ABSTRACT:

We have previously shown that cadmium, a metal that alters cellular redox status, induces CYP2A5 expression in nuclear factor (erythroid-derived 2)-like 2 (Nrf2) knockout mice but not in the wild-type (Nrf2+/+) mice. In the present studies, the potential role of Nrf2 in cadmium-mediated regulation of Cyp2a5 gene was investigated in mouse primary hepatocytes. Cadmium chloride (CdCl2) caused a time-dependent induction of the CYP2A5 at mRNA, protein, and activity levels, with a substantial increase observed within 3 h of exposure. Immunoblotting showed cadmium-dependent nuclear accumulation of Nrf2 within 1 h of exposure. Cotransfection of mouse primary hepatocytes with Cyp2a5 promoter–luciferase reporter plasmids and Nrf2 expression plasmid resulted in a 3-fold activation of Cyp2a5 promoter-mediated transcription relative to the control. Deletion analysis of the promoter localized the Nrf2 responsive region to an area from −2656 to −2339 base pair. Computer-based sequence analysis identified two putative stress response elements (StRE) within the region at positions −2514 to −2505 and −2386 to −2377. Chromatin immunoprecipitation and electrophoretic mobility shift assays showed that interaction of the more proximal StRE with Nrf2 was stimulated by CdCl2. Finally, site-directed mutagenesis of the proximal StRE in Cyp2a5 promoter-luciferase reporter plasmids abolished Nrf2-mediated induction. Collectively, the results indicate that Nrf2 activates Cyp2a5 transcription by directly binding to the StRE in the 5′-flanking region of the gene. This acknowledges Cyp2a5 as a potential target of the Nrf2 pathway in response to cellular stress.

The mouse CYP2A5 and its human ortholog CYP2A6 mediate the phase I metabolism of a diverse range of toxic compounds, including nitrosamines and aflatoxins (Su and Ding, 2004). These enzymes are predominantly expressed in hepatocytes but are also present in extrahepatic tissue, particularly in the nasal mucosa (Su and Ding, 2004). CYP2A5 enzyme is the major catalyst of coumarin 7-hydroxylase in mouse liver (Lang et al., 1989). Its regulation is complex and unique among other major cytochromes P450 (P450s). The Cyp2a5 gene is induced by classical inducers, such as phenobarbital (Wood and Conney, 1974), and by various hepatotoxic agents, including pyrazole, carbon tetrachloride, and metals (Seubert et al., 2002; Abu-Bakar et al., 2004; Su and Ding, 2004). Elevated CYP2A5 protein was also observed in spontaneous, transplanted, or chemically induced mouse hepatomas (Su and Ding, 2004).

Depending on the inducer, the activation of hepatic CYP2A5 can be achieved both by transcriptional and post-transcriptional mechanisms. Transcriptional induction of Cyp2a5 by 2,3,7,8-tetrachlorodibenzo-p-dioxin is mediated by the binding of a ligand-activated aryl hydrocarbon receptor (AHR) to the xenobiotic response element (XRE) site at the Cyp2a5 gene (Arpiainen et al., 2005). Pyrazole, a hepatotoxin, induces CYP2A5 by a post-transcriptional mechanism involving binding of heterogenous nuclear ribonucleoprotein A1 to the 3′-untranslated region of CYP2A5 mRNA, with subsequent stabilization of the mRNA (Glisovic et al., 2003).

However, given the structural diversity of the inducers, it is possible that induction of CYP2A5 is not directly related to the nature of the inducing agents, but instead may be an indirect consequence of a
specific cellular event associated with the pathogenesis of liver injury. For example, certain agents may disturb cellular redox status, a common denominator that may consequently induce the expression of CYP2A5 through activation of stress-related transcription factors, such as the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). In support of this hypothesis are the observations that 1) overexpression of CYP2A5 by pyrazole is related to alterations in cellular redox equilibrium (Gilmore and Kirby, 2004); 2) pyrazole treatment in mice increased Nrf2 protein in the liver (Gong and Cederbaum, 2006); and 3) cadmium, an agent that alters cellular redox status, induces CYP2A5 expression in Nrf2+/− mice but not in Nrf2−/− mice (Abu-Bakar et al., 2004).

Nrf2, a basic-leucine zipper protein, regulates coordinated activation of a battery of genes in response to oxidative stress. These include genes that encode phase II drug-metabolizing enzymes such as NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthase, and glutathione S-transferase (Kang et al., 2005); haem oxygenase-1 (HO-1) (Alam et al., 1999); and thioredoxin (Kim et al., 2001). Under normal conditions, Nrf2 exists in an inactive, cytosol-localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1 (Itoh et al., 1999). On cellular stimulation by stress agents, Nrf2 is dissociated from Keap1, which then leads to stabilization and nuclear translocation of Nrf2 by a, as yet, poorly characterized mechanism(s). However, cadmium alters cellular redox by reducing the intracellular ratio of glutathione to oxidized glutathione (Ryter and Choi, 2002) and activates dissociation of the Nrf2-Keap1 complex through p38 mitogen-activated protein kinase-mediated phosphorylation of Keap1 (Alam et al., 2000). The phosphorylation may take place either directly or indirectly through intermediary kinases, thus inhibiting rapid degradation of Nrf2 by ubiquitination (Stewart et al., 2003). In the nucleus, Nrf2 dimerizes with small Maf proteins (Itoh et al., 1995) or other basic-leucine zipper proteins, including Jun family members (Venugopal and Jaiswal, 1998). The resulting heterodimers, in turn, bind to cis-elements with similar core sequences, alternatively known as Maf recognition elements (Kataoka et al., 1994), antioxidant response elements (Rushmore et al., 1991), or stress response elements (SiRE) (Chai and Alam, 1996), to regulate target genes transcription.

The aim of the present study was to elucidate the potential role of Nrf2 in cadmium-mediated Cyp2a5 regulation. The results show existence of Nrf2 binding sites at the Cyp2a5 promoter and that cadmium activates binding of Nrf2 to a SiRE at about 2.4 kilobase upstream of the transcription start site of the Cyp2a5 promoter, which in turn up-regulates the CYP2A5. The present evidence represent a novel mechanism in the regulation of phase I xenobiotic-metabolizing gene.

### Materials and Methods

#### Chemicals and Antibodies

Cadmium chloride (CdCl₂); coumarin; collagenase; dexamethasone; insulin, transferrin, sodium selenite media supplement; gentamicin; 1% L-glutamine; HEPES; phenylmethylsulfonyl fluoride (PMSF); Igepal; Tween 20; spermidine; spermin; and leupeptin were from Sigma-Aldrich (St. Louis, MO). Rabbit IgG (sc-2027), rabbit polyclonal anti-Fos (sc-8047X) antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A monoclonal anti-HO-1 antibody and HO-1 (Hsp32) protein were from Stressgen Biotechnologies Inc. (Victoria, BC, Canada).

#### Isolation and Treatment of Primary Culture Hepatocytes

Hepatocytes were isolated from male DBA/2 mice (Möllegaard, Copenhagen, Denmark) aged 8 to 10 weeks. Livers were perfused with collagenase solution as described previously (Seglen, 1972). After filtration and centrifugation, the isolated hepatocytes were dispersed in Williams’ E medium containing 20 ng/ml dexamethasone; insulin, transferrin, sodium selenite media supplement (5 mg/l insulin, 5 mg/l transferrin, and 5 µg/l sodium selenite); 10 µg/ml gentamicin; 1% L-glutamine; and 10% decomplemented fetal calf serum at a density of 1.8 × 10⁶ cells/60-mm uncoated culture dish (Corning, Palo Alto, CA) and 3 × 10⁵ cells/well on 12-well plates. The cultures were maintained at 37°C in 5% CO₂ in a humidifier incubator. After 2 h of incubation, the medium was replaced with serum-free Williams’ E medium. The cultures were maintained for additional 24 h before treatment with 4 µM CdCl₂ solution or transient transfection. CdCl₂ was dissolved in normal saline solution.

#### Animals

Six 8- to 10-week-old DBA/2 male mice (Animal Resources Centre, Murdoch, WA, Australia) were divided into two groups of three mice in each group. They were housed in filter-top polycarbonate cages containing wood chip bedding and maintained in a 12-h light/dark cycle with free access to standard mouse chow and tap water. They were treated with a single i.p. injection of 16 µmol CdCl₂/kg b.wt. dissolved in normal saline solution. The animals in the control group were given normal saline only. The mice were sacrificed at 8 h after treatment by CO₂ overdose. The livers of individual animals were excised. All the experimental procedures were approved by, and conducted in accordance with, the animal experimentation guidelines of the Queensland Health Scientific Services Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

#### Isolation of Nuclear and Cytoplasmic Proteins

Nuclear and cytoplasmic extracts from primary hepatocytes were prepared as described previously (Geneste et al., 1996). Hepatocytes were washed and resuspended in phosphate-buffered saline. The cell suspension was centrifuged at 2000g for 30 s. The resulting pellet was resuspended in buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM PMSF, 10 µg/ml leupeptin, and 0.4% Igepal) and kept on ice for 1 h. The cell suspension was vortexed, homogenized, and centrifuged at 15,000g at 4°C for 10 min. The supernatant containing cytoplasmic proteins was aliquoted and stored at −80°C. The pellet containing the nuclei was resuspended in buffer B (20 mM HEPES-KOH, pH 7.6, 1.5 mM MgCl₂, 420 nM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 0.4% Igepal) and gently agitated for 30 min at 4°C. The suspension was centrifuged at 15,000g for 15 min at 4°C. The supernatant containing the nuclear proteins was aliquoted and stored at −80°C. Protein content was measured by Lowry method (Lowry et al., 1951).

#### Isolation of Nuclear Proteins from Mouse Liver

Briefly, fresh liver (about 1 g) was rinsed in ice-cold Tris-buffered saline and homogenized in homogenizing buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 1 mM EDTA, 2 M sucrose, 10% glycerc, 0.5 mM spermidine, 0.15 mM spermin, 0.5 mM DTT, 0.5 mM PMSF, and 0.5 µg/ml leupeptin). The homogenate was poured into a centrifuge tube containing equal volume of homogenizing buffer and centrifuged at 100,000g for 5 min at 44°C. The supernatant was discarded, and the pellet was washed by dissolving in 50 mM Tris, pH 8.0, 40% glycerol, 5 mM EDTA, and 5 mM MgCl₂ and centrifuged at 10,000g for 5 min at 4°C. The supernatant was discarded, and the intact nuclei were resuspended in 50 mM Tris, pH 8.0, 40% glycerol, 5 mM EDTA, and 5 mM MgCl₂, aliquoted; and stored at −80°C. Nuclear proteins were harvested from the intact nuclei by pelleting the suspended nuclei (centrifugation at 10,000g for 5 min at 4°C). The pellet was resuspended in buffer A (10 mM HEPES, pH 7.6, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerc, 0.5 mM DTT, and 0.5 mM PMSF) and stirred with magnetic stirrer for 30 min at 4°C. The suspension was homogenized by pestling (10 strokes × 2) and centrifuged at 15,000g for 5 min at 4°C. The nuclear proteins were obtained by dialyzing the nuclei (supernatant) against 100 volumes of buffer B (20 mM HEPES, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 20% glycerc, 0.5 mM DTT, and 0.5 mM PMSF) overnight at 4°C. This was done by pipetting the nuclei onto a nitrocellulose membrane (Millipore Corporation, Billerica, MA) floating over 100 volumes of buffer B. The dialyzed sample was centrifuged at 15,000g for 5 min at 4°C, and the supernatant was aliquoted and stored at −80°C.

#### RNA Extraction and mRNA Analysis

Total cellular RNA was extracted from primary mouse hepatocytes using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Messenger RNA levels were determined by Northern blotting. Total RNA (10 µg) was size-fractionated on a 1.2% agarose/formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences, Buckinghamshire, UK). The CYP2A5 cDNA and HO-1 cDNA were radiolabeled with [α-³²P]dCTP using the Megaprime labeling kit (Amersham Biosciences). Successive hybridizations were carried out on the same filter using the cDNA probes (1.7 × 10⁷ cpm of radiolabeled probe) at 65°C.
overnight in Church buffer (Church and Gilbert, 1984) (modified to contain 0.25 M phosphate buffer, 7% SDS, and 1 mM EDTA). The filter was washed twice for 5 min at room temperature in a buffer containing 2× standard saline citrate and 0.1% SDS and then once for 15 min at 65°C in a buffer containing 2× standard saline citrate and 1% SDS. To assess equal loading of the samples, the mRNA level of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was measured using the GAPDH cDNA (CLONTECH, Palo Alto, CA) as a probe. For the densitometric analysis, scanning of the film was performed with a Scanjet 3500c scanner (Hewlett Packard, Palo Alto, CA), and quantification was conducted using the software NIH Image 1.61 (http://rsb.info.nih.gov/nih-image/).

**Protein Analysis.** Protein levels were determined by Western blotting. Proteins (20 μg of cytosolic protein for detection of CYP2A5 and HO-1 proteins; 15 μg of cytosolic or nuclear protein for detection of Nrf2 protein) were separated by SDS-polyacrylamide gel electrophoresis (12%), electrophoretically transferred to nitrocellulose/polyvinylidene difluoride membranes (Pierce Biotechnology, Rockford, IL/Bio-Rad Laboratories, Hercules, CA), and blocked for 1 h in phosphate-buffered saline containing TWEEN 20 (0.1%) and nonfat milk (5%). Blots were incubated with the HO-1:1 (500 dilution) or Nrf2:1 (500 dilution) antibody for 3 h. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit (1:5000 dilution) or goat anti-mouse (1:5000 dilution) antibody. After further washing with phosphate-buffered saline, blots were incubated in commercial chemiluminescence reagents (Amersham Biosciences). The catalytic activity of CYP2A5 was determined by measuring covalent addition to primary hepatocytes and 100 μM covalent as substrate.

**Plasmids and Transient Transfection Assays.** The Cyp2a5 5′-3033/+10 fragment (Ulvia et al., 2004) and the 5′-truncated fragments of the Cyp2a5 promoter cloned in front of the luciferase cDNA in the pGL3-Basic vector (Promega, Madison, WI) were used in transient transfection assays. Promoter constructs were cotransfected either with the empty expression vector or with the mouse Nrf2 expression plasmid (pcDNA3-\textit{Nrf2}). Promoter constructs were cotransfected either with the empty expression vector or with the mouse Nrf2 expression plasmid (pcDNA3-\textit{Nrf2}). The catalytic activity of CYP2A5 was determined by measuring covalent addition to primary hepatocytes and 100 μM covalent as substrate.

**Site-Directed Mutagenesis.** The potential StRE sites at 2386 to 2377 bp, respectively, were analyzed by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) as instructed by the manufacturer. The former element was analyzed by using an existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the existing mutagenesis construct (Arpiainen et al., 2005).
Results

Cadmium Stimulates Induction of CYP2A5. Treatment of primary hepatocyte cultures with 4 μM CdCl₂ stimulated CYP2A5 mRNA and protein expression, as well as CYP2A5-mediated COH activity in a time-dependent manner (Figs. 1 and 2). The induction pattern correlates with that of the HO-1. An observable increase in HO-1 mRNA is typically between 1 and 2 h after exposure to CdCl₂ (Alam et al., 1989; Stewart et al., 2003). These observations are consistent with our previous in vivo findings in the mouse liver (Abu-Bakar et al., 2005).

Effect of Cadmium on Cellular Distribution of Nrf2. Cellular localization of Nrf2 protein was determined by Western blotting. Treatment of primary hepatocytes with 4 μM CdCl₂ caused a time-dependent increase of Nrf2 in the nucleus (Fig. 3). In our gel system, mouse Nrf2 migrates as a 75-kDa protein, which is larger than the predicted size of 68 kDa (Moi et al., 1994). The anomalous migration on SDS-polyacrylamide gel electrophoresis has been suggested to be caused by the abundance of acidic residues found in Nrf2 (Moi et al., 1994). Accumulation of Nrf2 (3-fold increase compared with control) in the nucleus was detected within 1 h after cadmium treatment, well before the observable increase in CYP2A5 and HO-1 mRNA. This is consistent with the observations of Stewart et al. (2003) that cadmium delays the ubiquitinated degradation of Nrf2, which in turn leads to hmx-1 gene activation in the mouse hepatoma cells.

Nrf2 Is Involved in the Transcriptional Regulation of the Cyp2a5 Gene. Involvement of Nrf2 in transcriptional regulation of the Cyp2a5 gene was studied by transient transfections. A series of 5’-truncated Cyp2a5 promoter-luciferase reporter plasmids were constructed and transfected into mouse primary hepatocytes. The cells were cotransfected with Nrf2 expression plasmid or empty control plasmid. Nrf2 response was detected with the constructs equal to or longer than 2656 bp but not with any of the shorter constructs. This suggests that Nrf2 responsive element(s) are present in the sequence region from 2656 to 2338.

Identification and Characterization of a Putative Nrf2 Binding Element. Cadmium-induced hmx-1 transcriptional activation is
mediated by Nrf2 binding to the StRE, which is present in multiple copies in the mouse hmox-1 promoter (Sikorski et al., 2004). We next searched for putative Nrf2 binding sites in the 5'-flanking region of the Cyp2a5 gene. A transcription factor binding site search was performed using the TFSEARCH program. The search exposed two regions of high sequence similarity with the consensus StRE [5'- (T/C)GCTGAGTCA-3']. The two potential StRE identified are at positions −2514 to −2505 and −2386 to −2377 of the Cyp2a5 5'-flanking region (Fig. 5A). The location of these binding sites is within the Nrf2 responsive region identified in the transfection experiments.

The difference between these putative and the consensus StRE sequence is only 2 bp (Fig. 5B).

Next, binding activity of Nrf2 to the two putative sites was tested. Electrophoretic mobility shift assays (EMSA) were performed with liver nuclear extracts from untreated and cadmium-treated mice, using the two potential Nrf2-binding sites as probes. Probe 1 encompassed sequences from −2597 to −2419 (distal putative StRE), and probe 2 encompassed sequences from −2467 to −2269 (proximal putative StRE). Figure 5C shows that a distinct, cadmium-dependent DNA-protein complex (open arrow) formed with both probes (lanes 3 and 11). The complex with the probe 1 was not competed by 100-fold excess of unlabeled consensus StRE oligo, nor was the complex supershifted with anti-Nrf2 antibody. This result suggests that the complex does not contain Nrf2. In contrast, the complex with the probe 2 was inhibited, although not totally abolished by competition with the unlabeled consensus StRE oligo. Moreover, anti-Nrf2 antibody was able to supershift this complex, which confirmed that the complex involves Nrf2 (closed arrow, lane 12).

The StRE binding sequences typically overlap with activator protein-1 binding motif. In our EMSA assay, anti-Fos antibody could induce supershifted complex, and anti-Jun antibody inhibited complex formation (Fig. 5C). Therefore, c-Fos/c-Jun dimer may compete with Nrf2 complex for binding. Alternatively, c-Fos and c-Jun may be potential heterodimerization partners for Nrf2 in the regulation of the Cyp2a5 gene.

Binding of Nrf2 to the Cyp2a5 promoter StRE was confirmed by ChIP experiment. Primary hepatocytes were treated with 4 μM CdCl₂ for 30 min or left untreated, and the fixed DNA-protein complexes were immunoprecipitated with anti-Nrf2 antibody. Extracted DNA fragments were amplified in real-time PCR with a primer pair spanning the proximal Cyp2a5 StRE (Fig. 5A). Anti-Nrf2 antibody precipitated Nrf2 bound to Cyp2a5 promoter only from cadmium-treated cells (Fig. 5D). This indicates that Nrf2 binds to Cyp2a5 promoter at proximal StRE in vivo, in true chromatin structure, and this binding is cadmium-dependent.

Proximal StRE Mediates Nrf2 Response of the Cyp2a5 Gene. To establish the functional significance of the identified proximal StRE element at −2467 to −2269, the core element critical for Nrf2 binding was mutated in the Cyp2a5−3033/+10-Luc construct. The distal StRE, which does not bind the Nrf2, was also mutated and used as a control. The mutant constructs were then cotransfected with the Nrf2 cotransfection on Cyp2a5-5’-luciferase construct activities in DBA/2 mouse primary hepatocytes. Luciferase activities were measured 48 h after transfection. The measured activities were normalized against cotransfected Renilla control plasmid (pRL-TK) activities. The values (n = 4) represent means ± S.D. Nrf2 response of each reporter construct is indicated by -fold of activity to control cotransfection with pcDNA3. The experiment was repeated three times, and similar results were obtained. Mean difference is significant from control group at *** p < 0.001; ** p < 0.01 (Student’s t test).
expression plasmid into mouse primary hepatocytes. Mutation of the proximal StRE (Cyp2a5-5/H11032-3033-2379mut) abolished the Nrf2 response in reporter gene assays, whereas mutation of the distal StRE (Cyp2a5-5/H11032-3033-2505mut) had no effect (Fig. 6). These results indicate that the identified proximal StRE is functional and mediates the activation of Cyp2a5 transcription by Nrf2.

Discussion

In a previous study using Nrf2-null and Nrf2-wild-type mice, we observed that treatment with 16 μmol CdCl2/kg b.wt. for 4 h does not induce hepatic CYP2A5 mRNA in the null mice. In contrast, a 3-fold induction was observed in the wild-type mice, which indicates that cadmium induction of CYP2A5 is Nrf2-dependent (Abu-Bakar et al., 2004). In the present studies, it is evident that Cyp2a5, a phase I

Fig. 5. Putative StRE at the Cyp2a5 promoter and effects of cadmium on DNA-protein binding on the promoter. A, a schematic representation of the -2613 to -2244 region in the Cyp2a5 promoter. The opened arrows indicate orientation of the two putative StRE. The closed arrows indicate orientation of primers for real-time PCR (used in ChIP assay) and EMSA probes. The boxed sequence represents the potential XRE element at position -2513 to -2490 that we found in our previous work (Arpiainen et al., 2005). B, the difference between putative and the consensus StRE sequence. The boxed region represents the region that contains the different base pairs, which are shown in bold. C, EMSA analysis of StRE-binding proteins/Cyp2a5 promoter interactions. Nuclear extracts from liver of untreated or cadmium-treated mice were incubated with radiolabeled probe 1 (lanes 2–8) or probe 2 (lanes 10–16) in an EMSA assay. Lanes 1 and 9 represent reaction mixtures without nuclear protein. When indicated, the reaction mixtures were incubated with anti-Nrf2 antibody (lanes 4 and 12), anti-Fos antibody (lanes 5 and 13), or anti-Jun antibody (lanes 6 and 14). The reactions with nuclear extracts were competed with 100-fold excess of unlabeled probe 1 or 2 (S) (lanes 7 and 15, respectively) and unlabeled consensus StRE (C) oligonucleotides (lanes 8 and 16). The opened arrow indicates DNA-protein complex, and the closed arrow indicates supershift complex. The experiment was repeated three times, and similar results were obtained. D, ChIP with Nrf2 antibody from hepatocytes. Murine primary hepatocytes were treated with 4 μmol CdCl2 or left untreated for 30 min. Anti-Nrf2 antibody (Ab) was used to precipitate fixed DNA-protein complexes from the cells. Rabbit IgG was used as a negative control. Extracted DNA fragments were amplified with specific primers (indicated in Fig. 5A) in real-time PCR, and the relative amounts of DNA copies were counted by comparing the sample fluorescence with the fluorescence values measured from total chromatin input dilution series. Fluorescence values were corrected with fluorescence signals of the passive reference dye (ROX).
detoxification enzyme encoding gene, is under the direct control of Nrf2 in response to cadmium-induced cellular stress. This is supported by the following observations: 1) the temporal pattern of cadmium-induced translocation of Nrf2 from the cytosol to the nucleus was consistent with concurrent expression of CYP2A5 and well-established Nrf2 target gene HO-1; 2) the transfection experiments with Cyp2a5 promoter constructs indicated that Nrf2 induces Cyp2a5 transcription and confined the Nrf2 responsive region to the 5' flanking sequence from −2656 to −2338; 3) computer-based searches revealed a putative StRE element at −2386 to −2377; 4) ChIP and EMSA showed that Nrf2 binds to the StRE after cadmium treatment; and 5) site-directed mutation of the StRE element totally abolished Nrf2 activation of Cyp2a5 promoter. Collectively, the results of the present study together with the results in Nrf2−/− null mice establish Cyp2a5 as the first identified P450 gene regulated by Nrf2.

The cellular detoxifying system uses phase I and II xenobiotic-metabolizing enzymes. The phase I metabolism by P450 enzymes may produce reactive oxygen species that activate Nrf2 nuclear translocation and transcription of antioxidant genes. For example, induction of CYP2E1 by ethanol is one of the central pathways by which ethanol generates a state of oxidative stress in hepatocytes. This in turn activates Nrf2-dependent HO-1 expression, which in turn protects cells against oxidative injury (Cederbaum, 2006; Gong and Cederbaum, 2006). Oxidative metabolism by P450 enzymes may also produce electrophile intermediaries that activate Nrf2-dependent transcription of phase II genes. Thus, P450s are not considered as Nrf2 target genes. However, the evidence presented in this report indicates a unique role for the CYP2A5 among the P450 enzymes.

The nature of that specific role is not apparent in this study. However, earlier studies showed that genes that are regulated by the Nrf2-StRE pathway encode proteins that help control the cellular redox status and defend the cell against oxidative damage. For example, Ho-1 catalyzes the degradation of haem to the potent antioxidants biliverdin and bilirubin (BR) (Choi and Alam, 1996). In 1990, Stocker (1990) proposed that induction of HO-1 represents an antioxidant defense operating at two different stages simultaneously by 1) decreasing the levels of the potential pro-oxidants haem and haemoproteins such as the cytochromes; and 2) increasing the cellular concentrations of potent antioxidants, biliverdin and BR. However, excess concentrations of BR are toxic and need to be dynamically controlled. BR is normally conjugated with glucuronic acid and excreted in the bile. When glucuronidation is impaired, oxidative BR metabolism offers an alternative degradation pathway (Schmid and Hammaker, 1963). We have previously shown that in vivo treatment of CdCl2 to DBA/2 mice caused coordinated induction of the HO-1 and CYP2A5 at mRNA, protein, and enzyme activity levels, whereas the total P450 content was reduced significantly (Abu-Bakar et al., 2004).

Enzyme kinetic analysis established that CYP2A5 plays an important role in microsomal BR oxidation, when BR levels were elevated following induction of HO-1 by cadmium (Abu-Bakar et al., 2005). Thus, we hypothesize that under the condition of oxidative stress exerted by cadmium, activation of Nrf2-dependent HO-1 leads to drastic elevation of BR concentration, which may overwhelm BR oxidant scavenging activity. This in turn creates a need for BR to be enzymatically metabolized. Transcriptional activation of Cyp2a5 gene through Nrf2 may thus function as part of the protective network that maintains dynamic control of BR levels and redox homeostasis.

Given that CYP2A5 is also induced by bifunctional agents, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Aarpiainen et al., 2005), it is plausible that this enzyme plays a role in the dynamic balance between electrophile-producing phase I and inactivating phase II enzyme systems to protect cellular homeostasis. A central example of this coordination is through interaction of the AHR-XRE and the Nrf2-StRE pathways. The AHR ligands are able to activate genes by interaction with XRE and therefore induce P450 genes especially in family 1. In addition, AHR is able to activate some phase II genes through XRE sequence motifs in their promoters, as well as indirectly through StRE sites via Nrf2. This process may involve several mechanisms. The phase I metabolism by P450 enzymes may produce electrophilic intermediaries and reactive oxygen species that activate Nrf2 nuclear translocation and the transcription of phase II genes. Furthermore, a recent study showed that AHR is able to activate Nrf2 gene transcription by binding to XRE of the Nrf2 promoter (Miao et al., 2005). Our previous work reported that Cyp2a5 is a direct target of AHR (Aarpiainen et al., 2005). Thus, it represents a gene controlled both by AHR and Nrf2. The two binding sites are located in relatively close proximity (less than 150 bp) of the Cyp2a5 promoter (Fig. 5A). This suggests that these binding sites may form a functional regulatory unit. It seems probable that AHR ligands may activate Cyp2a5 transcription directly through XRE and indirectly through StRE to enhance expression of CYP2A5 under conditions of oxidative stress.

AHR may play an additional role in CYP2A5-controlled BR homeostasis under oxidative stress conditions. BR has been reported to induce activation of the AHR-signaling system in hepatoma cells of mouse, rat, and human origin (Phelan et al., 1998). It is plausible the excess BR produced by HO-1 may cause substrate-mediated transcriptional regulation of the Cyp2a5 gene, where BR may serve as an endogenous ligand to the AHR, which then triggers the AHR-dependent XRE-mediated transcriptional activation of the Cyp2a5 gene. Furthermore, AHR may amplify Nrf2-mediated Cyp2a5 induction by increasing Nrf2 expression level.

In conclusion, the present findings show that the Cyp2a5 gene is under the direct control of Nrf2 through StRE site in the 5'-flanking region. Therefore, Cyp2a5 represents the first example of a P450 gene regulated by oxidative stress through Nrf2. Thus, we propose that the CYP2A5 is involved in the cellular network that maintains redox homeostasis to protect cells from oxidative stress.

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References


