease in the content of Nqo1 or Gst ya mRNA after the inhibition of their RNA synthesis.

Figure 5, A and B, shows that in untreated cells, the Nqo1 and Gst ya mRNAs are constitutively expressed (lane 1). However, pretreat-
ment of the cells with Act-D for 6 h significantly inhibited the constitutive Nqo1 and Gst ya mRNAs by approximately 23 and 27%, respectively (lane 2). In metal-treated cells, Hg²⁺/H₁₁₀₀₁ and Pb²⁺/H₁₁₀₀₁ alone significantly induced Nqo1 mRNA by approximately 85 and 40% and Gst ya mRNA by 170 and 70%, respectively (lanes 3 and 5). Cu²⁺/H₁₁₀₀₁, on the other hand, inhibited the constitutive expression of both genes (lane 7). Treatment of the cells with Act-D completely inhibited the metal-mediated induction of Nqo1 and Gst ya mRNAs (lanes 4, 6, and 8). Similar results were observed with TCDD in which the induction of Nqo1 (2-fold) and Gst ya (1-fold) mRNAs was completely inhibited by Act-D (lanes 9 and 10). These results suggest that heavy metals increased the Nqo1 and Gst ya mRNA levels by increasing their de novo RNA synthesis in a manner similar to what was observed with TCDD.

Induction of Nqo1 and Gst ya mRNAs in Response to Heavy Metals Requires a Labile Protein. Several studies have shown that Nrf2, a labile transcriptional protein, is required for the induction of Nqo1 and Gst ya genes by the XRE and ARE inducers (Ma et al., 2004; McWalter et al., 2004). To examine whether the effect of heavy metals on Nqo1 and Gst ya mRNAs requires de novo protein synthesis, we examined the effect of the protein synthesis inhibitor CHX at a concentration that has been shown to inhibit Nrf2 protein synthesis in Hepa 1c1c7 cells (Stewart et al., 2003; Ma et al., 2004). For this purpose, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg²⁺, 25 μM Pb²⁺, or 10 μM Cu²⁺ in the presence and absence of increasing concentrations of TCDD (0.1, 1, or 10 nM) for 24 h prior to assay. Nqo1 and Gst ya enzyme activities were determined spectrophotometrically using DCPIP and CHP as substrates, respectively. Values are presented as mean ± S.E.M. (n = 6). *, p < 0.05 compared with control; and +, p < 0.05 compared with TCDD.

![Fig. 4. Effect of heavy metals on the TCDD concentration-dependent induction of (A) Nqo1 and (B) Gst ya activities.](image-url)
Inhibition of Heavy Metal-Mediated Induction of Nqo1 and Gst ya mRNAs by CHX Is a Transcriptional Mechanism. Inhibition of the metal-mediated induction of Nqo1 and Gst ya mRNAs by CHX can be transcriptional, in which CHX inhibits RNA synthesis, or post-transcriptional, due to an increase in degradation rate. To distinguish between these possibilities, Hepa 1c1c7 cells were treated with either metals alone for 2 or 6 h, or with metals for 2 h followed by metals plus CHX for additional 4 h.

As shown in Fig. 7, CHX alone caused a significant decrease of the constitutive expression of Nqo1 and Gst ya mRNAs after 4 h of exposure (lane 2). In addition, metals increased the expression of Nqo1 and Gst ya mRNAs at 2 and 6 h in a time-dependent manner. On the other hand, the mRNA levels of either Nqo1 or Gst ya from cells treated with metals for 6 h plus CHX for 4 h (lanes 5, 8, and 11) was almost similar to the mRNA levels obtained from cells treated with metals alone for 2 h (lanes 3, 6, and 9). These results imply that CHX did not affect the level of existing Nqo1 or Gst ya mRNA; rather, it inhibited the de novo RNA synthesis, which is required by heavy metals for the induction of these genes. This suggests that heavy metals regulate the expression of Nqo1 and Gst ya mRNAs at the transcriptional level.

Induction of Nqo1 and Gst ya mRNAs by Heavy Metals Is a Transcriptional Mechanism. It has been shown that Nrf2 protein is degraded rapidly (t_{1/2} < 30 min) through the 26S proteasome pathway (Stewart et al., 2003; Chen and Kunsch, 2004; Ma et al., 2004). To further confirm the involvement of a transcriptional mechanism in the regulation of Nqo1 and Gst ya genes by heavy metals, we tested the hypothesis that inhibition of the proteasome-dependent degradation of Nrf2 by MG-132, a potent 26S proteasome inhibitor, would reverse the repressor effect of CHX on the metal-mediated induction of Nqo1 and Gst ya mRNAs. Therefore, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence and absence of either 10 μg/ml CHX or 10 μg/ml CHX plus 25 μM MG-132 at a concentration of MG-132 known to inhibit the proteasome-dependent degradation of Nrf2 protein in Hepa 1c1c7 cells (Ma et al., 2004).

As shown in Fig. 8A, the presence of MG-132 significantly increased the expression of Nqo1 mRNA compared with control (lanes 11, 12, 14, and 15). In addition, the expression of Nqo1 mRNA was increased when MG-132 was added with CHX (lanes 13, 16, 18, and 19). These results suggest that MG-132 reverses the repressor effect of CHX on the metal-mediated induction of Nqo1 mRNA.

Fig. 5. Effect of a RNA synthesis inhibitor on heavy metal-mediated induction of (A) Nqo1 and (B) Gst ya mRNAs. Hepa 1c1c7 cells were treated with 5 μg/ml Act-D, a RNA synthesis inhibitor, 30 min before exposure to 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [32P]-labeled cDNA probe specific for mouse Nqo1. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for Gst ya and GAPDH, which was used as a loading control. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± S.E.M., n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. +, p < 0.05 compared with control; +, p < 0.05 compared with the same treatment in the absence of Act-D.

Fig. 6. Effect of a protein synthesis inhibitor on heavy metal-mediated induction of (A) Nqo1 and (B) Gst ya mRNAs. Hepa 1c1c7 cells were treated with 10 μg/ml CHX, a protein synthesis inhibitor, 30 min before exposure to 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD for 6 h. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [32P]-labeled cDNA probe specific for mouse Nqo1. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for Gst ya and GAPDH, which was used as a loading control. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± S.E.M., n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. +, p < 0.05 compared with control; +, p < 0.05 compared with the same treatment in the absence of CHX.
In Fig. 8, A and B (lane 2), treatment of Hepa 1c1c7 cells with CHX alone caused a significant reduction in Nqo1 and Gst ya mRNA expression. MG-132 alone, in the absence of CHX, did not significantly alter the Nqo1 and Gst ya mRNA expression (data not shown), which is consistent with previously published data in Hepa 1c1c7 cells (Ma et al., 2004). However, in the presence of CHX, MG-132 significantly reversed the CHX-mediated inhibition of Nqo1 and Gst ya mRNAs to their control levels (lane 3). Importantly, treatment of the cells with metals plus CHX and MG-132 significantly reversed the CHX-mediated inhibition of the metal-inducible Nqo1 and Gst ya mRNAs (lanes 6, 9, and 12). A similar result was observed with TCDD (lanes 13–15). Taken together, these results confirm that heavy metals regulate the expression of Nqo1 and Gst ya genes by a transcriptional mechanism.

**Nrf2 Protein Is Required for the Induction of Nqo1 and Gst ya mRNAs by Heavy Metals.** To directly explore whether Nqo1 and Gst ya mRNAs can be induced by heavy metals in a Nrf2-dependent manner, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg2+ or Pb2+, 25 μM Cu2+, or 1 nM TCDD in the presence and absence of either 10 μg/ml CHX or 10 μg/ml CHX plus 25 μM MG-132. Nrf2 protein expression was then detected by Western blot analysis. Figure 8C shows that Hg2+ and Pb2+ significantly increased the level of Nrf2 compared with control, whereas Cu2+ and TCDD did not significantly alter Nrf2 level (Fig. 8C, lanes 1, 4, 7, 10, and 13). Furthermore, Hg2+ and Pb2+-mediated induction of Nqo1 and Gst ya mRNAs (Fig. 8, A and B, lanes 4 and 7) was associated with elevated Nrf2 level (Fig. 8C, lanes 4 and 7), suggesting that a Nrf2-dependent mechanism is involved. As expected, treatment of the cells with CHX alone or CHX plus metals significantly decreased the constitutive (Fig. 8C, lane 2) and metal-inducible (Fig. 8C, lanes 5 and 8) Nrf2 expression that was associated with a decrease in Nqo1 and Gst ya mRNA expressions (Fig. 8, A and B, lanes 2, 5, 8, and 11). Importantly, the CHX-mediated decrease of Nrf2 protein, Nqo1 and Gst ya mRNA levels were significantly reversed by MG-132 treatment (Fig. 8, A–C, lanes 3, 6, 9, and 12). These results imply that Nrf2 is required by heavy metals for the induction of Nqo1 and Gst ya mRNAs.

**Lack of a Post-Transcriptional Regulation of Nqo1 and Gst ya Genes by Heavy Metals.** To further investigate whether the effects of heavy metals on the Nqo1 and Gst ya mRNAs could be attributed to a post-transcriptional stabilization of the mRNA, an Act-D chase experiment was performed to determine the effect of heavy metals on the half-lives of both constitutive and inducible Nqo1 and Gst ya mRNAs. If metals stabilize the mRNA, we should observe an increase in the mRNA half-life. Act-D chase experiment revealed that the decay of Nqo1 and Gst ya
mRNA transcripts isolated from Hepa 1c1c7 cells pretreated with either vehicle or TCDD alone followed first-order kinetics, with approximate half-lives of 16.45 /H11006 2.52 and 10.90 /H11006 1.17 h (constitutive, Fig. 9, A and B) and approximately 17.88 /H11006 0.48 and 11.4 /H11006 0.45 h (inducible, Fig. 10, A and B). However, treatment of the cells with heavy metals did not significantly affect the half-lives of Nqo1 and Gst ya mRNAs of both vehicle- and TCDD-treated Hepa 1c1c7 cells, implying that all three metals did not change the stability of Nqo1 and Gst ya mRNAs. These results revealed that the modulation of Nqo1 and Gst ya mRNAs by heavy metals is not regulated by a post-transcriptional mechanism.

Lack of a Post-Translational Regulation of Nqo1 and Gst ya Genes by Heavy Metals. The sustained elevation of Nqo1 and Gst ya mRNAs with variable response to the catalytic activities (Fig. 4, A and B) prompted further investigation to examine whether heavy metals could modify the stability of Nqo1 and Gst ya proteins. Therefore, the effect of heavy metals on the constitutive and inducible Nqo1 and Gst ya protein half-lives was determined using CHX chase
experiments. Our results clearly showed that the half-lives of Nqo1 and Gst ya proteins were determined to be greater than 24 h, at both the constitutive (Fig. 11, A and B) and inducible (Fig. 12, A and B) levels. Furthermore, the stability of both proteins did not appear to be significantly altered by heavy metals up to 24 h (Figs. 11 and 12). The results of these experiments indicate a lack of a post-translational regulation of the Nqo1 and Gst ya by heavy metals.

Discussion

Nqo1 and Gst ya are phase II metabolizing enzymes that have been shown to play an essential role in the detoxification of xenobiotics and carcinogenic metabolites (Rushmore and Kong, 2002; Chen and Kunsch, 2004; Lee and Johnson, 2004; Xu et al., 2005). Several studies have demonstrated a complex regulation of NQO1 and GST Ya genes, in which the transcriptional activation of these genes are regulated by both XRE and ARE (Friling et al., 1990; Noda et al., 2003; Chen and Kunsch, 2004; Nioi and Hayes, 2004; Miao et al., 2005; Xu et al., 2005). Recently, we have shown that heavy metals differentially regulate the expression of Nqo1 and Gst ya genes at the constitutive and inducible levels (Korashy and El-Kadi, 2004), yet the exact mechanism has previously not been examined.

The present study demonstrates several important differences between heavy metals and TCDD on their effects on the kinetics of Nqo1 and Gst ya mRNA and activity levels. Hg2+/Hg2+ and Pb2+/Hg2+ show a rapid onset of induction compared with TCDD. However, TCDD shows a longer duration of induction, in which Hg2+/Hg2+ or Pb2+/Hg2+-mediated induction of Nqo1 and Gst ya mRNAs returned to the basal level at 24 and 12 h, respectively. In contrast, TCDD-mediated induction remained elevated for at least 24 h. Furthermore, the magnitude of the Hg2+/Hg2+-mediated induction of Nqo1 mRNA was similar to that observed with TCDD, whereas the induction of Gst ya mRNA by Hg2+ was 2-fold higher than that of TCDD.

Initially, we questioned whether the induction of Nqo1 and Gst ya mRNAs by heavy metals, particularly Hg2+/Hg2+ or Pb2+/Hg2+, is regulated by a transcriptional mechanism in which heavy metals increase de novo RNA synthesis and/or by a post-transcriptional stabilization of the
mRNA. Thus, a series of experiments were carried out to determine which molecular mechanisms are targeted by these heavy metals.

The transcriptional regulation of \textit{Nqo1} and \textit{Gst ya} genes by heavy metals was demonstrated by different approaches. First, we have shown that the RNA synthesis inhibitor Act-D abolished the heavy metal-mediated induction of \textit{Nqo1} and \textit{Gst ya} mRNAs. These results imply that metals increase de novo \textit{Nqo1} and \textit{Gst ya} RNAs synthesis in a manner similar to that observed with TCDD, which is known to induce these genes at the transcriptional level through the XRE-ARE pathway (Radjendirane and Jaiswal, 1999; Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). Second, the protein synthesis inhibitor CHX significantly inhibited the metal-mediated induction of \textit{Nqo1} and \textit{Gst ya} mRNAs through a transcriptional mechanism, in which CHX inhibited the newly synthesized \textit{Nqo1} and \textit{Gst ya} mRNAs but did not affect the existing mRNAs. Our results are in agreement with previous published studies in different cells lines that demonstrated that CHX inhibited the induction of \textit{Nqo1} (Lamb and Franklin, 2002; Ma et al., 2004) and \textit{GST Ya} (Eickelmann et al., 1995) mRNAs through a transcriptional mechanism. Furthermore, the inhibition of metal-mediated induction of \textit{Nqo1} and \textit{Gst ya} mRNAs by CHX was not due to a decrease in cell viability or general inhibitory effect on gene transcriptions, since the same concentration of CHX suppressed the heavy metal-mediated induction of \textit{Cyp1a1} mRNA in Hepa 1c1c7 cells (Korashy and El-Kadi, 2005).

Previously (Korashy and El-Kadi, 2004), we have shown that heavy metals were able to induce the \textit{Nqo1} and \textit{Gst ya} genes in both wild-type Hepa 1c1c7 and AhR-deficient cells. However, metals were more effective at the induction of both genes in wild type than in AhR-deficient cells, suggesting the involvement of XRE- and ARE-dependent mechanisms. In this regard, we have recently demonstrated that metals directly activate the AhR and hence the XRE-dependent pathway in Hepa 1c1c7 cells (Korashy and El-Kadi, 2005).

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FIG. 10. Effect of heavy metals on the TCDD-inducible (A) \textit{Nqo1} and (B) \textit{Gst ya} mRNA half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then incubated with 1 nM TCDD for 12 h. The cells were then washed three times with PBS and incubated in fresh media containing 5 \textmu M \textit{Hg}^{2+}, 25 \textmu M \textit{Pb}^{2+}, or 10 \textmu M \textit{Cu}^{2+} plus 5 \textmu g/ml Act-D, a RNA synthesis inhibitor. Total RNA (20 \mu g) was isolated at the indicated time points and then separated by electrophoresis on a 1.1% formaldehyde denaturing gel. The gel was then transferred to nylon membranes and hybridized with a 32P-labeled cDNA probe specific for mouse \textit{Nqo1}. The blots were subsequently stripped and rehybridized sequentially with cDNA probes specific for \textit{Gst ya} and GAPDH, which was used as a loading control. All mRNA decay curves were analyzed individually, and the half-lives were estimated from the slope of a straight line fitted by linear regression analysis (r^2 = 0.85) to a semilog plot of normalized mRNA amount to GAPDH level, expressed as a percentage of treatment at t = 0 (maximum, 100%) level versus time. The half-lives obtained from these independent experiments were then used to calculate the mean half-life (mean ± S.E.M., n = 3). One of three representative experiments is shown.
is a basic leucine zipper transcriptional protein that is degraded rapidly by the proteasome pathway (Chen and Kunsch, 2004; Jaiswal 2004; Ma et al., 2004). Noda et al. (2003) showed that only inducible, but not constitutive, Nqo1 and Gst-p gene expressions were abolished in Nrf2-null mice. Conversely, in AhR- and Nrf2-double knockout mice, both constitutive and inducible expression of Nqo1 and Gst-p genes was completely abolished. Similar results were observed in Nrf2-knockout mice embryonic fibroblast cells (Hayes et al., 2000; Jaiswal 2004; Ma et al., 2004; McWalter et al., 2004; Nioi and Hayes, 2004). These results clearly indicate that both AhR- and Nrf2-mediated pathways play an integral role in the regulation of Nqo1 and Gst ya genes. More recently, it has been shown that a cross-talk exists between AhR-XRE and Nrf2-ARE, in which Nrf2 gene expression is directly regulated through AhR activation (Ma et al., 2004; Nioi and Hayes, 2004; Miao et al., 2005). Moreover, Marchand et al. demonstrated that the NQO1 gene expression can be controlled by CYP1A1 activity, a phase I XME that is regulated mainly through an AhR-dependent pathway, through an oxidative stress-mediated pathway (Marchand et al., 2004; Radjendirane and Jaiswal, 1999).

The notion that a CHX-sensitive labile protein, Nrf2, mediates the regulation of Nqo1 and Gst ya genes by heavy metals is supported by several observations. Initially, the protein synthesis inhibitor, CHX, at a concentration shown to inhibit Nrf2 protein synthesis (Ma et al., 2004), blocks the constitutive and the metal-mediated induction of Nqo1 and Gst ya mRNAs. In addition, the inhibition of proteasome-dependent degradation of Nrf2 by MG-132, a 26S proteasome inhibitor, in the presence of CHX plus metals reverses the inhibitory effects of CHX on the induction of Nqo1 and Gst ya mRNAs by heavy metals. Most importantly, we have shown that heavy metals not only require Nrf2 for the induction of these genes but also increase the Nrf2 protein level. Thus, it is possible that the increases in Nqo1 and Gst ya mRNAs observed with Hg2+/H11001 and Pb2+/H11001 could be attributed to the ability of these metals to decrease the turnover rate of Nrf2 protein. In agreement with previous reports (Kwak et al., 2002; Ma et al., 2004), we demonstrated that blocking the Nrf2 degradation alone using MG-132 is not sufficient to induce the expression of basal Nqo1 or Gst ya mRNA, although it increases total cellular Nrf2 level. This effect could be attributed to the fact that MG-132 increases the level of total cellular Nrf2 but does not increase the nuclear level of Nrf2, which will be sequestered in the cytoplasm. Therefore, it has been

![Fig. 11. Effect heavy metals on the constitutive (A) Nqo1 and (B) Gst ya protein half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, then incubated in fresh media containing 5 μM Hg2+, 25 μM Pb2+, or 10 μM Cu2+ plus 10 μg/ml CHX, a protein synthesis inhibitor. Total cell lysate was collected at the indicated time points and then separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C and then incubated with primary antibodies against Nqo1 and Gst ya, sequentially, followed by 2-h incubation with secondary monoclonal antibody at room temperature. Antibody was detected using the enhanced chemiluminescence method. All protein decay curves were analyzed individually, and the half-lives were estimated from the slope of a straight line fitted by linear regression analysis (r² ≥ 0.85) to a semilog plot of normalized protein amount to β-actin level, expressed as a percentage of treatment at t = 0 (maximum, 100%) level versus time. One of three representative experiments is shown.](http://aspetjournals.org/doi/abs/10.1124/dmd-2017-6456)
hypothesized that a Nrf2 inducer is required to enhance the nuclear translocation of Nrf2 to transactivate the ARE-mediated genes. Moreover, the discrepancy in the effects of CHX in the presence and absence of MG-132 suggests that CHX does not affect the level of existing Nrf2 proteins; rather, it inhibits its de novo protein synthesis, which is required by heavy metals for the induction of Nqo1 and Gst ya genes (Kwak et al., 2002).

It is still unclear how heavy metals activate the Nrf2-ARE-mediated transcriptional induction of Nqo1 and Gst ya genes. However, it has been postulated that phosphorylation signaling pathways may contribute to the induction of Nqo1 and Gst ya genes by heavy metals. In this context, it has been demonstrated that mitogen-activated protein kinases and protein kinases phosphorylate Nrf2/Keap1 complex in the cytoplasm (Rushmore and Kong, 2002; Jaiswal, 2004), resulting in Nrf2 dissociation and nuclear translocation. In the nucleus, Nrf2 dimerizes with bZIP protein, which in turn transactivates the ARE on the promoter region of Nqo1 and Gst ya genes and, hence, initiates their gene transcription. In this respect, heavy metals have been shown to activate mitogen-activated protein kinases and protein kinases via the production of reactive oxygen species, which in turn activate the Nrf2-ARE pathway (Huang et al., 2002; Jonak et al., 2004; Kim et al., 2005).

To test the hypothesis that heavy metals may modulate Nqo1 or Gst ya genes at the post-transcriptional and/or post-translational levels, we assessed the turnover rates of their mRNA and protein. Our results showed that the constitutive and inducible expression of Nqo1 and Gst ya genes is long-lived mRNAs with estimated half-lives of approximately 17 and 11 h, respectively. These results are in agreement with previous studies that reported a half-life of 15 h for Nqo1 mRNA in Hepa 1c1c7 cells (Ma et al., 2004) and 14.5 h for Gst ya mRNA in human HepG2 cells (Eickelmann et al., 1995). However, Nqo1 and Gst ya mRNA half-lives in Hepa 1c1c7 cells treated with metals were not statistically different from their corresponding control mRNA, indicating that heavy metals do not regulate the Nqo1 and Gst ya genes at the post-transcriptional level. Furthermore, our results showed that constitutive and inducible Nqo1 and Gst ya are long-lived proteins with estimated half-lives > 24 h, which is in agreement with a previously published study (Siegel et al., 2001). In addition, the
stabilities of both proteins were not significantly altered by heavy metals up to 24 h, indicating the lack of post-translational regulation of the Nqo1 and Gst yα genes by heavy metals.

In conclusion, we have provided strong evidence that heavy metals regulate the expression of Nqo1 and Gst yα genes through AhR-XRE- and Nrf2-ARE-dependent transcriptional mechanisms. Although heavy metals are considered toxic and ranked highly as the most hazardous substances in the environment [Agency for Toxic Substances and Diseases Registry (ATSDR) list, available from http://www.atsdr.cdc.gov/exs/exs3.html], we have demonstrated that heavy metals may have cytoprotective properties by increasing the expression of Nqo1 and Gst yα genes, which are considered carcinogenesis-protecting enzymes.

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