Role of NADPH-cytochrome P450 reductase and cytochrome b5/NADH b5 reductase in variability of CYP3A activity in human liver microsomes

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Running title: Contribution of variability in CPR and $b_5/b_5$R to variability in CYP3A activity

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Number of text pages: 26

Number of tables: 1

Number of figures: 5

Number of references: 35

Number of words in the Abstract: 232

Number of words in the Introduction: 750

Number of words in the Discussion: 1,229

Abbreviations:

- Cytochrome $b_5$ ($b_5$)
- NADH $b_5$ reductase ($b_5$R)
- NADPH-cytochrome P450 reductase (CPR)
- Horseradish peroxidase (HRP)
- Human liver microsomes (HLMs)
- Half maximal (50%) inhibitory concentration of a substrate (IC$_{50}$)
- Immunoglobulin G (IgG)
- 6-propyl-2-thiouracil (PTU)
- Potassium cyanide (KCN)
- Sodium dodecyl sulfate (SDS)
- Tris buffered saline (TBS)
Abstract

NADPH-cytochrome P450 reductase (CPR) and cytochrome \(b_5\) \((b_5R)\) together with NADH \(b_5\) reductase \((b_5R)\) play important roles in cytochrome P450 (CYP) 3A-mediated drug metabolism via electron transfer. However, it is not clear whether variability in expression of these accessory proteins contribute to the known interindividual variability in CYP3A activity. CPR and \(b_5\) were measured in human liver microsomes (HLMs) by spectrophotometry and immunoblotting. HLMs from elderly (>46 years) male donors \((n = 11)\) averaged 27\% \((P = 0.034)\) and 41\% \((P = 0.011)\) lower CPR levels than young (<45 years) male donors \((n = 21)\) for spectrophotometric and immunoblot values, respectively. Similarly, HLMs from elderly male donors averaged 43\% \((P = 0.034)\) and 47\% \((P = 0.011)\) lower \(b_5\) levels than young male donors for spectrophotometric and immunoblot values, respectively. \(\alpha\)-Lipoic acid and 6-propyl-2-thiouracil (PTU) were evaluated for selectivity of inhibition of CPR and \(b_5R\) activities (respectively) using recombinant enzymes and HLMs, as well as for effects on CYP3A-mediated triazolam hydroxylation in HLMs with either NADH or \(\beta\)-NADPH. The results indicate that both compounds are relatively nonselective inhibitors of CPR and \(b_5R\) activities. Finally, we used multivariate regression analysis and showed that variability in CPR or \(b_5\) expression between HLMs does not contribute significantly to variability in CYP3A-mediated midazolam hydroxylation. Consequently, while aging is associated with decreased CPR and \(b_5\) expression in human livers, this effect does not contribute to CYP3A variability.
Introduction

NADPH-cytochrome P450 reductase (CPR) is an electron transfer di-flavoprotein (Porter and Kasper, 1986). It is widely expressed in all tissues and is most abundant in the endoplasmic reticulum of the liver (Simmons and Kasper, 1989). CPR is responsible for transferring electrons to many naturally-occurring electron acceptors, including cytochrome P450 enzymes and cytochrome b$_5$, etc. (Murataliev et al., 2004; Guengerich, 2005), as well as to some non-physiological electron acceptors which are often used to test enzymatic activity of CPR in in vitro assays.

CPR is obligatory for catalysis of many P450 reactions. In the endoplasmic reticulum membrane, CPR transfers the electrons from NADPH into the P450 catalytic cycle (Elmore and Porter, 2002). CPR is a required electron transfer partner for the catalytic activity mediated by CYP3A isoforms. Antibodies against CPR activity significantly impair CYP3A-mediated drug metabolism in a concentration-dependent manner (Yamazaki et al., 1996). In recombinant enzyme systems, CYP3A reactions cannot proceed without CPR. Increasing concentrations of CPR enhance the metabolism of testosterone in a concentration-dependent manner (Yamazaki et al., 1999; Yamazaki et al., 2002).

Compared with P450 enzymes, relatively little interindividual variation in gene expression of CPR has been found in adult human livers (Shephard et al., 1992). However, various studies have shown an aging related decline of CPR activity or its expression in rodents (Schmucker and Wang, 1983; Guo et al., 1993; Warrington et al., 2004), but not in rhesus monkeys (Maloney et al., 1986; Schmucker and Wang, 1987). Gender related differences in CPR activity have also been reported in mice. Guo et al. found that CPR activity was higher in male than in female mice ranging in age from 6 to 48 months (Guo et al., 1993). In humans, the effects of aging and gender on CPR are unclear. One study in human livers reported that CPR activity did not change with aging, while CPR protein
expression measured by immunoprecipitation showed a weakly negative correlation with aging in females, but not in males (Schmucker et al., 1990). In another study, CPR activity was found to decrease with aging while gender had no effect on CPR activity (George et al., 1995).

Cytochrome b5 (b5) and NADH b5 reductase (b5R) are ubiquitous electron transport proteins. In endoplasmic reticulum, b5 plays important roles in maintenance of normal cellular functions by transferring electrons to microsomal desaturases that synthesize unsaturated fatty acids, plasmalogens, and cholesterol (Vergeres and Waskell, 1995). b5R is responsible for transferring electrons from NADH to b5 in these reactions, although NADPH can also be used as the electron donor in some cases. b5 can also accept electrons from an alternative reductase, CPR (Guengerich, 2005).

b5/b5R participates in drug metabolism, augmenting the reactions mediated by some P450 isoforms, including CYP3A4. In recombinant systems, the metabolism of testosterone, nifedipine and midazolam, the three typical index substrates for CYP3A, is enhanced by addition of b5 and b5R (Holmans et al., 1994; Voice et al., 1999; Yamazaki et al., 1999; Yamazaki et al., 2002). In human liver microsomes (HLMs), b5 antisera inhibited the metabolism of testosterone and nifedipine, suggesting that b5 is an essential component in modulation CYP3A activity (Yamazaki et al., 1996).

Little is known about the effect of aging and gender on the expression of membrane-bound b5 and b5R. Previous study has shown that b5 content and b5R activity decrease with aging in human erythrocytes (Matsuki et al., 1981).

Large interindividual variability in CYP3A activity in vivo and in vitro has been observed for many years (Parkinson et al., 2004; Cotreau et al., 2005). Both in vivo and in vitro studies have shown a gender-specific and aging-dependent decrease of CYP3A-mediated drug metabolism (Patki et al., 2004; Cotreau et al., 2005). However, variations in CYP3A activity
in vitro are not completely explained by variation in CYP3A protein levels alone (Parkinson et al., 2004; Cotreau et al., 2005). Consequently, we hypothesized that variation of activity and/or protein content of CPR or \( b_5 \) might account in part for high inter-individual variability of CYP3A activity as well as for effects of aging and gender. Using a well-characterized bank of HLMs (n=46) we evaluated variability in CPR and \( b_5 \) levels with respect to donor age and gender. In rat liver microsomes \( \alpha \)-Lipoic acid and 6-propyl-2-thiouracil (PTU) have been reported to inhibit CPR (Slepneva et al., 1995) and \( b_5 \)R activities (Lee and Kariya, 1986), respectively. Consequently, we evaluated the selectivities of these compounds as CPR and \( b_5 \)R inhibitors using both recombinant enzymes and HLMs, and also determined the effects of these inhibitors on CYP3A-mediated triazolam hydroxylation in HLMs.
Materials and Methods

Reagents

PTU, cytochrome c (bovine heart), potassium cyanide (KCN), α-lipoic acid, β-NADPH, NADH, NADP⁺, isocitrate dehydrogenase, DL-isocitrate, and 50 mM potassium phosphate buffer (pH 7.5) were purchased from Sigma (St. Louis, MO). Human b5 antisera and purified recombinant human b5R were provided by Dr. L. Trepanier (University of Wisconsin-Madison, Madison, WI). Methods used to generate these reagents and establish antibody selectivity have been published previously (Kurian et al., 2004). Potassium phosphate monobasic, acetonitrile, and methanol were obtained from Fisher Scientific (Fairlawn, NJ). Laemmli’s sodium dodecyl sulfate (SDS) sample reducing buffer, Tris-glycine-SDS running buffer, transfer buffer, and Tris buffered saline (TBS)-Tween-20 buffer were purchased from Boston BioProducts (Worcester, MA). Polyvinylidene difluoride membranes and Tris-glycine-SDS polyacrylamide gradient gels (4-15% and 10-20%) were from Bio-Rad (Hercules, CA). Purified recombinant human CPR, b5, and BenchMark™ protein ladder were from Invitrogen (Carlsbad, CA). Rabbit polyclonal primary antibody to CPR was purchased from Abcam (Cambridge, MA). Goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) labeled secondary antibody was from PerkinElmer Life Sciences (Boston, MA). SuperSignal West Pico chemiluminescent substrate (for HRP) was from Pierce (Rockford, IL). Potassium ferricyanide was from ICN Biomedicals (Aurora, OH).

Human liver microsomes

Liver samples were obtained from the International Institute for the Advancement of Medicine (Exton, PA), or the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MI). The majority of livers were from organ transplant donors with a history of head trauma or cerebral ischemia. The others were either from biopsy or
autopsy specimens of apparently healthy tissue. All livers did not have known liver diseases. Donors included 32 males and 14 females with ages ranging from 2 to 75 years. They were 40 Caucasians, 4 African-Americans, and 2 Hispanics. The history of cigarette smoking and alcohol consumption were reported for 15 and 21 donors, respectively. Specific details of each liver donor are provided elsewhere (Hesse et al., 2004). Microsomes were prepared through ultracentrifugation and stored at -80°C until use as described previously (von Moltke et al., 1993).

**Measurement of CPR activity**

Concentrations of CPR in the set of 46 HLMs were determined using an activity assay that spectrophotometrically measured the rate of CPR-mediated reduction of cytochrome c by β-NADPH (Venkatakrishnan et al., 2000). The assays were performed using standard 1-cm disposable cuvettes (Fisher, Fairlawn, NJ) in a total reaction volume of 1 mL. Each reaction contained 330 mM KH₂PO₄ (pH 7.6), 1 mM KCN, 50 μM cytochrome c, and 15-35 μg microsomal protein. Reactions were initiated by the addition of 10 μL of a 4.2 mM solution of β-NADPH to the sample cuvette. The increase in absorbance at wavelength 550 nm was recorded at room temperature for 3 min using a dual beam spectrophotometer (Uvikon, Kontron, Zürich). The rate of reduction of cytochrome c was determined as the slope of the linear part of the absorbance-time curve. Calibration curves were generated using 0.14 to 7 pmol of purified recombinant human CPR. The concentrations of CPR in HLMs were calculated using the calibration curve. Each experiment was performed in duplicate and repeated twice. Assays without HLMs or without β-NADPH were used as negative controls. Intra- and inter-assay coefficients of variation were less than 10%.

**Measurement of b₅ concentrations by spectrophotometry**

Concentrations of cytochrome b₅ in 46 HLMs were determined by measuring the differential absorbance between NADH-reduced and oxidized microsomes (Venkatakrishnan
et al., 2000). Microsomes were diluted to 1 mg/mL in 100 mM KH$_2$PO$_4$ (pH 7.4) and divided between two 1 mL cuvettes. The absorbance of oxidized $b_5$ was measured at 410 nm. By addition of 5 µL of 20 mM β-NADH to the sample cuvette, $b_5$ was reduced and the absorbance was recorded at 425 nm. The concentration of $b_5$ ($C$) was calculated using the following equation based on the Beer-Lambert law:

$$C = \frac{A}{(\varepsilon \cdot L)}$$

where $A$ is the difference in absorbance between oxidized and reduced $b_5$, $L$ is the pathway length (1 cm), and $\varepsilon$ is the extinction coefficient (185 mM$^{-1}$cm$^{-1}$). Assays without HLMs or without addition of NADH were used as negative controls. Intra- and inter-assay coefficients of variation were less than 10%.

**Immuoquantitation of microsomal CPR and $b_5$ protein content**

Microsomal CPR and $b_5$ protein content were measured by quantitative immunoblot analysis as follows. Briefly, CPR and $b_5$ were separated on gradient denaturing polyacrylamide gels (4–12% and 10–20% gels, respectively). After electrophoresis, protein was transferred onto polyvinylidene difluoride membranes for 1h (CPR) or 2h ($b_5$) at 25 V. Blots were blocked with 5% nonfat milk in TBS-Tween-20 buffer for 1h, washed in TBS-Tween-20 buffer, and then incubated with primary antibody for 1h. After further washes and incubation in the HRP-conjugated secondary antibody for 1h, chemiluminescent substrate (West Pico, Pierce) for 5 min at room temperature, labeled bands were imaged by cooled CCD camera (Kodak Image Station 440, Kodak, Rochester, NY). A calibration curve was generated by plotting the band intensity versus various concentrations of purified recombinant CPR or $b_5$. The concentrations of CPR or $b_5$ in HLMs were calculated based on the band intensities and the slope value of the calibration curve. Each protein was measured twice. The inter-assay variability was less than 15%.

**Microsomal CYP3A activity and protein content**
Midazolam 1-hydroxylation activity (a validated CYP3A-specific index activity) as well as CYP3A4 protein content (by quantitative immunoblotting) was determined in the same set of 46 HLMs. These data and the analytical methods have been reported in detail previously (He et al., 2006).

**Inhibitory selectivities of α-lipoic acid and PTU**

The selectivities of α-lipoic acid and PTU as chemical inhibitors of CPR and b5R were tested using both purified recombinant enzyme systems and HLMs. CPR activity was measured spectrophotometrically as described above. Concentrations of α-lipoic acid (up to 2 mM) or PTU (up to 4 mM) prepared in methanol were added to 2-mL polypropylene tubes and evaporated to dryness in a 40°C vacuum oven. The reaction was started by addition of 1 mL of the reaction mixture contained either 40–60 µg microsomal protein or 2 nM purified recombinant CPR.

Inhibition of b5R activity by α-lipoic acid and PTU was determined from b5R-mediated reduction of potassium ferricyanide by NADH. The measurements were performed based on the methods described previously with some modification (Badwey et al., 1983). Various concentrations (up to 4 mM) of α-lipoic acid or PTU in methanol solution were evaporated to dryness in a 40°C vacuum oven before addition of the reaction mixture containing 50 mM KH₂PO₄ (pH 7.6), 0.5 mM KCN, 0.5 mM potassium ferricyanide, and 40–60 µg microsomal protein or 0.2 µg/mL purified recombinant b5R. Reactions were started by addition of 10 µL of a 20 mM solution of β-NADH to the sample cuvette. The inhibition by PTU required a pre-incubation of PTU with HLMs or b5R enzyme at 25°C for 15 min. The decreased absorbance at wavelength 420 nm was recorded at room temperature for 1 min using a dual beam spectrophotometer (Uvikon, Kontron, Zürich). The rate of reduction of potassium ferricyanide was determined as the slope of the linear part of the absorbance-time curve.

Each assay was performed in duplicate using individually prepared HLMs from 4
different donors. For purified recombinant CPR or \(b_5\)R experiments, assays were performed in triplicate. Activities were expressed as the percentage of control activity (no inhibitor added). Intra- and inter-assay coefficients of variation were both less than 15%.

**Effect of \(\alpha\)-lipoic acid and PTU on triazolam hydroxylation by HLMs**

Measurement of rates of triazolam 1- and 4-hydroxylations by HLMs was performed based on the method described previously with minor modification (von Moltke et al., 1996). Reaction volume was 250 µL, triazolam concentration was 250 µM, concentration of HLM protein was 0.1 mg/mL, and incubation time was 20 min. Control incubations with no cofactor, no protein, and no substrate were performed concurrently as negative control. For evaluation of effects of PTU (up to 4 mM) and \(\alpha\)-lipoic acid (up to 40 mM), HLMs from 4 different individuals (0.1 to 0.25 mg/mL) were used. PTU was pre-incubated with HLMs at 37°C for 15 min. NAD\(^+\) (0.5 mM), NADP\(^+\) (0.5 mM), or NAD\(^+\) plus NADP\(^+\) (0.5 mM each) were evaluated as cofactors.

**Statistical analysis**

Descriptive statistics (mean and SD) of microsomal protein contents of CPR or \(b_5\) were calculated for the entire set of HLMs as well as HLMs stratified by donor gender and age. For the purpose of stratification of HLMs by age, groups included those donors ≤ 45 years old (“young”) and those donors ≥ 46 years old (“elderly”). This resulted in HLMs from 21 young male, 11 elderly male, 7 young female, and 7 elderly female donors. Statistical tests (Sigmapost 3.0, Systat, San Jose, CA) included ANOVA (one-way and 2-way) on rank transformed data with post-hoc analysis by Student-Newman-Keuls multiple pairwise comparisons testing, Spearman rank order correlation, and simple and multiple linear regression on rank transformed data. A two-tailed \(P\) value of < 0.05 was considered to be statistically significant. IC\(_{50}\) values (inhibitor concentration resulting in 50% decrease in CPR, \(b_5\)R, or CYP3A activity) were determined from the curve-fits of the
concentration-inhibition data using GraphPad Prism software (GraphPad Software, Inc., San Diego CA).
Results

CPR and \( b_5 \) expression in the HLMs bank

CPR concentrations in the set of HLMs measured by spectrophotometry ranged from 0.022 to 0.170 nmol/mg protein (0.086 ± 0.035 nmol/mg protein) (Figure 1A), while immunoblotting yielded somewhat lower results ranging from 0.005 to 0.060 nmol/mg protein (0.028 ± 0.015 nmol/mg protein) (Figure 1B). CPR levels measured by the two assay methods were relatively well correlated (Spearman correlation coefficient \( R_s = 0.60, P < 0.001 \)). Concentrations of \( b_5 \) in the HLMs measured by spectrophotometry ranged from 0.007 to 0.66 nmol/mg protein (0.32 ± 0.18 nmol/mg protein) (Figure 2A), while immunoblotting results were higher, ranging from 0.25 to 2.59 nmol/mg protein (1.15 ± 0.56 nmol/mg protein) (Figure 2B). Levels of \( b_5 \) assayed by these two methods were somewhat poorly correlated (\( R_s = 0.41, P = 0.01 \)).

Effect of aging and gender on CPR and \( b_5 \) content of HLMs

CPR and \( b_5 \) content data were stratified by liver donor gender and age and then analyzed by 2-way ANOVA. As shown in Table 1 and Figure 1, microsomal CPR concentrations measured by either assay method were not associated with either aging (\( P > 0.05 \)) or gender (\( P > 0.05 \)). However, within the set of HLMs from male donors, elderly male donors (\( n = 11 \)) averaged 27% (\( P = 0.034 \)) and 41% (\( P = 0.011 \)) lower CPR levels than young male donors (\( n = 21 \)) for spectrophotometric and immunoblot values, respectively. There were no differences in microsomal CPR content measured by spectrophotometric or immunoblot methods between young and elderly females (\( P > 0.05 \)).

Regarding microsomal \( b_5 \) content (Table 1 and Figure 2), mean spectrophotometric values were 30% lower (\( P = 0.047 \)) in the elderly donor group compared with the young donor group (0.24 ± 0.19 versus 0.36 ± 0.16 nmol/mg protein, respectively). Although immunoblot-derived \( b_5 \) content also averaged 30% lower in the elderly donor group compared
with the young donor group (0.96 ± 0.45 versus 1.38 ± 0.57 nmol/mg protein, respectively),
the difference did not reach statistical significance ($P > 0.05$). Within the set of HLMs from
male donors, elderly male donors averaged 43% ($P = 0.034$) and 47% ($P = 0.011$) lower
microsomal $b_5$ levels than young male donors for spectrophotometric and immunoblot values,
respectively. There were no differences in microsomal $b_5$ content by either
spectrophotometric or immunoblot methods between young and elderly females ($P > 0.05$).

**Inhibitory selectivities of $\alpha$-lipoic acid and PTU**

$\alpha$-Lipoic acid and PTU were evaluated as selective inhibitors of CPR and $b_5R$,
respectively, using both recombinant enzymes and HLMs. As shown in Figure 3A, both
CPR and $b_5R$ activities by recombinant enzymes were inhibited equally well by $\alpha$-lipoic acid
with maximal effects (92 and 84% reduction in activity, respectively) observed at 2 mM
$\alpha$-lipoic acid concentration. Calculated IC$_{50}$ values were 0.14 ± 0.02 mM (mean ± SE) for
inhibition of recombinant CPR and 0.05 ± 0.05 mM for inhibition of recombinant $b_5R$ by
$\alpha$-lipoic acid. Similar results were obtained with HLMs, although the extent of inhibition
was somewhat less reaching only 76 and 54% reduction in CPR and $b_5R$ activities
(respectively) at 2 mM $\alpha$-lipoic acid concentration (Figure 3B).

PTU also inhibited both CPR and $b_5R$ activities in purified recombinant enzymes with
decreases of 51% and 78% (respectively) observed at 4 mM PTU concentration (Figure 4A).
PTU was also somewhat less inhibitory in HLMs, with only 16% reduction in CPR activity
and 41% reduction in $b_5R$ activity at the highest PTU concentration evaluated (4 mM)
(Figure 4B).

**Inhibition of triazolam hydroxylation in HLMs by $\alpha$-lipoic acid and PTU**

The effects of $\alpha$-lipoic acid (up to 40 mM) and PTU (up to 4 mM) on triazolam
hydroxylation activities were evaluated in HLMs in the presence of NADH alone, $\beta$-NADPH
alone, or a mixture of both NADH and $\beta$-NADPH. As shown in Figure 5, both $\alpha$-lipoic acid
and PTU inhibited the 1-hydroxylation of triazolam in a concentration-dependent manner. Similar results were obtained for effects on 4-hydroxytriazolam formation (data not shown). IC$_{50}$ values for $\alpha$-lipoic acid inhibition of 1-hydroxytriazolam formation were 4.71 ± 0.04 mM (estimate ± standard error of the estimate), 4.72 ± 0.03 mM, and 6.22 ± 0.03 mM for NADH alone, $\beta$-NADPH alone, or a mixture of both NADH and $\beta$-NADPH, respectively. IC$_{50}$ values for PTU inhibition were 0.43 ± 0.03 mM, 0.20 ± 0.02 mM, and 0.15 ± 0.02 mM for NADH alone, $\beta$-NADPH alone, or a mixture of both NADH and $\beta$-NADPH, respectively. Although the type of cofactor included did not influence the extent of inhibition of 1-hydroxytriazolam formation by $\alpha$-lipoic acid, PTU inhibited CYP3A activity to a greater extent in the presence of $\beta$-NADPH compared with incubations that excluded $\beta$-NADPH at all PTU concentrations evaluated ($P < 0.05$; Student-Newman-Kuels test) (Figure 5).

**Effect of CPR or $b_{5}$ on CYP3A activity in HLMs**

CYP3A-mediated 1-hydroxymidazolam formation rates and CYP3A protein contents of the 46 HLMs used here have been reported in detail previously (He et al., 2006). In this study, we used simple linear and multivariate regression approaches to evaluate the possible contribution of variable CPR and/or $b_{5}$ levels (by spectrophotometric and immunoblot methods) to the observed variability in CYP3A-mediated catalysis (1-hydroxymidazolam formation) with respect to CYP3A protein content. By simple linear regression both CYP3A protein content ($R^2 = 0.80$, $P < 0.001$) and CPR protein content ($R^2 = 0.20$, $P = 0.002$) were identified as potential predictors of CYP3A activity. However, incorporation of both these dependent variables into a multiple linear regression model indicated that CYP3A protein content accounted for the majority of observed CYP3A activity variability with a standardized regression coefficient of 0.88 ($P < 0.001$) compared with 0.02 ($P = 0.76$) for CPR protein content. Furthermore, the regression coefficient for this multivariate model ($R^2 = 0.80$, $P < 0.001$) was unchanged compared with the regression coefficient for the simple regression.
model that used CYP3A protein alone. Further evaluation of various other models incorporating CPR and/or \( b_5 \) (spectrophotometric and immunoblot measures) in addition to CYP3A protein also failed to improve prediction of CYP3A activity over CYP3A protein alone. We also evaluated whether CYP3A protein content was correlated with either of the CPR, or \( b_5 \) measures. We found a weak but statistically significant covariation between CYP3A protein and CPR protein content (Rs = 0.48; \( P < 0.001 \)), but not between CYP3A protein and CPR spectrophotometric content or either \( b_5 \) measure (Rs < 0.20; \( P > 0.05 \)).
Discussion

The initial goal of this study was to determine CPR and \( b_5 \) expression in 46 HLMs using two different methods (spectrophotometry and immunoblotting) and then to investigate the effects of aging and gender on the expression of these enzymes. The results indicate that expression of CPR and \( b_5 \) in HLMs both decline with aging in males in that CPR levels were 27–41% lower in elderly males and \( b_5 \) levels were 43–47% lower in elderly males. Although we did not observe a similar age related difference in female livers, the number of female HLMs (7 young, 7 elderly) available to us was somewhat smaller than the number of male donor HLMs (21 young, 11 elderly). Consequently, a lack of effect of aging on the expression of CPR and \( b_5 \) in female HLMs cannot be concluded with any certainty and will need to be confirmed by study of a larger number of female livers.

Although CPR levels measured by spectrophotometry were reasonably well correlated with immunoblot levels (Rs = 0.60), the CPR levels measured by immunoblotting were consistently lower than levels determined by the spectrophotometric method (Table 1). On the other hand, \( b_5 \) levels measured by immunoblotting were consistently higher than those determined by the spectrophotometric method and \( b_5 \) levels measured by these two methods were less well correlated (Rs = 0.41). While reasons for these discrepancies are not clear, we speculate that there may have been some inactivation of accessory proteins during liver sample collection and/or microsome preparation of HLM leading to lower \( b_5 \) spectrophotometric levels relative to immunoblot levels. Lower CPR immunoblot levels relative to spectrophotometric levels may be the result of less efficient antibody binding to CPR in HLMs versus purified recombinant CPR. Despite these inconsistencies, we found similar differences in \( b_5 \) and CPR levels between the young male and elderly male HLMs regardless of assay method.

A second goal of the study was to determine whether \( \alpha \)-lipoic acid and PTU could be
used as selective chemical inhibitors of CPR and $b_5R$ enzymatic activities, respectively. 

$\alpha$-Lipoic acid is a naturally occurring disulfide compound found in many foods, such as meats and vegetables (especially spinach) and is often used as a dietary supplement for antioxidant effects. $\alpha$-Lipoic acid has been shown previously to inhibit CPR activity both in human (Dudka, 2006) and rat (Slepneva et al., 1995) liver microsomes possibly through interaction with critical sulfhydryl (SH) groups in the CPR molecule (Yelinova et al., 1993). Our results confirmed that $\alpha$-lipoic acid inhibited CPR activity in both recombinant enzymes and HLMs. However we also observed inhibition of $b_5R$ activity by $\alpha$-lipoic acid. Although the extent of inhibition of $b_5R$ activity at 2mM $\alpha$-lipoic acid was somewhat less (by about 10% in recombinant enzymes and 20% in HLMs) than for inhibition of CPR activity, the magnitude of the difference would be insufficient to allow this compound to discriminate between the contributions of CPR and $b_5R$ to an observed reductase activity. As far as we are aware, no other study has reported the selectivity of $\alpha$-lipoic acid as a CPR inhibitor. Of note was that the inhibitory effects of $\alpha$-lipoic acid on CPR and $b_5R$ were less in HLMs than in recombinant enzymes (Figure 1). This may be due to the presence of alternative electron transfer routes in HLMs (but not in recombinant enzymes), which would compensate for inhibition of either CPR or $b_5R$ activity by $\alpha$-lipoic acid.

PTU is a drug used in the treatment of hyperthyroidism via inhibition of thyroperoxidase (Streetman and Khandarina, 2003). PTU has been reported to selectively inhibit $b_5R$ activity without reducing CPR activity in rat liver microsomes at concentrations up to 10 mM (Lee and Kariya, 1986), possibly via interaction with the NADH-binding site of the enzyme. We preincubated enzymes with PTU prior to NADH addition to enhance the inhibitory effect on $b_5R$ (Lee and Kariya, 1986). Despite this, PTU inhibition of HLMs was limited, showing maximal inhibition of $b_5R$ activity of about 40% in HLMs over 1 to 4 mM concentrations with somewhat more (78% inhibition) in recombinant enzyme at 4 mM concentration. PTU
was also found to inhibit CPR activity in recombinant enzyme (51% decrease at 4 mM), but only had minimal effect on HLMs (<20% decrease at 4 mM). Consequently, we conclude that PTU is not an efficacious or selective \( b_5 \)R inhibitor in HLMs. The discrepancy in our results from those of Lee and Kariya (1986) who found good reductase discrimination may relate to species differences in human (this study) versus rat (previous study) reductase enzymes. Again, the lower inhibitory effects of PTU on CPR and \( b_5 \)R in HLMs compared with recombinant enzymes might be the result of alternative electron transfer routes in HLMs.

Reductase-specific inhibitory antibodies have been used in previous work to demonstrate the contribution of CPR and \( b_5 \)R to microsomal CYP3A activities (Yamazaki et al., 1996). However these antibodies are not commercially available and as such would have less utility than chemical inhibitors.

We also evaluated effects of \( \alpha \)-lipoic acid and PTU on CYP3A activity in HLMs. Different cofactors were used with the rationale that incubation mixtures containing \( \beta \)-NADPH alone would be more susceptible to CPR inhibitors (i.e. \( \alpha \)-lipoic acid), while those containing NADH alone would be more susceptible to \( b_5 \)R inhibitors (i.e. PTU). Consistent with our previous finding of a lack of reductase selectivity, \( \alpha \)-lipoic acid inhibited CYP3A activity regardless of the cofactor included. In contrast, PTU was found to inhibit CYP3A activity to a greater extent in the presence of \( \beta \)-NADPH compared with incubations that lacked \( \beta \)-NADPH. However, this result is consistent with PTU being a CPR-selective inhibitor rather than being a \( b_5 \)R-selective inhibitor, as we had originally hypothesized. Regardless, these data provide further evidence that \( \alpha \)-lipoic acid and PTU are not reductase selective inhibitors in HLMs.

Finally, we used simple linear and multivariate regression approaches to evaluate whether variable CPR and/or \( b_5 \) expression in a HLM bank contributes to the observed variability of CYP3A activity in addition to that contributed by CYP3A protein content.
This might occur perhaps through enhancement of CYP3A catalytic function via more efficient electron transfer in microsomes containing higher amounts of CPR or \(b_5\) relative to CYP3A protein content. However, our data indicates that variable CPR or \(b_5\) expression across the HLMs bank does not account for CYP3A activity variability in addition to that already explained by CYP3A protein levels. We also found a weak but statistically significant correlation between CYP3A protein and CPR protein content but not between CYP3A protein and \(b_5\) protein content. This finding is consistent with coregulation of gene expression of CYP3A with CPR but not with \(b_5\).

In conclusion, the results of this study indicate that hepatic CPR and \(b_5\) activity and content decrease with aging particularly in male livers. However, these changes in expression and activity of the accessory proteins are not sufficient to account for aging related decreases in CYP3A activity \textit{in vitro} (Parkinson et al., 2004; Patki et al., 2004) and \textit{in vivo} (Cotreau et al., 2005).
Acknowledgments

We thank Charles L. Crespi (BD Biosciences), Scott R. Obach (Pfizer Inc.), Richard I. Shader (Pharmacology department in Tufts university), and Larry H. Cohen (Millennium Pharmaceuticals Inc.) for their helpful suggestions.
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Footnotes

Dr Court was supported by grants R01GM061834 and R21GM074369 from the National Institute of General Medical Sciences (NIGMS), National Institutes of Health (Bethesda, MD).

Drs. Greenblatt was supported by grants AG-17880 from the National Institute on Aging and AI-58784 from the National Institute on Allergy and Infectious Disease, National Institutes of Health (Bethesda, MD). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Dr. Trepanier was supported by R01GM61753 from the National Institute of General Medical Sciences (NIGMS), National Institutes of Health (Bethesda, MD).

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Legends for Figures

Figure 1. CPR content in HLMs

CPR content in the set of 46 HLMs was measured by spectrophotometry (A) and immunoblotting (B). Results are expressed as CPR content (nmol / mg microsomal protein) versus donor age in years. Data from females are represented as open circles and data from males are represented as filled black circles.

Figure 2. Cytochrome b5 content in HLMs

b5 content in the set of 46 HLMs was measured by spectrophotometry (A) and immunoblotting (B). Results are expressed as b5 content (nmol / mg microsomal protein) versus donor age in years. Data from females are represented as open circles and data from males are represented as filled black circles.

Figure 3. Inhibition of CPR and b5R activity by α-lipoic acid

Reductase activities were measured in the absence and presence of increasing concentrations of α-lipoic acid. CPR activities were measured using recombinant NADPH-cytochrome P450 reductase (A) and HLMs (B), while b5R activities were measured using recombinant cytochrome b5 reductase (A) and HLMs (B). Results are expressed as the percentage of control activities (incubations without α-lipoic acid) and represent the mean ± SD of either three replicates (for recombinant enzyme) or 4 separate experiments using HLMs from 4 different donors.

Figure 4. Inhibition of CPR and b5R activity by PTU

Reductase activities were measured in the absence and presence of increasing concentrations of PTU. CPR activities were measured using recombinant NADPH-cytochrome P450
reductase (A) and HLMs (B), while $b_5R$ activities were measured using recombinant cytochrome $b_5$ reductase (A) and HLMs (B). Results are expressed as the percentage of control activities (incubations without PTU) and represent the mean ± SD of either three replicates (for recombinant enzyme) or 4 separate experiments using HLMs from 4 different donors.

**Figure 5. Inhibition of triazolam 1-hydroxylation in HLMs by α-lipoic acid and PTU**

Triazolam 1-hydroxylation activities in HLMs were measured in the absence and presence of increasing concentrations of α-lipoic acid (left) or PTU (right). Cofactors included either NADH alone (black bar), β-NADPH alone (hatched bar), or a mixture of NADH plus β-NADPH (open bar). Results are expressed as the percentage of control activities and represent the mean ± SD of four experiments using HLMs from 4 different donors. *P < 0.05 by Student-Newman-Kuels test for NADH alone incubation versus β-NADPH alone and NADH/β-NADPH incubations measured at the same inhibitor concentration. Similar results were obtained for 4-hydroxylation of triazolam (data not shown).
Table 1. Mean (± SD) NADPH-cytochrome P450 reductase and cytochrome b<sub>5</sub> content in a set of 46 HLMs

<table>
<thead>
<tr>
<th>Assay method</th>
<th>NADPH-cytochrome P450 reductase content (nmol/mg)</th>
<th>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; content (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young males</td>
<td>Elderly males</td>
</tr>
<tr>
<td>Spectrophotometric</td>
<td>0.095 ± 0.033*</td>
<td>0.069 ± 0.037*</td>
</tr>
<tr>
<td>Immunoblot</td>
<td>0.033 ± 0.014*</td>
<td>0.019 ± 0.011*</td>
</tr>
</tbody>
</table>

"Young" group were liver donors with an age ≤45 years and "Elderly" group were donors ≥46 years
n = 21 (Young male), n = 11 (Elderly male), n = 7 (Young female), n = 7 (Elderly female)

*P < 0.05 for Young males versus Elderly males by Student-Newman-Kuels test
Figure 1

A. Spectrophotometric Method

B. Immunoblot Method
Figure 2.

A. Spectrophotometric Method

- $b_5$ Content (nmol/mg)
- Age (Years)

B. Immunoblot Method

- $b_5$ Content (nmol/mg)
- Age (Years)
Figure 3.

A. Recombinant System

B. Human Liver Microsomes
Figure 4.

A. Recombinant System

B. Human Liver Microsomes

PTU Concentrations (mM)

Reductase Activity (percentage of control)

- b5R activity
- CPR activity
Figure 5.

The figure shows a bar graph representing the percentage of control for 1-hydroxy-triazolam formation. The x-axis represents the concentration of lipoic acid and PTU in mM, while the y-axis represents the percentage of control. The bars are divided into three categories: Both, NADPH, and NADH.