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Evidence for induced microsomal bilirubin degradation by cytochrome P450 2A5

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Abstract

Oxidative metabolism of bilirubin (BR) – a breakdown product of haem with cytoprotective and toxic properties – is an important route of detoxification in addition to glucuronidation. The major enzyme(s) involved in this oxidative degradation are not known. In this paper, we present evidence for a major role of the hepatic cytochrome P450 2A5 (Cyp2a5) in BR degradation during cadmium intoxication, where the BR levels are elevated following induction of haem oxygenase-1 (HO-1). Treatment of DBA/2J mice with $CdCl_2$ induced both the Cyp2a5 and HO-1, and increased the microsomal BR degradation activity. By contrast, the total cytochrome P450 (CYP) content and the expression of Cyp1a2 were down-regulated by the treatment. The induction of the HO-1 and Cyp2a5 was substantial at the mRNA, protein and enzyme activity levels. In each case, the up-regulation of HO-1 preceded that of Cyp2a5 with a 5–10 h interval. BR totally inhibited the microsomal Cyp2a5-dependent coumarin hydroxylase activity, with an IC_{50} approximately equal to the substrate concentration. The 7-methoxyresorufin 7-*O*-demethylase (MROD) activity, catalyzed mainly by the Cyp1a2, was inhibited up to 36% by BR. The microsomal BR degradation was inhibited by coumarin and a monoclonal antibody against the Cyp2a5 by about 90%. Furthermore, 7-methoxyresorufin, a substrate for the Cyp1a2, inhibited BR degradation activity by approximately 20%. In sum, the results strongly suggest a major role for Cyp2a5 in the oxidative degradation of BR. Secondly, the coordinated up-regulation of the HO-1 and Cyp2a5 during Cd-mediated injury implicates a network of enzyme systems in the maintenance of balancing BR production and elimination.

Keywords: Cadmium; Cytochrome P450 2A5; Alternative pathway of bilirubin degradation; Haem oxygenase 1; Oxidative stress; Bilirubin

1. Introduction

The cytochrome P450 (CYP) enzymes constitute a superfamily of haemoproteins. They play a critical role in the detoxification and activation of xenobiotics, and are expressed in a tissue-selective manner. The liver is the main site of detoxification. The Cyp2a5 enzyme is the major catalyst of coumarin 7-hydroxylation in the mouse liver [1,2]. It is inducible by many structurally unrelated compounds, such as phenobarbital [3,4], pyrazole [5], porphyrinogenic agents [6] and metals [7–9]. Increased expression of Cyp2a5 has also been observed in spontaneous, transplanted or chemically induced mouse hepatomas, as judged by studies comparing induction in normal liver and in liver injuries resulting from chemical intoxication or microbial infection [10–18].

The induction of hepatic Cyp2a5-dependent 7-coumarin hydroxylase (COH) activity, by metals or agents known to affect cellular haem balance or by liver injuries was often observed with a decrease of total CYP content [6,19,20], as well as by a repression of other CYP forms [21]. The diminished total CYP content, in turn, has been shown to be associated with strong induction of the haem

Abbreviations: AhR, aryl hydrocarbon receptor; BNF, β-naphthoflavone; BR, bilirubin; Cd, cadmium; COH, 7-coumarin hydroxylase; CYP, cytochrome P450; EROD, 7-ethoxyresorufin 7-*O*-demethylase; HO-1, haem oxygenase-1; hmox-1, haem oxygenase-1 gene; 3-MC, 3- methylcholanthrene; MROD, 7-methoxyresorufin 7-*O*-demethylase; Nrf2, erythroid-related factor 2; ROS, reactive oxygen species; 3,4-TCB, 3,4,3′,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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oxygenase-1 (HO-1) [22], an enzyme that catalyses the rate-limiting step in haem catabolism. This enzymatic reaction produces the bile pigment biliverdin (BV) and its metabolite bilirubin (BR), a compound with both cytoprotective and toxic properties. At high concentrations, BR impairs mitochondrial function and inhibits various metabolic enzymes [23–25]. In contrast, at physiological concentrations, BR and its glucuronides are potent anti-oxidants [26–30].

Bilirubin oxidative metabolites (BOMs) have been found to be potent antioxidants in rat and human models, and the production of such metabolites is related to oxidative stress [30]. It is also believed that the oxidative metabolism of BR is an important alternative route of detoxification in the situation where the glucuronidation pathway of BR disposal is inhibited or absent [31,32]. The involvement of bilirubin oxidases in this alternative disposal mechanism has been suggested [31,33], and was shown to exist in the hepatic microsomal fractions of rats and mice [34,35]. The oxidative pathway was observed to be stimulated by inducers of the CYP1A, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [36]. Hence, CYP1A1 [36] and Cyp1a2, in particular [35], have been implicated in the inducibility of this pathway. However, their contribution to microsomal BR oxidation appeared to be minor and therefore other CYP isoforms may contribute to the major part of the constitutive and inductive microsomal BR oxidation [35].

We have previously shown that acute cadmium (Cd)[1] exposure resulted in time- and dose-dependent reduction of the total CYP content as well as strong induction of the Cyp2a5 in the mouse liver [20]. In this paper, we present evidence that under conditions of oxidative stress exerted by Cd, the Cyp2a5 is concurrently induced with the HO-1. Secondly, the coordinated inductions of these enzymes are related phenomena in which the Cyp2a5 plays a major role in the oxidative degradation of BR.

2. Materials and methods

2.1. Animals

Thirty 7–9-week-old DBA/2J male mice from the Animal Resources Centre, Western Australia, were divided into 10 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/12-h dark cycle with free access to standard mouse chow and tap water. The animals were treated with a single intraperitoneal injection of 16 μmol $CdCl_2$/kg body weight dissolved in normal saline solution. The animals in the control group were given normal saline only. They were sacrificed at 1, 2, 3, 4, 6, 8, 18, 24 and 48 h after treatment by CO_2 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.

[1] Cd is a strong inducer of HO-1.

2.2. Materials and chemicals

Cadmium chloride ($CdCl_2$) (purity 99%), coumarin, umbelliferone (7-hydroxycoumarin), resorufin, 7-methoxyresorufin, bilirubin, hemin chloride, nicotinamide adenine dinucleotide phosphate, β-NADPH, D-glucose-6 phosphate (G6P), glucose–phosphate dehydrogenase (G6PDH) (Type XV from Baker's yeast), BSA and glycine were from Sigma (Sydney, Australia). All other chemicals were reagent grade available from Sigma (Sydney, Australia).

2.3. Preparation of microsomes

Liver microsomal fractions were prepared by a modification [20] of the method described previously [37]. Protein concentrations of the microsomal samples (BSA as standard) were in the range of 22–35 μg/μl [38]. Total CYP content was determined according to the carbon monoxide difference spectra method [39].

2.4. Enzymes activity assay

Coumarin 7-hydroxylase (COH) and 7-methoxyresorufin 7-*O*-demethylase (MROD), activity was measured spectrofluorometrically by a modification [20] of the method previously described [40,41], using 100 μM final concentration of coumarin and 7-methoxyresorufin. This concentration was also used in the study of COH activity inhibition caused by addition of bilirubin. Ten micromolars of 7-methoxyresorufin or 7-ethoxyresorufin was used when the inhibition of MROD or 7-ethoxyresorufin 7-*O*-demethylase (EROD) caused by addition of bilirubin was studied. EROD activity was measured spectrofluorometrically, as described previously [41].

Haem oxygenase (HO) activity was measured by bilirubin generation using the modified method described by Maines [42]. Incubation of reaction mixture [160 mM potassium phosphate buffer (pH 7.4), 2 mM magnesium chloride, 0.8 mM NADPH, 2 mM G6P, 0.2 units G6PDH, 2 mg of cytosolic protein from mouse kidney (as a source of biliverdin reductase) and 0.1 mg microsomal protein] was done in the dark at 37 °C for 30 min with constant shaking. The reaction was started by addition of substrate hemin (20 μM) and terminated by addition of 1 ml chloroform on ice. The extracted bilirubin was measured by the difference in absorbance between 450 and 530 nm.

The concentration of bilirubin was calculated using an extinction coefficient of 58 mM^{-1} cm^{-1}.

2.5. Bilirubin disappearance activity

The rate of bilirubin metabolism by isolated microsomal fractions was studied as described previously [34] with a slight modification. Briefly, incubation mixture [potassium phosphate buffer, pH 7.4 (0.1 M), $MgCl_2$ (5 mM), EDTA (2 mM), 100 μl microsomal protein (5 μg/ml) from control or induced livers (corresponding to a concentration of cytochrome P450 ranging from 1.7 to 3.8 pmol/ml); in total volume of 2 ml] was placed in plastic disposable cuvettes. After 5 min pre-incubation, NADPH (1.5 mM) was added to both sample and reference cuvettes and absorbance was zeroed against reference cuvette at 440 nm (Cary 3E UV–visible Spectrophotometer, Varian Australia, Pty. Ltd.). Immediately, bilirubin in DMSO (20 μM final concentration) was added to the sample cuvette and absorbance at 440 nm was recorded as $t = 0$. The sample was then incubated for 10 min and the decrease in absorbance at 440 nm was recorded. The rate of bilirubin disappearance is expressed as pmol bilirubin disappearing/min, using a ε (mM) = 84.4 cm^{-1}, which was obtained experimentally under the conditions of the assay. An additional cuvette containing all the above ingredients without NADPH was run along the sample and reference cuvettes as control for the spontaneous disappearance of BR. All experiments were done in the dark and 2 mM (final concentration) of EDTA was added in all incubation mixture.

In the study of the inhibitory effect of the primary monoclonal anti-mouse Cyp2a5 antibody, the final concentration of microsomal protein used was 5 μg/ml (corresponding to 2.1 pmol/ml cytochrome P450). Increasing concentrations of the antibody were then added, to obtain concentration ratios to microsomal protein ranging from approximately 0.05 to 1.0. After 5 min incubation, 20 μM bilirubin (in DMSO) was added. The reaction was initiated by addition of NADPH and incubation at 37 °C for 20 min. The same condition was employed (with the addition of increasing concentrations of coumarin or 7-methoxyresorufin in the range of 0–50 μM) when the inhibitory effect of coumarin and 7-methoxyresorufin on the bilirubin-degrading activity of the induced liver microsomes was studied.

2.6. Northern blot

Total hepatic RNA was prepared with TRIzol® Reagent (Gibco, Sydney, Australia) according to the manufacturers recommendation. RNA was resuspended in 50 μl DEPC-treated water and the concentration was determined optically at 260 and 280 nm after dilution in water. Total liver RNA (20 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 (provided by Dr. Masahiko Negishi, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle, NC), Cyp1a2 cDNA (kindly provided by Dr. P. Honkakoski, University of Kuopio, Finland) and HO-1 cDNA (a kind gift from Dr. Jawed Alam, Dept. of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, USA) were radiolabelled with [α-^{32}P]dCTP using the Megaprime labelling kit (Amersham Biosciences). Successive hybridizations were carried on the same filter using the cDNA probes (1.7×10^7 cpm of radiolabelled probe) at 65 °C overnight in Church buffer [43] (modified to contain 0.25 M phosphate buffer, 7% SDS and 1 mM EDTA). The filter was washed 2× 5 min at room temperature in a buffer containing 2× SSC and 0.1% SDS and then 1× 15 min at 65 °C in a buffer containing 2× 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.

2.7. Western blot

Liver microsomal proteins (20 μg protein) were separated by SDS-PAGE (12%) and blotted onto a nitrocellulose membrane (Pierce Biotechnology, USA). A monoclonal anti-mouse haem oxygenase-1 antibody (Stressgen Biotech, Canada) and a primary monoclonal anti-mouse Cyp2a5 antibody were used in a 1:1000 dilution. All solutions contained 5% non-fat dry milk and the detection was performed with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology).

2.8. Statistical analysis

Data was analysed with the use of the SPSS statistical package (Version 11) for Windows. Comparisons of several groups were done with one-way ANOVA followed by the post hoc Dunnett's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Induction of Cyp2a5 and HO-1 by cadmium

The *Cyp2a5* and *hmox-1* genes were observed to be induced time-dependently by Cd in the liver (Fig. 1A). Elevation of HO-1 mRNA levels precedes that of the Cyp2a5 mRNA where maximum induction occurred 3 h post-treatment (31-fold increase compared to control) and decreased dramatically 6 h later. The Cyp2a5 mRNA levels gradually increased 3 h post-treatment to reach maximum at 8 h (35-fold increase compared to control) (Fig. 1B). In contrast, no significant change of Cyp1a2 mRNA levels was observed.

3.2. Cadmium increases protein levels and activity of Cyp2a5 and HO

Western blot analysis (Fig. 2A and B) showed that Cd also increased both HO-1 and Cyp2a5 proteins in a time-dependent manner. The up-regulation profiles of both proteins corresponded well to the respective mRNA levels, as shown in Fig. 1. Furthermore, the pattern of protein induction was in tandem with the enzymatic activities of COH (indices of Cyp2a5 activity) and HO, where the maximal induction was observed at 18 and 8 h, respectively (Fig. 3). No significant change was observed in the MROD activity (indices of Cyp1a2 activity).

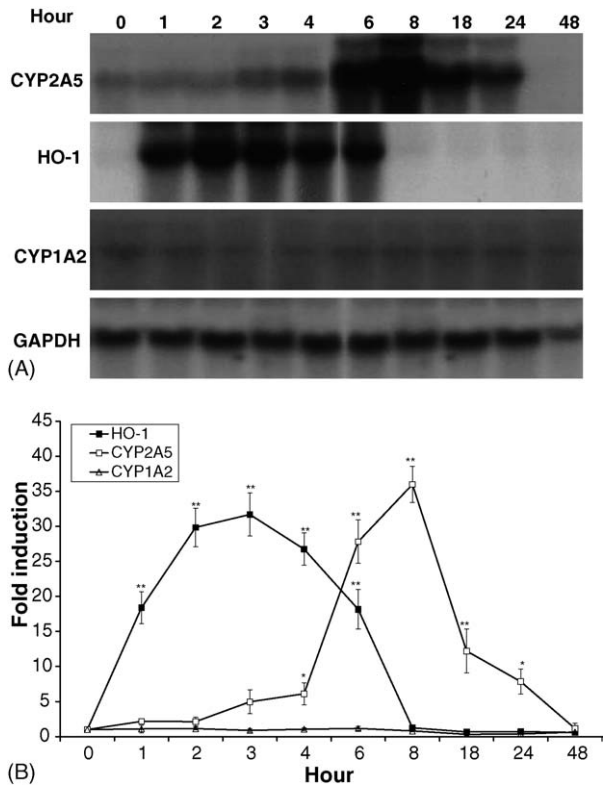

Fig. 1. Effect of $CdCl_2$ treatment on Cyp2a5, Cyp1a2 and HO-1 mRNA expression in the liver of DBA/2J mice. The animals were treated with 16 μmol $CdCl_2$/kg body weight for 0, 1, 2, 3, 4, 6, 8, 18, 24 or 48 h. (A) Twenty micrograms of total RNA isolated from liver of treated and control animals were electrophoresed, blotted and hybridised with mouse Cyp2a5, Cyp1a2 and HO-1 probes. GAPDH mRNA levels are shown as control for RNA loading. Each blot represents one of three blots of 10 samples (each blot showed the same pattern of induction). Electrophoresis of the samples (10 samples) was run on the same mini gel with two six-well combs positioned parallel to each other horizontally, i.e. one on the top of the gel and the other in the middle of the gel. The transfer of RNA to a nylon membrane was done from the same gel, hence the pre-hybridization and hybridization steps, as well as the autoradiography. The scanned film was cut in order to position all the samples in the same lane for comparison. (B) Densitometric quantification of Cyp2a5, Cyp1a2 and HO-1 mRNA blots. The values are normalized against GAPDH control levels. The mean ± S.D. of three normalized samples are compared with control groups (treatment with normal saline only). Difference to control group $^{**}p < 0.001$ and $^{*}p < 0.01$ (one-way ANOVA followed by Dunnet post hoc test).

The observation of time dependent reduction in total CYP content (approximately 65% during 48 h of treatment) (Fig. 3) and diminished expression of Cyp1a2 by Cd is in sharp contrast to Cd effects on the expression and activity of Cyp2a5. This suggests that Cd induction of the Cyp2a5 is a specific effect amongst the CYP isozymes.

3.3. Stimulation of microsomal bilirubin degradation by Cd treatment

To explore the possibility that the concurrent induction of Cyp2a5 and HO-1 by Cd may be related phenomena, microsomal bilirubin degradation activity was compared between Cd-treated and control DBA/2J male mice. Eighteen hours after Cd treatment, a strong (46%) reduction in total CYP was observed, with an equally strong reduction of MROD activity. In contrast, COH activity increased eight-fold. Most significantly, a 10-fold increase in the rate of bilirubin degradation was observed after Cd treatment compared to controls without treatment (Table 1).

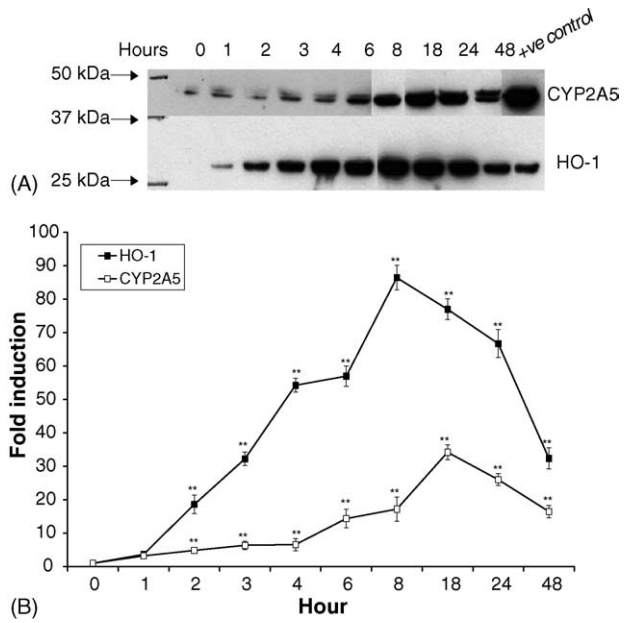

Fig. 2. Effect of $CdCl_2$ treatment on Cyp2a5 and HO-1 protein expression in the liver of DBA/2J mice. The mice were treated with 16 μmol $CdCl_2$/kg body weight for 0, 1, 2, 3, 4, 6, 8, 18, 24 or 48 h. (A) Western blot analysis of microsomal protein (20 μg total protein/lane) from livers of control and cadmium-treated mice probed with mouse anti-Cyp2a5 and anti-HO-1 antibodies. In vivo pyrazole-induced mouse liver microsomes were used as positive control for Cyp2a5 and HO-1 protein as positive control for HO-1. Each blot represents one of three blots of 10 samples (each blot showed the same pattern of induction). Electrophoresis of 10 samples was run on two mini gels side by side in the same electrophoretic chamber. The transfer of protein to a nylon membrane was also done side by side in the same chamber. All incubations for the two blots were done simultaneously and subjected to the same experimental environment. The scanned film was cut in order to position all the samples in the same lane for comparison. (B) Densitometric quantification of Western blot. Each data point represents the mean ± S.D. of three samples and are 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).

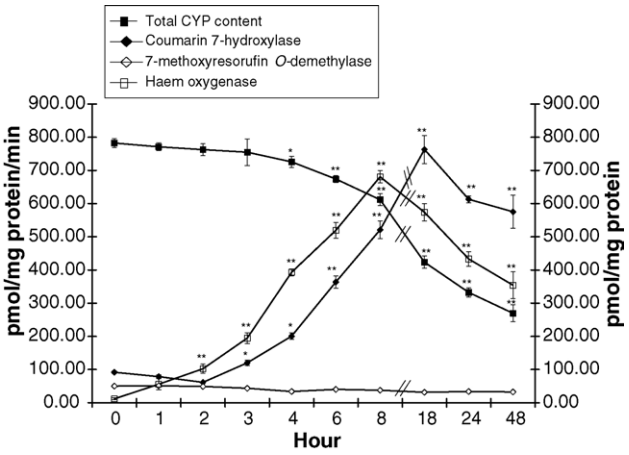

Fig. 3. Effect of $CdCl_2$ treatment on total CYP content and COH, MROD and HO activities in the liver of DBA/2J mice. Liver microsomes were isolated from control and treatment groups. The mice were treated with 16 μmol $CdCl_2$/kg body weight for 0, 1, 2, 3, 4, 6, 8, 18, 24 or 48 h. Time-dependent changes in COH, MROD and HO activities as well as the total cytochrome P-450 content in liver microsomes of control and treatment groups. The scale on the left refers to enzyme activities and the one on the right refers to total CYP content. The values represent mean ± S.D. of three samples. Mean difference is significant from control group at $^{**}p < 0.001$ and $^{*}p < 0.01$ (one-way ANOVA followed by Dunnet post hoc test).

3.4. Effect of bilirubin on MROD, EROD and COH activity of isolated liver microsomes

Previous study using the wild-type [*Cyp1a2* (+/+)] and knock-out [*Cyp1a2* (−/−)] mice demonstrated that the Cyp1a2 contributes to the constitutive microsomal BR degradation up to 20%, while Cyp1a1 contributed to the oxidation when challenged with an aryl hydrocarbon receptor (AhR) ligand, in vitro [35]. However, the main catalyst of BR degradation has so far remained unidentified. Our present observations show that the rate of BR degradation is increased in microsomes with induced Cyp2a5 and with reduced total CYP content (Table 1). We therefore explored the relationship between BR degradation and the Cyp2a5 enzyme in detail.

The dose–response curve for COH activities obtained with microsomes from induced mice, in the presence of various concentrations of BR, showed that inhibition of activity by BR is almost 100% with an IC_{50} of approximately 80 μM BR (Fig. 4A). The MROD activity was inhibited maximally by about 36% (Fig. 4B), which is similar to inhibition of EROD activity by BR (Fig. 4B).

A more detailed kinetic analysis showed that inhibition of the Cyp2a5 catalyzed COH activity by BR is of a mixed type that affected both the V_{max} and K_m, where the V_{max} is reduced and the K_m is increased (Fig. 5A). The K_m for coumarin 7-hydroxylation is 0.45 μM. This is similar to the published value of 0.5 μM [44]. The affinity of BR to the enzyme appears to be in the same range of coumarin, with K_i value of 0.7 μM (Fig. 5B).

Fig. 6A shows that a monoclonal antibody directed against Cyp2a5 inhibited the microsomal BR degradation of Cd-induced mice. Also, coumarin, a specific high affinity substrate for Cyp2a5, inhibited the BR degradation (Fig. 6B). The two dose-inhibition curves were found to be very similar. In both cases, the degradation activity was inhibited by up to 90% maximally. By way of contrast, 7-methoxyresorufin (a substrate for the Cyp1a2) inhibited BR degradation activity, maximally by about 17%. This observation is consistent with the findings of Zaccaro et al. [35] that the Cyp1a2 contributes to the constitutive BR degradation by approximately 20%. Inhibition of BR degradation activity by 7-ethoxyresorufin (a substrate for the Cyp1a1) could not be detected (data not shown). It seems therefore that in the Cd-induced livers the Cyp2a5 could be responsible for most of the BR degradation.

4. Discussion

The present results suggest a major role for Cyp2a5 in hepatic bilirubin metabolism during Cd intoxication. Previously, a bilirubin-degrading system which can be induced with TCDD, β-naphthoflavone (BNF), or 3-methylcholanthrene (3-MC) in vivo, has been described in the microsomal fraction of rat and mouse liver [34,35]. Such induction was markedly stimulated by addition of a planar polychlorinated biphenyl (PCB), 3,4,3′,4′-tetrachlorobiphenyl (3,4-TCB), to the microsomal incubation in vitro [36]. A study in different strains of mouse [35], including *Cyp1a2* null mutant mice, implicated both Cyp1a1 and Cyp1a2 in the BR-degrading activity of the induced liver microsomes. Cyp1a2 is believed to possess BR-degrading activity intrinsically,[2] whereas Cyp1a1 only requires it when challenged with 3,4-TCB. Addition of 3,4-TCB in vitro was also found to cause a dose-dependent inhibition of EROD activity of the induced microsomal fractions [36]. These observations led to the hypothesis that 3,4-TCB interacts with the induced Cyp1a1 as poor substrate, which may lead to increased production of reactive oxygen species (ROS) by an uncoupling mechanism. These could then be responsible for oxidation of important target molecules, such as bilirubin [45].

On the contrary, our study presents evidence for direct involvement of CYP enzyme in microsomal BR degradation, where BR may act as a substrate for the hepatic Cyp2a5, a major catalyst for BR degradation under the condition of substantial elevation of BR levels following induction of HO-1. This is evident in the 10-fold increased in the rate of BR degradation observed in Cd-induced microsomes, where the COH[3] and MROD[4] activity was markedly increased and decreased, respectively (Table 1). The argument is further supported by the enzyme kinetic

[2] CYP1A2 contributes to the constitutive microsomal BR degradation by about 20% [35].
[3] Indices of CYP2A5 activity.
[4] Indices of CYP1A2 activity.

Table 1
Cytochrome P450 content, COH, MROD and bilirubin degrading activity of liver microsomes from control and $CdCl_2$ treated mice

Treatment in vivo	Cytochrome P-450 (pmol/mg protein)	COH activity (pmol/(min mg protein)	MROD activity (pmol/min mg protein)	Rate of bilirubin degradation (pmol/min nmol cytochrome P450)
None	782.25 ± 13.86 (4)	91.90 ± 3.70 (4)	50.08 ± 2.18 (4)	765 ± 128 (6)
$CdCl_2$	423.50 ± 18.59 (4)*	763.15 ± 42.20 (4)*	31.57 ± 3.32 (4)	7755 ± 616 (6)*

Male DBA/2J mice either were untreated controls or were treated with 16 μmol $CdCl_2$/kg body weight for 18 h. Hepatic microsomal fractions were isolated for the estimation of total cytochrome P450 content, COH, MROD and bilirubin degrading activity (as described in Section 2). Results are given as mean ± standard deviation of the number of observations in parentheses.

* $p < 0.001$, compared with corresponding values of un-induced microsomes.

analysis which showed that: (a) BR is a high affinity substrate of the Cyp2a5 with a K_m in the same range as coumarin[5]; (b) BR causes a total dose-dependent inhibition of COH activity of the induced microsomal fractions; (c) both the antibody raised against Cyp2a5, and coumarin caused a dose-dependent inhibition of microsomal BR-degradation activity by approximately 90%. In contrast, BR does not markedly inhibit MROD or EROD activity, and 7-methoxyresorufin[6] inhibits microsomal BR-degradation activity by approximately 17%. In addition, microsomal BR-degradation activity does not seem to be inhibited by 7-ethoxyresorufin (data not shown). Thus, the findings clearly show that the Cyp2a5 is the main CYP enzyme responsible for the microsomal BR oxidation. This observation is consistent with earlier results suggesting that the Cyp1a2 and Cyp1a1 may have a minor contribution to the BR oxidation [35].

It should be noted though that the kinetic data were obtained using microsomal preparations instead of pure enzymes, which is why they should be interpreted with caution. Moreover, a preliminary study using microsomal fractions extracted from recombinant AH22 *Saccharomyces cerevisiae* yeast cells expressing Cyp2a5 [46] was conducted (unpublished observations). The study confirmed that the Cyp2a5 metabolises BR. Because the COH activity in the recombinant yeast microsomes is substantially lower than the COH activity in induced-liver microsomes (which is due to a relatively low expression levels of the Cyp2a5 protein), it was difficult to measure kinetic parameters reliably.

Although our observations strongly implicate Cyp2a5 as a major catalyst for microsomal BR oxidation, the mechanisms by which it is induced is not apparent in this study. Earlier observations in C57BL/6 strain of mouse – a strain that is genetically responsive to AhR ligand – showed that treatment by either 3-MC or BNF markedly induced microsomal BR-degrading activity [35]. The induction, however, was not enhanced when challenged with 3,4-TCB in vitro, despite marked elevation of EROD (indices for Cyp1a1 activity) and MROD activities [35]. This observation suggests existence of mechanism(s) other than the uncoupling of CYPs, in which subsequent production of ROS leads to oxidation of BR. Our recent study using the C57BL/6 strain, showed that treatment with AhR ligands (TCDD and 3-MC) strongly induced the hepatic Cyp2a5 [47]. We have also characterized two Ah-response elements at the Cyp2a5 promoter [47]. This study established that regulation of the Cyp2a5 gene involves an AhR-dependent pathway and could therefore explain the earlier

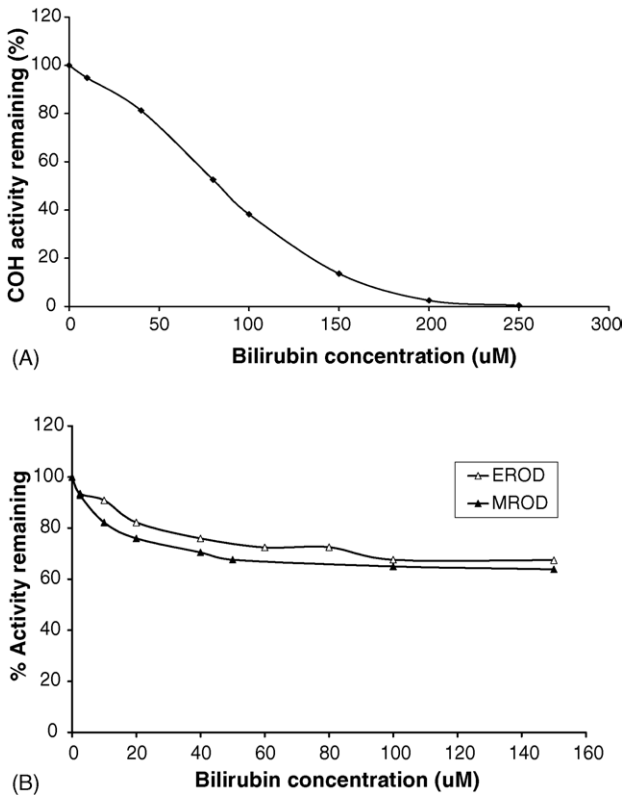

Fig. 4. Effect of bilirubin on cytochrome P450 monooxygenase activity in liver microsomes. Liver microsomes were isolated from mice treated with 16 μmol $CdCl_2$/kg bwt for 18 h (induced microsomes). Dose–response curve for (A) COH activity obtained with induced microsomes in the presence of various concentrations of bilirubin. The rate of COH was determined as described in Section 2, with concentration of coumarin 100 μM. Fifty micrograms per millilitres microsomal protein was used corresponding to 27 pmol cytochrome P450/ml of incubation mixture. (B) MROD or EROD activity obtained with induced microsomes in the presence of various concentrations of bilirubin. The rate of MROD and EROD was determined as described in Section 2, with concentration of 7-methoxyresorufin or 7-ethoxyresorufin 10 μM. Fifty μg/ml microsomal protein was used corresponding to 27 pmol cytochrome P450/ml of incubation mixture.

[5] Substrate specific for CYP2A5.
[6] Substrate for CYP1A2.

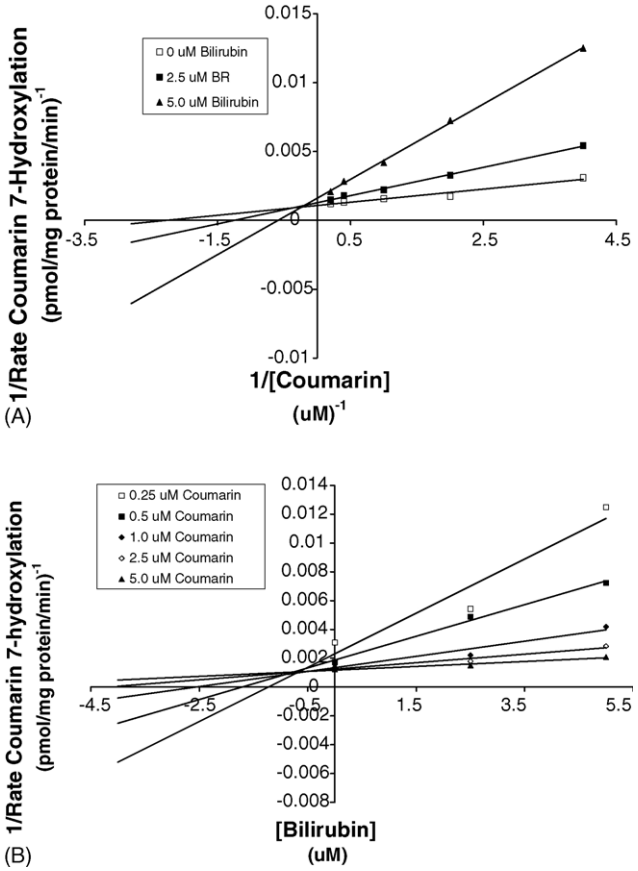

Fig. 5. Effect of bilirubin on the 7-hydroxylation of coumarin in DBA/2J liver microsomes. (A) Liver microsomes (50 μg/ml) from Cd-treated mice were incubated with various concentrations of coumarin in the presence of increasing amounts of bilirubin. The rates of coumarin 7-hydroxylation were determined by fluorescence spectroscopy. The double-reciprocal tended to intersect above the x axis, indicating BR is a mixed inhibitor. The K_m for coumarin 7-hydroxylation was estimated to be 0.45 μM. (B) The Dixon plot intersected above the x axis confirmed the competitive component of BR with K_i value of approximately 0.7 μM.

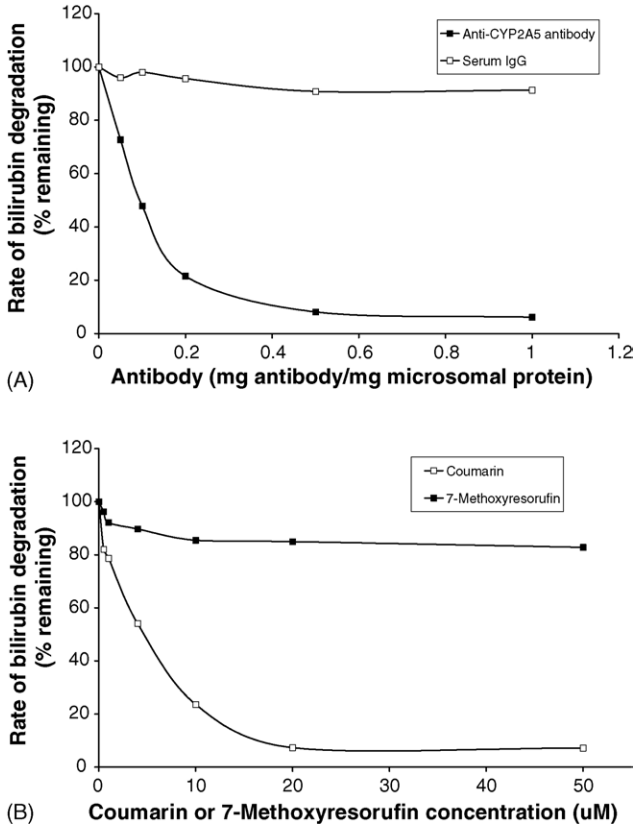

Fig. 6. Effects of anti-Cyp2a5 antibody, coumarin and 7-methoxyresorufin on microsomal bilirubin degradation activity. (A) Inhibition of bilirubin-degradation activity of induced microsomes caused in the presence of anti-Cyp2a5 antibody (■) and in the presence of serum IgG (□). The IgGs were added in increasing amount so as to achieve the antibody to microsomal protein concentration ratios indicated. (B) Inhibition of bilirubin-degradation activity of induced microsomes caused by addition of coumarin (specific substrate for Cyp2a5), or 7-methoxyresorufin (a substrate for the Cyp1a2). The bilirubin degrading activity were assayed as described in Materials and Methods in the presence of increasing coumarin and 7-methoxyresorufin concentrations. In both (A) an (B) 20 μM final concentration of BR was used. Bilirubin-degrading activity is expressed as a percentage of the corresponding values.

findings on the involvement of the AhR in the regulation of BR oxidation.

However, evidence suggests that factors other than the AhR may play a role in the induction of the BR oxidation as well. For example, the present results show a strong up-regulation of BR oxidation (catalyzed by the Cyp2a5) without an increase in the expression of the Cyp1a2 or the corresponding enzyme activity. These results were obtained using the DBA/2 strain of mouse, which possesses a mutated Ah gene and is therefore non-responsive to AhR ligands [48]. In addition, we recently have shown that the Cyp2a5 regulation is under the control of the nuclear erythroid-related factor 2 (Nrf2) [20], a general regulator of cell defence mechanism against oxidative stress. This observation is consistent with several earlier observations of Cyp2a5 up-regulation under the condition of oxidative stress [49–51], when other CYP forms were down-regulated. Indeed, this phenomenon was once again observed in the current study, which raises the question of whether the Cyp2a5 enzyme plays a role in cell defence against Cd-mediated injury.

In relationship to this, HO-1 induction by Cd has been suggested to represent a cellular defence against Cd-mediated injury, owing to the facts that: (a) acute Cd exposure increases production of ROS with subsequent rapid reduction of anti-oxidant enzymes[7] in mammals [52]; (b) elevation of HO enzymes was observed immediately after reduction of basal level of anti-oxidant enzymes [52]; (c) induction of HO-1 by Cd is strong and rapid [42]; (d) HO-1 catalyzed reaction produced compounds with anti-oxidant properties, including BR [53].

The consequent production of ROS is known to activate transcription factors associated with transcriptional regulation of *hmox-1* gene [54]. One of these factors is Nrf2. Cadmium, on the other hand, is known to retard the ubiquitinated degradation of Nrf2, leading to increased half-life of the protein and subsequent activation of Ho-1

[7] Superoxide dismutase, catalase and glutathione peroxidase.

transcription in Hepa cells [55]. Our own study, investigating the molecular mechanisms by which Cd influences the regulation of Cyp2a5 expression, demonstrates that Cd treatment to DBA/2 mice resulted in accumulation of Nrf2 in the nucleus (unpublished observations) within 8 h after treatment. These may explain the rapid and substantial increase in mRNA, protein and activity levels as depicted in Figs. 1–3. Such induction of HO-1 enhances haem degradation, which lead to shortage of haem in the cellular haem pool [56]. This could be the reason for diminished cellular content of the total CYP pool, and consequently, impaired CYP-dependent oxidative metabolism, which is reflected in reduced MROD activity (with no change in the corresponding mRNA levels) (Figs. 1 and 3). However, the elevation of COH activity and Cyp2a5 protein suggests complete incorporation of haem (from the haem pool) into the Cyp2a5 apoprotein, but not into other isoforms. When the cell is challenged by toxic dose of Cd and when the HO-1 is significantly induced, why is there preferential incorporation of limited free haem into the Cyp2a5 apoprotein rather than other CYP isoforms? This raises the question of whether the Cyp2a5 enzyme is involved in the oxidative metabolism of BR, either by producing antioxidants (BOMs) to protect the cell from further damage [30], or by contributing to the crucial step in balancing BR production and elimination, in order to maintain cellular integrity under conditions of oxidative stress. In support to these hypotheses, treatment of rats with an endotoxin [lipopolysaccharide (LPS)], which has been shown to stimulate ROS production, resulted in increased production of BOMs, which synergistically act as physiological antioxidants with ascorbic acid. [30]. In addition, our studies have shown that the LPS treatment of mice induces the hepatic Cyp2a5 (our unpublished observations), as does viral or parasitic infestation of the liver [49,50].

In conclusion, the current evidence suggest that several stress activated transacting factors may be involved in the regulation of the Cyp2a5 gene and hence bilirubin oxidation. The respective roles of these factors in the regulation are still an open question but their activation may depend on the type of challenge(s) the cells are exposed to. Lastly, the concurrent up-regulation of Cyp2a5 and HO-1 suggests a coordinated regulation of two different enzyme systems, as well as a physiological role of Cyp2a5, in cellular defence against Cd-mediated injury.

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