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Evidence That Cytochrome b_5 and Cytochrome b_5 Reductase Can Act as Sole Electron Donors to the Hepatic Cytochrome P450 System[S]

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ABSTRACT

We previously described the development of genetic models to study the in vivo functions of the hepatic cytochrome P450 (P450) system, through the hepatic deletion of either cytochrome P450 oxidoreductase [POR; HRN (hepatic reductase null) line] or cytochrome b_5 [HBN (hepatic cytochrome b_5 null) line]. However, HRN mice still exhibit low levels of mono-oxygenase activity in spite of the absence of detectable reductase protein. To investigate whether this is because cytochrome b_5 and cytochrome b_5 reductase can act as the sole electron donor to the P450 system, we crossed HRN with HBN mice to generate a line lacking hepatic expression of both electron donors (HBRN). HBRN mice exhibited exacerbation of the phenotypic characteristics of the HRN line: liver enlargement, hepatosteatosis, and increased expression of certain P450s. Also, drug metabolizing activities in vitro were further reduced relative to the HRN model, in some cases to undetectable levels. Pharmacokinetic studies in vivo demonstrated that midazolam half-life, C_{max}, and area under the concentration-time curve were increased, and clearance was decreased, to a greater extent in the HBRN line than in either the HBN or HRN model. Microsomal incubations using NADPH concentrations below the apparent K_m of cytochrome b_5 reductase, but well above that for POR, led to the virtual elimination of 7-benzyloxyquinoline turnover in HRN samples. These data provide strong evidence that cytochrome b_5/cytochrome b_5 reductase can act as a sole electron donor to the P450 system in vitro and in vivo.

Introduction

The cytochrome P450 (P450) monooxygenases comprise 70 to 80% of phase I xenobiotic metabolizing enzymes and are critical players in protecting organisms against damage from chemical insult, as well as maintaining cellular function and homeostasis through involvement in processes including steroidogenesis, bile acid production, cholesterol biosynthesis, vitamin D pathway, prostacyclin biosynthesis, long chain fatty acid and eicosanoid signaling, inflammatory pathways, and brain neurotransmitter synthesis (Romano et al., 1987; Nebert and Russell, 2002; Prosser and Jones, 2004; Miller, 2005; Ferguson and Tyndale, 2011).

More than 80% of prescribed drugs undergo P450-mediated phase I metabolism (Eichelbaum et al., 2006). Favorable absorption, distribution, metabolism, and excretion characteristics are critical determinants in the progression of new chemical entities (NCEs) through the drug development pipeline, and preclinical absorption, distribution, metabolism, and excretion uses both in vitro and in vivo models; however P450 metabolism is usually established solely by the in vitro route. Adverse drug reactions (ADRs), which are often associated with P450-dependent metabolism and are among the top five causes of drug-related deaths in the United States, are a major problem for the pharmaceutical industry. The root causes of ADRs fall into three groups: drug-drug interactions, reactive metabolite formation, and genetic polymorphisms of individual P450s (Eichelbaum et al., 2006). In humans, polymorphisms in genes such as *CYP2C9*, *2C19*, and *CYP2D6* lead to significant interindividual differences in both systemic drug exposure and prodrug activation in the patient population, and this can have repercussions including reduced efficacy and a range of ADRs (Johansson and Ingelman-Sundberg, 2011). Characterization of the metabolic fate of NCEs, allowing the re-engineering of efficacious molecules to design-out metabolic liabilities, is therefore of prime importance in the early preclinical phase of development.

Mammalian microsomal P450s function by catalyzing the insertion of one atom of molecular oxygen into a substrate molecule while reducing the other atom to water, a reaction

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ABBREVIATIONS: ADR, adverse drug reaction; AUC, area under the concentration-time curve; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; BQ, 7-benzyloxyquinoline; BR, benzyloxyresorufin; C_{max}, maximum plasma concentration; Cyb5, cytochrome b_5; Cyb5R, cytochrome b_5 reductase; HBN, hepatic cytochrome b_5 null; HRN, hepatic reductase null; HBRN, hepatic cytochrome b_5 and cytochrome P450 reductase null; LC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; MR, methoxyresorufin; NCE, new chemical entity; P450, cytochrome P450; POR, cytochrome P450 oxidoreductase; WT, wild-type.

that requires two electrons. Two proteins can transfer these electrons to the P450: the multidomain flavoprotein NADPH-cytochrome P450 oxidoreductase (POR), which has traditionally been attributed as providing the first (and often the second) electron (Paine et al., 2005), and cytochrome b_5 reductase (Cyb5R), which can also supply the second electron [via Cyb5 (cytochrome b_5)].

Studies aiming to elucidate the role of Cyb5 in P450-mediated metabolism using reconstituted systems in vitro have generated results that are difficult to interpret, so there is a clear need for a model that can provide information regarding interactions between P450s, POR, and Cyb5 in vivo and their respective roles in drug disposition. To that end, we and others have generated and characterized a conditional hepatic model of POR deletion—hepatic reductase null (HRN) (Gu et al., 2003; Henderson et al., 2003)—and an inducible conditional deletion in liver and gastrointestinal tract (Finn et al., 2007) as well as two models where Cyb5 has been either conditionally deleted in the liver [hepatic b_5 null (HBN)] (Finn et al., 2008) or knocked out globally (McLaughlin et al., 2010; Finn et al., 2011). These models have been used to investigate the P450 metabolism and resulting toxicity of many drugs and environmental carcinogens (Arlt et al., 2005, 2006, 2008, 2011; Pass et al., 2005; Stiborova et al., 2005, 2008; Finn et al., 2007, 2008; McLaughlin et al., 2010; Levova et al., 2011; Potega et al., 2011). Deletion of POR had a major effect on both in vitro and in vivo metabolism although in vitro P450 activity was not completely ablated (10% residual activity) (Gu et al., 2003; Henderson et al., 2003). We also demonstrated that deletion of Cyb5 can profoundly affect P450 metabolism in a tissue- and substrate-dependent manner (Finn et al., 2008; McLaughlin et al., 2010).

These data raised two questions: is the residual P450 activity observed in the HRN animals being driven by Cyb5/Cyb5R, and can the deletion of Cyb5 on a POR null background circumvent this? To address these questions we have generated a conditional hepatic POR and Cyb5 knockout mouse: hepatic b_5 reductase null (HBRN). Here we describe the initial characterization of these animals with respect to in vitro and in vivo P450 activity. The data presented provide evidence that Cyb5/Cyb5R can function as sole electron donors to the cytochrome P450 system in vivo.

Materials and Methods

Chemicals. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Poole, UK). NADPH was obtained from Melford Laboratories (Ipswich, UK). We purchased 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin, 7-benzyloxyquinoline (BQ), and hydroxy-tolbutamide from BD Gentest (Cowley, UK). Midazolam, 1-hydroxy midazolam, and 4-hydroxy midazolam were kind gifts from Roche (Burgess Hill, West Sussex, UK), and 1-hydroxy metoprolol and O-desmethyl-metoprolol were generous gifts from Astra Häsle (Mölndal, Sweden). Bupropion and hydroxybupropion were purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Generation of Hepatic Microsomal Cytochrome b_5 and Cytochrome P450 Oxidoreductase Null Mice. Hepatic reductase null (HRN [$POR^{lox/lox}$::Cre^{ALB}]) and hepatic Cyb5 null (HBN [$Cyb_5{}^{lox/lox}$::Cre^{ALB}]) animals were generated as described previously elsewhere (Henderson et al., 2003; Finn et al., 2008). HBRN ($Cytb_5{}^{lox/lox}$::$POR^{lox/lox}$ ± Cre^{ALB}) and wild-type (WT [$POR^{lox/lox}$::$Cyb_5{}^{lox/lox}$]) were generated by crossing the appropriate lines and thereafter maintained by crossing of homozygous pairs within each line. All lines used in this study were C57BL/6J (N6). All mice were kept under standard animal house conditions, with free access to food and water, and a 12-hour light/dark cycle. All animal work was performed on male 10-week-old mice in accordance with the Animal Scientific Procedures Act of 1986 and after local ethics review.

Preparation of Hepatic Microsomal Fractions. Microsomes were prepared from snap-frozen liver tissue harvested from 8- to 10-week-old male mice (five per genotype), as described previously elsewhere (Finn et al., 2008). Microsomes were stored at −70°C until required. Microsomal protein concentrations were determined using the Biorad Protein Assay Reagent (Bio-Rad Laboratories Ltd., Hertfordshire, UK). POR activity was estimated by NADPH-dependent cytochrome *c* reduction (Strobel and Dignam, 1978). The P450 content of mouse liver microsomes was determined by reduced carbon monoxide difference spectroscopy using the method of Omura and Sato (1964). The Cyb5 content was determined spectrophotometrically as described previously elsewhere (McLaughlin et al., 2010).

Immunoblotting. Western immunoblot analysis was performed as described previously elsewhere (Finn et al., 2008). Immunoreactive proteins were detected using polyclonal goat anti-rabbit, anti-mouse, or anti-sheep horseradish peroxidase immunoglobulins as secondary antibodies (Dako, Ely, UK), and were visualized using the Immobilon chemiluminescent substrate (Millipore, Watford, UK) on a LAS-3000 mini-imaging system (Fujifilm UK Ltd., London, UK). The densitometric analysis was performed using Multi Gauge V2.2 software (Fujifilm UK Ltd.).

In Vitro Fluorogenic Assay Incubations. Assays were performed essentially as described previously elsewhere (Finn et al., 2008) using 20 μg hepatic microsomes and 7-benzyloxy-4-trifluoromethylcoumarin (BFC; 40 μM); benzyloxyresorufin (BR) and methoxyresorufin (MR) (1 μM); 7-benzyloxyquinoline (BQ; 80 μM), and NADPH as a cofactor at a final concentration of 1 mM. Reactions were measured for 3 minutes at the recommended excitation and emission wavelengths for each probe by use of a Fluoroskan Ascent FL fluorometer (Labsystems, Basingstoke, Hampshire, UK). The turnover rates were calculated by use of authentic metabolite standards with the exception of BQ (7-hydroxy-4-trifluoromethylcoumarin for BFC; and resorufin for MR and BR).

In Vitro Probe Substrate Incubations. Midazolam, tolbutamide, and metoprolol assays were performed in triplicate for five samples per genotype, as described previously elsewhere (Finn et al., 2008), using midazolam (50 μM), tolbutamide (800 μM), and metoprolol (240 μM). Assays were allowed to proceed for 30 minutes before being stopped by the addition of 1 volume of ice-cold acetonitrile and ice incubation for 10 minutes. Samples were centrifuged for 8 minutes at 16,000*g* to remove particulate material before analysis by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The turnover was calculated based on authentic metabolite standards.

Bupropion assays were performed using 20 μg of hepatic microsomes and 250 μM substrate. Microsomal incubations were analyzed by LC-MS/MS using a Waters 2795 HPLC and Quattro Micro mass spectrometry system in positive electrospray ionization mode (Waters, Milford, MA). Multiple reaction monitoring data were acquired. The cone voltage and collision energy were optimized for each product (bupropion: cone voltage 28, collision energy 18, transition 240.43 > 184.26; 6-hydroxybupropion: cone voltage 26, collision energy 18, transition 256.40 > 238.31). A dwell time of 0.05 seconds was used between multiple reaction monitoring transitions. Bupropion and 6-hydroxybupropion were resolved in 2 minutes on an Acquity UPLC BEH C18 column, 2.1 × 75 mm, 1.7 μm column (Waters). The injection volume was 5 μl. The following elution program was used at a temperature of 30°C and a flow rate of 0.5 ml/min: eluent A, formic acid; eluent B, acetonitrile containing 0.1% formic acid; (1) 5% B held for 0.3 minutes, (2) linear gradient to 45% B over 0.35 minutes then held for a further 0.35 minutes, (3) linear gradient to 95% B in 0.05 minutes, held for 0.25 minutes, and (4) re-equilibration at 5% B for 0.25 minutes.

Midazolam Pharmacokinetics. WT, HBN, HRN, and HBRN mice ($n = 5$ for each genotype) were dosed orally with midazolam (2.5 mg/kg). Blood samples (10 μl) were taken from the tail vein of each mouse at 10, 20, 40, 60, 120, 240, 360, and 450 minutes after dosing. Midazolam analysis by LC-MS/MS and pharmacokinetic modeling was performed as described previously elsewhere (Finn et al., 2008). The data shown represent the mean ± S.E.M.

Results

Phenotype of HBRN ($Cyb_5^{lox/lox}$::$POR^{lox/lox}$::Cre^{ALB}) Mice. To establish the effect of simultaneous hepatic deletion of both microsomal Cyb5 and POR, we generated a mouse line with conditional knockouts of both genes. Mice lacking hepatic microsomal Cyb5 and POR were viable and exhibited no gross anatomic abnormalities. Both sexes were fertile, and offspring were born at expected Mendelian ratios. Postmortem examination of HBRN animals revealed that all tissues except the liver were normal in appearance. The livers of HBRN animals were pale, mottled, and enlarged relative to those of WT mice of the same genetic background, a phenotype indistinguishable from that observed in HRN mice (Henderson et al., 2003). When liver-to-body-weight ratios were calculated, those of the HRN and HBRN mice were significantly higher than those of the WT mice, both being increased by almost 50% (Fig. 1A). No change in the liver-to-body-weight ratio was observed in the HBN mice relative to the controls.

Liver sections stained with hematoxylin and eosin (Fig. 1B) indicated a progressive increase in lipid accumulation across the genotypes. The livers of HBN mice were only mildly affected, having a mottled appearance with pale areas surrounding the central veins, whereas the remainder of the liver parenchyma was normal in color. This appearance was more marked, being associated with centrilobular vacuolation in the HRN line; the phenotype of HBRN liver exhibited extensive vacuolation, with large vacuoles that were often larger than the size of an individual hepatocyte. This microvesicular and macrovesicular hepatic lipidosis is typical of steatotic liver.

Analysis of P450-Dependent Monooxygenase Components. As described previously elsewhere (Henderson et al., 2003; Finn et al., 2008), hepatic P450 concentrations measured by Fe^{2+}-CO versus Fe^{2+} difference spectra were significantly elevated (2.6-fold) in HRN animals compared with WT but unchanged in HBN (Fig. 1C). The increased expression observed in HBRN animals was similar to that seen in the HRN line, approximately 2.3-fold. The concentration of hepatic microsomal Cyb5 was reduced by 85 and 88% in HBN and HBRN livers, respectively, but interestingly was increased 2.7-fold in HRN livers (Fig. 1C). Hepatic POR activity, measured using cytochrome *c* as a surrogate electron acceptor, was unchanged in HBN animals compared with WT and undetectable in both HRN and HBRN livers (Fig. 1D). These results suggest that conditional deletion of hepatic POR leads to an increase in Cyb5 concentration whereas

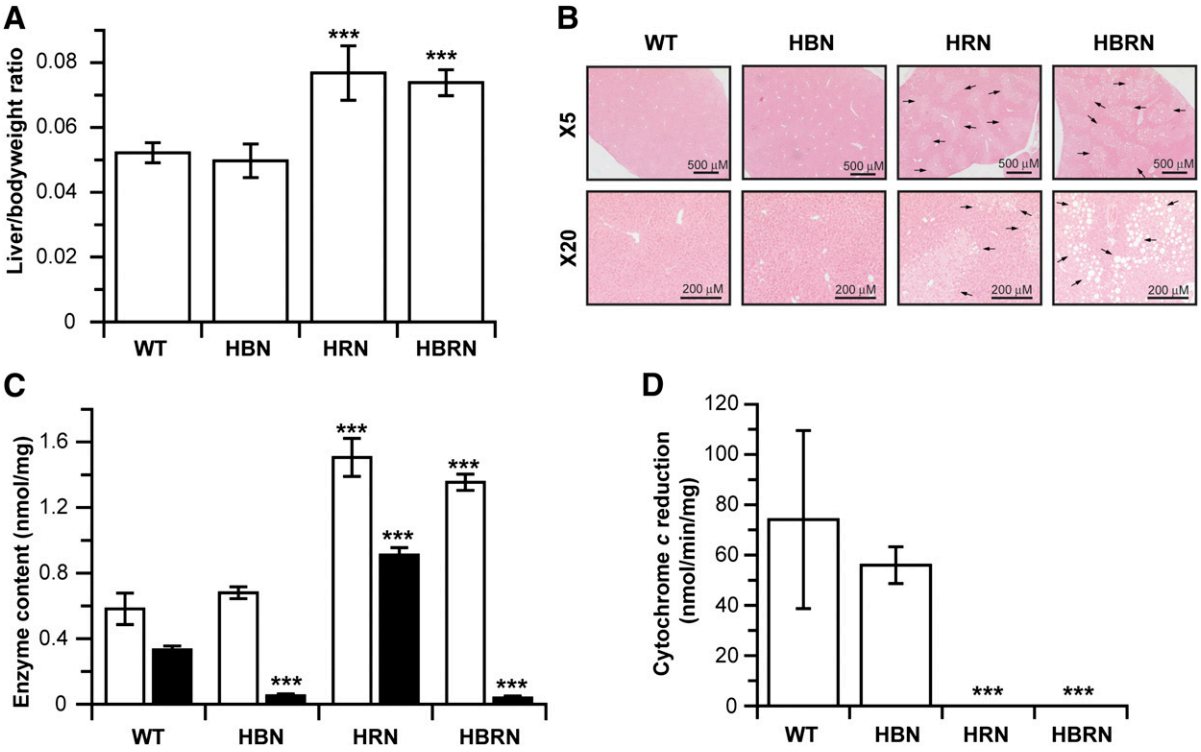

Fig. 1. Hepatic phenotype of HBRN mice compared with WT, HBN, and HRN. (A) Liver-to-body-weight ratio of WT, HBN, HRN, and HBRN mice (mean ± S.D.; $n = 5$ animals per genotype). (B) Representative hematoxylin and eosin stained sections of liver from WT, HBN, HRN, and HBRN mice. Original magnification: 5× (top row) and 20× bottom row). Arrows indicate areas of lipid deposition. (C) P450 (white bars) and cytochrome b_5 (black bars) content of hepatic microsomes from WT, HBN, HRN, and HBRN mice, measured by difference spectroscopy (mean ± S.D.; $n = 5$ animals per genotype). Statistical significance (comparing knockout animals to WT): ***$P \leq 0.001$. (D) POR activity of hepatic microsomes from WT, HBN, HRN, and HBRN mice as measured using cytochrome *c* as a surrogate electron acceptor (nmol/min/mg microsomal protein, mean ± S.D.; five animals per genotype, assayed in duplicate). Statistical significance (comparing knockout animals to WT): ***$P \leq 0.001$.

deletion of hepatic Cyb5 has no corresponding effect on POR expression.

Western blot analysis of hepatic Cyb5, POR, and P450 proteins in the various mouse lines is shown in Fig. 2, and the relative fold changes are shown in Table 1. Cyb5 and POR were not detectable in the HBN and HRN lines, and both proteins were absent from HBRN liver. Consistent with the data shown in Fig. 1, Cyb5 levels were increased approximately 3-fold in HRN liver, but conditional deletion of Cyb5 in the HBN liver had no corresponding effect on POR expression (Fig. 2).

As reported elsewhere, the expression of Cyp2b10 was increased in HRN mice, and indeed in all three conditional knockouts (2.3-fold, 8.6-fold, and 12.9-fold in the HBN, HRN, and HBRN, respectively; $P \leq 0.005$ in each case) (Table 1). The greater increase in expression in the HBRN line suggests a possible additive effect when both POR and Cyb5 are deleted ($P \leq 0.05$), but the interindividual variation in expression makes it difficult to evaluate this possibility. Cyp3a expression was also induced in the HBN, HRN, and HBRN lines (1.8, 2.6-, and 2.8-fold, respectively; $P \leq 0.005$); Cyp7a expression was also induced (14- to 15-fold; $P \leq 0.005$) in the HRN and HBRN lines. Conditional deletion of POR and/or Cyb5 had no effect on the hepatic expression of Cyp1a in any of the models (Fig. 2). The expression of Cyp2c, Cyp2d, Cyp2e, and Cyp4a proteins was slightly induced by these deletions (less than 2.5-fold).

In Vitro P450 Activities. In vitro P450 activities in hepatic microsomes from WT, HBN, HRN, and HBRN mice were determined using a panel of eight substrates, four of which are commonly designed as probes for specific P450s (Fig. 3A) and the other four being clinically used drugs (Fig. 3B). Deletion of hepatic Cyb5 had no effect on the O-dealkylation of BR and MR (Fig. 3A) but caused a significant, substrate-dependent reduction in turnover for five out of the eight substrates examined (BFC, BQ, midazolam, metoprolol, and tolbutamide) (Fig. 3, A and B). Interestingly, the rate of hydroxylation of bupropion by HBN liver microsomes was actually increased relative to the controls, possibly due to the increased levels of Cyp2b10. As observed previously, marked reduction of all these activities was observed in HRN liver microsomes. Importantly, in the HBRN line, the activities were further reduced (except in the case of tolbutamide, but the activities in both HRN and HBRN lines were very low for this substrate).

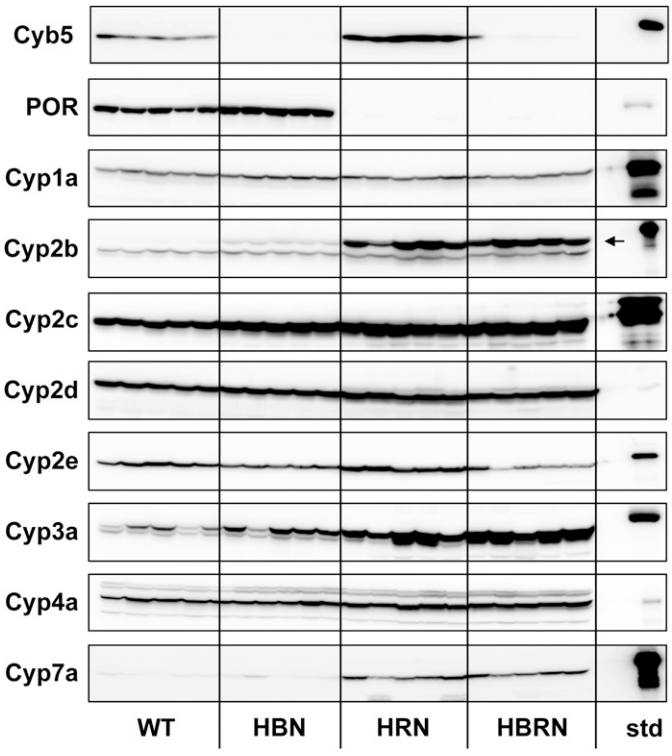

Fig. 2. Cytochrome b_5, cytochrome P450 oxidoreductase, and cytochrome P450 expression in livers from WT, HBN, HRN, and HBRN mice. Detection of hepatic expression of drug metabolizing enzymes by Western immunoblotting of individual microsomal samples (20 μg hepatic microsomes per lane). Further experimental details are given in the *Materials and Methods*. On Cyp2b blot, arrow indicates Cyp2b10 band. std, standard, recombinant P450 protein.

In general, two distinct patterns of effect were observed (Fig. 3A). Deletion of Cyb5 alone had no marked effect on microsomal activity toward MR and BR whereas deletion of POR almost abolished the corresponding activity. In contrast, deletion of either Cyb5 or POR markedly reduced activity toward BQ and BFC, and deletion of both genes had an additive effect, suggesting that both Cyb5 and POR are necessary for the metabolism of these substrates. These data suggest that the residual microsomal activity was at least in part due to cytochrome b_5 donating electrons directly into the P450 system.

The pattern of effects on the metabolism of drugs in clinical use generally reflected those observed with BQ and BFC (Fig. 3B). Deletion of either Cyb5 or POR, in spite of the increased expression of a number of P450 isozymes, significantly reduced the rate of metabolism of metoprolol (α-hydroxylation and O-demethylation), midazolam (1′- and 4-hydroxylation), and tolbutamide, while conditional deletion of both genes in the HBRN led to near-complete abolition of activity.

These results suggest that both Cyb5 and POR are required for maximal metabolism of these substrates, although the consequences of a single-gene deletion of POR were more severe than those of deletion of Cyb5 alone. Indeed, in the cases of metoprolol and tolbutamide, activity was almost entirely absent in HRN microsomes, with or without additional deletion of Cyb5, suggesting the electron transfer was entirely POR dependent.

TABLE 1

Relative changes in Cyb5, POR, and cytochrome P450 protein expression in WT, HBN, HRN, and HBRN livers

Statistical significance is shown for HBRN, HRN, or HBN compared with WT, and between HRN and HBRN; $n = 5$ for each genotype.

Protein	Fold Change Compared with WT			
	WT	HBN	HRN	HBRN
Cyb5	1 ± 0.2	0.004 ± 0.005*	3.1 ± 0.8*	0.2 ± 0.4*#
POR	1 ± 0.1	1.2 ± 0.08*	0.01 ± 0.01*	0.01 ± 0.01*
Cyp1a	1 ± 0.1	1.1 ± 0.06	1.1 ± 0.07	1.0 ± 0.05
Cyp2b10	1 ± 0.2	2.3 ± 0.2*	8.6 ± 2.0*	12.9 ± 2.5*&
Cyp2b	1 ± 0.2	1.4 ± 0.1†	1.9 ± 0.3†	2.4 ± 0.4*
Cyp2c	1 ± 0.05	1.6 ± 0.1*	1.7 ± 0.2*	1.6 ± 0.1*&
Cyp2d	1 ± 0.003	1.2 ± 0.002†	1.3 ± 0.004†	1.3 ± 0.001†
Cyp2e	1 ± 0.2	0.8 ± 0.05‡	1.4 ± 0.2‡	0.7 ± 0.2#‡
Cyp3a	1 ± 0.3	1.8 ± 0.4†	2.6 ± 0.4*	2.8 ± 0.2*
Cyp4a	1 ± 0.1	1.2 ± 0.2	1.3 ± 0.06†	1.4 ± 0.08*
Cyp7a	1 ± 0.4	1.7 ± 0.9	14.2 ± 3.8*	14.7 ± 2.7*

* Compared with WT, $P \leq 0.001$.
\# Compared with HRN, $P \leq 0.001$.
& Compared with HRN, $P \leq 0.05$.
† Compared with WT, $P \leq 0.01$.
‡ Compared with WT, $P \leq 0.05$.

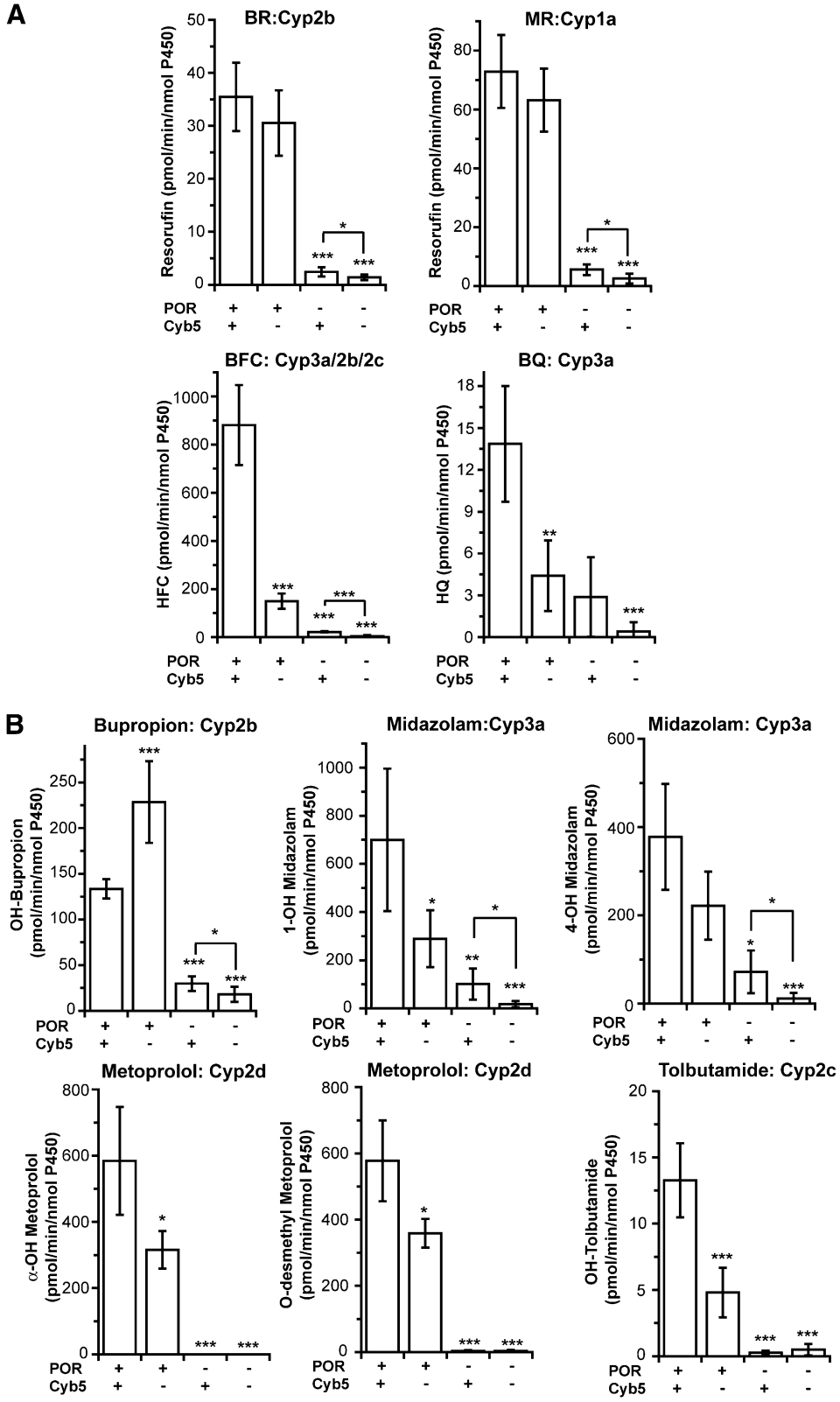

Fig. 3. Comparison of cytochrome P450-mediated in vitro metabolism using hepatic microsomes from WT, HBN, HRN, and HBRN mice. In vitro assays were performed as described in *Materials and Methods*. Assays were performed in triplicate on liver microsomes from five mice of each genotype, with NADPH as a cofactor at a final concentration of 1 mM. All data shown represent mean ± S.D. for five mice per group assayed in triplicate. Statistical significance: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$. (A) Benzyloxyresorufin dealkylation (BR; Cyp2b), methoxyresorufin dealkylation (MR; Cyp1a), 7-benzyloxy-4-trifluoromethylcoumarin dealkylation (BFC; Cyp3a/2b/2c), and 7-benzyloxyquinolone dealkylation (BQ; Cyp3a). (B) Bupropion 6-hydroxylation (Cyp2b), midazolam 1′- and 4-hydroxylation (Cyp3a), metoprolol α-hydroxylation and O–demethylation (Cyp2d), and tolbutamide hydroxylation (Cyp2c).

The exception to this pattern was bupropion (Fig. 3B). Hydroxylation of this substrate was actually increased in HBN liver microsomes ($P \leq 0.005$) relative to the activity observed in WT liver, although it was significantly reduced in HRN microsomes ($P \leq 0.005$) and further compromised in HBRN microsomes ($P \leq 0.05$).

The above data are presented as reaction rates normalized against total P450 concentration. When the activities were expressed per milligram of microsomal protein (Supplemental Table 1), the effects were still observed but were less pronounced. Whether this represents the existence of homeostatic mechanisms that modulate P450 expression

in an attempt to maintain total activities remains to be established.

In Vivo Pharmacokinetics of Midazolam. To determine whether deletion of Cyb5 together with POR further altered drug metabolism in vivo, the pharmacokinetics of orally administered midazolam were determined in WT, HBN, HRN, and HBRN mice. The elimination profiles of midazolam disappearance differed between WT and HBN mice, translating into significant alterations of pharmacokinetic parameters that were exacerbated in a genotype-dependent fashion (HBRN>HRN>HBN) (Fig. 4; Table 2). Profound changes in the pharmacokinetics were observed, with the half-life of midazolam extended by 1.2-, 2-, and 2.4-fold relative to WT in the three conditional knockout lines, respectively; the maximal plasma concentration (C_{max}) was increased by 3.3-, 5.5-, and 6.7-fold, and the clearance was decreased by 85, 95, and 97%. Midazolam exposure, as measured by the area under the concentration-time curve (AUC), showed an 8.5-, 19.6-, and 29-fold increase in the HBN, HRN, and HBRN models, respectively, compared with WT (Table 2). Furthermore, there was a significant increase in midazolam AUC between HRN and HBRN mice, although the observed increase in half-life between those two genotypes failed to reach statistical significance ($P = 0.06$).

To determine whether the electrons required for the residual P450-mediated activity observed in HRN liver microsomes were supplied by the (extremely low) levels of POR expressed in this line or by Cyb5R/Cyb5, we characterized the in vitro kinetics of POR in WT liver microsomes. This analysis indicated that murine hepatic POR has an apparent K_m for NADPH of 2.9 μM (Fig. 5A). The literature indicates that although Cyb5R is classed as NADH dependent, it does also have a low affinity for NADPH (K_m ~1 mM) (Roma et al., 2006). The in vitro assays illustrated in Fig. 3 were performed in the presence of 1 mM NADPH, meaning that the necessary electrons could be coming either from the residual POR or from Cyb5R/Cyb5.

The large difference in affinity for NADPH between the two enzymes was exploited to determine which enzyme is driving the reaction (Fig. 5B). If the P450-mediated activity in the HRN samples was driven by residual POR, titration of NADPH concentration from 1 mM down to 50 μM should have little effect on its rate, but if it is driven by Cyb5R, one would predict a significant reduction in turnover. In WT liver microsomes (containing both POR and Cyb5), reducing the concentration of NADPH down to 50 μM caused little change in the rate of BQ turnover because the lowest concentration tested was still significantly higher than K_m (2.9 μM). This is consistent with the role of POR as the electron donor in WT liver. In HRN samples, however, the activity observed was strongly dependent on the concentration of NADPH over the concentration range from 50–1000 μM. Reduction of the NADPH concentration to 50 μM caused a 94% reduction in BQ turnover (Fig. 5B). When NADH was used with HRN liver microsomes, there was little change in BQ turnover above a concentration of 100 μM, but a rapid falloff below that point consistent with the involvement of Cyb5R (K_m for NADH estimated at ~12 μM) as the source of electrons (Fig. 5B, inset). These data provide strong evidence that, in the absence of measurable levels of POR, electrons can be supplied to P450s from Cyb5R/Cyb5, rather than from residual hepatic POR.

Discussion

We previously used the HRN mouse line, a conditional hepatic model of POR deletion that profoundly reduces hepatic P450 activity (Henderson et al., 2003), to dissect the role of this enzyme system in the metabolism, disposition, and toxicity of drugs and foreign chemicals. It is important to note that the HRN model represents a genetic deletion of POR, with >99% of hepatocytes being negative on immunohistochemistry (Pass et al., 2005). However, despite the complete absence of detectable hepatic POR in this model, measurable

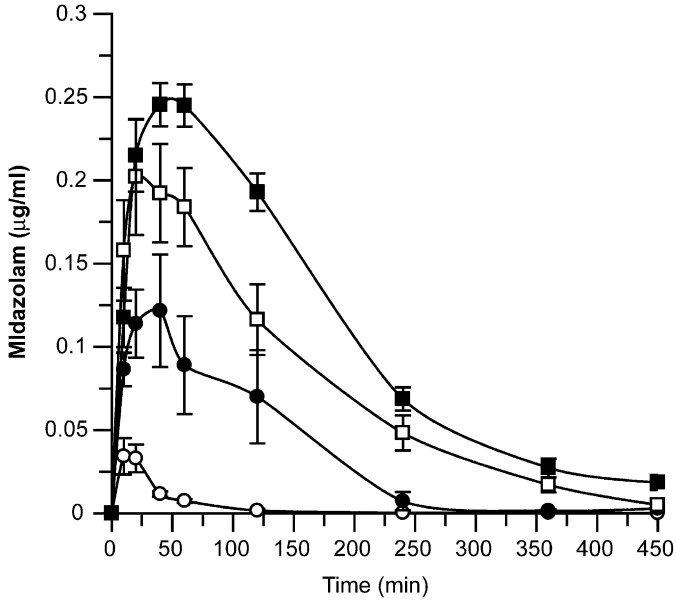

Fig. 4. In vivo pharmacokinetic profiles of midazolam in WT, HBN, HRN, and HBRN mice. Midazolam was administered orally to WT, HBN, HRN, and HBRN mice ($n = 5$ per group) at a dose of 2.5 mg/kg. ○ WT; ● HBN; □ HRN; ■ HBRN.

TABLE 2
Midazolam disposition in WT, HBN, HRN, and HBRN mice: comparison of pharmacokinetic parameters

	Half-Life	C_{max}	$AUC_{0\rightarrow 450\ min}$	AUC_{∞}	Clearance
	min	*μg/ml*	*min·μg/ml*	*min·μg/ml*	*ml/min/kg*
WT	37.6 ± 23.6	0.04 ± 0.02	1.73 ± 0.53	1.76 ± 0.52	1541 ± 505
HBN	43.9 ± 7.2	0.13 ± 0.07*	14.9 ± 11.6*	15.0 ± 11.7*	263 ± 126[&]
HRN	74.5 ± 19.6[#]	0.22 ± 0.07[&]	34.0 ± 11.4[&]	34.5 ± 11.4[&]	82.5 ± 40.4[&]
HBRN	89.2 ± 21.9[#]	0.26 ± 0.02[&]	48.4 ± 4.10[&†]	51.3 ± 5.66[&†]	49.5 ± 5.09[&]

* Compared with WT, $P \leq 0.05$.
[&] Compared with WT, $P \leq 0.001$.
[#] Compared with WT, $P \leq 0.01$.
[†] Compared with HRN, $P \leq 0.05$.

P450-dependent enzyme activities could still be detected in HRN liver microsomes (Henderson et al., 2003). In this study, we have interbred the HRN line with mice lacking hepatic Cyb5 to create the HBRN model to investigate the role of Cyb5 in this residual activity.

As with the HRN (Henderson et al., 2003) and HBN (Finn et al., 2008) models, the dual knockout of POR and Cyb5 had no deleterious effects on mouse fecundity, litter size, or pup development, and no gross physical phenotype was observed. The steatotic liver characteristic of POR deletion (Gu et al., 2003; Henderson et al., 2003; Finn et al., 2007) was even more pronounced in the HBRN animals than in the HRN model. This appeared to be an additive effect of the double knockout, because HBN mice also develop this phenotype after about 16 weeks of age (unpublished data). In assays using almost 1 mg of protein (~40-fold more than normal) from HBN or HBRN hepatic microsomes, we could not detect reduction of cytochrome *c* (unpublished data).

Consistent with our previous findings and those of others, indicating that Cyb5 can have positive, negative, or no effect on the function of the cytochrome P450 system (Zhang et al., 2005; Finn et al., 2008), the in vitro activities of hepatic microsomal fractions from the three models differed in a substrate-specific manner. Deletion of Cyb5 alone had no marked effect on microsomal activity toward MR and BR, as observed previously elsewhere (Finn et al., 2008; McLaughlin et al., 2010), whereas deletion of POR almost abolished the corresponding activity. This suggests that Cyb5 is not essential for the O-dealkylation of these substrates, which are metabolized predominantly by Cyp1a2 and Cyp2b10, respectively (Nerurkar et al., 1993). Interestingly, the metabolism of a further Cyp2b10 substrate—bupropion—was actually increased in HBN samples, probably reflecting the increased expression of Cyp2b10. In contrast, deletion of either Cyb5 or POR markedly reduced activity toward the other probe substrates and drugs used in this study, and deletion of both genes further increased this effect, suggesting that both Cyb5 and POR are necessary for the metabolism of these substrates and that in the absence of POR, Cyb5/Cyb5R can provide electrons into the mammalian P450 system.

Consideration of the redox potentials of Cyb5 (+20 mV) and ferric substrate-bound cytochrome P450 (−237 mV) shows that it would be thermodynamically impossible for Cyb5 to provide the first electron in the P450 catalytic cycle. However, the redox potential of Cyb5R (−265 mV) is such that it could support this reaction. Given that the redox potential of oxyferrous cytochrome P450 is also approximately +20 mV, it is feasible that Cyb5 can supply the second electron into the catalytic cycle. Further evidence for the role of Cyb5/Cyb5R was provided by exploiting the different affinities of POR and

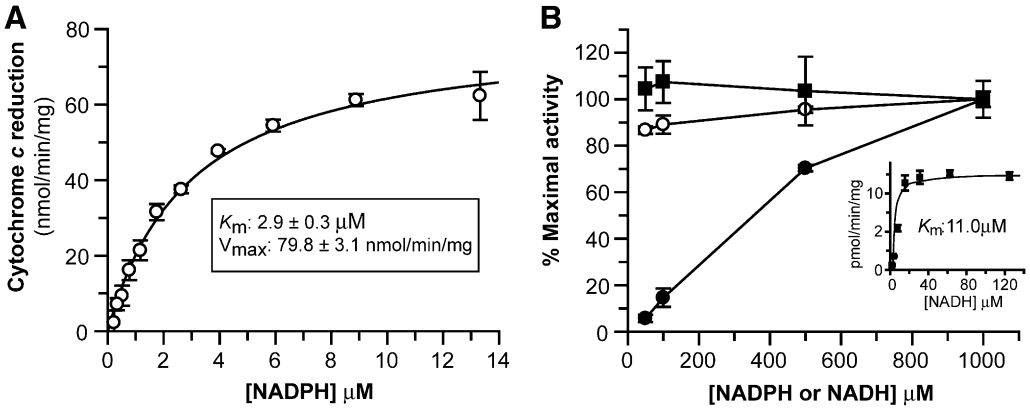

Fig. 5. Dependence of hepatic enzyme activities on cofactor concentration in HRN mice. (A) Dependence of cytochrome *c* reduction on NADPH concentration in WT hepatic microsomes. Assays were performed in triplicate under conditions of linearity for time and protein. Standard deviations for K_m and V_{max} are from the fit of the curve as calculated using the Michaelis-Menten equation (GraFit v5; Erithacus Software, Horley, UK). (B) Dependence of 7-benzyloxyquinoline metabolism on cofactor concentration. Assays in WT with NADPH (○), HRN with NADPH (●) and HRN with NADH (■) hepatic microsomes were performed in triplicate. Percentage of maximal activity was calculated using the following rates as 100% activity: WT with NADPH, 6.6 ± 0.4 pmol/min/mg; HRN with NADPH, 9.9 ± 1.1 pmol/min/mg; and HRN with NADH, 21.7 ± 1.7 pmol/min/mg. Data points represent the mean ± S.D. *Inset*: dependence of BQ oxidation on NADH concentration in HRN hepatic microsomes (mean ± S.D. for triplicate samples); standard deviation for K_m is from the fit of the curve as calculated using the Michaelis-Menten equation (GraFit v5).

Cyb5R for NADPH; we were able to show that in the absence of POR, BQ metabolism became highly dependent on NADPH concentration. Furthermore, in HRN samples, titration of NADH caused BQ activity to drop sharply at cofactor concentrations below 100 μM, with the K_m of the reaction consistent with that of Cyb5R for NADH. These data suggest both that residual POR is not the electron donor catalyzing substrate metabolism and also provide strong evidence that Cyb5R is the electron source under such circumstances.

A number of mechanisms have been proposed for the possible interactions between Cyb5 and P450 to explain the modifier action of the former on the latter. These include direct transfer of a rate-limiting electron; the formation of a ternary complex that allows near-simultaneous transfer of two electrons between POR and P450; improved reaction coupling; and direct effector actions (Schenkman and Jansson, 2003). It seems that where inhibitory effects are observed they are due to competition between Cyb5 and POR, such as for a binding site on the proximal surface of CYP2B4 whereby formation of a Cyb5-P450 complex prevents ferric P450 from accepting an electron from POR and initiating the catalytic cycle. Where stimulatory effects are observed, they are due to an increase in the rapidity and efficiency of catalysis in the presence of Cyb5 compared with POR; where no effect is observed, this represents a balance between these two opposing effects (Zhang et al., 2008).

The findings that in the absence of POR, hepatic Cyb5 mediates reactions in vivo was demonstrated by using the probe drug midazolam, metabolized predominantly by Cyp3a proteins in WT mice. As reported previously, the half-life, C_{max}, and AUC of midazolam were significantly increased, and clearance was substantially decreased, in the HBN and HRN models (as compared with WT). However, deletion of both genes produced a further significant increase in C_{max}, half-life, and AUC and a reduction in clearance. The induction of Cyb5 in HRN and HBRN would serve to amplify its role in the disposition of midazolam under these circumstances. Furthermore, as these effects are evident after oral administration of the compound, the data suggest that the consequences of differences in hepatic metabolism are superimposed on any intestinal first-pass effects on clearance. In almost all cases, the dual deletion of both Cyb5 and POR almost completely abrogates all cytochrome P450 activities, suggesting that other electron donors can at best only play a very minor role in hepatic P450 functions; the new HBRN model thus provides a more authentic hepatic-P450 null phenotype.

The cost of drug development is rising exponentially (Collier, 2009), and the failure rate for the development of NCEs is 80 to 90% (Cuatrecasas, 2006), principally due to toxicity and lack of efficacy. The use of in vitro technologies is both time- and cost-effective, allowing the metabolic profile of a NCE to be determined early in the preclinical development process and causing the contribution from ADRs to candidate attrition to fall over the last 10 years (Plant, 2004); however, ADRs are still a significant reason for failure in phases II and III of development. One possible reason for this is that potential ADRs associated with P450 metabolism are not always detected in vitro during early preclinical development.

The HBRN model described here, along with the HBN (Finn et al., 2008), complete Cyb5 knockout (McLaughlin et al., 2010; Finn et al., 2011), HRN (Henderson et al., 2003), inducible hepatic POR knockout (Finn et al., 2007), and gut POR knockout (Zhang et al., 2009) models, is a powerful tool in determining the impact of P450-mediated metabolism on the in vivo disposition and efficacy of drugs as well as on the toxicokinetics of parent compounds and their metabolites. The use of such models to screen NCEs for P450-mediated metabolism and toxicity would also address the lack of in vivo data in the early preclinical development of new drugs. The U.S. Food and Drug Administration has recommended that murine-human species differences in drug metabolism and disposition should be identified and characterized early as possible during the drug development process. The availability of mouse models that are humanized for the major drug metabolizing P450s, such as CYP3A4 and CYP2D6 (Yu et al., 2004; van Herwaarden et al., 2007; Felmlee et al., 2008; Hasegawa et al., 2011; van Waterschoot and Schinkel, 2011; Scheer et al., 2012), means that the HBRN model could be used in conjunction with such mice, allowing the identification of NCEs that are metabolized by human P450s in vivo before the initiation of clinical trials.

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Authorship Contributions

Participated in research design: Henderson, Wolf.
Conducted experiments: McLaughlin.
Contributed new reagents or analytic tools: Henderson.
Performed data analysis: McLaughlin.
Wrote or contributed to the writing of the manuscript: Henderson, McLaughlin, Wolf.

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