HEPATIC CYTOCHROME P450 REDUCTASE-NULL MICE AS AN ANIMAL MODEL TO STUDY ELECTRON TRANSFER PATHWAYS IN CHOLESTEROL SYNTHESIS AND CYP2E1-MEDIATED DRUG METABOLISM

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By
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Lexington, Kentucky

Director: Dr. Todd D. Porter, Associate Professor of Pharmaceutical Sciences
College of Pharmacy
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NADPH-cytochrome P450 reductase (CPR) is a flavoprotein containing both FAD and FMN and functions as the electron donor protein for several oxygenase enzymes found on the endoplasmic reticulum of eukaryotic cells, including cytochrome P450s involved in drug metabolism and cholesterol biosynthesis.

As many as three enzymes in the cholesterol biosynthetic pathway have been demonstrated, or proposed, to use CPR as a redox partner: squalene monooxygenase, which converts squalene to 2,3-oxidosqualene; lanosterol demethylase, a cytochrome P450 (CYP51); and 7-dehydrocholesterol reductase, the final step in cholesterol synthesis. In yeast CPR can be replaced by the NADH-cytochrome b₅ pathway, but this has not been demonstrated in animals or plants.

My studies with hepatic cytochrome P450 reductase-null mice have revealed a second microsomal reductase for squalene monooxygenase that was not previously detected. Studies carried out with hepatocytes from CPR-null mice demonstrate that this second reductase is active in whole cells and leads to the accumulation of 24-dihydrolanosterol, indicating that lanosterol demethylation, catalyzed by CYP51, is blocked. These results demonstrate that this second reductase plays a significant role in supporting squalene monooxygenase but not cytochrome P450-mediated reactions.

7-Dehydrocholesterol reductase (E.C. 1.3.1.21) catalyzes the reduction of the 7-8 double bond of 7-dehydrocholesterol to yield cholesterol. It has been suggested that cytochrome-P450 reductase is required for this reaction. My studies show that 7-dehydrocholesterol reductase is enzymatically active in CPR-null microsomes, with activity equal to or greater than that found in preparations from wild-type mice.

Mammalian cytochrome b₅, which can accept electrons from either cytochrome P450 reductase or NADH-cytochrome b₅ reductase, is known to be involved in augmenting some P450-dependent monooxygenase reactions. Cytochrome P450 2E1 has been found to exhibit reasonable rates of turnover via an NADH–cytochrome b₅ pathway in reconstituted enzyme systems and in
heterologous hosts. Using microsomes from hepatic CPR-null mice, I have determined that NADH-dependent CYP2E1 activity in the absence of NADPH-dependent activity constituted approximately 10% of CYP2E1 activity observed in microsomal preparations with NADPH from wild-type mice. However, little or no CYP2E1 activity could be detected in primary hepatocytes isolated from CPR-null mice.

KEYWORDS: Cytochrome P450 reductase, Squalene monooxygenase, Lanosterol 14alpha-demethylase, 7-Dehydrocholesterol reductase, CYP2E1

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November 2, 2006
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CHAPTER ONE: CYTOCHROME P450 REDUCTASE

Introduction

NADPH-cytochrome P450 reductase (CPR) is a flavoprotein containing both FAD and FMN and functions as the electron donor protein for several oxygenase enzymes found on the endoplasmic reticulum of eukaryotic cells. Figure 1.1 is a composite illustration showing NADPH-cytochrome P450 reductase and its electron partners. Among these oxygenases, the cytochromes P450s are a family of enzymes involved in the metabolism of many drugs and xenobiotics and in the synthesis of steroid hormones and other lipid signaling molecules; heme oxygenase catalyzes the first step in the degradation of heme to bilirubin; and squalene monooxygenase has been identified as the rate-limiting enzyme in the committed pathway for sterol biosynthesis. CPR has also been suggested to donate electrons to 7-dehydrocholesterol reductase in the sterol synthesis pathway, and to cytochrome $b_5$, which supports both sterol synthesis and the fatty acid desaturase and elongase pathways. Cytochrome $b_5$ has been shown to enhance some P450 reactions and several mechanisms for this effect have been proposed.
Figure 1.1: NADPH-Cytochrome P450 reductase and its electron partners.
Expression and regulation

Mammalian NADPH-cytochrome P450 reductase (CPR) was first discovered by Horecker in 1950 (Horecker 1950) as an NADPH-specific cytochrome c reductase. Later studies showed that this flavoprotein is a membrane-bound protein anchored to the endoplasmic reticulum by a NH$_2$-terminal hydrophobic peptide (Black, French et al. 1979). Rat and human cytochrome P450 reductase cDNAs were cloned in the 1980’s. Human cytochrome P-450 reductase is encoded by a single gene located on human chromosome 7(pter-q22), while a single copy of mouse cytochrome P450 reductase gene is located on chromosome 6 (Simmons, Lalley et al. 1985; Yamano, Aoyama et al. 1989).

Cytochrome P450 reductase is a house-keeping gene widely expressed in various tissues with highest level in the liver, where the cytochrome P450 system is highly expressed. The level of CPR expression is mainly regulated at the transcriptional level by the pituitary-thyroid axis. Thyroid hormone is necessary to maintain CPR expression in hepatocytes. In steroidogenic tissues, such as adrenal gland, a high level of CPR expression appears to be regulated by adrenocorticotropic hormone (ACTH) (O'Leary, Beck et al. 1994; O'Leary, Li et al. 1997).

Structure of cytochrome P450 reductase

Cytochrome P450 reductase belongs to a growing family of mammalian diflavin reductases including nitric oxide synthase, methionine synthase reductase, and cytosolic protein NR1 (Gutierrez, Munro et al. 2003). These diflavin reductases contain one molecule each of FMN and FAD. It has been suggested that cytochrome P450 reductase had evolved by an ancestral gene fusion event which brings two flavins in close proximity for efficient electron transfer. The NADPH/FAD domain has a strong structural resemblance to ferredoxin-NADP reductase and the FMN domain appears homologous with flavodoxins (Porter and Kasper 1986; Porter 1991). The three-dimensional structure of the cytochrome P450 reductase was determined by X-ray crystallography (Wang, Roberts et al. 1997). Cytochrome P450 reductase is composed
of four structural domains including the FMN-binding domain, the FAD- and the NADP-binding domains, and a domain which acts as a flexible hinge region to orchestrate interaction between the two flavin-containing domains for electron transfer, as shown in figure 1.2. An NH$_2$-terminal membrane-binding domain anchors the protein to the cytosolic side of the endoplasmic reticulum. Two recent studies suggest that two FMN-binding domains exist on CPR molecule which allows the FMN to "swing" in and out in relation to the rest of the CPR molecule and to shuttle electrons from FAD to CPR partners (Lamb, Kim et al. 2006). The movement is consistent with previous biochemical findings that describe the ease with which FMN dissociates from the enzyme, which would coincide with FMN release and binding (Hall, Vander Kooi et al. 2001).
Figure 1.2: Ribbons structure illustration of NADPH-cytochrome P450 reductase with bound FAD, FMN and the adenine portion of NADPH. This figure was adapted from Wang et al (Wang, Roberts, 1997)
Electron Transfer and Interaction

Cytochrome P450 reductase is a diflavin protein containing both FAD and FMN in equal proportions. Cytochrome P450 reductase binds NADPH and accepts a pair of electrons through its FAD moiety. Electrons are then transferred to the FMN moiety and then to P450 enzymes or other electron acceptors. Studies have shown that CPR functions as an electron bridge coordinating electron supply from NADPH, an obligatory two-electron donor, to one electron acceptors, by stabilizing the one-electron reduced form of the flavin cofactors FAD and FMN (Vermilion, Ballou et al. 1981). Under physiologic conditions the enzyme was proposed to cycle between the 1- and 3-electron reduced states, as shown in Figure 1.3 (Vermilion and Coon 1978).
Figure 1.3: Electron flow into NADPH-cytochrome P450 reductase from NADPH by hydride transfer. The reductase cycles between a three- and a one-electron reduced state. FAD accepts two electrons from NADPH, FMN acts as a one-electron donor/acceptor and shuttles a single electron to the downstream partner.
CPR primarily interacts with its downstream electron acceptor proteins through electrostatic charge pairing. Multiple acidic carboxylate groups of CPR pair with basic amino acids on the various electron acceptor proteins (Nisimoto 1986). Figure 1.4 illustrates, in cartoon format, the interaction of NADPH-cytochrome P450 reductase and cytochrome P450s. It has been shown that cytochrome P450 forms a dipole across the molecule, with the positive charge at the proximal face of the protein where the heme makes its closest approach to the surface of CPR. There is evidence for an additional hydrophobic component responsible for bringing the two proteins close enough together for electron transfer (Nadler and Strobel 1988; Hasemann, Kurumbail et al. 1995). Other electron acceptor proteins, such as cytochrome \( b_5 \), heme oxygenase, and squalene monooxygenase probably interact by the same mechanism (Ono, Ozasa et al. 1977; Enoch and Strittmatter 1979). Site-directed mutagenesis studies have shown that CPR mutations differentially affect the interaction with P450 and cytochrome c, suggesting that these two proteins bind to CPR in different manners. Studies with cytochrome \( b_5 \) indicate that its binding site on CPR is also distinct from that of P450 (Tamburini and Schenkman 1986). These studies indicate that each redox partner has a slightly different interaction with CPR.
Figure 1.4: Diagram illustrating the interaction of NADPH-cytochrome P450 reductase and cytochrome P450s. Adapted from the version drawn by Porter (http://www.uky.edu/Pharmacy/ps/porter/CPR_enzymology.htm).
The catalytic turnover of cytochrome P450s in some cases can be synergistically increased by cytochrome b\textsubscript{5}. Cytochrome b\textsubscript{5} can be reduced by either NADH-dependent cytochrome b\textsubscript{5} reductase or NADPH-dependent cytochrome P450 reductase. The exact mechanism by which cytochrome b\textsubscript{5} affects P450-dependent reactions remains controversial.

**Liver-specific deletion of cytochrome P450 reductase**

The cytochrome P450 reductase gene has been deleted in *Saccharomyces cerevisiae* and CPR-null yeast are able to survive by using the cytochrome b\textsubscript{5} NADH-dependent b\textsubscript{5} reductase system to supply both electrons to its downstream partners (Lamb, Kelly et al. 1999). However, complete loss of the P450 reductase gene results in embryonic lethality at day 11 of gestation in mouse, further characterized by developmental abnormalities in the neural tube, cardiac, eye and limb (Shen, O'Leary et al. 2002). These results emphasize the important role P450 reductase plays in providing electrons to enzymes important for development, including many involved in cholesterol synthesis and breakdown, retinoic acid metabolism, and heme catabolism. Mice with a conditional deletion of hepatic cytochrome P450 reductase have been generated from two groups individually using Cre/loxP system (Gu, Weng et al. 2003; Henderson, Otto et al. 2003; Wu, Gu et al. 2003; Weng, DiRusso et al. 2005). These two laboratories independently developed a mouse model with liver-specific deletion of the Cpr gene by cross-breeding the Cpr\textsuperscript{lox} mice with transgenic mice having liver-specific Cre expression (Albumin-Cre). Hepatic CPR-null mice were able to survive and breed. Deletion of the P450 reductase gene in the liver of adult mice by the Cre/lox system appears to lead to an impaired ability to both generate cholesterol and to eliminate it through bile acid synthesis, and is characterized by elevated hepatic lipids, reduced serum cholesterol levels and reduced bile flow. Furthermore, hepatic reductase-null mice completely lost microsomal P450 function in the liver, resulting in a five-fold induction of P450 enzymes with essentially no activity. Phenobarbital administration in hepatic reductase-null mice induced prolonged sleep at a dose that produced no sleep in wild-type animals. Dosing of acetaminophen led to a 90% drop in hepatic glutathione levels and a rise in serum
indicators of liver failure in wild-type animals, whereas glutathione levels and biomarkers of liver health remained stable in P450 reductase-null animals (Gu, Cui et al. 2005).

The relationship between hepatic phenotype and changes in gene expression in liver-specific cytochrome P450 reductase-null mice has been performed using gene microarray analysis by two different groups (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005). Overall, hepatic mRNA levels in control and CPR-null mice exhibited a number of marked differences including genes involved in pathways of lipid/sterol metabolism and cytochrome P450s. In the hepatic CPR-null mouse model, induction of several cytochrome P450 genes was reported, including CYP1A2, CYP2A5, CYP2B10, CYP3A11, and CYP3A13 mRNAs. CYP2E1 mRNA was expressed abundantly in mouse liver, although the mRNA of CYP2E1 level was not affected by hepatic CPR-deletion. The expression of certain P450 mRNAs was decreased in CPR-null livers. The expression level of Cyp7b1 (oxysterol 7α-hydroxylase), which is involved in bile acid synthesis, decreased by 50%. The mRNA levels of most of the enzymes involved in cholesterol biosynthesis were increased significantly in the CPR-null livers. The expression level of 3-hydroxy-3-methylglutaryl-CoA reductase, which catalyzes the rate-limiting step of cholesterol biosynthesis, increased by 1.56-fold. The transcription levels of squalene monooxygenase, 7-dehydrocholesterol reductase and CYP51, which have been assumed to require cytochrome P450 reductase for activity, have been shown to be increased by 2-fold in one hepatic CPR-null model (Wang, Chamberlain et al. 2005), but were not significantly affected in the CPR-null liver generated from another lab. (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005)
PART ONE: ELUCIDATION OF AN ALTERNATIVE ELECTRON TRANSFER PATHWAY IN CHOLESTEROL BIOSYNTHESIS USING CPR-NULL MICE AS A MODEL
CHAPTER TWO: CYTOCHROME P450 REDUCTASE-DEPENDENT ELECTRON TRANSFER IN THE CHOLESTEROL BIOSYNTHESIS PATHWAY

Introduction

Cholesterol is an important component of the membrane of cells and the precursor for the synthesis of vitamin D and the various steroid hormones. Recent research shows that cholesterol has an important role as a signaling molecule for development and maintaining normal cell functions. The developmental gene sonic hedgehog (Shh) plays an important role in brain and limb development. This protein has to be covalently linked to cholesterol for its activity (Roux, Wolf et al. 1997; Lewis, Dunn et al. 2001). Cholesterol-rich plasma membrane microdomains, termed "membrane rafts" including caveolae, have been implicated in a variety of cellular processes, including signal transduction, endocytosis, transcytosis and cholesterol trafficking (Barnett-Norris, Lynch et al. 2005). Cholesterol plays an important role in maintaining the boundary area and the properties of the membrane microdomains (Hao, Mukherjee et al. 2001; Maxfield 2002). However, elevated serum cholesterol levels can alter membrane function and are closely associated with atherosclerotic plaques.

Liver is the primary site for maintaining cholesterol homeostasis, by several mechanisms including the regulation of cholesterol biosynthesis, uptake through lipoprotein receptors, release into the blood by lipoproteins, and the storage by esterification as well as the degradation and the conversion into bile acids (Dietschy, Turley et al. 1993; Kang and Davis 2000).

Studies performed by Bloch et al in early 1950’s revealed that all the carbon atoms of cholesterol are derived from acetate. Subsequent investigations elucidated a number of enzymes and proteins involved in cholesterol biosynthesis and its regulation. In general, cholesterol biosynthesis pathway can be divided into two segments, an upper isoprenoid pathway (the mevalonate pathway), and a downstream, or “committed” pathway, illustrated in Figure 2.1. Whereas the isoprenoids synthesized in the mevalonate portion of the pathway are incorporated into many cellular components,
including proteins and RNA, the downstream pathway, beginning with squalene, is dedicated specifically to cholesterol biosynthesis in somatic cells.

Cytochrome P450 reductase has a multipart role in sterol synthesis, as shown in Figure 2.1. In addition to its coupling to squalene monooxygenase, CPR serves as the redox partner for lanosterol demethylase (CYP51), the enzymatic step that follows the formation of lanosterol. CPR can also serve as an alternative electron donor to cytochrome b5, which is the redox partner for sterol 4α-methyl oxidase and sterol Δ5-desaturase (Risley 2002), and CPR may be the redox partner for 7-dehydrocholesterol reductase, the final step in cholesterol synthesis (Nishino and Ishibashi 2000). In yeast CPR can be replaced by the NADH-cytochrome b5 pathway (Lamb, Kelly et al. 1999), but this has not been demonstrated in animals or plants.
Figure 2.1: A scheme of the cholesterol biosynthetic pathway. Cytochrome P450 reductase is suggested to be required for three enzymatic reactions: 1) Squalene Monooxygenase; 2) Lanosterol 14α-Demethylase; 3) 7-Dehydrocholesterol Reductase.
Squalene monooxygenase

Squalene monooxygenase (E.C. 1.14.99.7) is a 64-kDa enzyme bound to the endoplasmic reticulum of eukaryotic cells (Yamamoto and Bloch 1970). First characterized by Yamamoto and Bloch in 1970s, squalene monooxygenase catalyzes the second committed step in cholesterol synthesis from farnesyl pyrophosphate, and converts squalene to 2,3-oxidosqualene by inserting an oxygen atom across the 2,3-double bond, as shown in Figure 2.2.
Figure 2.2: The squalene monooxygenase reaction.

\[
\text{Squalene Monooxygenase} \quad +\text{O}_2 \quad \rightarrow \quad \text{Squalene} +\text{H}_2\text{O}
\]

\[
\text{NADPH} \quad \rightarrow \quad \text{NADP}^+
\]
Assays with microsomal squalene monooxygenase require FAD, NADPH, O$_2$, and the cytosolic fraction. Although the epoxidation of an alkene is a reaction more typical of the cytochrome P450s, squalene monooxygenase is not a P450. Squalene monooxygenase shows no absorption at 450nm and is not inhibited by common P450 inhibitors including carbon monoxide and metyrapone (Yamamoto and Bloch 1970; Ono and Bloch 1975; Ono, Nakazono et al. 1982). The cytosolic fraction was later found to contribute three essential components to the enzymatic activity: supernatant protein factor (SPF), phospholipids, and FAD (Tai and Bloch 1972; Saat and Bloch 1976).

Squalene monooxygenase has a loosely-bound FAD (flavin) group. The addition of FAD to microsomal reactions reconstituted with purified SPF and phospholipids resulted in full activation of squalene monooxygenase (Tai and Bloch 1972; Saat and Bloch 1976).

Assays with purified or recombinant squalene monooxygenase require the further addition of NADPH-cytochrome P450 reductase (CPR) (Ono, Takahashi et al. 1980), an electron transfer protein more readily recognized for its role as the redox partner for the microsomal cytochromes P450, a ubiquitous family of hemeprotein monooxygenases involved in steroid synthesis and drug and xenobiotic metabolism. The unusual requirement for an electron transfer partner differentiates squalene monooxygenase from all other known flavoprotein monooxygenases. Figure 2.3 is a diagram illustrating the requirement of electron transfer for the squalene monooxygenase reaction.
Figure 2.3: Diagram representation of squalene monooxygenase reaction: requirement of electron transfer to the cofactor FAD group.
The third component of the supernatant fraction required for the monooxygenase activity is supernatant protein factor (SPF). SPF, a heat-labile cytoplasmic protein, when added to microsomes along with anionic phospholipids such as phosphatidylserine or phosphatidylglycerol, was able to activate squalene monooxygenase activity as much as the cytosolic fraction alone (Tai and Bloch 1972; Saat and Bloch 1976). The cDNA of SPF was cloned in 2001 by Shibata et al (Shibata, Arita et al. 2001). This protein was found to belong to the CRAL/TRIO family of lipid-binding proteins that includes Sec14p, α-tocopherol transfer protein (αTTP), and cellular retinol binding protein (CRALBP) (Porter 2003). The role of SPF in the squalene monooxygenase reaction remains unclear. Early studies revealed that SPF did not stimulate squalene monooxygenase when the enzyme was solubilized. Early work on SPF suggested that it catalyzed the transfer of squalene into and between intracellular membranes, and that this transfer enhanced the activity of squalene monooxygenase (Caras, Friedlander et al. 1980; Friedlander, Caras et al. 1980; Chin and Bloch 1984); Friedlander et al (Friedlander, Caras et al. 1980) demonstrated that SPF promotes the transfer of squalene from trypsin-treated microsomes that lack squalene monooxygenase activity to normal, enzymatically active microsomes in a temperature-dependent process, despite the fact that SPF binds only weakly to microsomes (Caras, Friedlander et al. 1980). Although squalene transfer is bidirectional, transfer to membranes containing squalene monooxygenase is about twice as efficient as that to membranes that lack this enzyme (Kojima, Friedlander et al. 1981; Fuks-Holmberg and Bloch 1983). Despite these early studies, it has not been possible to demonstrate high affinity binding of squalene or 2,3-oxidosqualene to SPF, and SPF shows greatest affinity for phosphatidylinositol and α-tocopherylquinone (Panagabko, Morley et al. 2003; Stocker and Baumann 2003). The dependency of squalene monooxygenase on anionic phospholipids for maximal activity raises the possibility that an anionic phospholipid, such as phosphatidylinositol, is the physiologic ligand for SPF (Tai and Bloch 1972; Chin and Bloch 1984). The mechanism by which SPF stimulates microsomal squalene monooxygenase remains unknown. Studies have shown that SPF also activates the other enzymes involved in cholesterol synthesis including oxidosqualene cyclase and HMG-CoA reductase (Saat and Bloch 1976; Mokashi, Singh et al. 2005). Oxidosqualene cyclase converts the product of
squalene monooxygenase, 2,3-oxidoqualene, to the first sterol in the pathway, lanosterol. HMG-CoA is the major regulatory step for cholesterol biosynthesis pathway. Early studies revealed that the SPF and phospholipids could be replaced by the nonionic detergent Triton X-100, which has been used in most in-vitro squalene monooxygenase activity assays. It was proposed that Triton X-100 solubilizes the microsomes and removes the requirement for intermembrane or intramembrane squalene transport (Tai and Bloch 1972).

The expression of squalene monooxygenase is highly regulated transcriptionally by cholesterol. Nagia et al (Nagai, Sakakibara et al. 2002; Ono 2002) identified the sterol regulatory element (SRE) sequence and nuclear factor Y (NF-Y) binding sequence in the promoter of the gene of squalene monooxygenase, both of which play a critical role in sterol-mediated regulation of the gene. They showed that the sterol regulatory element-binding protein (SREBP-2) activates transcription of the gene. SREBP and its regulatory proteins are found in the endoplasmic reticulum and require proteolysis to be released from the membrane to find its way into the nucleus where it can upregulate transcription of cholesterologenic genes (Brown and Goldstein 1997).

**Lanosterol 14α-demethylase**

More than 1000 genes of the cytochrome P450 superfamily are found in species of all of the kingdoms of biology. CYP51, encoding lanosterol 14α-demethylase, is the only known P450 expressed in prokaryotes and eukaryotes and considered to be an ancestor of the P450 superfamily (Nelson 1999).

Lanosterol 14α-demethylase catalyzes the oxidative removal of the 14α-methyl group of lanosterol and 24-methylene-24,25-dihydrolanosterol in yeast and fungi, obtusifoliol in plants, and 24,25-dihydrolanosterol in mammals to give α^{14,15}-desaturated intermediates in ergosterol, phytosterol, and cholesterol biosynthesis, respectively. During the catalytic cycle lanosterol undergoes three successive monooxygenation reactions: The methyl group is first converted to an alcohol, then to an aldehyde, and then removed as formic acid without the release of the substrate, each step requiring
one molecule of molecular oxygen and one molecule of NADPH (Rozman and Waterman 1998), as illustrated in Figure 2.4.
**Figure 2.4: The lanosterol 14α-demethylase reaction.** The methyl group is removed by a three-step process catalyzed by lanosterol 14α-demethylase (CYP51) without release of intermediates. Each step requires cytochrome P450 reductase (CPR) and one molecule of oxygen.
In animals, lanosterol 14α-demethylase is the only P450 in the cholesterol synthesis pathway. Its activity is strongly inhibited by carbon monoxide and azole compounds. Lanosterol 14α-demethylase is a housekeeping gene expressed in virtually all animal cells with high expression level in the liver, the site for cholesterol synthesis. However, the highest level of expression of lanosterol 14α-demethylase was found in testes. This is because the product of lanosterol 14α-demethylase is the potent meiosis-activating sterol, 4,4-dimethylcholesta-8(9),14,24-trien-3β-ol (also known as FF-MAS) (Rozman and Waterman 1998).

Like other cholesterolgenic genes, lanosterol 14α-demethylase responds to cholesterol negative feedback regulation through binding of SREBPs to a sterol regulatory element (SRE) in the CYP51 promoter region. Similar to squalene monooxygenase, the predominant SREBP-isof orm responsible for regulation of CYP51 expression in mammalian cells is SREBP-2 (Rozman, Fink et al. 1999; Fon Tacer, Kalanj-Bognar et al. 2003). The CYP51 promoter also contains a cAMP regulatory element (CRE) known to bind cAMP regulatory proteins CREB/CREM. The CYP51 response to cAMP has been observed to be independent of lipid composition and might play a role in cross-talk of cholesterol biosynthesis with other important biological processes (Rozman, Fink et al. 1999; Halder, Fink et al. 2002). In hepatic cytochrome P450 reductase-null mice, the expression level of lanosterol 14α-demethylase increased by 2-fold (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005).

The structure of lanosterol 14α-demethylase in *M. tuberculosis* was solved by Podust et al in 2001. Lanosterol 14α-demethylase exhibits the typical P450 fold but contains differences that define the substrate access channel which runs roughly parallel to the cofactor heme (Podust, Poulos et al. 2001). Like many other cytochrome P450s, the activity of human lanosterol 14α-demethylase requires cytochrome P450 reductase. However, it has been reported that the activity of lanosterol 14α-demethylase was augmented by the membrane-bound cytochrome *b*5 in an *in vitro* reconstituted system in the presence of cytochrome P450-reductase (Lamb, Kaderbhai et al. 2001).
7-Dehydrocholesterol reductase

7-Dehydrocholesterol reductase (E.C. 1.3.1.21), the final enzyme of cholesterol synthesis, catalyzes the reduction of the $\Delta^7$ double bond of 7-dehydrocholesterol and 7-dehydrodesmosterol to yield cholesterol and desmosterol, respectively, as shown in Figure 2.5. Desmosterol is a 27-carbon sterol that differs from cholesterol due to the presence of a C24–25 double bond in the side chain, which is reduced by 24-sterol reductase to generate the end product cholesterol (Waterham and Wanders 2000).
Figure 2.5: Terminal enzymatic reactions in cholesterol synthesis. 7-Dehydrocholesterol reductase (7DHCR) reduces the C7–8 double bond of both 7-dehydrocholesterol and 7-dehydrodesmosterol to yield cholesterol and desmosterol, respectively. NADPH is required for the reaction. 24-Sterol reductase reduces the C24–25 double bond in desmosterol to yield cholesterol.
Shefer et al. (Shefer, Salen et al. 1998) characterized the substrate specificity of 7-dehydrocholesterol reductase. Although less efficient than the reduction of 7-dehydrocholesterol, this group demonstrated that 7-dehydrocholesterol reductase can reduce the C7–8 double bond in the B-ring of ergosterol, 7-dehydrositosterol, and 7-dehydroepicholesterol. Ergosterol reduction to brassicasterol has been used as a surrogate assay for the activity of the enzyme in both reconstituted microsomes and in cell cultures (Honda, Tint et al. 1996; Honda, Tint et al. 1998; Shefer, Salen et al. 1998).

Although experimental data on the membrane topology of 7-dehydrocholesterol reductase is unavailable, it has been proposed to be a multi-transmembrane protein located in endoplasmic reticulum, based on the predicted amino acid sequence. Fitzky et al. (Fitzky, Moebius et al. 2001) predicted a model with nine transmembrane spanning domains containing the putative sterol-sensing domain (Fitzky, Moebius et al. 2001). Waterham and Wanders (Waterham and Wanders 2000) proposed a more conservative model with six transmembrane spanning domains which differs from Fitzky’s model in orientation of the carboxy terminus relative to the cytosol, and in the localization of the extramembranous (Waterham, Wijburg et al. 1998). The potential sterol-sensing domain (SSD) in rat and human 7-dehydrocholesterol reductase has high homology with the SSD of sterol cleavage activating protein (SCAP). SSDs have been identified in a number of other proteins, including HMG-CoA reductase, sterol cleavage activating protein, and Niemann–Pick type C protein. All of these proteins are involved in cholesterol synthesis or homeostasis (Bae, Lee et al. 1999; Fitzky, Moebius et al. 2001), whereas the SCAP motif binds sterols is essential for the cleavage of SREBP to release the nuclear transcriptional factor to activate the sterol regulating genes (Brown and Goldstein 1997).

The conversion of 7-dehydrocholesterol to cholesterol requires NADPH which cannot be substituted by NADH as a reducing factor. It has been reported that the presence of molecular oxygen was not necessary for the conversion of 7-dehydrocholesterol to cholesterol in rat liver homogenate, indicating cytochrome P450 is not involved. Wilton et al. (Wilton, Munday et al. 1968) purposed that there was a direct transfer of hydrogen from NADPH to the 7α-position of cholesterol, and that the 8β-hydrogen was derived from water.
It has also been suggested that cytochrome-P450 reductase activity is required for the enzyme to catalyze reduction of 7-dehydrocholesterol in a reconstituted microsomal system; however, this reaction mechanism is distinct from that of cytochrome P450s. Specific inhibitors of the cytochrome P450s, carbon monoxide andazole compounds, had no effect on the activity of 7-dehydrocholesterol reductase (Nishino and Ishibashi 2000). EDTA, o-phenanthroline and KCN inhibited the activity of the enzyme, indicating the requirement of a metal ion for the enzymatic reaction (Nishino and Ishibashi 2000). Nishino et al further reported that ferric ion restored the reductase activity in EDTA-treated microsomes, suggesting that 7-dehydrocholesterol reductase may be an iron-containing enzyme. Studies done by Scallen et al (Scallen, Noland et al. 1985) suggested that sterol carrier protein 2 (SCP2) activates the conversion of 7-dehydrocholesterol to cholesterol. It also has been suggested that SCP-2 is necessary to transfer certain water-insoluble cholesterol precursors and metabolic products between one compartment and the next compartment in a cascade of enzyme reactions. It has been suggested SCP2 binds 7-dehydrocholesterol and may increase substrate availability to the reductase (Scallen, Noland et al. 1985).

Shefer et al (Shefer, Salen et al. 1998) suggested that the activity of 7-dehydrocholesterol reductase is regulated by phosphorylation/dephosphorylation. In the reconstituted liver microsomal system, addition of alkaline phosphatase resulted in decreased 7-dehydrocholesterol reductase activity, whereas treatment with NaF, a non-specific phosphatase inhibitor, increased the activity of the enzyme. In vivo regulation of 7-dehydrocholesterol reductase activity by phosphorylation/dephosphorylation has not been demonstrated. Multiple potential phosphorylation sites are predicted in 7-dehydrocholesterol reductase based on amino acid sequence of this protein. Bae et al. (Bae, Lee et al. 1999) identified multiple potential protein kinase C phosphorylation and potential tyrosine kinase phosphorylation sites in rat 7-dehydrocholesterol reductase, some of those are conserved in the human, murine, rat, and zebrafish proteins. The potential tyrosine phosphorylation site corresponding to amino acids 450–458 is contained within the sterol reductase 2 motif in rat 7-dehydrocholesterol reductase. The sterol reductase 2 motif also exists in the yeast genes involved in ergosterol synthesis (Correa-Cerro and Porter 2005).
As expected for enzymes involved in cholesterol synthesis, the expression of 7-dehydrocholesterol reductase is induced by sterol deprivation. Bae et al (Bae, Lee et al. 1999) identified a sterol regulatory element (SRE) sequence and two nuclear factor Y (NF-Y) binding sequences in the promoter of the gene for 7-dehydrocholesterol reductase, indicating sterol mediated regulation of the gene (Bae, Lee et al. 1999). A deficiency of 7-dehydrocholesterol reductase activity because of genetic mutation in humans has recently been found to cause Smith-Lemli-Opitz syndrome (SLOS). SLOS is an autosomal recessive disorder with various developmental abnormalities and is characterized by elevated 7-dehydrocholesterol in serum body fluids and tissues (Correa-Cerro and Porter 2005).
Experimental rationale

Cytochrome P450 reductase has a multipart role in sterol synthesis. In reconstituted microsomal systems, cytochrome P450 reductase appears to be required for three enzymatic reactions in cholesterol synthesis pathway. It has been reported that cytochrome P450 reductase was essential for the activity of solubilized squalene monooxygenase by Triton X-100 (Ono, Ozasa et al. 1977). Cytochrome P450 reductase serves as the redox partner for lanosterol demethylase (CYP51), the enzyme that removes the 14-methyl group from lanosterol. Cytochrome P450 reductase may also serve as the redox partner for 7-dehydrocholesterol reductase, the final step in cholesterol synthesis (Nishino and Ishibashi 2000). In yeast CPR can be replaced by the NADH-cytochrome b5 pathway (Lamb, Kelly et al. 1999), but this has not been demonstrated in animals or plants. In conditional-null mice, in which the CPR gene is deleted in the liver during maturation, adult animals exhibit an 80% decrease in plasma cholesterol levels and accumulation of lipid droplets in the livers, suggesting a loss in the ability to synthesize cholesterol and accumulation of intermediate(s) in the liver. Given that the three enzymes proposed to require cytochrome P450 reductase have no structural similarity and contain different cofactors (squalene monooxygenase is a FAD-dependent enzyme, lanosterol demethylase is a heme-binding protein, and the cofactor of the intergral membrane protein 7-dehydrocholesterol reductase is unknown), the purpose of this study is to determine if additional electron transfer partner(s) exist for the above-mentioned enzymes. The cytochrome P450 reductase-null mouse is an excellent model to study alternative electron transfer for these enzymes in cholesterol biosynthesis.
Specific Aims

Specific Aim 1: Determine if cytochrome P450 reductase is required for the activity of squalene monooxygenase in cholesterol biosynthesis. Squalene monooxygenase requires a second enzyme to provide the electrons necessary for catalysis, and for many years it has been assumed that cytochrome P450 reductase (CPR) is this requisite electron donor protein. The possibility that additional electron transfer partners might exist for squalene monooxygenase has not been explored. The objective of this project is to determine if other redox proteins can substitute for CPR in hepatic CPR-null mice. Squalene monooxygenase activity will be determined by 2,3-oxidosqualene formation from $^{14}$C-squalene in the presence of an oxidosqualene cyclase inhibitor (to prevent feed-through of the product). Squalene and 2,3-oxidosqualene formation will be monitored in hepatocytes using $^{14}$C-mevalonate as the precursor. Oxidosqualene formation will demonstrate that the reductase is functional in whole cells, and, when compared to oxidosqualene formation in wild-type cells, will give an indication of the extent to which it contributes to squalene monooxygenase activity.

Specific Aim 2: Determine if the cytochrome P450 reductase is required in other steps, including lanosterol demethylase and 7-dehydrocholesterol reductase, in the cholesterol biosynthesis pathway. To determine if CPR is an essential component of cholesterol synthesis, $^{14}$C-mevalonate incorporation into cholesterol will be measured in hepatocytes isolated from CPR-null mice; if cholesterol synthesis is blocked, the CPR-dependent enzyme will be identified from the intermediate that accumulates in these cells. To determine if CPR participates in the specific enzyme reactions noted above, the activities of these enzymes will be determined in CPR-null microsomes and in hepatocytes.
CHAPTER THREE: MATERIALS AND METHODS

Animals

CPR liver-specific knockout mice (Alb-Cre\textsuperscript{+/−}/Cpr\textsuperscript{lox+/+}) were generated by crossing mice expressing liver-specific Cre under control of the an albumin promoter (Alb-Cre) with two lines of mice bearing a conditional CPR allele (Cpr\textsuperscript{lox+/+}) (Gu, Weng et al. 2003; Wu, Gu et al. 2003). Littermates lacking the Alb-Cre transgene (Alb-Cre\textsuperscript{−/−}/Cpr\textsuperscript{lox+/+}) were designated as wild-type. Two to four month old mice from null and wild-type littermate groups on mixed C57BL/6 (75%) and 129/Sv (25%) genetic background were used in the studies. Animals were normally maintained in a temperature-, humidity-, and light-controlled facility (70-72°F, 48-52% humidity, 12-hr light/dark cycle) and were allowed free access to water and food. Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Preparation of microsomal and cytosolic fractions

Animals were killed by CO\textsubscript{2} asphyxia, the livers promptly removed, and microsomal and cytosolic fractions were prepared by standard procedures (Singh, Mokashi et al. 2003). In brief, approximately 3 livers were minced thoroughly with scissors, transferred into 4 volumes by sample weight of chilled homogenization buffer (100 mM Tris-HCl buffer, pH 7.4, containing 1.0 mM EDTA) and homogenized using a homogenizer. All subsequent steps were carried out at 4 °C. The samples then were centrifuged at 10,000g for 20 min. The supernatant was collected and centrifuged at 100,000g in for 60 min. The upper lipid layer was removed and the cytosolic supernatant collected. The microsomal pellet was resuspended in chilled homogenization buffer (100mM Tris-HCl buffer, pH 7.4, containing 1.0 mM EDTA) and the 100,000g centrifugation for 60 min was repeated. The microsomal fraction was resuspended at ~15 mg of protein/ml in 100 mM Tris-HCl, 1mM EDTA, (pH 7.4) and the cytosolic fraction (100,000g supernatant) was diluted to ~15 mg of protein/ml. Protein content was determined by Coomassie Plus assay reagent kit (Pierce). Samples were stored at −80°C.
Expression and purification of human supernatant protein (SPF)

The human SPF cDNA was cloned and expressed in the pTYB4 expression vector (New England Biolabs). SPF protein was purified following the protocol for expression of intein fusion proteins with the IMPACT T7 system as follows. SPF expression was induced in \textit{E. coli} ER2566 cells overnight with 1 mM isopropyl-\(\alpha\)-thiogalactopyranoside at 30 °C with slow shaking. All subsequent steps were carried out at 4 °C. Cells were broken in a French pressure cell in buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.1 mM EDTA, and the lysate was cleared by 12,000 \(\times\) \(g\) centrifugation for 30 min followed by digestion with 1 µg of DNA nuclease for 1 h. The cleared lysate was loaded onto a chitin affinity column (2-ml bed volume), washed with 40 ml of lysis buffer, and incubated overnight at 4 °C in 4 ml of buffer containing 30 mM \(\beta\)-mercaptoethanol to promote cleavage of the intein-SPF bond. SPF was eluted with 10-20 ml of the same buffer, which was then replaced by centrifugal dialysis with 20 mM Tris-HCl, pH 7.4, and the sample was stored at 80 °C. The purified protein retains four amino acids (Leu-Glu-Pro-Gly) at the C terminus that are derived from the intein fusion.

Squalene monooxygenase assays

Microsomal squalene monooxygenase activity was determined with mouse liver microsomes (400 µg/incubation) in a final volume of 0.2 ml as described previously (Singh, Mokashi et al. 2003). In brief, 400 µg of microsomal protein, 30 µM FAD, 40 µM \([^{14}\text{C}]\)squalene, 10 µg of phosphatidylglycerol, and 0.3 mM AMO 1618 (Calbiochem) to inhibit oxidosqualene cyclase in 200 µl of 20 mM Tris-HCl buffer, pH 7.4, with 1 mM EDTA. Reactions were started by the addition of NADPH to 1 mM, incubated in a 37 °C water bath for 1 h, and were stopped by the addition of 0.5 ml of 10% KOH in methanol after the incubation volume was brought to 1 ml with water. The tubes were capped, and after saponification at 80 °C for 1 h the neutral lipids were extracted with 3 ml of petroleum ether. After removing the solvent by centrifugal evaporation, the lipids were resuspended in 50 µl of petroleum ether and spotted onto silica thin-layer plates. Lipids were fractionated with 5% ethyl acetate in hexane, visualized, and quantified by
electronic autoradiography (Packard Instant Imager). Purified recombinant SPF was added at 0.8 µg unless indicated otherwise. Radiolabeled $^{14}$C-squalene was synthesized by SRI International (Menlo Park, CA) and used at 7 mCi/mmol.

**Affinity chromatography of SPF to nucleotide affinity resins**

To determine the binding of SPF to nucleotide affinity resins, 4 µg of SPF in 40 µl of 50 mM potassium phosphate buffer, pH 7.5, was mixed with approximately the same volume of 2’,5’-ADP-agarose or β-NADP-agarose and incubated at room temperature for 10 min. The resin was pelleted in a microfuge, the supernatant removed, and the resin washed 3 times with 0.5 ml of buffer. Elution was carried out with 40 µl of 10 mM NADPH or FAD in phosphate buffer. All supernatant fractions were analyzed by SDS-polyacrylamide gel electrophoresis with immunoblotting as described below.

**Isolation of primary hepatocytes**

Hepatocytes were prepared from 12-16-week old mice as follows: Mice were anesthetized with urethane and the liver was perfused via the portal vein with 50 ml of liver perfusion medium (Gibco) followed by 30 ml of liver digestion medium (Gibco). The liver was removed and transferred into a tissue culture plate and the liver capsule was removed. Hepatocytes were dissociated by blunt dissection and twice-filtered through a double layer of gauze. The cells were pelleted, washed twice, counted, and viability determined by trypan blue exclusion. Hepatocytes were allowed to attach to 35-mm tissue culture plates (BD Primaria™) in William’s E Medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. After 3 hr the cells were washed, the medium replaced with fresh medium, and the cells were used immediately in radiolabeling assays or incubated overnight prior to cytological analysis. Lipids were visualized by fixing the hepatocytes in 10% formalin and staining with hematoxylin-eosin and oil red O following standard procedures.
Radiolabeling of squalene, 2,3-oxidosqualene, and cholesterol in primary hepatocytes

Primary hepatocytes, prepared as described above, were incubated for 3 hr in media to which 1 µCi/ml of $^{14}$C-mevalonate was added, after which the cells were washed twice with phosphate-buffered saline, harvested by scraping, resuspended, and lysed by sonication in 1 ml 0.05 M Tris-HCl (pH 7.5) buffer. Inhibitors of cholesterol synthesis were added to the media prior to the addition of radiolabel as indicated in the figure legends: AMO 1618 (Calbiochem), 1.5 mM; terbinafine (TCI America), 60 µM; ketoconazole (Sigma Chemical), 10 µM; squalestatin (zaragozic acid A, Sigma Chemical), 66 µM. Radiolabeled squalene and 2,3-oxidosqualene were extracted and quantified by electronic autoradiography as described above for squalene monooxygenase assays. Cholesterol synthesis was measured as follows: Lipids were extracted into chloroform:methanol (2:1), the solvent was removed by evaporative centrifugation, and the lipids were resuspended and spotted onto silica thin layer plates. Chromatography was carried out in petroleum ether:ethyl ether:acetic acid (60:40:1), lipids were identified by co-chromatography of authentic standards visualized by iodine-vapor staining, and quantified by electronic autoradiography.

Extraction of lipids and gas chromatographic-mass spectrometric analysis

Lipids from hepatocytes incubated in the absence of radiolabel were prepared as described above, fractionated by thin-layer chromatography, and visualized by iodine-vapor staining. The desired lipid was scraped from the plate, extracted into chloroform:methanol (2:1), dried, and derivatized with trimethylsilane. For the analysis of lipids from the livers of wild-type and CPR$^{-/-}$ mice, 20 mg of liver tissue was homogenized in 1 ml of water, followed by the addition of 2 ml of alcoholic potassium hydroxide (4.5 M KOH in 60% ethanol) and the samples were saponified at 90°C for 1 h. The samples were diluted with 1 ml of 95% ethanol and saponification continued for 1 hr, after which the lipids were extracted into 5 ml of $n$-heptane, dried by centrifugal evaporation, and derivatized with trimethylsilane. Mass spectra were acquired by the
University of Kentucky Mass Spectrometry Facility. GC-MS was performed on a Trace Gas Chromatograph with a DB5-ms (Agilent/J&W) 30 m x 0.25 mm (0.25 µm df) column coupled to a ThermoFinnigan PolarisQ ion-trap mass spectrometer. Electron impact ionization mass spectra were recorded at 70 eV and a mass spectral search program was used to identify compounds. Authentic 24-dihydrolanosterol (Steraloids, Inc.) was used to confirm the lipid assignment.

7-Dehydrocholesterol reductase assay

Microsomes (800 µg of protein) were incubated in a final volume of 500 µl buffer (pH 7.3) containing 100 mM K$_2$HPO$_4$, 1 mM DTT, 30 mM nicotinamide, 0.1 mM EDTA and NADPH generating system: 3.4 mM NADP$^+$, 30 mM glucose-6-phosphate, 0.3 IU glucose-6-phosphate dehydrogenase and ergosterol solubilized with 15 µl of a 13% solution of β-cyclodextrin (Pharmatec Inc., Alachua, FL). The reaction was initiated by the addition of NADPH regenerating system and continued for 1hr at 37°C with vigorous shaking. The reaction was stopped by adding 1ml of 1 N ethanolic NaOH and 500 ng of stigmasterol as an internal recovery standard. Stigmasterol (24-ethylcholest-5,22-diene-3β-ol) is a plant sterol which is not synthesized by humans and is identical to brassicasterol except that it has an ethyl instead of a methyl group at C-24. The mixture was saponified at 90°C for 1hr, extracted with 3 ml n-hexane and evaporated under a stream of nitrogen. Trimethylsilyl-ether derivatives were formed for gas–liquid chromatography–mass spectrometry. Quantitation was carried out by gas–liquid chromatography–mass spectrometry with selected-ion monitoring (SIM) using a ThermoFinnigan PolarisQ ion-trap mass spectrometer. Mass spectra were acquired by the University of Kentucky Mass Spectrometry Facility. GC-MS was performed on a Trace Gas Chromatograph with a DB5-ms (Agilent/J&W) 30 m x 0.25 mm (0.25 µm df) column coupled to a ThermoFinnigan PolarisQ ion-trap mass spectrometer. Electron impact ionization mass spectra were recorded at 70 eV and a mass spectral search program was used to identify compounds. The multiple ion detector was focused on $m/z$ 363 for ergosterol, $m/z$ 380 for brassicasterol, and $m/z$ 484 for 7-dehydro sitosterol.
Gel electrophoresis and immunoblotting

Microsomal or cytosolic proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and electroblotted to nitrocellulose filters. Immunodetection was carried out with goat anti-rabbit cytochrome P450 reductase, rabbit anti-human squalene monooxygenase, or chicken anti-human SPF as the primary antibody and alkaline phosphatase-conjugated IgG as the secondary antibody. Proteins were visualized by bromochloroindolyl phosphate/nitro-blue tetrazolium staining. Reductase antibody was a gift from Dr. M. J. Coon (University of Michigan); antibodies to squalene monooxygenase and SPF were prepared by commercial suppliers to the purified recombinant proteins provided by our laboratory. For immunoquantitation, 25 µg of protein was fractionated by electrophoresis and electroblotted to nitrocellulose. The membrane was blocked with 0.05% Tween-20 and 5% defatted milk and then incubated in this same buffer with antibody to squalene monooxygenase, SPF, or HMG-CoA reductase (Upstate USA, Inc., or Santa Cruz Biotechnology, Inc.). The immunoblots were developed with a secondary antibody conjugated to horseradish peroxidase and visualized and quantified by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce) on a Kodak Image Station.
CHAPTER FOUR: RESULTS AND CONCLUSION

The cytochrome $b_5$ pathway does not support squalene monooxygenase in mouse liver microsomes

Gene deletion studies revealed that CPR is not essential to ergosterol synthesis in yeast (Sutter and Loper 1989), and subsequent studies demonstrated that the three enzymes thought to be dependent on CPR, squalene monooxygenase, lanosterol 14α-demethylase (CYP51) and sterol Δ22-desaturase (CYP61), could be supported efficiently by the microsomal NADH-dependent cytochrome $b_5$ electron transport pathway (Lamb, Kelly et al. 1999). To determine if the $b_5$ pathway was similarly effective in mammalian cells, we monitored 2,3-oxidosqualene synthesis in liver microsomes from hepatic CPR$^{-/-}$ mice. As shown in Figure 4.1, only NADPH supported squalene monooxygenase activity, and only in microsomes from wild-type mice; neither NADPH nor NADH supported squalene monooxygenase activity in microsomes from animals lacking CPR. These results indicate that, unlike in yeast, the cytochrome $b_5$ pathway cannot support squalene monooxygenase in mouse liver. This conclusion was also reached by Ono and Bloch (Ono and Bloch 1975) in studies with partially purified enzymes.
Figure 4.1: The cytochrome $b_5$ pathway does not support squalene monooxygenase in mouse liver microsomes. Squalene monooxygenase activity was measured in microsomes prepared from livers of CPR$^{-/-}$ or wild-type animals in the presence of Triton X100 and NADPH or NADH. Lane 1: CPR$^{-/-}$ liver microsomes in the presence of 1 mM NADPH; Lane 2: CPR$^{-/-}$ liver microsomes in the presence of 1 mM NADH; Lane 3: Wild-type liver microsomes in the presence of 1 mM NADPH; Lane 4: Wild-type liver microsomes in the presence of 1 mM NADH.
Addition of cytosol supports activity of squalene monooxygenase in CPR\(^{-/-}\) microsomes

Studies have shown that squalene monooxygenase requires the cytosolic fraction for its activity. The non-ionic detergent Triton X100 can replace supernatant protein factor (SPF). It has been shown that non-ionic detergent Triton X-100 effects enzymatic reactions in a complex manner and that the detergent can activate some reactions and inhibit the others. To exclude the inhibitory effects of Triton X-100 on the possible alternative reductase and the possibility of a cytosolic reductase in liver, the 100,000xg supernatant fraction was added back to microsomes from CPR\(^{-/-}\) and wild-type animals. Addition of cytosol from either CPR\(^{-/-}\) or wild-type animals to CPR\(^{-/-}\) microsomes in the presence of NADPH but not NADH restored up to 40% of the squalene monooxygenase activity seen in wild-type microsomes (Figure 4.2). Essentially no activity is obtained with CPR-null microsomes in the absence of cytosol and NADPH, indicating a requirement for cytosol and NADPH-dependence. In addition, virtually no cytochrome P450 reductase exists in cytosol fraction, as detected by Western blotting for cytochrome P450 reductase (Figure 4.3), suggesting this stimulation is not due to residual cytochrome P450 reductase in cytosol fraction. Cytosol can not be replaced by Triton X-100 in CPR\(^{-/-}\) microsomes, suggesting that it is inhibitory effect on the unknown reductase for squalene monooxygenase. Western blot analysis also shown at least a 95% reduction in CPR expression in CPR-null microsomes, as shown in Figure 4.3, similar to the results performed by Gu et al. (Gu, Weng et al. 2003). Total CPR-deletion in CPR-null livers was not expected to occur since this tissue also contains several other cell types besides hepatocytes such as liver endothelial cells and Kupffer cells.
Figure 4.2: Stimulation of squalene monooxygenase activity in mouse liver microsomes upon addition of cytoplasmic fraction. Squalene monooxygenase activity was measured in CPR<sup>−/−</sup> or wild-type liver microsomes (400 µg total protein) in the presence of 1 mM NADPH. Lane 1: CPR<sup>−/−</sup> liver microsomes; Lane 2: CPR<sup>−/−</sup> liver microsomes and CPR<sup>−/−</sup> liver cytosol (2 mg total protein); Lane 3: CPR<sup>−/−</sup> liver microsomes and wild-type liver cytosol (2 mg total protein); Lane 4: Wild-type liver microsomes; Lane 5: Wild-type liver microsomes and wild-type liver cytosol (2 mg total protein).
Figure 4.3: Levels of CPR expression in cytoplasmic and microsomal fractions determined by immunoblotting with microsomes and cytosols. Lane 1: Prestaining protein marker; Lane 2: CPR standard (purified protein, 1 µg); Lane 3: 20 µg of total protein from CPR<sup>−/−</sup> mouse liver microsomes (upper panel) or cytosol (lower panel); Lane 4: 20 µg of total protein from wild-type mouse liver microsomes (upper panel) or cytosol (lower panel). Immunodetection was carried out with goat anti-rabbit cytochrome P450 reductase as the primary antibody and alkaline phosphatase-conjugated IgG as the secondary antibody. Proteins were visualized by bromochloroindolyl phosphate/nitro-blue tetrazolium staining.
An SPF-dependent squalene monooxygenase reductase is present in CPR⁻⁻ microsomes

Squalene monooxygenase activity measured in hepatic CPR-null microsomes showed a dose-dependent pattern with CPR-null liver cytosol (Figure 4.4A). Although initially this suggested the presence of a cytosolic reductase, further studies suggested that this activity is due to the presence of SPF, a cytosolic protein that stimulates squalene monooxygenase, HMG-CoA reductase, and cholesterol synthesis (Tai and Bloch 1972; Ferguson and Bloch 1977; Mokashi, Singh et al. 2005). The addition of purified recombinant SPF to CPR-null microsomes abolished the dose-dependent response of squalene monooxygenase activity versus the concentrations of cytosol proteins (Figure 4.4B).
Figure 4.4: Addition of supernatant protein factor (SPF) abolished the dose-dependent response of squalene monooxygenase activity versus the concentrations of cytosol proteins. A) Squalene monooxygenase activity was measured in hepatic CPR-null microsomes in dose-dependent pattern with CPR-null liver cytosol proteins. B) Squalene monooxygenase activity was measured in hepatic CPR-null microsomes with various concentrations of CPR-null liver cytosol proteins in the presence of bacterial expressed purified SPF (0.8ug). Squalene monooxygenase activity was measured with CPR-null liver cytosol proteins. Each point represents the mean and standard error of 3 experiments carried out in duplicate.
A.

B.
Furthermore, we demonstrated that the addition of purified recombinant SPF to microsomes from CPR$^{-/-}$ or wild-type animals increased squalene monooxygenase activity by 2-4-fold. This increase is similar to that seen upon the addition of cytosol to CPR$^{-/-}$ microsomes, but is somewhat less than the stimulation obtained with 0.1% Triton X100 in wild-type microsomes. This detergent is unable to stimulate squalene monooxygenase activity in microsomes from CPR$^{-/-}$ animals even in the presence of SPF, suggesting that the stimulatory effect of this detergent is on CPR. SPF was considerably less effective in stimulating squalene monooxygenase when NADH was the reductant, further suggesting that the squalene monooxygenase reductase was NADPH-specific (Figure 4.5).
Figure 4.5: An SPF-dependent squalene monooxygenase reductase is present in CPR−/− microsomes. Squalene monooxygenase activity was measured in CPR−/− liver microsomes in the presence of NADPH or NADH and Triton X-100 (TX100) or purified recombinant SPF (0.8 µg). Each value represents the mean ± S.E. of three experiments carried out in duplicate.
SPF is not involved in NADPH-binding

To exclude the possibility that SPF was serving as the NADPH-dependent reductase, SPF was added to purified, recombinant squalene monooxygenase: no squalene monooxygenase activity was obtained in this reconstituted system in the absence of CPR. Addition of SPF to detergent-activated wild-type microsomes also provided no additive effect. Affinity chromatography experiments provided further evidence against a redox role for SPF, as SPF did not bind to 2’,5’-ADP agarose or β-NADP-agarose, and washing with NADPH or FAD did not elute SPF protein (Figure 4.6). Consistent with this finding, SPF shows no structural features characteristic of NADPH-dependent reductases (Stocker, Tomizaki et al. 2002).
**Figure 4.6: The binding of SPF to nucleotide affinity resins.** 4 µg of purified SPF was mixed with 2',5'-ADP-agarose or β-NADP-agarose affinity resin and incubated at room temperature for 10 min. The resin was pelleted in a microfuge, the supernatant removed, and the resin washed. Elution was carried out with 40 µl of 10 mM NADPH or FAD in phosphate buffer. All supernatant fractions were analyzed by SDS-polyacrylamide gel electrophoresis with immunoblotting. Lane 1: Molecular markers; Lane 2: Elution of 2',5'-ADP-agarose resin with FAD; Lane 3: Elution of 2',5'-ADP-agarose resin with NADPH; Lane 4: Supernatant from the incubation with 2',5'-ADP-agarose resin; Lane 5: Purified SPF; Lane 6: Supernatant from the incubation with β-NADP-agarose resin; Lane 7: Elution of β-NADP-agarose resin with FAD; Lane 8: Elution of β-NADP-agarose resin with NADPH.
Antibody to P450 reductase does not block the SPF-dependent reductase

Because conditional-null animals often have very low levels of the targeted gene product due to incomplete deletion during development, as well as due to expression in other cell types (i.e., macrophages, vascular tissue), the possibility was considered that the activity present in CPR-null hepatic microsomes was due to low levels of CPR from other sources. To exclude this possibility that residual CPR might play a role in the SPF-dependent squalene monooxygenase activity, antibodies to CPR were added to microsomes from wild-type and CPR-null animals, and squalene monooxygenase activity was measured in the presence of SPF. As shown in Figure 4.7, antibody to CPR reduced squalene monooxygenase activity in wild-type microsomes by more than 60%, to a level equal to that obtained with microsomes from CPR\(^{-/-}\) animals. Antibody to CPR had no effect on squalene monooxygenase activity in CPR\(^{-/-}\) animals, demonstrating that residual CPR was not responsible for the activity present in these preparations. Addition of antibody to cytochrome P450 2E1 had no effect on activity in either microsomal preparation, ruling out a nonspecific effect of antibody on squalene monooxygenase activity, and greater amounts of antibody to CPR had no further effect on activity in either preparation. These results indicate that residual CPR in microsomes from CPR\(^{-/-}\) animals is not responsible for the squalene monooxygenase activity seen in these preparations.
Figure 4.7: Antibody to P450 reductase does not block the SPF-dependent reductase. Squalene monooxygenase activity was measured in CPR<sup>−/−</sup> or wild-type liver microsomes in the presence of cytosol and 0.2 mg/ml polyclonal antibody to CPR or to cytochrome P450 2E1 (CYP2E1). Antibodies were added to the incubations 30 min prior to the addition of NADPH to start the reaction. Each value represents the mean ± S.E. of three experiments carried out in duplicate.
In order to evaluate whether squalene monooxygenase is active in the livers of CPR-/- mice, primary hepatocytes were isolated from CPR-/- mice and wild-type mice. The hepatocytes isolated from CPR-/- mice appear bulky under the microscope in comparison to wild-type hepatocytes. The cells were stained with oil red O which stains the neutral lipids, including sterols and sterol esters (Figure 4.8). The results of oil red O staining showed that the hepatocytes from CPR-/- mice contains multiple lipid droplets covering approximately 30% area of the cells, indicating the extensive accumulation of sterols and neutral lipids. In contrast, there were virtually no sterols and neutral lipids observed in wild-type hepatocytes. These results indicate that the capacity of the CPR-/- cells to remove the neutral lipids is attenuated.
Figure 4.8: Primary hepatocytes from A) wild-type and B) hepatic CPR-null mice were stained with Oil Red O. Hepatocytes were prepared from mice at age of 12-16 wks, incubated overnight, fixed in 10% formalin and stained with Oil Red O following standard procedures Gill's hematoxylin solution was used for counterstaining the nuclei.
A) wild-type hepatocytes

B) CPR\textsuperscript{−/−} hepatocytes
Squalene monooxygenase is active in CPR<sup>−/−</sup> hepatocytes

To exclude the possibility that the squalene monooxygenase reductase activity found in CPR<sup>−/−</sup> microsomes was an artifact of the preparation, hepatocytes were isolated from CPR<sup>−/−</sup> mice and 2,3-oxidosqualene formation from <sup>14</sup>C-mevalonate was monitored by thin-layer chromatography. To prevent oxidosqualene from being converted to lanosterol, AMO 1618, a cell-permeable inhibitor of oxidosqualene cyclase, was added to these incubations. As shown in Figure 4.9, 2,3-oxidosqualene accumulates in CPR<sup>−/−</sup> cells (os, lane 1). The addition of terbinafine, an inhibitor of squalene monooxygenase, decreases the oxidosqualene band while causing squalene to accumulate (sq, lane 2). In untreated cells, both squalene and 2,3-oxidosqualene were further metabolized and do not accumulate (lane 3). This offers strong evidence that squalene monooxygenase is enzymatically active in CPR-null hepatocytes. Similar results are obtained with wild-type cells (lanes 4-7), although the lack of squalene accumulation in these cells in the presence of an oxidosqualene cyclase inhibitor (lane 4) suggests that squalene monooxygenase is more active in wild-type than in CPR<sup>−/−</sup> cells. Furthermore, to confirm that these two bands are indeed squalene and oxidosqualene, zaragozic acid A (squalestatin), an inhibitor of squalene synthase, was added into the medium to prevent the synthesis of both pre-sterols (lane 6), leading to the disappearance of both squalene and 2,3-oxidosqualene.
Figure 4.9: Squalene monooxygenase is active in CPR-/- hepatocytes. 2,3-Oxidosqualene formation from 14C-mevalonate was monitored by electronic autoradiography of thin-layer chromatograms. A, lipids were extracted from CPR-/- hepatocytes incubated in the presence of AMO 1618 (lanes 1) with terbinafine (lane 2) or untreated cells (lane 3); os, oxidosqualene; sq, squalene. B, lipids were extracted from wild-type hepatocytes incubated in the presence of AMO 1618 (lanes 4-6) with terbinafine (lane 5) or squalestatin (lane 6). Lane 7 contains lipids from untreated cells.
**Cholesterol biosynthesis is blocked in CPR<sup>−/−</sup> hepatocytes**

Cholesterol synthesis in hepatocytes isolated from CPR<sup>−/−</sup> mice and wild-type mice was monitored by incubating with \(^{14}\)C-mevalonate in the absence of cholesterol synthesis inhibitors. Lipids were extracted into chloroform: methanol (2:1) and cholesterol biosynthesis was monitored by thin-layer chromatography. In Figure wild-type animals. Addition of cytosol from either CPR<sup>−/−</sup> or wild-type animals to CPR<sup>−/−</sup> microsomes in the presence of NADPH but not NADH restored up to 40% of the squalene monooxygenase activity seen in wild-type microsomes (Figure 4.2). Essentially no activity is obtained with CPR-null microsomes in the absence of cytosol and NADPH, indicating a requirement for cytosol and NADPH-dependence. In addition, virtually no cytochrome P450 reductase exists in cytosol fraction, as detected by Western blotting for cytochrome P450 reductase (Figure 4.3), suggesting this stimulation is not due to residual cytochrome P450 reductase in cytosol fraction. 4.10, lipids from CPR<sup>−/−</sup> cells exhibit a prominent band with a mobility characteristic of methyl-sterols (lane 1, ms). Labeled cholesterol is not evident in these cells, but is prominent in lipids from wild-type cells (lane 2, chol). In lipids from wild-type cells treated with ketoconazole, an inhibitor of lanosterol demethylase, cholesterol labeling decreased and a band corresponding to lanosterol appeared (lane 3, ms). The methyl-sterol that accumulates in CPR<sup>−/−</sup> cells closely resembles lanosterol in mobility.
Figure 4.10: Cholesterol biosynthesis is blocked in CPR\(^{+/−}\) hepatocytes and an unknown sterol is accumulated. Cholesterol formation from \(^{14}\)C-mevalonate was monitored by electronic autoradiography of thin-layer chromatograms in the absence of cholesterol synthesis inhibitors. Lipids were extracted from CPR\(^{+/−}\) (lane 1) or wild-type (lanes 2, 3) hepatocytes. Lane 3 contains lipids from cells incubated with ketoconazole. \(ms\), Methyl-sterol; \(chol\), cholesterol.
24-Dihydrolanosterol accumulates in CPR<sup>−/−</sup> hepatocytes

To identify the methyl-sterol that accumulates in CPR<sup>−/−</sup> cells, lipids from unlabeled CPR<sup>−/−</sup> hepatocytes were fractionated by thin-layer chromatography and the methyl-sterol region on the plate was isolated and subjected to gas-chromatographic mass-spectrometric analysis. As shown in Figure 4.11, the gas chromatogram revealed a principal peak at 27.64 min (panel A) which corresponds to 24-dihydrolanosterol by mass-spectrometric analysis (panel B). Analysis of a 24-dihydrolanosterol standard yielded a similar retention time at 27.71 min and an identical ion pattern (panels C and D), confirming the identity of this sterol. The peak at 27.30 min in the dihydrolanosterol standard (E) is lanosterol, as determined by mass spectrometric analysis.
4.11: The unknown sterol is identified as 24-dihydrolanosterol. Lipids from CPR^-/- hepatocytes were fractionated by thin-layer chromatography and the methyl-sterol region was eluted and further fractionated by gas chromatography (A). The peak at 27.64 min in panel A was subjected to mass-spectrometric analysis (B), yielding 24-dihydrolanosterol (inset). Gas chromatographic analysis of authentic 24-dihydrolanosterol yielded a peak at 27.71 min (C), which yielded an ion spectral pattern (D) identical to that in panel B. The peak at 27.30 min in the 24-hydrolanosterol standard (C) is lanosterol, as determined by mass-spectrometric analysis (E).
24-Dihydrolanosterol accumulates of in CPR-null livers

Neutral lipids from the wild-type and CPR\(^+\) livers were extracted and analyzed by GC/MS to determine the levels of cholesterol and of the following cholesterol precursors: squalene, lanosterol, and 24-dihydrolanosterol.

As shown in Figure 4.12, a total ion chromatogram shows that cholesterol is the predominant component in both cells types (panel A, E), indicating that the CPR\(^+\) hepatocytes were able to maintain cholesterol level by uptake from blood circulation even though the synthesis of cholesterol was abolished. In addition, attenuation of the synthesis of bile acids in CPR\(^+\) hepatocytes might contribute to the homeostasis of cholesterol. Despite the same level of cholesterol in CPR\(^+\) and wild-type livers, Gu et al found a significant decrease in bile acid synthesis in CPR-null mice (Gu, Weng et al. 2003), indicating that the P450s (CYP7A1, CYP8B1 and CYP27A1) involved in bile acid synthesis are inactivated as their obligatory electron donor, cytochrome P450 reductase, has been deleted in the hepatocytes.

Comparing panel C to panel G, markedly elevated levels of 24-dihydrolanosterol were found in CPR-null livers, but this sterol metabolite was not above the background level in lipids from wild-type mice. Lanosterol was not above the background level in lipids from both wild-type and CPR-null livers with ion chromatogram of m/z 393 and retention time at 27.30 min as an indicator for this molecule. In addition, GC/MS also failed to pick up squalene above the background level in lipids from both wild-type and CPR-null livers when comparing with authentic squalene standard. The results were confirmed by co-injection with lipids from wild-type and CPR-null livers.

In summary, the analysis of lipids from the livers of CPR\(^+\) and WT animals demonstrated an abundance of 24-dihydrolanosterol in CPR\(^+\) livers but not in the lipids from WT mice. Lanosterol was not prominent in hepatocyte lipids from wild-type and CPR\(^+\) mice, suggesting that this sterol intermediate is readily converted to dihydrolanosterol from lanosterol by sterol \(\Delta 24\)-reductase (Bae and Paik 1997). The accumulation of dihydrolanosterol in CPR\(^-\) livers demonstrates that squalene monooxygenase is active in CPR\(^-\) hepatocytes and indicates that cholesterol synthesis.
is interrupted at lanosterol demethylase, a cytochrome P450 evidently dependent on CPR.
Figure 4.12: Gas chromatographic-mass spectral analysis of sterols isolated from the livers of CPR<sup>−/−</sup> and wild-type livers. Sterols from CPR<sup>−/−</sup> and wild-type mice livers were extracted and analyzed by gas chromatography-mass spectrometric analysis. A) Total ion chromatogram of the mixture of sterols from CPR<sup>−/−</sup> mice liver. B) The highest peak at 24.71 min has been identified as cholesterol. C) Ion chromatogram of m/z 395 of sterols from CPR<sup>−/−</sup> mice liver shows the peak of 24-dihydrolanosterol at 27.63 min. D) The peak at 27.63 min has been identified as 24-dihydrolanosterol. E) Total ion chromatogram of the mixture of sterols from wild-type mice liver. F) The highest peak at 24.71 min has been identified as cholesterol. G) Ion chromatogram of m/z 395 of sterols from wild-type mice liver did not show the peak of 24-dihydrolanosterol at 27.63 min above background. H) Gas chromatographic analysis of authentic squalene yielded a peak at 19.77 min. I) The ion spectral pattern of squalene. J) Coinjection of sterols from CPR<sup>−/−</sup> and wild-type mice livers: total ion chromatogram; K) coinjection of sterols from CPR<sup>−/−</sup> and wild-type mice livers: ion chromatogram of m/z 395.
Co-injection: Total ion

m/z = 395-396
7-Dehydrocholesterol reductase is active in CPR<sup>−/−</sup> microsomes

The conversion of ergosterol to brassicasterol was used to determine the 7-dehydrocholesterol reductase activity in CPR<sup>−/−</sup> and wild-type microsomes (Honda, Tint et al. 1996). Figure 4.13 shows a representative GC/MS analysis obtained by incubation of ergosterol with microsomes while stigmasterol was used as an internal standard. TMS-ether derivatives of brassicasterol, ergosterol and stigmasterol have the retention time at 25.42, 26.45 and 27.21 min, respectively. The calibration curve for the weight ratio of brassicasterol and stigmasterol was established by focusing the ion detector on m/z 380 for brassicasterol (ion species: brassicasterol-(CH3)2-SiOH) and m/z 484 for stigmasterol (ion species: stigmasterol+).

As shown in Figure 4.14A, the activity of 7-dehydrocholesterol reductase in CPR<sup>−/−</sup> microsomes is slightly higher than that in wild-type microsomes. Although an antibody for 7-dehydrocholesterol reductase is not available for Western blotting, it has been shown the mRNA level of 7-dehydrocholesterol reductase increased by two-fold as compared to that in wild-type livers (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005). These results offer strong evidence that 7-dehydrocholesterol reductase is enzymatically active in CPR-null microsomes. In addition, the activity of 7-dehydrocholesterol reductase has been reported to be regulated by phosphorylation/dephosphorylation. The presence of Mg<sup>2+</sup> and ATP significantly increases the activity of 7-dehydrocholesterol reductase (Shefer, Salen et al. 1998). When we measured the activity of 7-dehydrocholesterol reductase with and without ATP, 10-fold lower activity was observed in the absence of ATP in CPR<sup>−/−</sup> and wild-type microsomes, which is in agreement with the findings of Shefer et al (Figure 4.14).
Figure 4.13: A representative gas-chromatogram using ergosterol as substrate for 7-dehydrocholesterol reductase. Peaks of product (brassicasterol) and ergosterol catalyzed by 7-dehydrocholesterol, internal standard (stigmasterol) and substrate (ergosterol) were shown at m/z= 380, 484 and 363, respectively.
Figure 4.14: Comparison of 7-dehydrocholesterol reductase activity in CPR\(^+\) and wild-type microsomes. Brassicasterol synthesis was measured in hepatic CPR-null and wild-type microsomes in dose-dependent pattern with different concentrations of ergosterol A) in the presence of ATP; B) in the absence of ATP. Each point represents the mean and standard error of 2 experiments.
**Lipid accumulation in CPR<sup>−/−</sup> cells alters the expression of cholesterolgenic enzymes**

Despite the block to cholesterol synthesis, lipid droplets accumulate in CPR<sup>−/−</sup> hepatocytes, consistent with the hepatic lipidosis characteristic of these mice (Gu, Weng et al. 2003; Henderson, Otto et al. 2003). This lipid accumulation apparently leads to the down-regulation of two key cholesterolgenic enzymes, squalene monooxygenase (Figure 4.15) and HMG-CoA reductase (Figure 4.16) by half. In contrast, the expression of SPF is doubled, consistent with its role in facilitating electron transfer to squalene monooxygenase by squalene monooxygenase reductase (Figure 4.17). The decrease in HMG-CoA reductase expression contrasts with the modest increase (~1.5-fold) reported for HMG-CoA reductase mRNA levels in these mice (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005). Similarly, although squalene monooxygenase protein levels were decreased, squalene monooxygenase mRNA levels were increased 2-fold (Weng, DiRusso et al. 2005) or unchanged (Wang, Chamberlain et al. 2005). SPF gene expression was not examined in these two studies. These results indicate that translational and/or post-translational regulatory mechanisms are likely operative for these key cholesterolgenic enzymes.
Figure 4.15: Quantitative analysis of immunoblot analysis of squalene monooxygenase. Images were acquired with 2 min exposures of immunoblots containing twofold serial dilutions of CPR⁻/⁻ and wild-type microsomes from 20 µg to 2.5 µg total protein. Standard curves were generated with mean band intensity of squalene monooxygenase from wild-type microsomes and the mean band intensities from CPR⁻/⁻ microsomes were fit into the curve. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background.
Figure 4.16: Quantitative analysis of immunoblot analysis of HMG-CoA reductase. Images were acquired with 2 min exposures of immunoblots containing twofold serial dilutions of CPR\textsuperscript{-/-} and wild-type microsomes from 20 \( \mu \)g to 2.5 \( \mu \)g total protein. Standard curves with mean band intensity of HMG-CoA reductase from wild-type microsomes were generated and the mean band intensities from CPR\textsuperscript{-/-} microsomes were fit to the curve. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background.
Figure 4.17: Quantitative analysis of immunoblot analysis of SPF. Images were acquired with 2 min exposures of immunoblots containing twofold serial dilutions of CPR$^{-/-}$ and wild-type cytosol from 10 µg to 1.25 µg total protein. Standard curves with mean band intensity of SPF from wild-type cytosol were generated and the mean band intensities from CPR$^{-/-}$ cytosol were fit to the curve. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background.
Conclusion and discussion

The selective deletion of hepatic cytochrome P450 reductase results in a marked decrease in plasma cholesterol levels even while lipids accumulate in the liver (Gu, Weng et al. 2003; Henderson, Otto et al. 2003, and present data). This contrasting effect on lipids reflects the essential role of CPR in both the synthesis and degradation of cholesterol. While it was anticipated that cholesterol synthesis would be interrupted by the loss of CPR, it was not anticipated that squalene monooxygenase activity would remain partially intact in these cells. A requirement for CPR in squalene epoxidation was first suggested by Ono and Bloch (Ono and Bloch 1975) and Ono et al. (Ono, Ozasa et al. 1977) in studies with partially purified enzymes, and later confirmed by Ono et al. (Ono, Takahashi et al. 1980) in a reconstituted system with the purified enzymes. Why was squalene monooxygenase reductase, the enzyme we uncovered here, not recognized in these early studies? The answer probably lies in the use of Triton X-100 to solubilize the microsomes for fractionation and enzyme purification. My studies indicate that Triton X-100 prevents the reduction of squalene monooxygenase by this second reductase, both in the presence and absence of SPF. As this nonionic detergent was included in all assays used to fractionate and purify squalene monooxygenase, it is not surprising that a detergent-sensitive reductase that also requires SPF for activity was not discovered. I believe the present studies convincingly demonstrate the existence of this reductase, but I have no information as to its properties (other than NADPH-specificity) and do not know of any microsomal redox proteins that might fulfill this role.

The mechanism by which SPF facilitates this reaction remains unresolved. SPF was first characterized as a squalene transfer protein (Friedlander, Caras et al. 1980), but more recent studies are equivocal on this role. SPF clearly belongs to the CRAL/TRIO family of lipid binding proteins, which includes Sec14p and α-tocopherol transfer protein (Stocker, Tomizaki et al. 2002; Porter 2003). However, SPF shows greatest binding affinity for phosphatidylinositol, whereas squalene is a relatively poor ligand for this protein (Panagabko, Morley et al. 2003). The ability of anionic phospholipids to stimulate squalene monooxygenase (Tai and Bloch 1972) raises the
possibility that phosphatidylinositol is the physiologic ligand for this cytosolic protein. Why anionic phospholipids are stimulatory in this system and how SPF and these lipids interact with squalene monooxygenase and squalene monooxygenase reductase is unclear; indeed, the stimulation of squalene monooxygenase by SPF may simply reflect its ability to recruit and/or activate squalene monooxygenase reductase as an additional electron donor in this pathway. My studies indicate that SPF does not bind to nucleotide cofactor affinity resins nor directly catalyze electron transfer to squalene monooxygenase. Nonetheless, SPF shows some unusual characteristics, including the ability to be activated by phosphorylation (Singh, Mokashi et al. 2003), to hydrolyze GTP (Habermehl, Kempna et al. 2005), to suppress tumor cell growth via inhibition of phosphatidylinositol-3-kinase/Akt signaling (Kempna, Zingg et al. 2003; Ni, Wen et al. 2005), and to stimulate HMG-CoA reductase and cholesterol synthesis when overexpressed in cultured cells (Mokashi, Singh et al. 2005), the latter of which requires an interaction with Golgi (Mokashi and Porter 2005).

As demonstrated herein, 24-dihydrolanosterol levels are greatly above background in the livers of hepatic CPR\(^{-/-}\) mice. The accumulation of this sterol indicates that lanosterol demethylation, a cytochrome P450-mediated reaction, is blocked, and lanosterol is serving as a substrate for sterol \(\Delta 24\)-reductase. Studies with freshly isolated hepatocytes from CPR\(^{-/-}\) mice further demonstrate the \textit{de novo} synthesis and accumulation of this methyl-sterol. I thus conclude that, while squalene monooxygenase is able to obtain electrons from the newly discovered reductase in the absence of CPR, lanosterol demethylase does not have a significant alternative redox partner. This is in contrast to yeast, where ergosterol synthesis is minimally maintained in the absence of CPR by the cytochrome b\(_5\) electron transport pathway (Lamb, Kelly et al. 1999). The alternative electron donor for squalene monooxygenase in yeast has not been established, but has been assumed to be cytochrome b\(_5\). The present studies may induce a re-examination of this assumption, but it should be noted that a clear ortholog of SPF does not exist in Saccharomyces.

It has also been suggested that mammalian 7-dehydrocholesterol reductase, the final step in cholesterol synthesis, is dependent on CPR, based on several indirect assays (Nishino and Ishibashi 2000). My experiments with this enzyme in reconstituted
CPR⁻/⁻ microsomal systems do not support this hypothesis. Surprisingly, the activity of 7-dehydrocholesterol reductase was approximately twice as high as in CPR⁺/⁺ microsomes as compared to that of wild-type microsomes. Given that the mRNA level of 7-dehydrocholesterol reductase increased by two-fold as compared to that in wild-type livers, I expected that 7-dehydrocholesterol reductase would be as enzymatically active in CPR-null microsomes as same as in wild-type microsomes. As with squalene monooxygenase, the activity of 7-dehydrocholesterol reductase is NADPH-dependent, ruling out a role for cytochrome b₅ in these reactions. Unlike squalene monooxygenase, the activity of 7-dehydrocholesterol reductase in CPR⁻/⁻ microsomes can be increased by the addition of ATP to the same extent as in wild-type microsomes, indicating that 7-dehydrocholesterol reductase activity is regulated in part by phosphorylation.

Under normal circumstances a block to cholesterol synthesis in CPR⁻/⁻ hepatocytes would be expected to up-regulate cholesterol synthesis via the SREBP pathway. Indeed, a modest increase (~1.5-fold) in HMG-CoA reductase mRNA was reported in the livers of hepatic CPR⁻/⁻ animals, with similar increases in the mRNAs for many, but not all, cholesterologenic enzymes co-regulated by the SREBP pathway (Wang, Chamberlain et al. 2005; Weng, DiRusso et al. 2005). However, this increase is considerably less than might be expected, likely reflecting the concomitant suppression of SREBP processing by the inability of the CPR⁻/⁻ liver to eliminate extra-hepatic cholesterol as bile acids. In fact, my studies reveal that HMG-CoA reductase and squalene monooxygenase protein levels are decreased in the livers of CPR⁻/⁻ mice, suggesting that secondary, post-translational mechanisms for the regulation of these enzymes prevail in CPR⁻/⁻ hepatocytes. Interestingly, it has been shown that 4,4'-dimethylsterols, including lanosterol and 24-dihydrolanosterol, are potent regulators of HMG-CoA reductase enzyme stability (Song, Javitt et al. 2005). These cholesterol precursors, including lanosterol and 24-dihydrolanosterol, promote the formation of a reductase-Insig complex which is then recognized by a microsomal E3 ligase, gp78, leading to the ubiquitination and subsequent degradation of HMG-CoA reductase (Song, Sever et al. 2005). The 50% decrease in HMG-CoA reductase protein levels in CPR⁻/⁻ livers found herein suggests that the accumulation of 24-dihydrolanosterol promotes the degradative of HMG-CoA reductase, but HMG-CoA reductase activity was
not measured in these experiments, and further studies will be needed to confirm that these changes in enzyme levels reflect changes in total enzyme activity. Interestingly, squalene monooxygenase is not known to be subject to post-translational regulation in this manner, but our finding that protein levels were similarly decreased suggests that this enzyme may also be regulated by this mechanism, particularly in light of the 2-fold increase in squalene monooxygenase mRNA levels found by Weng et al. (Weng, DiRusso et al. 2005). The 2-fold increase in SPF protein levels suggests an up-regulation of this protein in response to the loss of CPR, which would facilitate the reduction of squalene monooxygenase by the squalene monooxygenase reductase uncovered here.

Cholesterol is an important membrane component as well as a precursor for the synthesis of the steroid hormones and bile acids. On the other hand, high blood cholesterol level is a major risk factor for coronary heart diseases. Therefore, the synthesis and utilization of cholesterol must be tightly regulated in order to prevent abnormal deposition within the body. Statins, HMG-CoA reductase inhibitors, are the main treatment for lowering cholesterol. A major side effect of statins includes muscle weakness. Understanding the unique requirements for electron transfer for different steps in cholesterol synthesis might lead to new treatment for lowering plasma cholesterol level with fewer side effects.
PART TWO: STUDY OF THE STIMULATORY EFFECT OF CYTOCHROME $b_5$ IN THE ACTIVATION OF CYP2E1 USING THE HEPATIC CYTOCHROME P450 REDUCTASE-NULL MOUSE MODEL
Introduction

Cytochrome $b_5$ is a ubiquitous electron transfer protein capable of accepting and transferring a single electron with a redox midpoint potential of 20 mV (Porter 2002). Several $b_5$ isoforms have been isolated, including a membrane-bound endoplasmic reticular form of $b_5$ which is involved in lipid desaturation; a mitochondria membrane-bound form of $b_5$ with unknown function; and a cytosolic form whose function is to reduce hemoglobin in red blood cells. Although the amino acid sequence of the heme-binding domains of the microsomal and mitochondrial cytochromes $b_5$ reveals that only 58% of the amino acids are conserved, they arise from the same gene by different mRNA splicing, whereas the soluble cytochrome $b_5$ is expressed from a separate gene (Slaughter, Williams et al. 1982; Lederer, Ghrir et al. 1983; Schafer and Hultquist 1983).

The form of cytochrome $b_5$ associated with the endoplasmic reticulum has been purified from rat microsomes in the presence of detergents (Carlsen, Christiansen et al. 1988). The 17 kDa protein is composed of two domains: a hydrophobic tail which anchors the protein to the membrane, and a hydrophilic portion, the heme binding domain, which is active in redox reactions. The solution-NMR structure of $b_5$ shows that cytochrome $b_5$ consists of 6 helices and 5 $\beta$-strands. The heme is inserted into a hydrophobic pocket within the catalytic region between four $\alpha$-helices with a heme edge exposed to the environment, while the heme iron is coordinated with two completely conserved histidine side chains, H68 and H44. The ligation of the fifth and sixth coordination position of the heme iron to histidines prevents its direct interaction with molecular oxygen (Figure 5.1). However, the protein is able to serve as an electron transfer intermediate between reductases and oxidative enzymes (Arnesano, Banci et al. 1999; Schenkman and Jansson 2003).

The endoplasmic reticular-bound cytochrome $b_5$ normally accepts an electron from NADH-cytochrome $b_5$ reductase and donates the electron to a variety of acceptors involved in the biosynthesis of desaturated lipids and cholesterol. Alternatively,
cytochrome $b_5$ can accept an electron from NADPH-cytochrome P450 reductase and participate in drug metabolism by interacting with some cytochrome P450s (Dailey and Strittmatter, 1980; Fukushima, Grinstead et al. 1981; Aoyama, Nagatak et al. 1990).
Figure 5.1: Ribbon structure illustration of solution NMR structure of the B form of oxidized rat microsomal cytochrome $b_5$ with cofactor heme. The heme iron is coordinated with histidine side chains of H68 and H44. Protein data bank (1BFX), viewed with CN3D viewer (Arnesano, Banci et al. 1999).
Catalytic cycle of cytochrome P450

The catalytic cycle of cytochrome P450s has been studied extensively. The general P450 reaction scheme is presented in Figure 5.2. The resting form of cytochrome P450s is a six-coordinated low spin ferric state. The iron-containing heme of P450 is bound to the enzyme through four porphryin nitrogens and a cysteine, which gives rise to the characteristic absorbance maximum at 450 nm when reduced and complexed with CO. A water molecule forms the exchangeable distal ligand trans to the proximal cysteine. The binding of substrate generates the five-coordinate high spin ferric state, which results in a significant increase in the redox potential of the heme (-330 to -173mV). This allows the electron-donor partner to transfer an electron to the iron to generate the ferrous iron. Molecular oxygen then becomes bound to the ferrous iron center to give the ferrous-dioxy complex. Addition of the second electron to the ferrous-dioxy complex yields a ferric-peroxide complex leading to the splitting of the oxygen-oxygen bond, with the release of one atom as water. The remaining oxygen-iron complex forms the reactive FeO$_3^{3+}$ which is able to oxidize substrate. Uncoupled turnover of P450s produces superoxide and H$_2$O$_2$ (Guengerich, 2001).
**Figure 5.2: The catalytic cycle of cytochrome P450 enzymes.** The iron in brackets represents the prosthetic heme group of P450s and RH represents a substrate. Molecular oxygen is split, one atom is utilized to oxidize substrate, and the other atom is reduced to water (Guengerich, 2001).

\[
[\text{Fe}^{+3}] + \text{RH} \rightarrow [\text{Fe}^{+3}][\text{RH}] \rightarrow [\text{Fe}^{+3}][\text{RH}]_{\text{low spin}} \rightarrow [\text{Fe}^{+3}][\text{RH}]_{\text{high spin}}
\]

\[
[\text{Fe}^{+3}][\text{ROH}] \rightarrow [\text{Fe}=\text{O}][\text{RH}] \rightarrow [\text{Fe}^{+2-}\text{O}_2^-][\text{RH}] \rightarrow [\text{Fe}^{+2-}\text{O}_2^-][\text{RH}]
\]

\[
\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{O}_2^{-}
\]
The ability of NADH to stimulate drug metabolism when added to liver microsomes suggested that the $b_5$ reductase/cytochrome $b_5$ pathway might also contribute to P450-mediated reactions (Conney, Brown et al. 1957; Hildebrandt and Estabrook 1971), a hypothesis supported by studies in which antibodies to cytochrome $b_5$ prevented the stimulation by NADH (Mannering, Kuwahara et al. 1974; Noshiro, Harada et al. 1979). The parallel observation that cytochrome P450 reductase was able to transfer electrons to cytochrome $b_5$ (Enoch and Strittmatter 1979), has complicated efforts to define the role of the NADH-dependent pathway in P450-mediated metabolism.

An initial study by Hildebrandt and Estabrook (Hildebrandt and Estabrook 1971) proposed that cytochrome $b_5$ could contribute the second electron to P450 in the catalytic cycle. However, the recent demonstration by Guengerich and colleagues that $b_5$ devoid of heme could also stimulate certain P450 reactions called into question the role of electron transfer in the stimulation by this hemeprotein (Yamazaki, Johnson et al. 1996; Yamazaki, Shimada et al. 2001). Although this has been demonstrated most clearly for CYP3A4, stimulation by apo-$b_5$ has been now been confirmed for a variety of P450s, including 2C9, 4A7, and 17 (Porter 2002). In contrast, CYP2E1 differs from these P450 isoforms in that it appears to require electron transfer from cytochrome $b_5$ for the stimulatory effect (Yamazaki, Gillam et al. 1997; Yamazaki, Nakamura et al. 2002). Yamazaki et al. (Yamazaki, Nakano et al. 1996) found that a reconstituted system containing NADH, cytochrome $b_5$ reductase, cytochrome $b_5$, and CYP2E1, but lacking cytochrome P450 reductase metabolized 7-ethoxycoumarin at about one-quarter the rate obtained in the presence of P450 reductase, demonstrating that both electrons could be derived from the NADH-dependent $b_5$ pathway in vitro. Studies with microsomes using antibodies to P450 reductase supported this finding (Yamazaki, Nakano et al. 1996). It has also been reported that CYP 2E1 in a reconstituted system containing NADH, $b_5$ reductase and cytochrome $b_5$ catalyzes chlorzoxazone 6-hydroxylation at about half of the rate obtained with P450 reductase (Yamazaki, Nakano et al. 1996).

It has been shown that the coexpression of cytochrome $b_5$ with CYP2E1 and P450 reductase in heterologous systems significantly enhances CYP2E1-mediated metabolism with isolated membrane preparations and in in vivo assays (Patten and
Koch 1995; Wang, Patten et al. 1996). Moreover, studies with the Ames mutagenicity assay showed that the NADH-dependent electron transport pathway is fully able to support CYP2E1 activity in the absence of cytochrome P450 reductase (Cooper and Porter 2001). In this system all electrons are derived from NADH and must pass through cytochrome b<sub>5</sub> to reach CYP2E1. Omitting either b<sub>5</sub> reductase or b<sub>5</sub> prevented activity, as indicated by a lack of nitrosamine-induced revertants in this assay. The presence of activity with this system clearly demonstrates that cytochrome b<sub>5</sub> is capable of providing both the first and second electron to CYP2E1 in whole cells.

Consistent with the experimental data indicating an interaction between cytochrome b<sub>5</sub> and cytochrome P450 reductase, modeling studies by Gao et al (Gao, Doneanu et al. 2006) shows that electrostatic interactions are the main stabilizing force for the interaction of CYP2E1 and cytochrome b<sub>5</sub>, and contribute to the proper relative orientations of the prosthetic groups necessary for the electron transfer process. The negatively charged residues located on the surface region where the b<sub>5</sub> heme group protrudes toward the solvent contribute to electrostatic interactions, along with the positively charged residues across the the proximal face of CYP2E1 where the buried CYP2E1 heme group comes closest to the solvent (Gao, Doneanu et al. 2006). The heme groups of the two proteins were positioned close to each other at a near 90° angle, supporting the direct electron transfer between these two proteins.
CYP2E1 is a major cytochrome P450 enzyme in liver that catalyzes a variety of xenobiotic hydroxylations including that of drugs and carcinogens. The stimulatory effect of cytochrome \( b_5 \) on CYP2E1 has been thought to result from direct electron transfer of one or both required electrons to this P450. Transfer of the second electron to oxyferrous P450 via cytochrome \( b_5 \) could be from either NADH-cytochrome \( b_5 \) reductase or NADPH-cytochrome P450 reductase. However, to date studies have been done only with purified enzyme systems or by addition of purified \( b_5 \) into P450 reductase-bearing microsomes. In the present studies, hepatic cytochrome P450 reductase-null mice, in which the expression of cytochrome P450 reductase is eliminated during maturation, were used to evaluate the role of cytochrome \( b_5 \) in CYP2E1-mediated drug metabolism using chlorzoxazone as a probe.

Chlorzoxazone is metabolized mainly by CYP2E1 in liver microsomes of various experimental animals and humans and has been used as a probe drug for CYP2E1-mediated metabolism. Although the biotransformation of chlorzoxazone by rat liver microsomes was fit to a biphasic kinetic profile, suggesting the involvement of multiple isoforms (Court, Von Moltke et al. 1997), and chlorzoxazone can be metabolized by CYP 2E1 and CYP1A1 using rat cDNA-expressed CYPs (Warrington, Court et al. 2004), the majority of kinetic data for liver microsomes from different species, including mouse, dog and human, followed a one-enzyme Michaelis–Menten model, indicating that CYP2E1 is the major P450 responsible for the chlorzoxazone biotransformation in these animals (Court, Von Moltke et al. 1997). Its principal metabolite, 6-hydroxychlorzoxazone, is easily detected by HPLC.
Specific Aims

1) NADH- and NADPH-mediated activities of CYP2E1 were determined using chlorzoxazone as a probe for CYP2E1 catalytic activity in CPR−/− and wild-type microsomes. Kinetic analysis was performed, providing an insight into the mechanism of stimulation by b5.

2) To evaluate whether b5 stimulation is physiologically relevant and takes place in the CPR-null liver cells, primary hepatocytes from CPR−/− mice and wild-type mice were isolated and chlorzoxazone metabolism was determined.
CHAPTER SIX: MATERIALS AND METHODS

Chemicals

Chlorzoxazone (CLZ), 6OH-chlorzoxazone (6OH-CLZ), phenacetin and other chemicals and solvents were purchased from Sigma. (St Louis, MO, USA). CLZ, 6OH-CLZ, and phenacetin were dissolved in methanol and stored at -20 °C. For incubations, appropriate volumes of CLZ in methanol were added to the incubation tubes and evaporated to dryness under reduced pressure in a vacuum oven at room temperature.

Animals

CPR liver-specific knockout mice (Alb-Cre^+/−/Cpr^lox+/+) were generated by crossing mice expressing liver-specific Cre under control of the an albumin promoter (Alb-Cre) with two lines of mice bearing a conditional CPR allele (Cpr^lox+/+) (Gu, Weng et al. 2003; Wu, Gu et al. 2003). Littermates lacking the Alb-Cre transgene (Alb-Cre^-/-/Cpr^lox+/+) were designated as wild-type. Two to four month old mice from null and wild-type littermate groups on mixed C57BL/6 (75%) and 129/Sv (25%) genetic background were used in the studies. Animals were normally maintained in a temperature-, humidity-, and light-controlled facility (70-72°F, 48-52% humidity, 12-hr light/dark cycle) and were allowed free access to water and food. Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the respective universities.

Preparation of Microsomes

Animals were killed by CO2 asphyxia, the livers promptly removed, and microsomal and cytosolic fractions prepared by standard procedures. The microsomal fraction was resuspended at ~15 mg of protein/ml in 100 mM phosphate buffer, 1mM EDTA, (pH 7.6), aliquoted, and stored at -80 °C. Frozen microsomes were thawed on ice immediately prior to use. All microsomes used in these studies were frozen and thawed.
once only. Protein content was determined by Coomassie Plus assay reagent kit (Pierce).

**Chlorzoxazone metabolism**

In vitro incubations were performed as described with minor modifications (Court, Von Moltke et al. 1997). Briefly, disposable glass culture tubes containing CLZ (0±1000 mM) were prepared with phosphate buffer (100 mM; pH 7.6), and thawed microsomal protein (400 ug total protein per tube) was added. The tubes were vortexed and placed in a 37 °C agitating water bath. Reactions were started by addition of 100 ul of an NADPH regenerating system (0.5 mM NADP⁺, 3.75 mM glucose-6-phosphate and 1 unit/mL glucose-6-phosphate dehydrogenase) or 100 ul of an NADH regenerating system (0.5 mM NAD⁺, 3.75 mM formic acid and 1 unit/mL formic acid dehydrogenase) to a final reaction volume of 0.5 ml. After 20 min at 37 °C, the reaction was stopped by addition of 100 ul 43% H₃PO₄, vortexed, and immediately cooled on ice. After addition of 5 mg phenacetin as an internal standard, the mixture was extracted twice with 1 ml of ethyl acetate. The organic layer was transferred to microcentrifuge tubes and evaporated under a stream of nitrogen. 120 µl of mobile phase (50 mM KH₂PO₄ in water:acetonitrile (75:25 v/v)) was added and vortexed, and the tubes were centrifuged for 15 min at 16,000 rpm. The supernatant was then transferred to vials for high-performance liquid chromatography (HPLC). The chromatography apparatus (Shimazu, Japan) consisted of a C18 column (u Bondapack, 300mm X 3.9mm I. D., Waters, Milford, MA, U.S.A.) with 50 mM KH₂PO₄ in water:acetonitrile (75:25 v/v) mobile phase at a flow rate of 1.0 ml/min. Eluants were monitored by ultraviolet absorption at 295 nm using a variable-wavelength detector. Retention times for 6OH-CLZ, phenacetin, and CLZ were approximately 7.03, 13.29, and 19.56 min, respectively. Product peak identity was verified by demonstrating co-elution with the purified 6OH-CLZ standard and disappearance upon exclusion of cofactors or microsomes from the incubation mixture. For each run, a calibration curve was prepared using a series of concentrations of pure 6OH-CLZ dissolved in the HPLC mobile phase with the internal standard. Actual
concentrations of metabolite were calculated by linear regression of the calibration curve.

**Isolation of primary hepatocytes**

Hepatocytes were prepared from 12-16-week old mice as follows: Mice were anesthetized with urethane and the liver was perfused via the portal vein with 50 ml of liver perfusion medium (Gibco) followed by 30 ml of liver digestion medium (Gibco). The liver was removed and transferred into a tissue culture plate and the liver capsule was removed. Hepatocytes were dissociated by blunt dissection and twice-filtered through a double layer of gauze. The cells were pelleted, washed twice, counted, and viability determined by trypan blue exclusion. Hepatocytes were allowed to attach to 35-mm tissue culture plates (BD Primaria™) in William’s E Medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. After 3 hr the cells were washed by PBS, the medium replaced with 2 ml of Hank’s Balanced Salt Solution (HBSS) and the cells were incubated with chlorzoxazone for 45 min. Cells were scraped from plates and transferred with HBSS solution into 10 ml of glass tubes and sonicated for 1 min. Chlorzoxazone and its metabolite were extracted with ethyl acetate as described above.

**Gel electrophoresis and immunoblotting**

Microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels (CYP2E1 and cytochrome P450 reductase) or 15% gels (cytochrome b\(_5\)), and then electroblotted to nitrocellulose filters. Immunodetection was carried out with goat anti-rat CYP2E1, goat anti-rabbit cytochrome P450 reductase, and goat anti-human b\(_5\) as the primary antibody and horseradish peroxidase-conjugated IgG as the secondary antibody. Reductase antibody was a gift from Dr. M. J. Coon (University of Michigan); antibodies to CYP2E1 (Daichi Pure Chemicals) and cytochrome b\(_5\) (Oxford Biomedical Research) were purchased from commercial suppliers. For immunoquantitation, 25 µg of protein was fractionated by electrophoresis.
and electroblotted to nitrocellulose. The membrane was blocked with 0.05% Tween-20 and 5% defatted milk and then incubated in this same buffer with antibody. The immunoblots were developed with a secondary antibody conjugated to horseradish peroxidase and visualized and quantified by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate, Pierce) on a Kodak Image Station.

**Data analysis**

Formation of 6-OH chlorzoxazone was fit to a single-enzyme Michaelis–Menten model using nonlinear regression: \( V = \frac{V_{\text{max}}S}{(K_m+S)} \), where \( V \) is the reaction velocity, \( S \) is the substrate concentration, \( V_{\text{max}} \) is the maximum reaction velocity and \( K_m \) is the substrate concentration corresponding to 50% \( V_{\text{max}} \).

**Statistical analysis**

The points were fit using nonlinear regression with the computer program GraphPad PRISM (San Diego, CA). The differences between the CPR+/- group and the wild-type group were compared using a one-way analysis of variance. For both statistical tests, an alpha value was set at \( P<0.05 \).
CHAPTER SEVEN: RESULTS AND DISCUSSION

NADH stimulates CYP2E1 activity in wild-type microsomes

Chlorzoxazone was used as a probe for the CYP2E1 activity in CPR⁻/⁻ and wild-type microsomes. Figure 7.1 shows a representative HPLC analysis obtained by incubation of chlorzoxazone with wild-type microsomes. Phenacetin was used as an internal standard. 6-Hydroxychlorzoxazone, phenacetin and chlorzoxazone have the retention time at 7.03, 13.29 and 19.57 min, respectively. The calibration curve was generated by the height:height ratio of 6-Hydroxychlorzoxazone:phenacetin.

As shown in Figure 7.2, NADH- and NADPH-dependent formation of 6-OH chlorzoxazone was evaluated in liver microsomes from CPR⁻/⁻ mice and wild-type mice. The reactions were performed in the presence of 3.7 mM NADPH and/or 3.7mM NADH in the presence of an NADPH regenerating system and/or an NADH regenerating system, as described under Materials and Methods. With wild-type microsomes, NADPH served as an effective electron donor for chlorzoxazone 6-hydroxylation. Addition of NADH along with NADPH doubled the rate of chlorzoxazone hydroxylation; however, inclusion of only NADH supported only a very low rate of hydroxylation (~5%) of that obtained with NADPH.
Figure 7.1: A representative HPLC chromatogram. Peaks of product (6OH-chlorzoxazone), internal standard (phenacetin) and substrate (chlorzoxazone) are shown at 7.03, 13.29 and 19.57 min, respectively.
Figure 7.2: Effect of NADH and NADPH on hydroxylation of chlorzoxazone in wild-type microsomes. Increasing concentrations of chlorzoxazone were incubated with pooled liver microsomes from wild type mice (n=6) for 20 min at 37°C in the presence of NADPH, NADH, or NADPH with NADH. Data was fit to one-site binding model using Prism. Assays were performed in triplicate and values represent the mean±s.e.
NADH supports low CYP2E1 activity in CPR⁻/⁻ microsomes

As shown in Figure 7.3, in microsomes from CPR⁻/⁻ livers NADH supports chlorzoxazone metabolism at a rate approximately 20% of that obtained in wild-type microsomes with NADPH, slightly greater than that seen in wild-type microsomes with NADH. Thus in both wild-type and CPR⁻/⁻ microsomes NADH supports a low rate of CYP2E1 activity. Addition of NADPH to NADH increased the chlorzoxazone hydroxylation activity in CPR-null microsomes by about one third, but was not statistically different (P>0.05) from the rate obtained with NADH alone. The use of NADPH alone did not support chlorzoxazone hydroxylation activity, consistent with the absence of cytochrome P450 reductase in these microsomes. Maximal catalytic activity in CPR⁻/⁻ liver microsomes in the presence of NADPH and NADH is approximately 15% of the chlorzoxazone hydroxylation observed in wild-type microsomes with the same cofactors.
Figure 7.3: Effect of NADH and NADPH on hydroxylation of chlorzoxazone in CPR^{-/-} microsomes. Increasing concentrations of chlorzoxazone were incubated with pooled liver microsomes from CPR^{-/-} mice (n=6) for 20 min at 37°C in the presence of NADPH, NADH, or NADPH with NADH. Data was fit to one-site binding model using Prism. Assays were carried out in duplicate and values represent the mean±s.e.
CYP2E1 and cytochrome $b_5$ expression in CPR$^{-/-}$ microsomes

Protein expression of CYP2E1 increased by approximately 4-fold in CPR$^{-/-}$ microsomes, as compared to that in wild-type microsomes (Figure 7.4). In addition, cytochrome $b_5$ expression increased by approximately 40% in CPR$^{-/-}$ microsomes (Figure 7.5). This may explain the slightly greater chlorzoxazone hydroxylation with NADH in CPR$^{-/-}$ microsomes, compared to that with NADH in wild-type microsomes. Expression of cytochrome P450 reductase was undetectable in the CPR$^{-/-}$ microsomes for these assays (Figure 7.6).
Figure 7.4: Quantitative immunoblot analysis of CYP2E1 in CPR<sup>−/−</sup> and wild-type microsomes. Images acquired with 2 min exposures of immunoblots containing two fold serial dilutions of CPR<sup>−/−</sup> and wild-type microsomes from 20 µg to 2.5 µg total protein. Standard curves were generated with mean band intensity of CYP2E1 from wild-type microsomes and the mean band intensities from CPR<sup>−/−</sup> microsomes were fitted to the curve. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background.
Figure 7.5: Quantitative immunoblot analysis of cytochrome $b_5$ in CPR$^{-/-}$ and wild type microsomes. Images acquired with 2 min exposures of immunoblots containing twofold serial dilutions of CPR$^{-/-}$ and wild-type microsomes from 20 µg to 2.5 µg total protein. Standard curves were generated with mean band intensity of cytochrome $b_5$ from wild-type microsomes and the mean band intensities from CPR$^{-/-}$ microsomes were fitted to the curve. Images were acquired on a Kodak MM2000 gel documentation station and analyzed for mean intensity above background.
Figure 7.6: Immunoblot analysis of cytochrome P450 reductase in CPR−/− and wild-type microsomes. Images acquired with 2 min exposures of immunoblots containing two-fold serial dilutions of CPR−/− and wild-type microsomes from 20 µg to 2.5 µg total protein.
Kinetic analysis of chlorzoxazone 6-hydroxylation in the CPR\textsuperscript{−/−} and wild-type microsomes

The effects of NADH and NADPH on the kinetic parameters of chlorzoxazone hydroxylation in CPR\textsuperscript{−/−} and wild-type microsomes were calculated using a one-enzyme Michaelis–Menten model using Prism one-site binding model (Figure 7.2 and Figure 7.3). From Table 7.1, with wild-type microsomes, the apparent Km for chlorzoxazone and Vmax were determined to be 13.01 µM and 1.278 nmol/min/mg protein, respectively, in the presence of NADPH. Addition of NADH along with NADPH doubles the apparent Km to 28.16 µM, and increased the apparent Vmax to 2.315 nmol/min/mg protein. With NADH in wild-type microsomes, apparent Km and Vmax were determined to be 24.01 µM and 0.1135 nmol/min/mg protein, respectively. In CPR\textsuperscript{−/−} microsomes in the presence of NADH, the apparent Km, Vmax were 61.07 µM and 0.24 nmol/min/mg protein. Addition of NADPH along with NADH results in a slight increase in apparent Km and Vmax, however, there are no statistically significant differences between the two groups (p>0.05). There is no chlorzoxazone hydroxylation detected with NADPH alone, consistent with the lack of cytochrome P450 reductase in CPR\textsuperscript{−/−} microsomes.
Table 1: Kinetic analysis of NADH-dependent and NADPH-dependent chlorzoxazone 6-hydroxylation in CPR<sup>−/−</sup> and wild-type microsomes. Data represent mean ± s.e of duplication determinations. Numbers in parentheses indicate percent of the activities.

<table>
<thead>
<tr>
<th>Liver ID</th>
<th>Cofactor(s)</th>
<th>Km (µM)</th>
<th>Vmax (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>NADH</td>
<td>24.01±4.99</td>
<td>0.1135±0.005 (5%)</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>13.01±2.28</td>
<td>1.278±0.0385 (55%)</td>
</tr>
<tr>
<td></td>
<td>NADH+NADPH</td>
<td>28.16±3.61</td>
<td>2.315±0.0627 (100%)</td>
</tr>
<tr>
<td>CPR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>NADH</td>
<td>61.07±18.19</td>
<td>0.245±0.020 (11%)</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>NADH+NADPH</td>
<td>33.68±7.93</td>
<td>0.330±0.017 (14%)</td>
</tr>
</tbody>
</table>
Antibody to cytochrome $b_5$ inhibits NADH-dependent chlorozoxazone hydroxylation

As shown in Figure 7.7, in order to evaluate the role of cytochrome $b_5$ in the NADH-dependent chlorozoxazone hydroxylation, antibody to cytochrome $b_5$ was incubated with CPR$^{-/-}$ microsomes prior to assay. Antibody to cytochrome $b_5$ reduced the CYP2E1-mediated activity in CPR$^{-/-}$ microsomes by approximately 25% at the highest concentration of antibody tested, indicating the involvement of cytochrome $b_5$ in the NADH-supported chlorozoxazone hydroxylation. However, antibody to cytochrome $b_5$ failed to fully inhibit this reaction at the highest concentration used, suggesting that either it is not fully able to prevent the interaction between cytochrome $b_5$ and CYP2E1, or that the NADH-dependent activity is mediated in part by another unrecognized electron transfer protein.
Figure 7.7: Antibody to cytochrome $b_5$ inhibits the NADH-dependent hydroxylation of chlorzoxazone. 6-Hydroxylation of chlorzoxazone was measured in CPR$^{-/-}$ liver microsomes in the presence of NADH and polyclonal antibody (0.2 mg/ml) to $b_5$. Antibody was added to the incubations 30 min prior to the addition of NADH to start the reaction. Each value represents the mean ± S.E. of two experiments carried out in duplicate.
Chlorzoxazone hydroxylation in CPR\textsuperscript{−/−} hepatocytes

Although my studies revealed that NADH could support CYP2E1-mediated hydroxylation of chlorzoxazone in microsomes from CPR\textsuperscript{−/−} livers, I wanted to determine if this activity was present in whole cells. Primary hepatocytes are a physiological milieu that contains normal concentration of enzymes and cofactors, as well as the cellular regulatory mechanisms to regulate enzymatic reactions. In comparison with many other in vitro techniques, such as purified enzymes or subcellular fractions, primary hepatocytes are clearly closer to the in vivo situation in terms of drug metabolism. Therefore, hepatocytes were isolated from hepatic CPR\textsuperscript{−/−} mice and the synthesis of 6-hydrochlozoxazone was measured. The formation of 6-hydroxychlorzoxazone is negligible in these hepatocytes as compared to that in wild-type hepatocytes; at the highest concentration, chlorzoxazone hydroxylation activity was less than 1% of wild-type cells, suggesting that the activity found in CPR\textsuperscript{−/−} microsomes was not representative of whole cells. However, many factors could contribute to the discrepancy of drug metabolism using microsomes and hepatocytes. One possibility is that the abnormal morphology of CPR\textsuperscript{−/−} hepatocytes could make the substrate, chlorzoxazone inaccessible to the endoplasmic reticulum and therefore inhibit its metabolism.
Figure 7.8: Hydroxylation of chlorzoxazone in CPR\(^{-}\) and wild-type primary hepatocytes. Hepatocytes were prepared from mice at an age of 12-16 wks. Medium was replaced by Hank’s Balanced Salt Solution with different concentrations of chlorzoxazone for 45 min. Samples were performed in duplicate.
Conclusion and discussion

The cytochrome P450-dependent monooxygenase system is responsible for many biological processes, including the synthesis of steroid hormones, cholesterol, and the biotransformation and detoxication/activation of xenobiotics. NADH-dependent stimulation of drug metabolism in liver microsomes suggested that the \( b_5 \) reductase/cytochrome \( b_5 \) pathway might also contribute to P450-mediated reactions, as reviewed by Porter (Porter 2002). Cytochrome \( b_5 \) has been shown to stimulate cytochrome P450-mediated drug metabolism with different P450 isoforms and their substrates. Cytochrome P450 2E1 has been reported to exhibit a reasonable turnover rate via an NADH–cytochrome \( b_5 \) pathway (Warrington, Court et al. 2004). Because most studies that support this postulate have been carried out in vitro with reconstituted P450 systems, my studies were undertaken to determine what role the NADH-cytochrome \( b_5 \) pathway played in CYP2E1-mediated drug metabolism in CPR\(^{-/-}\) microsomes and in CPR\(^{-/-}\) hepatocytes.

My studies have demonstrated CYP2E1 activity in microsomes, but not hepatocytes, from hepatic CPR-null mice using chlorzoxazone as a probe for CYP2E1 catalytic activity. Microsomes from hepatic CPR\(^{-/-}\) mice have NADH-dependent CYP2E1 activity but no NADPH-dependent CYP2E1 activity, suggesting residual cytochrome P450 reductase is not responsible for chlorzoxazone 6-hydroxylation found in CPR\(^{-/-}\) microsomes. This NADH-dependent CYP2E1 activity in CPR\(^{-/-}\) microsomes contributes approximately 10% of CYP2E1 activity observed in microsomal preparations with NADPH from wild-type mice and is approximately twice as high as that seen with NADH in wild-type microsomes, which can be explained by the increase in the protein expression of CYP2E1 and cytochrome \( b_5 \), as my data showed. In hepatic CPR\(^{-/-}\) livers, the expression level of liver cytochrome \( b_5 \) increased by ~40% and CYP2E1 expression level increased ~4-fold compared to that in wild-type livers.

The kinetic parameters for the metabolism of chlorzoxazone by microsomes from CPR\(-/-\) and wild-type livers have been determined in my studies. Vmax was greatest with wild-type microsomes in the presence of NADH+NADPH (2.3 nmol/min/mg protein). The Vmax obtained with only NADPH was approximately 55% of the rate
obtained with both cofactors, confirming the stimulatory effect of NADH, and presumably the cytochrome b<sub>5</sub> electron transfer pathway, on CYP2E1 activity. However, the rate with NADH alone was only about 5% of the rate obtained with both cofactors, indicating that the NADH-b<sub>5</sub> pathway alone was unable to substantially support CYP2E1 activity. This finding is consistent with literature reports. With CPR<sup>-/-</sup> microsomes the Vmax obtained in the presence of both cofactors was less than 15% of the combined cofactor rate obtained in wild-type microsomes, and the rate with NADH alone was only slightly less than the rate obtained in the presence of both cofactors. This finding suggests that NADPH had very little impact on CYP2E1 activity in the absence of CPR. The approximately two-fold greater Vmax for CPR<sup>-/-</sup> microsomes with NADH, relative to wild-type microsomes, may reflect the four-fold higher levels of CYP2E1 and 30% higher levels of cytochrome b<sub>5</sub> in CPR<sup>-/-</sup> microsomes. Km values for chlorzoxazone did not differ greatly between wild-type and CPR<sup>-/-</sup> microsomes in the presence of either cofactor or combination, and probably do not reflect significant differences. It would not be expected that the Km for chlorzoxazone would be affected by the presence or absence of CPR, or by the cofactor included in these studies.

The reaction mechanism for P450-mediated drug metabolism has been proposed as a Ping-Pong Bi-Bi mechanism, in which the binding of the second electron is dependent on the previous binding from electron donor(s) (Masters et al., 1965; Hiwatachi & Ichikawa, 1979; Mayer & Durrant, 1979; Crankshaw et al., 1979). These and other early studies show that the rate-limiting step in the P450-mediated monooxygenase reaction is input of a second electron. The role of cytochrome b<sub>5</sub> was suggested to be to provide the second electron more rapidly from NADH than could be provided from NADPH via cytochrome P450 reductase (Hilderbrandt and Estabrook, 1971). The inability of the NADH-dependent b<sub>5</sub> pathway to drive CYP2E1 activity at rates seen with NADPH in wild-type preparations probably stems from the redox potentials of ferrous cytochrome b<sub>5</sub> (+20 mV) (Velick and Strittmatter 1956) and ferric P450 (approx. -300 mV) (Guengerich 1983): this difference would impede input of the first electron to P450 and, subsequently, overall turnover. However, as cytochrome b<sub>5</sub>
facilitates the second electron transfer, the apparent V\text{max} increased by 2-fold in the presence of both cofactors (Figure 7.9).
Figure 7.9: Redox potential control of the electron flow in microsomal P450-dependent system. This figure was adapted from Sevrioukova et al (Sevrioukova, Li et al, 1999).
In hepatocytes, my studies suggested that the NADH-cytochrome $b_5$ reductase/ $b_5$ pathway has little ability to catalyze CYP2E1 activity in the absence of cytochrome P450 reductase. Assuming NADH levels are unlikely to be limiting in hepatocytes, the reason for this inability of the $b_5$ pathway to mediate electron transfer to CYP2E1 in whole cells, as compared to microsomes, is unclear. These results suggest that cytochrome P450-reductase might be absolutely essential for chlorzoxazone metabolism in vivo although several mechanisms including substrate accessibility to CYP2E1 might be the issue for the differing results from microsomes and primary hepatocytes. More detailed research is needed to draw the final conclusion.

Overall, my studies were carried out with CPR$^{-/-}$ microsomes and hepatocytes, focusing on the cytochrome $b_5$ and CYP2E1 interactions in endoplasmic reticular-membrane electron transport processes with physiological relevance. Future studies with hepatic CPR-null mice are likely to more clearly elucidate the various roles of cytochrome $b_5$ in the many monooxygenase reactions that contribute to the therapeutic and toxicological effects of drugs and xenobiotics.
 CHAPTER EIGHT: SUMMARY OF RESEARCH

SUMMARY OF RESEARCH

NADPH-cytochrome P450 reductase (CPR) is a flavoprotein containing both FAD and FMN and functions as the electron donor protein for several oxygenase enzymes found on the endoplasmic reticulum of eukaryotic cells, including the cytochrome P450s involved in drug metabolism and sterol biosynthesis. Germ-line deletion of the CPR gene in mice is lethal during embryogenesis, but it is not clear if this is due to a loss of cholesterol synthesis or to disruption of other developmental pathways dependent on this widely-used reductase. In conditional-null mice, in which the CPR gene is deleted in the liver during maturation, adult animals exhibit an 80% decrease in plasma cholesterol levels, suggesting a loss in the ability to synthesize cholesterol in the liver.

Squalene monooxygenase is a microsomal enzyme that catalyzes the first oxidative step in cholesterol biosynthesis, converting squalene to 2,3(s)-oxidosqualene, the immediate precursor to lanosterol. Unlike other flavoprotein monooxygenases that obtain electrons directly from NAD(P)H, squalene monooxygenase requires a second enzyme to provide the electrons necessary for catalysis. For many years it has been assumed that NADPH-cytochrome P450 reductase, the redox partner for the microsomal cytochrome P450 monooxygenase system, is this requisite electron donor protein. My studies with hepatic cytochrome P450-reductase-null mice have revealed a second microsomal reductase for squalene monooxygenase that was not previously detected; this second reductase is dependent upon supernatant protein factor, a cytosolic protein that stimulates squalene monooxygenase, for activity. Inhibition studies with antibody to P450 reductase indicate that this second reductase supports up to 40% of the monooxygenase activity that is obtained with microsomes from normal mice. Studies carried out with hepatocytes from CPR-null mice demonstrate that this second reductase is active in whole cells and leads to the accumulation of 24-dihydrolanosterol, indicating that lanosterol demethylation, a cytochrome P450-mediated reaction, is blocked. These results suggest that this SPF-dependent
reductase plays a significant role in supporting squalene monoxygenase but not P450-mediated reactions.

7-Dehydrocholesterol reductase, the final enzyme of the cholesterol synthesis, catalyzes the reduction of the $\Delta^7$ double bond of 7-dehydrocholesterol and 7-dehydrodesmosterol to yield cholesterol and desmosterol, respectively. It has been suggested that cytochrome-P450 reductase activity is required for this enzyme activity. My study shows that 7-dehydrocholesterol reductase is enzymatically active in CPR-null microsomes with slightly greater activity than that found in wild-type mice, and the activity is greatly stimulated by the addition of ATP. Thus, CPR is not required for the activity of 7-dehydrocholesterol reductase.

It has been reported that NADH could provide a synergism for some NADPH-supported drug metabolism, indicating the involvement of cytochrome $b_5$ in cytochrome P450-mediated monoxygenations. With some substrates, some cytochrome P450s have shown an obligatory requirement for cytochrome $b_5$. Among them, cytochrome P450 2E1 has been found to exhibit reasonable rates of turnover via an NADH–cytochrome $b_5$ pathway in vitro. My studies have revealed CYP2E1 activity with hepatic CPR-null mice using chlorzoxazone as a probe for CYP2E1 catalytic activity. Microsomes from hepatic CPR-null mice have shown NADH-dependent CYP2E1 activity and no NADPH-dependent CYP2E1 activity. This NADH-dependent CYP2E1 activity contributes approximately 10% of CYP2E1 activity observed in microsomal preparations with NADPH from wild-type mice. However, the formation of 6-OH-chlorzoxazone is negligible in primary hepatocytes from CPR$^{-/-}$ mice. In these mice, the expression level of liver cytochrome $b_5$ is only slightly increased, but CYP2E1 expression level increased 3-5 fold compared with that in wild-type mice. These results indicate that in whole cells there is less effective direct electron transfer of both required electrons from NADH–cytochrome $b_5$ reductase to P450 than in microsomal preparation from these animals. Future studies with hepatic CPR-null mice are likely to more clearly elucidate the various roles of cytochrome $b_5$ in the many monoxygenase reactions that contribute to the therapeutic and toxicological effects of drugs and xenobiotics.

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REFERENCES


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