REGULATION OF CYTOCHROME P450 2A5 GENE BY THE TRANSCRIPTION FACTOR NRF2 (NUCLEAR FACTOR (ERYTHROID-DERIVED 2)-LIKE 2

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Abbreviations: CYP2A5, mouse cytochrome P450 2A5; CYP2A6, human cytochrome P450 2A6; CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; ANOVA, analysis of variance; XRE, xenobiotic response element; hnRNP A1; heterogenous nuclear ribonucleoprotein A1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; bZIP, basic-leucine zipper; NQO1, NAD(P)H:quinone oxidoreductase; γ-GCS, γ-glutamylcysteine synthase; GST, glutathione S-transferase; HO-1, haem oxygense-1; MARE, Maf recognition element; ARE, antioxidant response element; StRE, stress response element; CdCl₂, cadmium chloride; ROS, reactive oxygen species; GSH, glutathione; GSSG, oxidized glutathione; MAPK, mitogen-activated protein kinase; BV, biliverdin; BR, bilirubin; hmox-1, haem oxygenase-1 gene.
ABSTRACT

We have previously shown that cadmium, a metal that alters cellular redox status, induces cytochrome P450 2A5 (CYP2A5) expression in nuclear factor (erythroid-derived 2)-like 2 wildtype (Nrf2 +/+ ) mice but not in the knockout (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. Co-transfection 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 localised the Nrf2 responsive region to an area from -2656 to –2339 bp. Computer-based sequence analysis identified two putative stress response elements (StREs) 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 the first phase I xenobiotic-metabolizing gene identified under the control of the StRE-Nrf2 pathway with a potential role in adaptive response to cellular stress.
INTRODUCTION

The mouse cytochrome P450 2A5 (CYP2A5) and its human orthologue, 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-hydroxylation in mouse liver (Lang et al., 1989). Its regulation is complex and unique among other major cytochrome P450s (CYPs). It can be induced by structurally unrelated compounds and also by several chemicals and pathophysiological conditions that usually repress other CYP isoforms. 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 (Su and Ding, 2004; Seubert et al., 2002; Abu-Bakar et al., 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 TCDD is mediated by the binding of a ligand-activated AHR/ARNT complex to the XRE (xenobiotic response element) site at the Cyp2a5 distal promoter (Arpiainen et al., 2005). Pyrazole, a hepatotoxin, induces CYP2A5 by a post-transcriptional mechanism involving binding of heterogenous nuclear ribonucleoprotein A1 (hnRNP 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: (a) over-expression of CYP2A5 by pyrazole is related to alterations in cellular redox equilibrium (Gilmore and Kirby, 2004); (b) pyrazole treatment in mice increased Nrf2 protein in the liver (Gong and Cederbaum, 2006); and (c) 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 (bZIP) 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 (NQO1), γ-glutamylcysteine synthase (γ-GCS), and glutathione S-transferase (GST) (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, cytoplasm-localised state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1 (Itoh et al., 1999). Upon 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 characterised mechanism(s). However, cadmium alters cellular redox by reducing intracellular ratio of glutathione (GSH) to oxidised glutathione (GSSG) (Ryter and Choi, 2002) and activates dissociation of the Nrf2-Keap1 complex through p38 MAPK-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 bZIP 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 MAREs (Maf recognition elements) (Kataoka et al., 1994), AREs (antioxidant response elements) (Rushmore et al., 1991) or StREs (stress response elements) (Choi 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 StRE at about 2.4 kb upstream of the transcription start site of the Cyp2a5 promoter, which in turn upregulates 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, ITS (insulin, transferrin, sodium selenite) media supplement, gentamicin, L-glutamine, Hepes, phenylmethylsulfonyl-fluoride, Igepal, Tween 20, spermidine, spermin, and leupeptine were from Sigma-Aldrich. Rabbit IgG (sc-2027), rabbit polyclonal anti-Nrf2 (sc-13032) and anti-Jun (sc-44X) antibodies, and mouse monoclonal anti-Fos (sc-8047X) antibody were from Santa Cruz Biotechnology Inc. (CA, USA). A monoclonal anti-HO-1 antibody and HO-1 (Hsp32) protein were from Stressgen Biotechnologies Inc. (Victoria, Canada).

Isolation and Treatment of Primary Cultures Hepatocytes. Hepatocytes were isolated from male DBA/2 mice (Möllegaard, Copenhagen, Denmark) aged 8-10 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, ITS (5 mg/l insulin, 5 mg/l transferrin, 5 µg/l sodium selenite), 10 µg/ml
gentamicin, 1% L-glutamine, and 10% decomplemented fetal calf serum at a density of 1.8 x 10^6
cells/60-mm uncoated culture dish (Corning, Palo Alto, CA), and 3 x 10^5 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 of CdCl₂ solution or transient
transfection. Cadmium chloride was dissolved in normal saline solution.

**Animals.** Six 8-10 weeks old DBA/2 male mice (Animal Resources Centre, Western
Australia) were divided into 2 groups of three mice in each group. They were housed in filter-top
polycarbonate cages containing wood chip bedding and maintained in a 12-hour light/dark cycle
with free access to standard mouse chow and tap water. They were treated with a single
intraperitoneal injection of 16 µmol CdCl₂/kg body weight 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 2000 g 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 phenylmethylsulfonyl-fluoride,
10 µg/ml leupeptine, and 0.4% Igepal) and kept on ice for 1 h. The cell suspension was vortexed,
homogenised, and centrifuged at 12,000 g 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 mM NaCl, 0.2 mM
EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl-fluoride, and 0.4% Igepal) and
gently agitated for 30 min at 4°C. The suspension was centrifuged at 15,000 g 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 TBS, and homogenised in homogenizing buffer (10 mM Hepes (pH 7.6), 15 mM
KCl, 1 mM EDTA, 2M sucrose, 10% glycerol, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM
DTT, 0.5 mM PMSF, 10 µg/ml leupeptin). The homogenate was poured into a centrifuge tube
containing equal volume of homogenizing buffer and centrifuged at 100,000 g for 50 min, at
4°C. The supernatant was discarded and the pellet was washed by dissolving in TGEM [50 mM
Tris (pH 8.0), 40% glycerol, 5 mM EDTA, 5 mM MgCl₂] and centrifuged at 10,000 g for 5 min
at 4°C. The supernatant was discarded and the intact nuclei were resuspended in TGEM,
aliquoted and stored at –80°C. Nuclear proteins were extracted from the intact nuclei by pelleting
the suspended nuclei (centrifugation at 10,000 g 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% glycerol,
0.5 mM DTT, 0.5 mM PMSF] and stirred with magnetic stirrer for 30 min at 4°C. The
suspension was homogenised by pestling (10 strokes x 2) and centrifuged at 15,000 g 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% glycerol, 0.5
mM DTT, 0.5 mM PMSF] overnight at 4°C. This was done by pipetting the nuclei onto a
nitrocellulose membrane (Millipore) floating over 100 volumes of buffer B. The dialyzed sample was centrifuged at 15,000 g 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, U.K.). The CYP2A5 cDNA and HO-1 cDNA were radiolabelled 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 x 10^7 cpm of radiolabelled 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 2 x 5 min at room temperature in a buffer containing 2 x SSC and 0.1% SDS and then 1 x 15 min at 65°C in a buffer containing 2 x SSC and 1% SDS. To assess equal loading of the samples, the mRNA level of the house keeping 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 cytosolic protein for detection of CYP2A5 and HO-1 proteins; 15 µg cytosolic or nuclear protein for detection of Nrf2 protein) were separated by SDS-PAGE (12%), electrophoretically transferred to nitrocellulose/PVDF membranes (Pierce Biotechnology, USA/Bio-Rad)
Laboratories, USA), and blocked for 1 h in phosphate-buffered saline containing Tween 20 (0.1%) and non-fat milk (5%). Blots were incubated with the HO-1 (1:500 dilution), CYP2A5 (1:1000 dilution), or Nrf2 (1:500 dilution) antibody for 1 h. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000 dilution) or goat anti-mouse (1:5,000 dilution) antibody. After further washing with phosphate-buffered saline, blots were incubated in commercial chemoluminescence reagents (Amersham Biosciences). The catalytic activity of CYP2A5 was determined by measuring coumarin 7-hydroxylase (COH) as described previously (Aitio, 1978) using 50 µg of cytoplasmic extracts from primary hepatocytes and 100 µM of coumarin as substrate.

**Plasmids and Transient Transfection Assays.** The $Cyp2a5$ 5'-3033 - +10 fragment (Ulvila 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, USA), were used in transient transfection assays. Promoter constructs were co-transfected either with the empty expression plasmid pcDNA3 or with the mouse Nrf2 expression plasmid (pcDNA3-mNrf2). To prepare a positive control for the luciferase activity assays, a functional StRE site (5′-GATCTTTTATGCTGAGTTGGTTT-3′, core sequence underlined) from the $hmox-1$ promoter region was cloned between the cloning sites KpnI and XhoI in the pGL3-TK plasmid, which contained the thymidine kinase promoter from pRL3-TK plasmid (Promega, Madison, WI, USA) subcloned between the cloning sites BgllII and HindIII. Mouse primary hepatocytes were transiently transfected after 24 h of culturing, using Tfx-20 reagent (Promega) according to the manufacturer’s protocol, in Opti-MEM I medium (Invitrogen). Cells in each well were transfected with 0.5 µg of the $Cyp2a5$ 5’ reporter construct, 0.1 µg of pcDNA3 or pcDNA3-
mNrf2, and 0.2 µg of pRL3-TK. The transfected cells were cultured 48 h before measuring the luciferase activities by the Dual–Luciferase Reporter Assay System (Promega).

**Electrophoretic mobility shift assay.** Double-stranded DNA corresponding to either the −2597 to −2419 region (probe 1) or the −2467 to −2269 region (probe 2) of the 5′-flanking Cyp2a5 promoter were generated by PCR. Primers 1 and 2 were used to amplify probe 1, while primers 3 and 4 were used to amplify probe 2. The sequences of the primers are shown in Table 1. The single-stranded oligonucleotides were obtained from Sigma-Genosys (Sydney, Australia). The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Vic, Australia) following the manufacturer’s protocol. The PCR-derived oligonucleotides (probes 1 and 2) were 5′-end labeled with [γ-32P]ATP (3000 Ci/mm) using a 5′ end labeling kit (Promega) and purified using the QIAquick nucleotide removal kit (QIAGEN). Ten µl of binding buffer [4% glycerol, 5 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC) (Amersham Biosciences)] and 10 µg of liver nuclear extract (NE) from control or Cd-treated mice were incubated at room temperature for 10 minutes. After incubation, 1 x 10⁵ cpm of labeled DNA was added and the mixtures were incubated for an additional 20 min at room temperature. In antibody supershift assays, 1 µl of anti-Nrf2, anti-Fos, or anti-Jun, antibody was added to the reaction mixture and incubated on ice for 60 min. For electrophoresis separation of the DNA-protein complexes, the samples were loaded onto a pre-electrophoresed, non-denaturing 4% polyacrylamide (60:1 acrylamide: bisacrylamide) gel in 0.5 X TBE (44 mM Tris-HCl, pH 8/44 mM boric acid/1mM EDTA). The samples were electrophoresed at 10 mA for 270 min, after which the gel was dried and autoradiographed.
Competition binding assay was conducted to assess the specificity of protein-DNA interactions. In this assay, 100-fold molar excess of unlabeled oligonucleotides was added to the incubation mixture. The double stranded consensus NF-E2 oligonucleotide 5’-TGGGAACCTGTGAGCTGGAG-3’ (core StRE sequence is underlined) was used as positive control for Nrf2 specific binding.

**Chromatin immunoprecipitation (ChIP) Assay.** Protein-DNA complexes were immunoprecipitated from murine primary hepatocytes (cultured in 175 cm² flasks). Two hours after liver perfusion, cells were either treated with 4 µM CdCl₂ or maintained in serum-free culture medium for 30 minutes. Chromatin immunoprecipitations were done according to Väisänen et al. (Väisänen et al., 2004). Briefly, transcription factors were cross-linked to DNA by treatment of the cells with 1% formaldehyde for 10 minutes at 37°C. The cells were then washed and pelleted, and were resuspended in 2 ml of SDS Lysis Buffer. Lysates were sonicated to shear the chromatin to DNA length of between 200 and 1000 bp. The lysate was pre-cleared with 50% salmon sperm DNA–Protein A agarose slurry (Upstate, Charlottesville, VA, USA) and fractionated into 200 µl aliquots. Fifty µl was reserved as the total chromatin input sample. Two hundred µl aliquots were diluted 10-fold in ChIP Dilution Buffer and incubated with 1 µg of anti-Nrf2 antibody or rabbit IgG (negative control) overnight at 4°C. Immunocomplexes were collected with 100 µl of 50% Protein A agarose slurry and eluted with 500 µl of elution buffer. Protein-DNA crosslinks were reversed by overnight incubation at 65°C and remaining proteins were digested with Proteinase K. After washing and elution, DNA was purified by phenol/chloroform/isoamylalcohol extraction and resuspended in 60 µl of H₂O. Confirmation of Nrf2 binding site was performed by quantitative real-time PCR (AmpliQ Universal Real Time PCR Master Mix Kit, Ampliqon, Copenhagen, Denmark). Five µl of DNA solution was used as
template in 20 µl PCR reactions containing 10 µl of the 2X master mix, 2 µl of Green DNA Dye (1:2000), and 200nM of each PCR primer (see Table 1). Samples were incubated at 95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s in a Mx3000P QPCR system (Stratagene). The specificity of the PCR-products was confirmed by melting curve analysis and size (agarose gel electrophoresis).

**Site-directed Mutagenesis.** The potential StRE sites at –2514 to –2505 and –2386 to –2377 bp respectively, were analysed by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) as instructed by the manufacturer. The former element was analysed by using an existing mutagenesis construct (Arpiainen et al., 2005). Mutagenesis of the latter element was performed by using the Cyp2a5 5′–3033 - +10 –luciferase reporter plasmid as template and by priming with mutated oligonucleotides –2401 CGTGACTTCAGTTTCTGCTCT

TCT

A

TCCATGCGTCTGAAAAGAAG –2357 (bold; mutated bases, underlined; core sequence). Presence of the mutated bases was confirmed by sequencing.

**Statistical analysis.** Student’s t test was used for comparisons between two groups. Comparisons of several groups were done with one-way ANOVA followed by the least significant difference post hoc test. Differences were considered significant when p < 0.05.

**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 coumarin 7-hydroxylase activity in a time-dependent manner (Figures 1 & 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 (Figure 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/PAGE has been suggested to be due to 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. (Stewart et al., 2003) that Cd delays the ubiquitinated degradation of Nrf2, which in turn leads to *hmox-1* gene activation in the mouse hepatoma (Hepa) cells.

**Nrf2 is Involved in the Transcriptional Regulation of the Cyp2a5 Gene.** Involvement of Nrf2 in transcriptional regulation of 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 co-transfected with Nrf2 expression plasmid or empty control plasmid. Nrf2 co-transfection induced the longest Cyp2a5-3033 to +10-Luc construct 3.5 fold relative to control (Figure 4). 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 Characterisation of a Putative Nrf2 Binding Element.** Cd-induced *hmox-1* transcriptional activation is mediated by Nrf2 binding to the StRE, that 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 StREs identified are at positions –2514 to –2505 and –2386 to –2377 of the Cyp2a5 5’-flanking region (Figure 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 two base pairs (Figure 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 Cd-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) was formed with both probes (lane 3 and 11). The complex with the Probe 1 was not competed by 100-fold excess of unlabelled 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 unlabelled 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 AP-1 binding motif. In our EMSA assay anti-Fos antibody could induce supershifted complex and anti-Jun antibody inhibited complex formation (Figure 5C). Therefore c-Fos/c-Jun dimer may compete with Nrf2 complex for
binding. Alternatively, c-Fos and c-Jun may be potential heterodimerisation partners for Nrf2 in the regulation of Cyp2a5 gene.

Binding of Nrf2 to the Cyp2a5 promoter StRE was confirmed by chromatin immunoprecipitation 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 (Figure 5A). Anti-Nrf2 antibody precipitated Nrf2 bound to Cyp2a5 promoter only from cadmium treated cells (Figure 5D). This indicates that Nrf2 binds to Cyp2a5 promoter at proximal StRE in vivo, in true chromatin structure and this binding is cadmium dependent.

The 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 to +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 co-transfected with the Nrf2 expression plasmid into mouse primary hepatocytes. Mutation of the proximal StRE (Cyp2a5-5′-3033-2379mut) abolished the Nrf2 response in reporter gene assays while mutation of the distal StRE (Cyp2a5-5′-3033-2505mut) had no effect (Figure 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 body weight for 4 h does not induce hepatic CYP2A5 mRNA
in the null mice. In contrast, a 3-fold induction was observed in the wildtype 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 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: (a) 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; (b) 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; (c) computer based searches revealed a putative StRE element at -2386 to -2377; (d) chromatin immunoprecipitation and electrophoretic mobility shift assays showed that Nrf2 binds to the StRE after cadmium treatment; and (e) 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 cytochrome P450 gene regulated by Nrf2.

The cellular detoxifying system uses phase I and II xenobiotic metabolizing enzymes. The phase I metabolism by cytochrome P450 enzymes may produce reactive oxygen species (ROS) 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 cytochrome P450 enzymes may also produce electrophilic intermediary metabolites that activate Nrf2-dependent transcription of phase II genes.
Cytochrome P450s are thus not considered as Nrf2 target genes. However, the evidence presented in this report indicates a unique role for the CYP2A5 among the CYP 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 catalyses the degradation of haem to the potent antioxidants biliverdin (BV) and bilirubin (BR) (Choi and Alam, 1996). In 1990, Roland Stocker (Stocker, 1990) proposed that induction of HO-1 represents an antioxidant defence operating at two different stages simultaneously by, (i) decreasing the levels of the potential pro-oxidants haem and haemo-proteins such as the cytochromes; and, (ii) increasing the cellular concentrations of potent antioxidants, BV and BR. However, excess concentrations of BR are toxic and need to be dynamically controlled. Bilirubin is normally conjugated with glucuronic acid and excreted in the bile. When glucuronidation is impaired, oxidative bilirubin 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, while the total CYP content was reduced significantly (Abu-Bakar et al., 2005). 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). We thus hypothesize that under the condition of oxidative stress exerted by cadmium, activation of Nrf2-dependent HO-1 leads to drastic elevation of bilirubin 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 bi-functional agents, such as TCDD (Arpiainen 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 enzymes systems to protect cellular homeostasis. A central example of this coordination is through interaction of the AHR-XRE and the Nrf2-StRE pathways. The aryl hydrocarbon receptor ligands are able to activate genes by interaction with XRE, and therefore induce CYP 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 cytochrome P450 enzymes may produce electrophilic intermediary metabolites and ROS 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 XREs of the Nrf2 promoter (Miao et al., 2005). Our previous work reported that Cyp2a5 is a direct target of AHR (Arpiainen et al., 2005). It thus 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 (Figure 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.

Aryl hydrocarbon receptor may play an additional role in CYP2A5 controlled BR homeostasis under oxidative stress conditions. Bilirubin has been reported to induce activation of the AHR-signalling 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. Cyp2a5 therefore represents the first example of a cytochrome P450 gene regulated by oxidative stress through Nrf2. We thus propose that the CYP2A5 is involved in cellular network that maintains redox homeostasis to protect cells from oxidative stress.

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Gong P and Cederbaum AI (2006) Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protect against oxidative stress caused by CYP2E1. *Hepatology* 43:144-152.


FOOTNOTES

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1 These authors contributed equally to this work
**FIGURE LEGENDS**

**Fig. 1.** Time-course effect of CdCl₂ on CYP2A5 and HO-1 mRNA expression in DBA/2 mouse hepatocytes. The hepatocytes were treated with 4 µM CdCl₂ or vehicle (normal saline) for 0, 1, 3, 6, or 12 hours. (A) 10 µg of total RNA were electrophoresed, blotted, and hybridised with mouse CYP2A5, HO-1, or GAPDH probes. GAPDH mRNA levels are shown as control for RNA loading. In vivo pyrazole-induced and Cd-induced mouse liver was used as positive control for CYP2A5 and HO-1 mRNA, respectively. (B) Densitometric quantification of CYP2A5 and HO-1 mRNA blots. The values were normalized against GAPDH control levels. The means ± SD of three normalized samples were compared with control groups (treatment with normal saline only). Difference to control group **p < 0.001 (One-way ANOVA followed by the least significant difference post hoc test).

**Fig. 2.** Effects of CdCl₂ treatment on CYP2A5 and HO-1 protein expression and coumarin 7-hydroxylase activity DBA/2 mouse hepatocytes. The hepatocytes were treated with 4 µM CdCl₂ or vehicle (normal saline) for 0, 1, 3, 6, or 12 hours. (A) Western immunoblotting of cytoplasmic extracts (20 µg total protein/lane) from control and cadmium-induced hepatocytes probed with anti-CYP2A5 or anti-HO-1 antibody. The protein samples were transferred onto nitrocellulose membrane. In vivo pyrazole-induced mouse liver microsomes and Hsp32 protein were used as positive control for CYP2A5 and HO-1, respectively. The band at 50 kDa corresponds to CYP2A5 protein as indicated by the positive control. (B) Densitometric quantification of Western blot. For CYP2A5, the upper band (50 kDa) was quantified. Each data point represents the mean ± SD of three samples and was normalized against the control levels. Mean difference is significant from control group at **p < 0.001 (One-way ANOVA followed
by Dunnet Post Hoc test). (C) Time-dependent changes in coumarin 7-hydroxylase (COH) activity (indicates CYP2A5 activity) in control and cadmium-induced hepatocytes. The values represent means ± SD of three samples. Mean difference is significant from control group at **p < 0.001 (One-way ANOVA followed by the least significant difference post hoc test).

![Fig. 3](#) Time-course effect of CdCl₂ on cellular distribution of Nrf2 in DBA/2 mouse hepatocytes. The hepatocytes were treated with 4 μM CdCl₂ or normal saline for 0, 1, 3, 6, or 12 hours. (A) Western immunoblotting of nuclear and cytosolic proteins (15 μg total protein/lane) from control and Cd-induced hepatocytes stained with mouse anti-Nrf2 antibody. The protein samples were transferred onto PVDF membrane. (B) Densitometric quantification of Western blot. Each data point represents the mean ± SD of three samples and was normalized against the control levels. Mean difference is significant from control group at **p < 0.001 (One-way ANOVA followed by the least significant difference post hoc test).

![Fig. 4](#) Effect of Nrf2 co-transfection 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 co-transfected Renilla control plasmid (pRL-TK) activities. The values (n = 4) represent means ± SD. Nrf2 response of each reporter construct is indicated by fold of activity to control co-transfection 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).
**Fig. 5.** Putative StREs 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 2 putative StREs. 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 Cd-treated mice were incubated with radiolabelled Probe 1 (lane 2-8) or Probe 2 (lane 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), or 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 unlabelled Probe 1 or 2 (S) (lane 7 and 15, respectively) and unlabelled 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) Chromatin immunoprecipitation (ChIP) with Nrf2-antibody from hepatocytes. Murine primary hepatocytes were treated with 4 µM 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 figure 5A) in real-time PCR and the relative amounts of DNA copies were counted by comparing the sample fluorescence to the fluorescence values measured.
from total chromatin input dilution series. Fluorescence values were corrected with fluorescence signals of the passive reference dye (ROX).

**Fig. 6.** Effect of mutagenesis of potential Nrf2 binding elements on *Cyp2a5*-5’- luciferase activities in Nrf2 co-transfection studies. The potential StRE sites were mutated as described: 

CTCAC/GTCA → CTATA/TCTA in *Cyp2a5*-5’-3033-2505mut and *Cyp2a5*-5’-3033-2379mut respectively, with the mutated bases in bold and the underlined bases representing the core sequence. The names of the mutated constructs refer to the location of the central mutated bases on *Cyp2a5*-5’-promoter. Luciferase activities were measured 48 h after transfecting the cells and the measured activities were normalized against co-transfected *Renilla* control plasmid (pRL-TK) activities. The values (n = 4) represent means + SD. Nrf2 response of each reporter construct is indicated by fold of activity to control co-transfection with pcDNA3. The experiment was repeated three times, and similar results were obtained. Mean difference is significant from control group at **p < 0.01** (Student's t test).
Table 1: Oligonucleotides used for amplifications in EMSA and Real-time PCR experiments.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EMSA:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Probe 1</strong></td>
<td></td>
</tr>
<tr>
<td>Primer 1 (forward primer)</td>
<td>5’- GGTCTCTGTCTATGCTGTGGC-3’</td>
</tr>
<tr>
<td>Primer 2 (reverse primer)</td>
<td>5’-CATGAACCAGGAGTCTAGTCG-3’</td>
</tr>
<tr>
<td><strong>Probe 2</strong></td>
<td></td>
</tr>
<tr>
<td>Primer 3 (forward primer)</td>
<td>5’-TTCACATGTCTGCTCAGG-3’</td>
</tr>
<tr>
<td>Primer 4 (reverse primer)</td>
<td>5’-AGGCAGGTCTCTTCTATCGTC-3’</td>
</tr>
<tr>
<td><strong>Real-time PCR:</strong></td>
<td></td>
</tr>
<tr>
<td>Cyp2a5 5’-2446 FW</td>
<td>5’-GACTCGAGCAGGACTGCC-3’</td>
</tr>
<tr>
<td>Cyp2a5 5’-2319 RV</td>
<td>5’-AGAGCTCGGAAGAGATCAAG-3’</td>
</tr>
</tbody>
</table>
**FIGURE 5**

(A) 

```plaintext
-3033

probe 1 (-2597)

-2613 ACCCCTGCCCCCCTTCCGGGTATTTGTCTATGCTGTGGCCCTGCTCTTAAT
CACTAAGAAAGGGAGACTGGGAGGCCATCAAAGAGAGACCTTCCCCAAAGCCCC

probe 2 (-2467)

TGCTCAGCTCAAGCAGCTCTCGGAAGGGGAGATGCTTGGCTGCTGTGGTCTC

distal StRE

ACATGTCTCTCGCCAGGTGACTGCAGGGCAGTGACTCCCGTGGTTCTGATCTGTGCTGT

PCR primer (-2446)

GTCTCCCCACGTGACTTCATCTGCTCTTGCTCTGTCAACTCCATGCGTCTGAAAAG

proximal StRE

AAGTCAAAGAAGACAAAATAAAACTTGTATCTGTCTTCCCGAGCTCTTCCAGCTCGGT

PCR primer (-2319)

TCCCATTTGCCCCCTGTGCGAGATTATGAGAAACCTGCTGCATTCACTCAGGGT

probe 2 (-2269)

CTTTCTGGGTTGC -2244
```

(B) 

<table>
<thead>
<tr>
<th>Consensus StRE sequence</th>
<th>5’-T GCT [GAG] TCA -3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal StRE</td>
<td>-2515 T GCT [CAC] TCA -2506</td>
</tr>
<tr>
<td>Proximal StRE</td>
<td>-2387 T GCT [CTG] TCA -2378</td>
</tr>
</tbody>
</table>
(C) FIGURE 5

| Cd      | - | - | + | + | + | + | + | + | - | - | + | + | + | + | + |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ab      | - | - | - | - | Nrf2 | Fos | Jun | - | - | - | Nrf2 | Fos | Jun | - | - | - |
| Comp    | - | - | - | - | - | S | C | - | - | - | - | - | - | S | C | - |

(D)

Relative quantity (% of total chromatin input)

- IgG
- Nrf2 Ab

Control

Cadmium chloride
FIGURE 6

-3033  

LUC  

3.5 x **

-3033  -2505mut  

LUC  

3.6 x **

-3033  -2379mut  

LUC  

1.2 x

pcDNA3  

pcDNA3-mnrf2

Relative Luciferase Activity