Human NADPH-P450 oxidoreductase modulates the level of cytochrome P450 CYP2D6 holoprotein via haem oxygenase-dependent and -independent pathways

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NADPH-P450 oxidoreductase (CPR) is essential for the activity of cytochrome P450 (P450). Previous studies demonstrated that CPR regulates the levels of various P450 isoforms *in vitro*. We investigated the mechanistic basis for this regulation. By transfection of Chinese hamster ovary DUKXB11 cells we obtained the cell line DUKX/2D6, which expressed human CYP2D6, a P450 isoform. Subsequently, DUKX/2D6 cells were transfected with human CPR cDNA to generate the cell line DUKX/2D6/CPR-3. Expression of recombinant CPR decreased the level of spectrally detectable CYP2D6 holoprotein in DUKX/2D6/CPR-3 cells by 70 %, whereas the level of immunodetectable apoprotein remained unchanged. Addition of the radical scavenger DMSO increased levels of CYP2D6 holoenzyme in DUKX/2D6/CPR-3 cells but not in DUKX/2D6 cells. A similar effect was noted when cells were grown in the presence of hemin. Importantly, combined treatment with DMSO and hemin increased levels of CYP2D6 holoenzyme in DUKX/2D6/CPR-3 but not in DUKX/2D6 cells even further than either treatment alone. None of these treatments affected the level of immunodetectable CYP2D6. This demonstrates that expression of CPR increases production of damaging radicals but also that CPR may alter haem homoeostasis. In agreement with this, the activity of haem oxygenase, a rate-limiting enzyme in haem metabolism, was compared with that in DUKX/DHFR control cells (expressing dihydrofolate reductase), and was 3-fold higher in DUKX/2D6/CPR-3 but similar in DUKX/2D6 cells. Furthermore, treatment of cells with sodium arsenite increased levels of haem oxygenase concomitant with a marked decrease of spectrally detectable CYP2D6 and a rise in levels of ferritin, which sequesters free iron released from the destruction of haem. These data demonstrate that CPR regulates P450 activity by supplying electrons and also by altering P450 levels via radical- and haem oxygenase-mediated pathways.

Key words: drug metabolism, oxidative stress, pharmacokinetics, regulation of P450, toxicology.

INTRODUCTION

Cytochrome P450s (P450s) are a superfamily of haemoproteins that catalyse the oxidation of a wide variety of endogenous and xenobiotic chemicals, including therapeutic drugs and carcinogens [1]. P450s have been found in all tissues but are localized mainly in the liver. P450-dependent metabolism requires two protein components, P450 and NADPH-P450 oxidoreductase (CPR; EC 1.6.2.4). Both enzymes are embedded in the membrane of endoplasmic reticulum, and CPR shuttles electrons from NADPH to P450 [2].

Expression of *P450* genes is regulated at the transcriptional and post-transcriptional levels [3,4]. The latter includes mRNA stabilization and altered degradation of proteins [5]. Haem, as a prosthetic group of P450, also regulates the synthesis and degradation of P450s [6]. Non-physiological repression of several P450 isoenzymes occurs in hepatocyte cultures that otherwise would form an ideal model for drug-metabolism studies. Some compounds induce expression of *P450* genes or stabilize P450 protein, whereas immunostimulants such as interleukins and lipopolysaccharide are involved in the down-regulation of P450 in animals and cultured hepatocytes [7–9]. However, the mechanisms of the down-regulation of P450 by immunostimulants are still not well known.

Previously it has been shown by several groups that the levels of human P450 isoforms CYP2A6 [10], CYP2E1 [11] and CYP2D6 [12] that had been expressed in a baculovirus system were significantly reduced upon the expression of recombinant CPR. We observed a similar effect of CPR on human P450 isoform CYP3A4 in recombinant mammalian cell lines [13]. Furthermore, our study showed that CYP3A4 was not saturated with this ancillary factor even at a CPR/CYP3A4 ratio that was more favourable than that seen in liver [14]. The various *in vitro* studies suggested that CPR controls not only the activity but also the cellular level of P450s. Because the effects of CPR on P450 were seen with different isoforms in different expression systems, they are likely to be of physiological relevance. In the present investigation we attempted to elucidate the mechanism by which CPR controls the level of cellular P450. We also wanted to extend the significance of our findings with CYP3A4 in mammalian cells to another pharmacologically important P450 isoform, namely CYP2D6.

EXPERIMENTAL

Materials

DNA-restriction and -modification enzymes were purchased from Gibco-BRL, Life Technologies (Paisley, U.K.).

Abbreviations used: CPR, NADPH-P450 oxidoreductase; DHFR, dihydrofolate reductase; HO, haem oxygenase; HO-1, HO type 1; P450, cytochrome P450; MTX, methotrexate; CHO, Chinese hamster ovary.

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(±)Bufuralol and 1′-hydroxybufuralol standard were kindly provided by Hoffmann LaRoche (Basel, Switzerland) and rabbit anti-[haem oxygenase (HO)] serum was kindly provided by Dr Brian Murphy (SRI International, Menlo Park, CA, U.S.A.). BSA, sodium arsenite and hemin chloride were obtained from Sigma (Poole, Dorset, U.K.). Hemin chloride was dissolved in DMSO (unless otherwise noted) to make a stock solution of 5 mg/ml. Methotrexate (MTX) was from Lederle (Gosport, Hampshire, U.K.).

Construction of expression plasmid

To modify the 5′ end of CYP2D6 cDNA to a consensus sequence NNRNNAUGG required for initiation of eukaryotic protein biosynthesis [15]. PCR amplification was carried out using a forward primer A (5′-CCGGAATTCCGACCATGGGGCTA-GAAGCACTCGTG-3′; initiation codon underlined), and a reverse primer B (5′-GTGTTCTGGAAGTCCACATGC-3′; covering a unique *Xma*I site in CYP2D6 cDNA), and using wild-type CYP2D6 cDNA in plasmid pMP201 [12] as a template. The PCR product was digested with *Eco*RI and *Xma*I, and the 120 bp fragment was inserted into the *Eco*RI and *Xma*I sites of pMP201 to construct plasmid pL2D6-2. The modified CYP2D6 cDNA was then digested with *Xho*I and *Xba*I, and ligated into the *Xho*I/*Xba*I-digested mammalian expression vector pDHFR [13] to generate the plasmid pDHFR/2D6.

Cell culture, DNA transfection and establishment of cell lines

Cell culture and DNA transfection were carried out as described previously [13]. The Chinese hamster ovary (CHO) cell line DUKXB11 was used for the expression of the recombinant proteins. After transfection with expression plasmid pDHFR/2D6, cells were selected for the $DHFR^+$ phenotype (where DHFR is dihydrofolate reductase) by growth in nucleoside-deficient medium [16]. $DHFR^+$ clones were pooled, and grown on increasing concentrations of MTX (0.02 and 0.1 μM) for amplification of transfected CYP2D6 cDNA. Cell clones that survived on 0.1 μM MTX selection were isolated. Ten of the isolated colonies were further selected with 0.3 μM MTX. The resulting cell lines were analysed for CYP2D6 expression by immunoblotting. Subsequently, a cell line expressing a high level of CYP2D6 was stably transfected with plasmid pcDNA/HR [13], which contains a full-length human CPR cDNA, and selected with G418 (Gibco-BRL; 800 μg/ml) and MTX (0.3 μM). After isolation of resistant clones, the concentration of G418 was changed to 400 μg/ml. The homogeneity of the cell line was assured by repeated subcloning. The designation of the cell lines is as follows: DUKX, DUKXB11 parental cells; DUKX/DHFR, DUKXB11 cells transfected with plasmid carrying the DHFR selection marker; DUKX/2D6, DUKB11 cells transfected with a plasmid carrying the CYP2D6 cDNA; DUKX/CPR, DUKXB11 transfected with a plasmid carrying the CPR cDNA; DUKX/2D6/CPR, DUKX/2D6 cells subsequently transfected with the CPR cDNA; DUKX/2D6/CPR-3, a subclone of DUKX/2D6/CPR cells.

Spectral quantification of P450 in total cellular lysate

Cells in two 175 cm^2 tissue-culture flasks (at 60–70 % confluence) were grown in either the presence or absence of hemin (5 μg/ml) and DMSO (0.1 %, v/v; 0.14 M) for 1 day. Cells were harvested by trypsin treatment, resuspended in 1 ml of water and solubilized by adding 1 ml of a buffer containing 0.2 M Tris/HCl, pH 7.4, 10 mM CHAPS (Sigma), 2 mM EDTA and 20 % glycerol. After an aliquot of cell lysate was removed for protein determination, spectrally active P450 was determined as described in [13].

Immunochemical detection of CYP2D6, CPR, HO and ferritin

Cells were harvested and lysed by sonication as described in [13]. Proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane and probed as detailed previously [12]. Primary antibodies were rabbit anti-(human CYP2D6), anti-(human CPR) and anti-[human HO type 1 (HO-1)] [17]. Ferritin was detected by immunoblotting as described in [18] using an anti-(human ferritin) antibody (Sigma). Secondary antibody was horseradish peroxidase-linked donkey anti-rabbit antiserum. Immunoblots were developed using the enhanced chemiluminescence (ECL) Western-blotting kit (Amersham).

Determination of catalytic activities of CYP2D6 and CPR in cultured cells

Briefly, cells were seeded at a density of 4×10^5 cells/60 mm culture dish in 3 ml of culture medium without G418 and MTX and incubated at 37 °C for 2 days. Cells were cultured for 1 day and the test compound was added directly into the culture medium. Cells were then cultured for a further 24 h before adding (±)bufuralol (20 mM stock in water) into cultured medium to a final concentration of 30 μM. After 1 h of incubation at 37 °C, an aliquot of the medium (0.3 ml) was transferred to a polypropylene tube containing 15 μl of 60 % perchloric acid, mixed and kept on ice for 5 min. The mixture was centrifuged at 12000 ***g*** for 5 min and the supernatant was transferred to a micro-vial. Metabolites were separated by HPLC and 1′-hydroxybufuralol was quantified by comparison with an authentic standard [12]. Subsequently, cells were then harvested by trypsinization and lysed by sonication. Protein concentration was determined as described above. The bufuralol-1′-hydroxylase activity was expressed as pmol/min per mg of total cellular protein. Activity of CPR was measured in total cellular protein as described previously [13]; 1 unit of CPR activity corresponded to the amount needed to reduce 1 nmol of cytochrome *c* per min.

Determination of catalytic activities of CYP2D6 and CPR in a reconstituted system

His-tagged CYP2D6 and native CPR were expressed in *Escherichia coli* and purified as described in [19–21]. Purified CYP2D6 (50 pmol) was mixed with varying amounts of CPR in the presence of 20 μg of a 1:1 (w/w) mixture of L-α-dilauryl-phosphatidylcholine and L-α-dioleoylphosphatidylcholine. After incubation for 5 min at 20 °C, P450 activity was determined using the bufuralol-1′-hydroxylase assay.

Enzyme activity of HO

HO activity in cells was measured by determining the formation of bilirubin as described in [22]. Cells (2×10^7) were harvested by trypsin treatment and resuspended in 0.3 ml of 10 mM sodium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 2 mM dithiothreitol and 1 mM EDTA. Cells were lysed by freeze–thawing three times followed by sonication using an MSE Soniprep (two 5 s bursts at an amplitude of 12 μm with sample kept on ice). The cell lysate was centrifuged at 12000 ***g*** for 2 min at 4 °C. The supernatant was transferred to an Eppendorff tube, and the protein concentration was determined. A reaction mixture (400 μl) containing cellular lysate (1–2 mg), rat liver cytosol (2 mg) as a source of biliverdin reductase, hemin (30 μM), glucose-6-phosphate (2 mM), NADPH (1 mM) and glucose-6-

phosphate dehydrogenase (0.12 unit) was incubated at 37 °C for 1 h in the dark. Bilirubin was extracted twice with chloroform. The chloroform extracts were combined (1 ml) and the difference in absorbance at 464 nm and 530 nm was determined. A molar absorption coefficient of $0.04\ nM^{-1} \cdot cm^{-1}$ for bilirubin was used for calculation. The rate of bilirubin formation is expressed as pmol of bilirubin formed/h per mg of total cellular protein.

RESULTS

High levels of CPR are needed for CYP2D6 enzyme activity in mammalian cells

Initially a cell line expressing CYP2D6 alone, designated DUKX/2D6, was established by transfecting plasmid pDHFR/2D6 into DUKXB11 cells. To achieve a high expression of CYP2D6, the copy number of transfected CYP2D6 cDNA was amplified by stepwise selections with MTX (final concentration of 0.3 μM) as described in the Experimental section. Expression of CYP2D6 in DUKX/2D6 cells was analysed by immunoblotting (Figure 1). As a control, DUKX11 cells were stably transfected with empty vector pDHFR and selected with 0.3 μM MTX. This cell line was designed DUKX/DHFR. Western-blot assays with total cellular protein from cell lines DUKXB11 and DUKX/DHFR yielded a faint band with a slightly higher mobility than CYP2D6 detected in human liver microsomes (Figure 1, compare lanes 1 and 2 with lane 6). However, the DUKX/2D6 cell line yielded a strong signal with the same mobility as CYP2D6 in human liver microsomes. These results

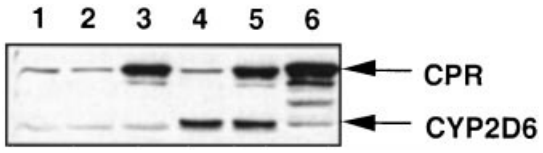

Figure 1 Immunoblot analysis of CYP2D6 and CPR proteins

Whole cell lysate was prepared from the recombinant cell lines as described in the Experimental section. Total cellular protein (20 μg) was electrophoresed on a SDS/10% polyacrylamide gel and transferred to a nitrocellulose membrane. CYP2D6 and CPR proteins were detected using both rabbit anti-CYP2D6 and anti-CPR IgGs. The lanes contained protein from the following cell lines: lane 1, DUKXB11; lane 2, DUKX/DHFR; lane 3, DUKX/CPR; lane 4, DUKX/2D6; lane 5, DUKX/2D6/CPR-3; lane 6, human liver microsomes (10 μg).

Table 1 P450 contents and activities of CYP2D6 and CPR in recombinant cell lines

P450 content was determined by Fe^{2+} versus Fe^{2+}-CO difference spectrophotometry in total cellular lysates. CPR activity was determined by measuring the reduction of cytochrome *c*. The CPR content was calculated from the turnover number of pure CPR towards cytochrome *c* ($3100\ min^{-1}$). Parental DUKXB11 cells did not contain spectrally detectable P450 (detection limit, approx. 2 pmol/mg of protein).

Cell line ...	DUKX/2D6	DUKX/2D6/CPR	DUKX/2D6/CPR-3
P450 content (pmol/mg)	23 ± 4	18 ± 1	8 ± 3
CPR activity (units/mg)	22 ± 3	145 ± 10	239 ± 21
CPR content (pmol/mg)	7	45	75
CPR/P450 ratio	0.3	2.5	9.4
CYP2D6 activity (pmol/mg per min)	147 ± 12	388 ± 52	439 ± 33
Catalytic-centre activity (min^{-1})	6.4	21.5	54.9

Table 2 Effects of radical scavengers and modulators of HO on spectrally active CYP2D6

Cell lines were cultured as described in the Experimental section. After addition of DMSO (0.1%, 0.14 M) and/or hemin (5 μg/ml, in 0.01% ammonia solution) the cells were cultured for an additional 24 h. Subsequently, total cellular lysate was prepared and used for the spectral determination of P450.

	CYP2D6 content (pmol/mg of protein)	
Treatment	DUKX/2D6	DUKX/2D6/CPR-3
None	23 ± 4	8 ± 3
DMSO	22 ± 3	27 ± 1
Hemin	Not determined	16 ± 4
DMSO + hemin	26 ± 3	49 ± 2

were also paralleled by the bufuralol-1′-hydroxylase activity, which was not detectable in either DUKXB11 or DUKX/DHFR cells but was moderate (147 ± 12 pmol/min per mg of total cellular protein) in DUKX/2D6 cells (Table 1).

As described previously [13], the level of endogenous CPR is low in DUKXB11 cells. Immunoblotting (Figure 1) showed that this was also true for the DUKX/DHFR and the DUKX/2D6 cell lines. The enzyme activity of CPR in DUKX/2D6 cells was only 22 units/mg, corresponding to 7 pmol of CPR/mg of total cellular protein (Table 1). To characterize the effect of CPR on the expression level and the enzyme activity of CYP2D6, human CPR cDNA was stably transfected into DUKX/2D6 cells. The resulting cell line was designated DUKX/2D6/CPR. Activity of CPR in this cell line was elevated about 7-fold to 145 units/mg of protein and the ratio of CPR to P450 was 2.5. Even though the CPR/P450 ratio in this cell line was already relatively high, a further increase of the ratio to 9.4 in cell line DUKX/2D6/CPR-3, a subclone of DUKX/2D6/CPR cells, led to a further increase in the catalytic-centre activity of CYP2D6 from 21.5 to $54.9\ min^{-1}$ (Table 1). Similarly, in a reconstituted system containing increasing amounts of purified CPR and a constant amount of CYP2D6 as well as phospholipids, half-maximal bufuralol-1′-hydroxylase activity was reached at a CPR to CYP2D6 ratio of 4. At a ratio of approx. 7 the P450 became fully saturated with CPR (results not shown).

Regulation of P450 homoeostasis by CPR

P450s are a superfamily of haemoproteins. Incorporation of haem is necessary for the formation of P450 holoenzyme, which can be quantified by Fe^{2+} versus Fe^{2+}-CO difference spectrophotometry. To analyse the effects of CPR on the level of CYP2D6 holoprotein, spectral quantification of CYP2D6 was carried out in detergent-solubilized cellular lysates of CHO cells. Importantly, it was found that, upon expression of CPR, the level of CYP2D6 was reduced to 30% of that found in cells without recombinant CPR (Table 1; compare P450 levels in DUKX/2D6 and DUKX/2D6/CPR-3 cells). However, this effect was not due to a decrease of CYP2D6 apoprotein, as determined by immunoblotting (Figure 1, compare lane 4 with lane 5).

To investigate the mechanism(s) by which CPR modulated the level of intracellular P450, we treated the recombinant cell lines with DMSO (0.1%, 0.14 M), which has been described to act as a scavenger of hydroxyl radicals [23]. Treatment for 1 day increased the level of spectrally active P450 in DUKX/2D6/CPR-3 cells 3-fold, whereas it did not alter P450 levels in DUKX/2D6 cells (Table 2). Similarly, the P450 level in DUKX/2D6/

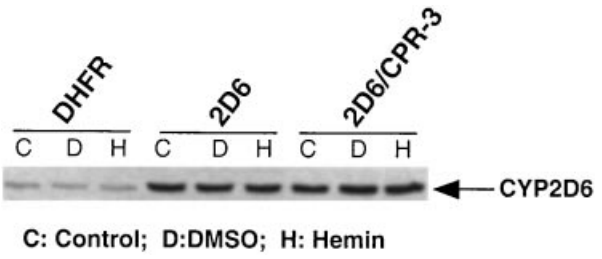

Figure 2 Effect of hemin and DMSO treatment on immunodetectable CYP2D6

Cells were treated with hemin (5 μg/ml) or DMSO (0.1%, 0.14 M) for 1 day prior to the preparation of whole cell lysate. CYP2D6 was detected by Western blot; 20 μg of total cellular protein per lane was analysed. Designation of cell lines is as in the text, but the prefix DUKX/ has been omitted.

CPR cells was also increased by treatment with hemin. DMSO and hemin acted either additively or synergistically, as treatment with these two compounds increased the level of spectrally active P450 by 6-fold (Table 2). However, this effect was again only seen in DUKX/2D6/CPR-3 cells, and not in DUKX/2D6 cells. Interestingly, even though DMSO and hemin increased the level of spectrally active CYP2D6 in the cell line that overexpressed CPR, treatment with these compounds did not alter the level of immunodetectable CYP2D6 (Figure 2).

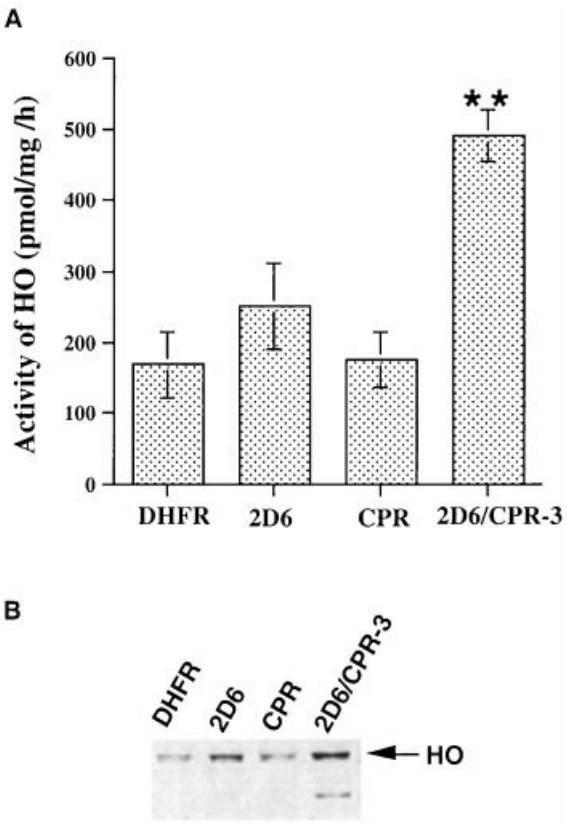

Figure 3 Analysis of HO protein and enzyme activity in cell lines

(**A**) Enzyme activity of HO was measured in different cell lines. Values represent the means $\pm$ S.E.M. of three independent determinations. **, significantly different ($P < 0.001$) from the HO activity in DUKX/DHFR cells. (**B**) Western-blot assay for HO protein expressed in the cell lines. Each lane contains 20 μg of total cellular protein. Designation of cell lines is as in the text, but the prefix DUKX/ has been omitted.

Table 3 Effects of DMSO and hemin on a sodium arsenite-induced decrease of spectrally active CYP2D6

Cells were treated with sodium arsenite (SA; 5 μM) in the presence or absence of either DMSO or hemin (concentrations as given in Table 2) for 24 h. Subsequently, spectrally active P450 was determined in cell lysates. ND, not detectable (limit of detection, approx. 2 pmol/mg of protein).

	CYP2D6 content (pmol/mg of protein)	
Treatment	DUKX/2D6	DUKX/2D6/CPR-3
None	40 $\pm$ 9	11 $\pm$ 4
SA	14 $\pm$ 8	ND
SA + DMSO	18 $\pm$ 6	13 $\pm$ 6
SA + hemin	40 $\pm$ 13	42 $\pm$ 8

Induction of HO by co-expression of CYP2D6 and CPR in CHO cells

Having established that hemin treatment prevented the CPR-induced decrease of spectrally active CYP2D6, we tried to characterize the underlying mechanism. It has been reported that HO catalyses the oxidation of haem to biliverdin via a CPR-dependent reaction [24]. Therefore, we investigated whether CPR modulates the level of immunodetectable HO-1 and its activity in the various cell lines. Compared with the basal level in DUKX/DHFR cells, HO activity and protein level were not changed significantly upon expression of CPR (Figure 3; compare DUKX/DHFR and DUKX/CPR cells). Expression of CYP2D6 (cell line DUKX/2D6) resulted in a clear increase in the level of HO-1 protein (Figure 3B) and a small but non-significant increase of HO activity (Figure 3A). Upon expression both of CPR and CYP2D6 (cell line DUKX/2D6/CPR-3), HO activity was increased 3-fold, which was also reflected by an increase of immunodetectable HO-1. These results suggested that increased levels of HO could be partially responsible for the lower level of spectrally active CYP2D6 in cells, which in addition to this P450 isoform express CPR.

Effects of modulators of HO on CYP2D6 levels

As shown above, hemin treatment led to an increase in spectrally active CYP2D6 in DUKX/2D6/CPR-3 cells (Table 3). However, we were able to show that hemin also increased the level of immunodetectable and enzymically active HO-1 (Figure 4B and Table 4). It should be noted that DMSO had no effect on the level of HO-1 in the recombinant cell lines (results not shown). To verify that HO is able to decrease the level of CYP2D6 in the recombinant cell lines, we induced HO-1 with sodium arsenite. As shown in Figure 4(B), this treatment led to a marked increase of immunodetectable HO-1 in DUKX/2D6 and DUKX/2D6/CPR-3 cells, which was paralleled by a marked increase of HO activity in both cell lines (Table 4). Concomitantly, this treatment reduced the level of spectrally active CYP2D6 in DUKX/2D6/CPR-3 cells to non-detectable levels (Table 3) but had no effect on the amount of immunodetectable CYP2D6 or CPR (Figure 4A). DMSO only slightly ameliorated the effects of sodium arsenite on spectrally active P450 in DUKX/2D6 cells,

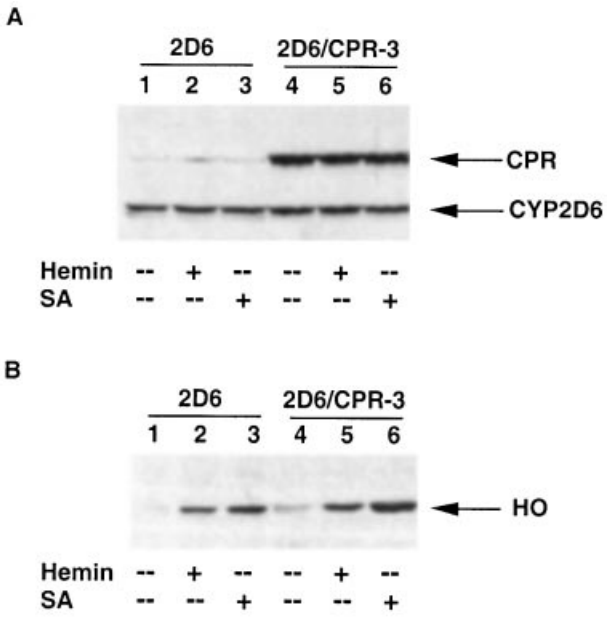

Figure 4 Effects of hemin or sodium arsenite on immunodetectable CYP2D6, CPR and HO-1

DUKX/2D6/CPR-3 and DUKX/2D6 cells were treated with either hemin (5 μg/ml) or sodium arsenite (SA; 5 μM) for 1 day prior to the preparation of total cell lysate. (**A**) Western-blot assay for both CYP2D6 and CPR proteins in cells with or without treatment. Each lane contains 20 μg of total cellular protein. (**B**) Western-blot analysis for HO protein. Designation of cell lines is as in the text, but the prefix DUKX/ has been omitted.

Table 4 Effects of modulators of HO on its activity in recombinant cell lines

The cell lines were treated with hemin (5 μg/ml) or sodium arsenite (5 μM) for 24 h. Subsequently, total cellular lysate was prepared and HO activity was determined as described in the Experimental section.

	HO activity (pmol of bilirubin/h per mg of protein)		
Cell line	No treatment	Hemin	Sodium arsenite
DUKX/DHFR	169 ± 46	821 ± 36	542 ± 51
DUKX/CPR	176 ± 38	1425 ± 66	4093 ± 1017
DUKX/2D6	251 ± 59	1588 ± 318	1400 ± 140
DUKX/2D6/CPR-3	490 ± 36	2894 ± 170	3931 ± 208

whereas it did so completely in DUKX72D6/CPR-3 cells (Table 3). In the presence of hemin, sodium arsenite had little effect on spectrally active P450 in DUKX/2D6 cells. In DUKX/2D6/CPR-3 cells, hemin even increased the level of spectrally active CYP2D6 by 4-fold, despite the presence of sodium arsenite (Table 3).

Degradation of either protein-bound or free haem upon induction of HO in the various cell lines was detected by determining the level of ferritin, which sequesters free iron. Whereas treatment with sodium arsenite had little effect on the level of ferritin in cells that did not express any recombinant protein or CPR only, it increased ferritin levels in DUKX/2D6/CPR-3 cells and to a much lesser extent in DUKX/2D6 cells (Figure 5). Treatment with hemin led to a marked increase of ferritin in all cell lines.

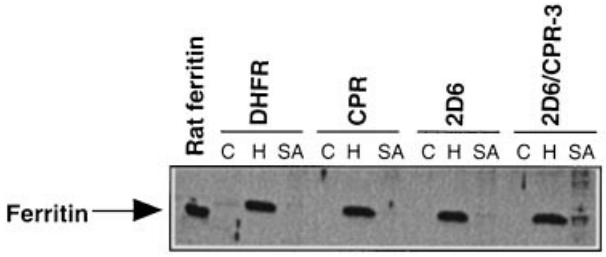

Figure 5 Effects of modulators of HO on ferritin levels in the recombinant cell lines

Cells were treated with either hemin (H; 5 μg/ml) or sodium arsenite (SA; 5 μM) for 1 day prior to preparation of total cellular lysate. Per lane, 20 μg of total cellular protein was loaded. Ferritin protein was detected by immunoblotting as described in the Experimental section. Designation of cell lines is as in the text, but the prefix DUKX/ has been omitted. C, control.

DISCUSSION

P450s catalyse the oxidative biotransformation of endogenous and exogenous substrates. Therefore, inhibition of P450 enzyme activity or repression of P450 holoenzyme would severely affect pharmacokinetic properties of drugs and prolong drug action. It has been found that CPR is essential for the catalytic activities of both HO and P450s. Therefore, CPR may also indirectly modulate the level of P450 holoprotein via HO-dependent haem degradation. Indeed, recent data have shown that co-expression of CPR with P450 results in a depression of P450 holoenzyme [5,10,11,13]; however, the mechanism responsible is still not known.

In the present study we investigated the effects of CPR on the post-translational regulation of P450 by evaluating the effect of recombinant human CPR on the level and the enzyme activity of CYP2D6 in CHO cells. Expression of recombinant CPR yielded cell lines which displayed a physiologically meaningful CPR activity similar to that found in human liver (200 units/mg of protein [14]). Despite this, the data presented in Table 1 show clearly that even at a CPR/CYP2D6 ratio of 2.5, the P450 is not yet saturated with this ancillary factor. It should be noted that the ratio of CPR to total P450 (comprising several P450 isoforms) in the liver is approx. 0.25 [14]. However, even though overexpression of CPR improved the CPR/P450 ratio in the recombinant cell lines, this was traded off against a decrease in the amount of spectrally active P450 (Table 1). This is in agreement with other studies, which had found that the levels of spectrally active CYP2A6 [10], CYP2D6 and CYP3A4 [12,13] were diminished upon expression of CPR in baculovirus and CHO expression systems. Importantly in the present study, we found that the repression of spectrally active CYP2D6 was not caused by a decrease of CYP2D6 apoprotein (Figure 1).

It is known that electrons can be transferred from NADPH via CPR and P450s to oxygen to form reactive oxygen species such as superoxide anion and hydrogen peroxide. This process has been termed uncoupling [25]. Hydrogen peroxide can yield hydroxyl radicals in a Fenton-type reaction. Some P450s, such as CYP2E1, have shown a high degree of uncoupling that resulted in damage to cellular components, including CYP2E1 [26,27]. To investigate whether a similar mechanism could explain the decrease of spectrally active P450 upon overexpression of CPR, we treated cells with the radical scavenger DMSO [23]. Importantly, this treatment increased levels of spectrally active CYP2D6 in DUKX/2D6/CPR-3 cells (Table 2) but not in cells that expressed only CYP2D6 (DUKX/2D6 cells). Interestingly,

radical-induced changes in the level of spectrally active CYP2D6 did not reduce the level of CYP2D6 apoprotein (Figure 2), which suggests that its half-life is similar to that of the holoenzyme. However, modification of CYP2D6 levels by reactive oxygen species is not the only mechanism by which CPR decreased the level of spectrally active P450 in the recombinant cell lines, since treatment with hemin also prevented the CPR-induced decrease of CYP2D6 levels (Table 2). Furthermore, hemin and DMSO acted either additively or synergistically to increase P450 levels in DUKX/2D6/CPR-3 cells but not in the cell line that expressed CYP2D6 only. This result suggested that upon overexpression of CPR either the cellular level of free hemin became limiting for P450 biosynthesis or that CPR either directly or indirectly caused degradation of the prosthethic haem group of CYP2D6. Because HO controls the degradation of hemin to biliverdin and has also been shown to degrade prosthethic haem in some but not all haemoproteins, we determined whether the expression of recombinant proteins (CYP2D6 or CPR) caused a change in the expression and the activity of endogenous HO. The activity of HO was highest in DUKX/2D6/CPR-3 cells, which also contained the lowest level of spectrally active CYP2D6 (Tables 1 and 4). It is highly likely that the form which was induced upon co-expression of CYP2D6 and CPR was HO-1, because only this enzyme and not HO-2 has been found to be inducible [18]. In addition, the antibody used in our studies was directed against purified HO-1 [17] and reacted mostly only with a single protein in immunoblotting experiments (Figures 3 and 4). HO-1 and HO-2 are separable by SDS/PAGE and their size difference is approx. 3 kDa [28], which is less than for the two proteins reacting with anti-HO-1 antibodies in DUKX/2D6/CPR-3 cells (Figure 3). The involvement of HO in the CPR-induced decrease of CYP2D6 was supported further by the observation that increased levels of HO after induction of this enzyme with sodium arsenite in DUKX/2D6/CPR-3 cells (Figure 4 and Table 4) also resulted in a marked decrease of spectrally detectable P450 (Table 3). Even though hemin itself also increased HO levels, this treatment did not decrease the levels of this P450, most probably because hemin as a substrate of HO acts as a competitive inhibitor to prevent degradation of haemoproteins by HO and/or replenishes intracellular hemin, which is degraded by the action of HO. Interestingly, DMSO also ameliorated the impact of sodium arsenite treatment on the level of spectrally active CYP2D6 (Table 3). There are two explanations for this: (i) sodium arsenite is known to generate oxygen radicals [23], and in the presence of DMSO the damaging effect of these on P450 would be prevented, or (ii) quenching of oxygen radicals by DMSO may have prevented the induction of HO-1, as has been reported [29], leading to less HO-1-mediated degradation of haem. We did not investigate these two possibilities. However, concerning the mechanisms by which CPR down-regulates spectrally active P450, it is important to note that DMSO treatment alone did not alter the levels of HO-1 (results not shown). It is therefore highly likely that the DMSO-induced increase of P450 levels in DUKX/2D6/CPR-3 but not in DUKX/2D6 cells (Table 2) was due directly to quenching of radical species derived from the action of CPR and not indirectly due to altered HO-1 levels.

In summary, our data show that activity of the pharmacologically important CYP2D6 is critically dependent on the level of the ancillary factor CPR. This suggests that the level of CYP2D6 as detected by immunological methods in tissues may be a poor indicator of CYP2D6 enzyme activity. Secondly, our data demonstrate that CPR regulates the level of the CYP2D6 holoenzyme in tissues by contributing indirectly to its degradation. However, other P450 isoforms also appear to be subject to this regulation, as shown by previous work, including ours [13]. Here we demonstrate that two mechanisms explain the down-regulation of P450 holoenzyme levels by CPR. One is most likely to be increased production of hydroxyl radicals, possibly by an uncoupled P450 reaction cycle, and the other is an increased activity of HO.

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