

REGULATION OF CYP1B1 AND CYP2S1 EXPRESSION IN EXTRAHEPATIC TISSUES IN RODENTS

by

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ABSTRACT

Cytochrome P450 (CYP) enzymes play an important role in the metabolism of xenobiotics and physiological substances that are vital to the normal biological functions. It is important to understand the tissue distribution and factors that regulate the expression of CYP enzymes in hepatic and extrahepatic tissues. In the present study the regulation of two extrahepatic CYP enzymes, CYP1B1 and CYP2S1, was investigated. Rat testicular CYP1B1 protein expression is developmentally regulated and suppressed by hypophysectomy. The hormonal regulation of testicular CYP1B1 expression was examined in mouse MA-10 and rat R2C Leydig tumor cells in the present study. CYP1B1 mRNA levels and protein kinase A (PKA) activity were increased 4.2-fold and 2.7-fold, respectively, after treatment of MA-10 cells with luteinizing hormone (LH). Similarly, LH increased CYP1B1 protein levels by 30% in R2C cells. Treatment with a PKA activator mimicked the inductive effect of LH on CYP1B1 expression in MA-10 and R2C cells. In contrast, treatment with PKA inhibitors attenuated the LH-elicited increases in CYP1B1 mRNA and protein levels and PKA activity and decreased basal CYP1B1 expression. Collectively, the results suggest that testicular CYP1B1 expression is regulated by LH through a PKA-mediated pathway. In separate experiments, estradiol benzoate decreased CYP1B1 mRNA levels in MA-10 cells. Treatment of adult rats and Leydig cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most potent AhR agonist, did not induce testicular CYP1B1 expression, indicating that testicular CYP1B1 is not regulated by the AhR pathway.

CYP2S1 mRNA was detected in rat liver, lung, and other extrahepatic tissues. There was no sex-dependent difference in constitutive CYP2S1 mRNA expression in adult rats. Antibody against rat CYP2S1 was prepared using an antipeptide approach and was able to detect CYP2S1 protein in microsomes of rat lung, stomach and kidney. Rat CYP2S1 mRNA levels were induced

in lung, stomach and jejunum by treatment with 3-methylcholanthrene (up to 3-fold) and in lung, liver and kidney by treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (up to 7-fold). However, CYP2S1 protein levels were not increased after treatment of rats with 3-methylcholanthrene, benzo[a]pyrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Thus, rat CYP2S1 expression at the level of mRNA, but not protein, is sensitive to treatment by AhR agonists.

The results obtained in the present study provide insight into the regulation of extrahepatic CYP1B1 and CYP2S1 by hormones and environmental pollutants. I show for the first time that CYP2S1 mRNA is expressed in most tissues whereas CYP2S1 protein has a limited tissue distribution in rats.

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LIST OF ABBREVIATIONS

8-Br-cAMP	8-bromo-cyclic adenosine monophosphate
8-Br-cGMP	8-bromo-cyclic guanosine monophosphate
3-MC	3-methylcholanthrene
ACTH	adrenocorticotrophic hormone
AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
BaP	benzo[a]pyrene
b.w.	body weight
cAMP	3',5'-cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CREB	cAMP response element binding protein
CYP	cytochrome P450
DMBA	7,12-dimethylbenzanthracene
DMSO	dimethyl sulfoxide
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis-(2-aminoethyl ether) tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating hormone
GH	growth hormone
Go 6983	(3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione)
H89	N-[2-(<i>p</i> -bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
KCl	potassium chloride
KLH	keyhole limpet hemocyanin
LC/MS	liquid chromatography/mass spectrometry
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
mg	milligram
ml	milliliter
µg	microgram
µl	microliter
MOPS	4-morpholinepropanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NBT	<i>p</i> -nitro-blue tetrazolium chloride
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMSF	phenylmethanesulphonylfluoride
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor

Rp-CPT-cAMP	8-(4-chlorophenylthio)-cAMP, Rp isomer
s.c.	subcutaneous
SD	Sprague-Dawley
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF-1	steroidogenic factor-1
Sulfo-SMCC	sulfosuccinimidyl-4-(<i>N</i> -maleimidomethyl)cyclohexane-1-carboxylate
T3	3,5,3'-triiodothyronine
T4	thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)aminoethane
UV	ultraviolet
XRE	xenobiotic response element

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*This Thesis is Dedicated to
My Mother and Wife*

1. INTRODUCTION

1.1. Cytochrome P450 Enzymes

Cytochrome P450 (CYP) enzymes are a superfamily of heme proteins that are present in a wide variety of organisms including bacteria, fungi, plants, insects, fish, birds and mammals. In mammals, CYP enzymes are present in the greatest concentration in liver. CYP enzymes are also found in lung, kidney, heart, adrenal gland, ovary, testis, small intestine, brain and other extrahepatic tissues (Ding and Kaminsky, 2003; Pavek and Dvorak, 2008). Within these tissues, CYP enzymes are localized predominantly in the smooth endoplasmic reticulum of cells. A subset of CYP enzymes are found in the mitochondria of cells (Omura, 2006). CYP enzymes demonstrate complex sex-, tissue- and development-dependent expression patterns that are regulated by hormones or growth factors (Bandiera, 1990; Lewis, 2001; Shimada et al., 2003; Waxman et al., 1985).

Martin Klingenberg (1958) first observed the absorption of light at 450 nm by a pigment in rat liver microsomes treated with the reducing agent, sodium dithionite. In later studies, Omura and Sato (1964a; 1964b) identified the pigment as a hemoprotein and named it cytochrome P450. The name cytochrome P450 describes the ability of this pigment to produce a unique spectrum with a maximum absorbance at 450 nm when chemically reduced and complexed with carbon monoxide (Figure 1.1). The enzymatic function of CYP enzymes was first described by Estabrook et al. (1963), in a study that demonstrated that CYP enzymes present in the microsomal fraction of adrenal cortex catalyzed the 21-hydroxylation of 17-hydroxyprogesterone. Shortly thereafter, the seminal work of Cooper et al. (1965), demonstrated that CYP enzymes are also involved in the hydroxylation of various foreign substances (also called xenobiotics) including, codeine, acetanilide, and monomethyl-4-aminopyrine.

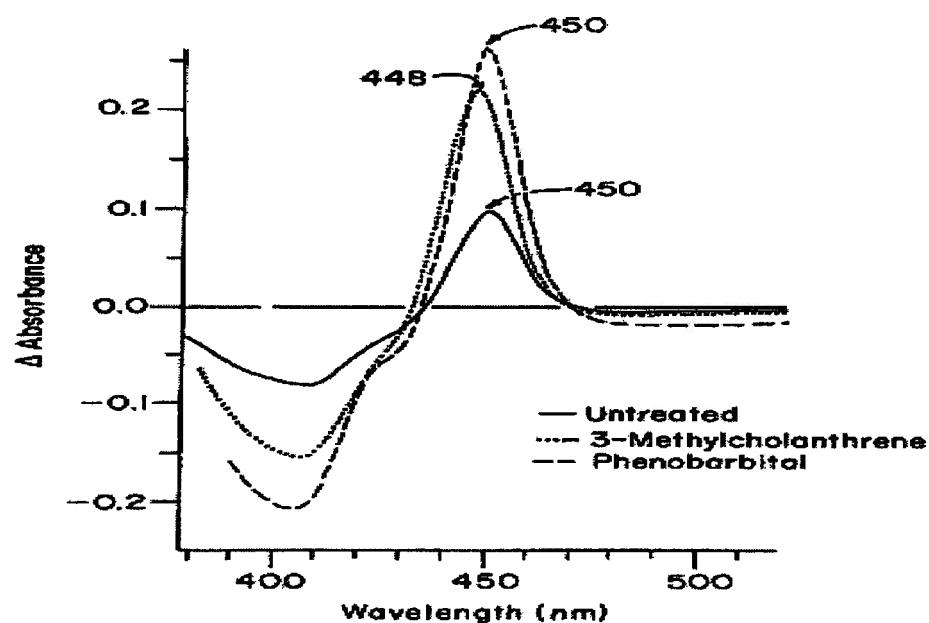
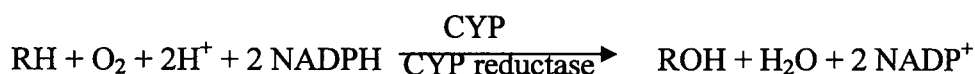


Figure 1.1: Carbon monoxide difference spectra of hepatic microsomal CYP enzymes. Livers were excised from untreated rats and animals treated with phenobarbital or 3-methylcholanthrene, and microsomes were prepared. A small amount of sodium dithionite was added to the microsomal samples (diluted to 1mg/ml with 0.1 M phosphate buffer) to reduce the heme groups. The reaction mixture was divided into two spectrophotometer cuvettes and the contents of one cuvette were gassed for 30 sec with carbon monoxide. An Aminco-Chance DW2 spectrophotometer was used to record the difference spectrum between 380 and 510 nm (taken from Hasler et al., 1999).

1.1.1. CYP Reaction Cycle

CYP enzymes contain a heme prosthetic group, present as protoporphyrin IX, that is attached to a polypeptide chain (apoprotein) consisting of approximately 500 amino acids. The iron atom in the heme prosthetic group is located in the center of the protoporphyrin ring and is part of the catalytic site of CYP enzymes (Hasler et al., 1999). The iron atom in the resting state is in the oxidized or ferric form (Fe^{3+}). The reduced or ferrous form (Fe^{2+}) is able to bind to oxygen or carbon monoxide. The N-terminal domain of CYP enzymes consists of hydrophobic amino acids that anchor the protein in the membrane (Lewis, 1996). CYP enzymes contain a conserved cysteine residue in the C-terminal region of the polypeptide chain to which the iron atom of the heme prosthetic group is coordinated. The heme (Fe^{2+})-thiolate complex is responsible for the signature absorbance band at 450 nm of the reduced CYP-carbon monoxide complex (Lewis, 1996). Hence, CYP enzymes are also known as heme-thiolate proteins.

CYP enzymes use molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH), or in some cases, reduced nicotinamide adenine dinucleotide (NADH), as reducing agents in the oxidative biotransformation of various substrates (RH). The products of the reaction are the respective monooxygenated product (ROH) and water (Hasler et al., 1999; Lewis, 1996), as shown by the following general reaction equation:



The reaction cycle by which mammalian CYP enzymes catalyze the oxidative conversion of a substrate is shown in Figure 1.2. Substrate binds to the protein portion of the CYP enzyme to form a substrate-enzyme complex. The Fe^{3+} heme group of the CYP-substrate complex is then reduced to the ferrous form by the addition of one electron from NADPH. Molecular oxygen binds to the heme iron of the CYP (Fe^{2+})-substrate complex and forms a ternary complex of CYP (Fe^{2+})-substrate-oxygen. A second electron from NADPH reduces the ternary complex and

forms a two-electron reduced intermediate. Molecular oxygen bound to the heme iron is reduced by the second electron from NADPH and forms a reactive oxygen species. The substrate is oxidized when the reactive oxygen species formed from molecular oxygen is incorporated into the substrate by a chemical rearrangement reaction (Hasler et al., 1999; Lewis, 1996; Yasui et al., 2005). The enzyme and oxidized substrate dissociate to generate free CYP enzyme (Fe^{3+}), which subsequently initiates the oxidation of another substrate molecule (Hasler et al., 1999; Lewis, 1996; Yasui et al., 2005).

CYP enzymes play important roles in the oxidative, reductive and peroxidative metabolism of xenobiotic compounds such as drugs, food-derived chemicals, organic solvents, pesticides and other environmental toxicants and endogenous compounds such as sex steroids (testosterone, estrogen), bile acids, vitamins, and fatty acids (Chang and Kam, 1999). Most of the CYP-catalyzed reactions are oxidative in nature and thus, oxidation is the main focus of this discussion. CYP enzymes catalyze a wide variety of oxidative biotransformation reactions including carbon hydroxylation, heteroatom oxidation, heteroatom dealkylation, and epoxidation of aliphatic and aromatic substances (Guengerich, 2001). In CYP-catalyzed oxidative reactions, an oxygen atom is incorporated in the molecule. Aliphatic and aromatic hydroxylation reactions catalyzed by CYP enzymes involve formation of primary, secondary, and tertiary alcohols. The CYP (FeO)³⁺-complex abstracts a hydrogen atom from the substrate, leading to generation of CYP (FeOH)³⁺ and an alkyl radical, and consequently, the alkyl radical undergoes an oxygen-rebound reaction to form an alcohol (ROH) and Fe^{3+} (Guengerich, 2001; Ortiz de Montellano, 1995). In heteroatom oxidation reactions, the substrate is converted to a monooxygenated product where an atom of oxygen is attached to nitrogen or sulfur. In heteroatom oxidation, the mechanism involves transfer of a single electron from heteroatom to CYP (FeO)³⁺, leading to formation of CYP (FeO)²⁺ and subsequent shifting of the oxygen atom to the heteroatom to form the final oxidized product and Fe^{3+} (Guengerich, 2001). Heteroatom dealkylation (e.g. *N*-

dealkylation, *O*-dealkylation, *S*-dealkylation) involves removal of an alkyl group attached to the heteroatom. For example, dealkylation of amines, ethers and sulfides is a major pathway in the biotransformation of xenobiotics. In heteroatom dealkylation, the reaction is initiated by the formation of the CYP (FeO)²⁺ complex (as described above), which then acquires a proton to form the CYP (FeOH)³⁺ complex and an unstable intermediate (carbon-based radical). Subsequently, the intermediate undergoes an oxygen-rebound reaction to give the dealkylated final product (RX) and Fe³⁺ (Guengerich, 2001; Ortiz de Montellano, 1995). In epoxidation reactions, the substrate is converted to a highly reactive electrophile, which can potentially bind to the endogenous nucleophilic moieties such as DNA and proteins to activate toxic pathways (Guengerich, 2001; Guengerich, 2003; Ortiz de Montellano, 1995). The substrates of CYP-catalyzed epoxidation reactions include alkenes and arenes. For epoxidation reactions, one proposed mechanism is oxidation of arenes, leading to epoxide formation, followed by generation of a ketone intermediate, which subsequently undergoes a rearrangement reaction to give an aromatic phenol (Guengerich, 2001; Ortiz de Montellano, 1995).

CYP enzymes are also capable of catalyzing reductive reactions in mammals. Reduction reactions are unusual considering the fact that CYP (Fe²⁺) exhibits high binding affinity for molecular oxygen (Guengerich, 2001). The CYP-catalyzed reductive reactions take place when the oxygen tension in the tissue is very low (Guengerich, 2001; Ortiz de Montellano, 1995). It has been suggested that the binding of certain substrates to the CYP enzyme may block the interaction of the oxygen atom with the CYP enzyme (Guengerich, 2001). Substrates that undergo CYP-catalyzed reduction include halocarbons, azo and nitro compounds. In these reactions, electrons are shifted to the substrate from the CYP (Fe²⁺), leading to reductive dehalogenation, aromatic nitro reduction or azo reduction of the substrates (Guengerich, 2001). Occasionally, the epoxides of alkenes are reduced back to the parent compounds (Sugiura et al., 1980).

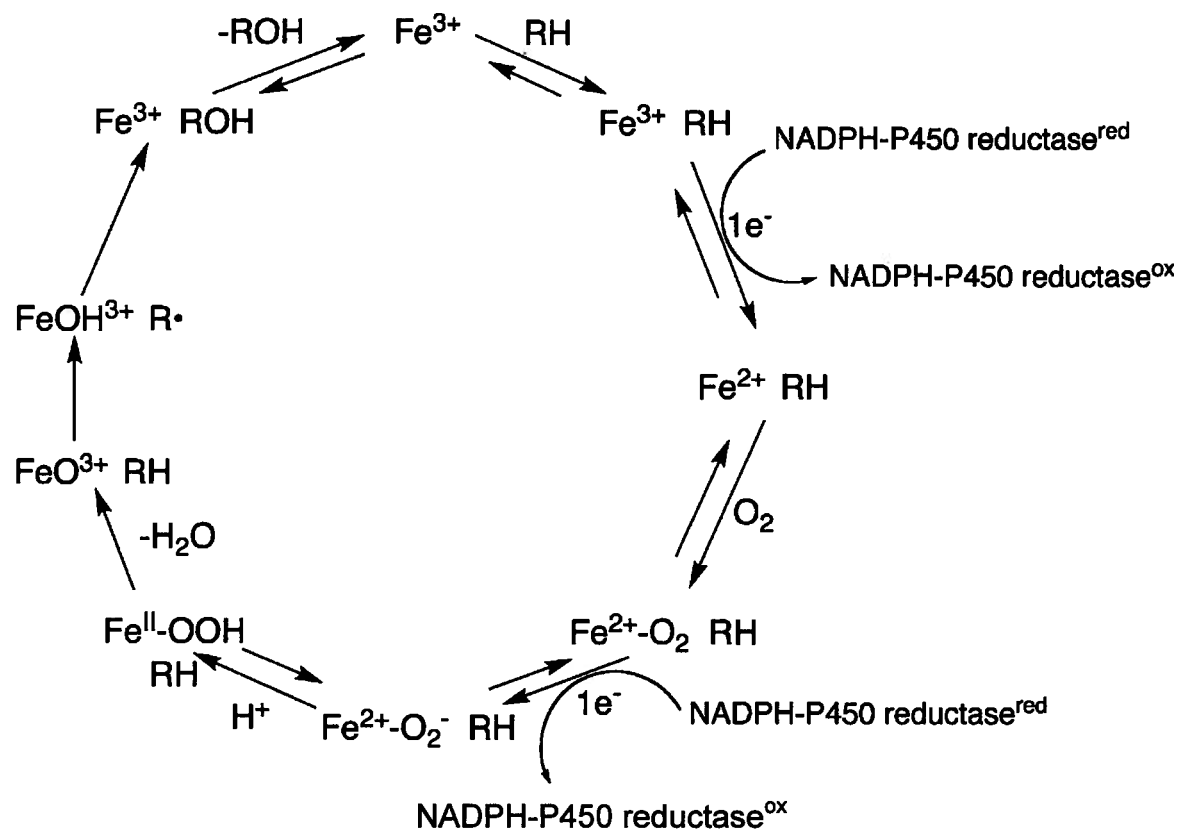


Figure 1.2: CYP-dependent mixed-function oxidase (oxygenase) reaction cycle and enzymatic intermediates (taken from Guengerich, 2008). Abbreviations: RH- substrate; ROH- monooxygenated product.

1.1.2. Effect of CYP-Mediated Biotransformation

Physiologically, animals are not designed to allow facile excretion of lipophilic compounds. Hence, lipophilic endogenous and xenobiotic compounds need to be biotransformed to more hydrophilic substances before they are eliminated from the body. In mammals, CYP proteins are the predominant enzymes involved in the biotransformation of lipophilic compounds (Lewis, 2001). CYP-catalyzed oxidative biotransformation is a process that converts lipophilic xenobiotics and endogenous compounds into more water soluble metabolites by exposing or introducing a functional group into the lipophilic substrate molecule (Guengerich, 2001).

The major consequences of CYP-catalyzed biotransformation reactions are detoxification (or deactivation) and bioactivation. Detoxification of endogenous and xenobiotic compounds can lead to loss of pharmacological activity. A vast majority of the therapeutically active compounds are metabolized to inactive metabolites by CYP enzymes before being excreted from the body. For example, phenobarbital, a widely used sedative drug, is metabolized to *p*-hydroxyphenobarbital via an aromatic hydroxylation reaction, leading to loss of sedative activity (Williams, 2002). Similarly, deactivation of potentially harmful toxins by CYP enzymes acts as a defense mechanism of the body. Toxicity resulting from exposure to pharmaceuticals and environmental toxicants may be reduced or lost after CYP-mediated metabolism of the compound in humans and rodents. For example, the cardiotoxic effects of terfenadine, an antihistaminic prodrug, is abolished after its metabolism by CYP enzymes in humans (Kivisto et al., 1994). Polycyclic aromatic hydrocarbons (PAHs), an important chemical class of environmental and occupational toxicants, are known to increase their own metabolism mediated by CYP enzymes, leading to decreased toxicity, after exposure to xenobiotics (Ma et al., 2007; Nebert et al., 2004). The absence of genes encoding PAH-metabolizing CYP enzymes leads to more toxic responses in CYP1A1-knockout mice after exposure to benzo[a]pyrene (BaP), a procarcinogen found in cigarette smoke (Uno et al., 2004).

In bioactivation reactions, a pharmacologically inactive or weakly active compound is converted into a more active metabolite. CYP-mediated bioactivation can have two outcomes: increased therapeutic activity or increased toxicity. The therapeutic effect occurs from formation of a more active metabolite that has beneficial pharmacological activity. For example, codeine, a synthetic opioid, is converted to the active compound, morphine, by a CYP-mediated reaction (Williams, 2002). Similarly, imipramine, an active compound that is used as an antidepressant, is metabolized to its more active metabolite, desipramine, by a CYP-mediated reaction (Nielsen and Brosen, 1993; Yang et al., 1999). However, formation of reactive metabolite intermediates by CYP-mediated reactions may also initiate xenobiotic-induced toxicity (Lewis, 1996). In toxic bioactivation reactions, pro-toxic compounds are converted to electrophilic oxygen radicals and other reactive oxygen species (e.g. superoxide, peroxide, hydroxyl radicals), which can form stable covalent adducts with macromolecules such as DNA and proteins, leading to toxic effects (Baillie, 2006; Guengerich, 2001; Guengerich, 2006; Zhou et al., 2005). A well known example of an adverse drug reaction is provided by acetaminophen. The reactive metabolite of acetaminophen, N-acetyl-*p*-benzoquinone-imine, covalently binds to cytosolic and mitochondrial proteins in liver, leading to liver necrosis (Hinson et al., 2004). In recent years, the importance of reactive metabolites in adverse drug reactions has been recognized and screening of drug metabolites for potential toxicity has become a routine practice in the pharmaceutical industry (Evans et al., 2004).

1.1.3. Nomenclature and Classification of CYP Enzymes

To date, approximately 400 CYP enzymes have been identified in various species including mammals, birds, insects, plants, fungi and bacteria (Denisov et al., 2005; Lewis, 1996; Nelson et al., 1996; Nelson et al., 2004). Due to the increasing number of CYP enzymes identified by various investigators, it was necessary to implement a systematic naming system. The CYP nomenclature system was first proposed by Nebert and Gonzalez (1987). The CYP nomenclature system is based on amino acid or nucleotide sequence identity and organizes CYP enzymes into families and subfamilies. The nomenclature of the CYP enzymes starts with the CYP abbreviation followed by an Arabic number (e.g. 1, 2, 3) denoting the family, a letter defining the subfamily (e.g. A, B, C), and a numeral (e.g. 1, 2, 3) denoting the individual protein (Nelson et al., 1996). For example, CYP1A1 is a member of family 1, a member of subfamily A, and is identified within this subfamily as enzyme 1. According to the classification system, CYP enzymes with amino acid sequences that exhibit greater than or equal to 40% identity are assigned to the same family (Nelson et al., 1993; Nelson et al., 1996). The same subfamily is assigned if more than 55% identity is observed in the amino acid sequences of CYP enzymes (Nelson et al., 1993; Nelson et al., 1996).

At present, 57 CYP enzymes have been identified in humans and classified into 18 families and 43 subfamilies (Hasler et al., 1999; Hoffman et al., 2001; Ingelman-Sundberg, 2004) (Figure 1.3). There are 83 CYP enzymes identified in rats and 103 CYP enzymes identified in mice (Lewis, 1996; Nebert and Gonzalez, 1987; Nelson et al., 2004). CYP enzymes belonging to families 1, 2, 3 and 4 are involved in the biotransformation of xenobiotics, including drugs, environmental toxicants, and endogenous compounds, including hormones, vitamins and fatty acids (Denisov et al., 2005; Elbekai and El-Kadi, 2006; Hoffman et al., 2001). CYP enzymes belonging to families 5, 7, 8, 11, 17, 19, 21, 27 and 51 play important roles in the biosynthesis and breakdown of endogenous substances (e.g. hormones, bile acids, eicosanoids).

The classification of human CYP enzymes in various families and their general roles are represented in Figure 1.3.

The enzymes in the human CYP1 family include CYP1A1 and CYP1A2 from the CYP1A subfamily and CYP1B1 from the CYP1B subfamily. The basal expression of CYP1 enzymes in liver and extrahepatic tissues is low, or undetectable in some cases. CYP1A enzymes are highly inducible after exposure to PAHs such as 3-methylcholanthrene (3-MC), β -naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, an environmental toxicant) (Lewis, 1996; Nebert et al., 2004; Okey, 1990). PAH procarcinogens are the main substrates of CYP1A1, CYP1A2 and CYP1B1 enzymes and have been extensively studied for their potential role in chemical carcinogenesis (Nebert and Dalton, 2006; Nebert et al., 2004).

The CYP2 family consists of fifteen subfamilies (A to H, J, K, R to U and W) in humans and rats and contains the largest number of CYP enzymes. The main subfamilies of the CYP2 family are CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP2J. The expression of enzymes belonging to subfamilies CYP2C, CYP2D and CYP2E are mainly hepatic, whereas enzymes from subfamilies CYP2A, CYP2B and CYP2J are expressed mainly in extrahepatic tissues. In comparison with CYP1A enzymes, fewer CYP2 family enzymes are inducible by treatment with xenobiotics (Lewis, 2001; Rendic and Di Carlo, 1997). The substrates metabolized by CYP2 enzymes are very diverse, including drugs, alcohol, steroids, fatty acids and vitamins (Lewis, 2001; Rendic and Di Carlo, 1997).

CYP3A is the only subfamily within the CYP3 family. CYP3A enzymes are expressed in liver and intestine and are induced by glucocorticoids, phenobarbital and rifampicin (Rendic and Di Carlo, 1997). Enzymes belonging to the CYP3A subfamily are involved in the metabolism of a substantial number of therapeutic agents.

The CYP4 family consists of five subfamilies, CYP4A, CYP4B, CYP4F, CYP4V and CYP4X. CYP4A and CYP4F enzymes are expressed in liver and are induced by clofibrate and

phthalates. The substrates of CYP4A and CYP4F enzymes include fatty acids and eicosanoids (Rendic and Di Carlo, 1997).

The substrates and inducers of major CYP enzymes from CYP1, CYP2, CYP3 and CYP4 families are summarized in Table 1.1.

CYP enzymes belonging to the CYP11, CYP17, CYP19 and CYP21 families play vital roles in the biosynthesis of sex steroids, glucocorticoids and mineralocorticoids in steroidogenic tissues such as adrenal gland, testis and ovary (Lewis and Lee-Robichaud, 1998; Payne and Hales, 2004). The CYP11 family is represented by two subfamilies, CYP11A and CYP11B. CYP11A1 is the only enzyme in the CYP11A subfamily, which is involved in the side-chain cleavage of cholesterol in the first step of steroid biosynthesis (Payne and Hales, 2004). The CYP11B subfamily consists of CYP11B1 and CYP11B2, which are involved in the hydroxylation of progesterone and corticosterone, respectively (Payne and Hales, 2004). CYP17A1 is the only member of the CYP17 family. Progesterone and pregnenolone are the substrates of CYP17A1 (Lewis and Lee-Robichaud, 1998; Payne and Hales, 2004). Similarly, the CYP19 family is represented by CYP19A1, which is involved in the aromatization of androgens to estrogens (Lewis and Lee-Robichaud, 1998). CYP21A1 is the sole member of the CYP21 family and catalyzes reactions important in the biosynthesis of corticoids (Payne and Hales, 2004). The CYP-mediated pathways involved in the biosynthesis of androgens, estrogens and corticoids are summarized in Figure 1.4.

Human CYP Families	General Roles
CYP1	Polycyclic aryl hydrocarbons
CYP2	Drugs, Alcohol, Steroids
CYP3	Drugs, Antibiotics, Flavonoids
CYP4	ω -oxidation of fatty acids
CYP5	Thromboxane synthase
CYP7	7 α -Hydroxylase, Bile acids
CYP8	Prostacyclin synthase, Bile acids
CYP11 [*]	Cholesterol side-chain cleavage Aldosterone synthesis
CYP17	Steroid 17 α -hydroxylase, Steroid C17/21 lyase
CYP19	Estrogen Biosynthesis, Aromatase
CYP21	Progesterone 21-hydroxylase
CYP24 [*]	Vit D degradation
CYP26	Retinoic acid hydroxylase
CYP27 [*]	Bile acid synthesis
CYP39	α -Hydroxylation of 24-OH cholesterol
CYP40 [*]	Vit D ₃ - 1 α hydroxylase
CYP46	Cholesterol 24-hydroxylase
CYP51	Cholesterol biosynthesis

Figure 1.3: Classification of human CYP enzymes in various families and their general roles (modified from Hasler et al., 1999). * Mitochondrial Enzyme.

Table 1.1: Substrates and inducers of major human CYP enzymes (compiled from Lewis et al., 1996; Rendic and Di Carlo, 1997; Hrycay and Bandiera, 2008).

Family	Enzyme	Substrates	Inducers
CYP1	CYP1A1	BaP, diethylstilbestrol, DMBA	PAHs, cigarette smoke, omeprazole
	CYP1A2	Aromatic amines, PAHs, acetaminophen, caffeine	cigarette smoke, cruciferous vegetables, PAHs
	CYP1B1	DMBA, estradiol	TCDD, PAHs
	CYP2A6	Coumarin, nicotine	phenobarbital
CYP2	CYP2B6	Cyclophosphamide, testosterone, methoxychlor	phenobarbital, rifampicin, troglitazone
	CYP2C8	Rosiglitazone, retinoic acid, taxol, trimethoprim	rifampicin, phenobarbital
	CYP2C9	Diclofenac, tolbutamide, warfarin, piroxicam, phenytoin	rifampicin, phenobarbital, hyperforin
	CYP2C19	Omeprazole, S-mephenytoin, diazepam, fluconazole	phenobarbital, rifampicin, ritonavir
	CYP2D6	Propranolol, dextromethorphan, ondasteron, fluoxetine	unknown
	CYP2E1	Chlorzoxazone, paracetamol, ethanol, chloroform	ethanol, isoniazid, benzene
	CYP2F1	Skatole	unknown
	CYP2J2	Arachidonic acid, testosterone, vitamin D	unknown
	CYP2R1	Vitamin D	unknown
	CYP2S1	unknown	TCDD, PAHs, <i>all-trans</i> -retinoic acid
CYP3	CYP3A4	Azole antifungals, bile acids, erythromycin, testosterone, carbamazepine, tamoxifen, retinoic acid, simvastatin	carbamazepine, dexamethasone, rifampicin
CYP4	CYP4A11	Arachidonic acid, eicosanoic acids, prostaglandins	clofibrate

Abbreviations: BaP, benzo[a]pyrene; DMBA, 7,12-dimethylbenzanthracene; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

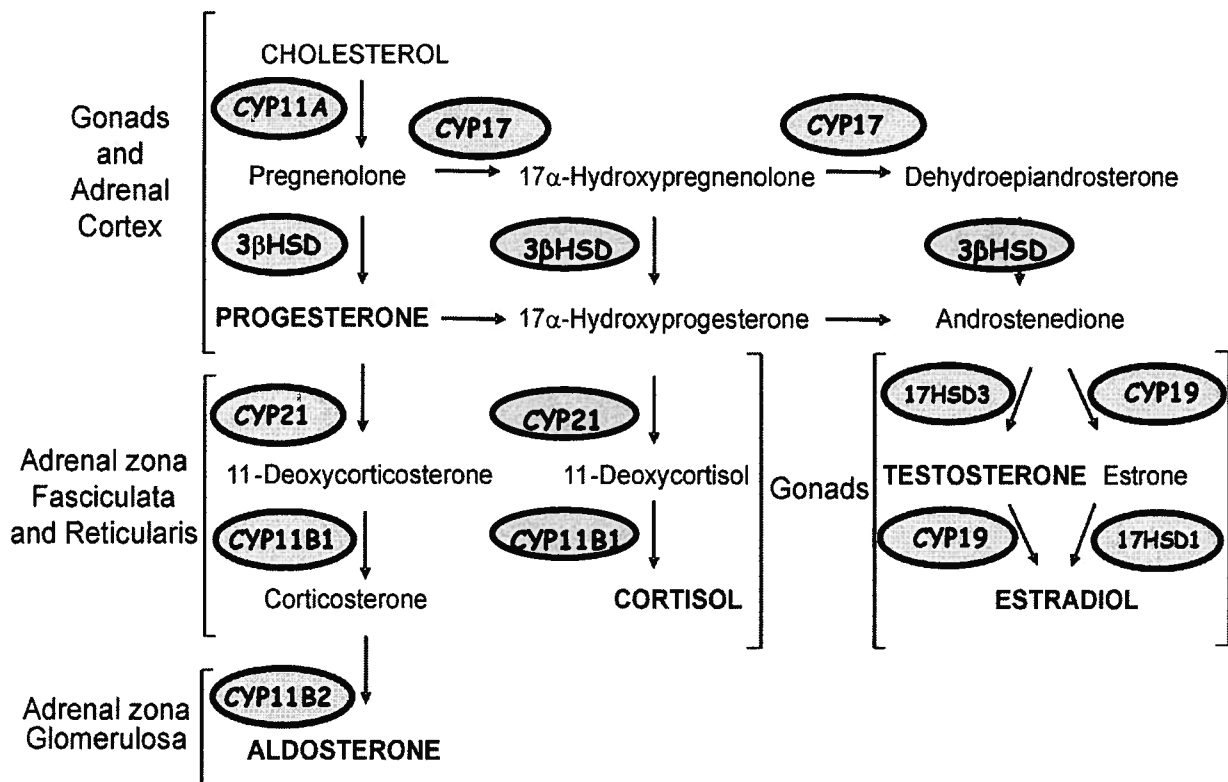


Figure 1.4: The steroidogenesis pathways in the adrenal gland, testis and ovary involve enzymes from the following CYP families, CYP11, CYP17, CYP19 and CYP21 (modified from Payne and Hales, 2004). Abbreviations: 3 β -HSD, 3 β -hydroxysteroid dehydrogenase-isomerase; 17HSD3, 17- ketosteroid reductase.

1.1.4. Tissue Distribution of CYP Expression

The total CYP content (expressed as nmol of total CYP per milligram of microsomal protein) of extrahepatic tissues is much lower than that of liver in humans, rats and most (if not all) mammals (Table 1.2). However, many of the CYP enzymes are preferentially expressed in extrahepatic tissues. Enzymes belonging to the CYP1A, CYP1B, CYP2A, CYP2E, CYP2F, CYP2J, CYP2S and CYP4F subfamilies are expressed in lung, stomach, small intestine, kidney, skin, brain, heart, spleen, thymus, placenta and steroidogenic tissues (adrenal gland, testis and ovary) (Bieche et al., 2007; Ding and Kaminsky, 2003; Hrycay and Bandiera, 2008; Pavek and Dvorak, 2008) (Table 1.3). The introduction of human and mouse genomic sequences along with highly sensitive molecular biology and analytical techniques has enabled the identification of novel extrahepatic CYP enzymes in recent years. In general, the clinical importance of extrahepatic CYP enzymes is much less than hepatic CYP enzymes. However, tissue-specific expression of CYP enzymes contributes to the differential absorption and elimination of therapeutic agents, as well as bioactivation of procarcinogens in different organs in the body.

The majority of CYP enzymes are expressed in liver, which is the primary site of drug metabolism *in vivo*. The protein levels of enzymes in the CYP1A, CYP2C and CYP3A subfamilies represent approximately 60% of the total CYP content of liver in humans and rodents (Lewis, 2001). In human liver, CYP1A2, CYP2C8, CYP2C9 and CYP3A4 are expressed at much higher levels compared to other CYP enzymes and these four enzymes are involved in the metabolism of >55% of currently marketed drugs (Lewis, 2001). Hepatic CYP3A4 (and CYP3A5) protein levels alone constitute >28% of the total CYP content of liver and catalyze the biotransformation of a large number of therapeutic agents. Inter-individual variation in the expression of hepatic CYP2C, CYP2D and CYP3A enzymes in humans is large and can result in a difference in the range of 20- to 1000-fold in the levels of a single CYP enzyme. For example, hepatic CYP2D6 protein levels differ by 1000-fold in humans (Rendic and Di Carlo, 1997).

Several CYP enzymes including CYP1A1, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2E1, and CYP2J2 mRNA and protein have been detected in human lung (Ding and Kaminsky, 2003; Pavek and Dvorak, 2008). The total CYP content in lung is considerably lower than in liver, but the metabolic capacity of the lung should not be ignored for the following reasons. First, unlike other organs of the body (except heart), the total cardiac output of blood has to go through the lung, thus exposing all circulating foreign and endogenous compounds to pulmonary CYP enzymes. Second, xenobiotics introduced into the body via intravenous, intramuscular, or inhalation routes circulate through the lung before reaching other organs, including liver. Activation of certain procarcinogen substrates is almost the same in both organs (Shimada et al., 1996b). In addition, the vascular surface area of pulmonary veins is very large, thus providing ample opportunity for circulating foreign compounds to undergo pulmonary metabolism. Thus, CYP enzymes in the lung play a clinically significant role in the metabolism of some xenobiotics (Hukkanen et al., 2002). Evidence from studies that utilize human lung preparations, pulmonary cell lines, and animal models shows that lung tissue has the capacity to convert chemicals to their reactive metabolites (Gerde et al., 1998; Hecht, 1999). For example, BaP, an important tobacco procarcinogen, is activated by CYP enzymes in human lung (Autrup, 1990; Hecht, 1999).

A large number of CYP enzymes from the CYP1, CYP2, CYP3 and CYP4 families are expressed in kidney, small intestine and brain. Expression of CYP enzymes in these tissues is beyond the scope of the present discussion and has been reviewed in the references cited here (Kaminsky and Zhang, 2003; Meyer et al., 2007; Pavek and Dvorak, 2008).

CYP enzymes are also expressed in steroidogenic tissues such as adrenal gland, testis and ovary and are involved mainly in the biosynthesis of endogenous compounds (Denisov et al., 2005). It is known that some CYP enzymes are expressed exclusively in steroidogenic tissues. Interestingly, the total CYP content of the adrenal gland is the highest among all extrahepatic

tissues. The total CYP content of testis and ovary, however, is very low compared to liver (Table 1.2). Mammalian testis is composed of mainly two types of cells, Leydig cells (also known as interstitial cells), which are the primary site of testosterone biosynthesis, and Sertoli cells, which are the site of spermatogenesis (Baker and O'Shaughnessy, 2001). CYP enzymes expressed in testis can be broadly categorized in two groups, namely, xenobiotic metabolizing CYP enzymes and steroidogenic CYP enzymes. Xenobiotic metabolizing CYP enzymes such as CYP1A1, CYP1B1, CYP2A1 and CYP2E1 have been detected at the mRNA and protein levels in rat testis, specifically in Leydig cells (Jiang et al., 1998; Lee et al., 2007; Leung et al., 2009; Seng et al., 1996). Catalytic activity of testicular CYP1B1, CYP2A1, and CYP2E1 has also been reported (Jiang et al., 1998; Otto et al., 1992; Seng et al., 1991). CYP1B1 is expressed at high levels in the adrenal gland and testis in rats (Bhattacharyya et al., 1995; Leung et al., 2009). A putative role for testicular CYP enzymes belonging to the CYP1 and CYP2 families in chemical-induced testicular dysfunction has been proposed (Schuppe et al., 2000; Sharpe, 2001), but the physiological role of these enzymes is yet to be determined.

Steroidogenic CYP enzymes that are involved in the biosynthesis of sex steroids are expressed in testis, ovary and adrenal gland. Cholesterol side-chain cleavage CYP enzyme (P450_{scc} or CYP11A1) is a mitochondrial steroidogenic enzyme that is constitutively expressed in human, rat, and mouse testis, ovary and adrenal cortex (Fukuzawa et al., 2004; Guo et al., 2007; Luo et al., 2001; Moore et al., 1991). CYP11A1 catalyzes the rate-limiting step (i.e. the conversion of cholesterol to pregnenolone) in the biosynthesis of testosterone, estrogen and adrenocorticoids (Guo et al., 2007) (Figure 1.4). CYP17A1 is a unique steroidogenic enzyme that is expressed in adrenal, testis, ovary, and placenta (Payne, 1990). CYP17A1 exhibits 17 α -hydroxylase and 17,20-lyase activity in testosterone biosynthesis (Payne, 1990). CYP19A1, an enzyme involved in the conversion of androstenedione and testosterone to estrogens, is expressed in mammary, ovary, testis, brain, adipose tissue, bone and human placenta (Yanase et

al., 2001). In testis, expression of CYP11A1 and CYP17A1 is Leydig cell-specific, whereas CYP19A1 is expressed both in Sertoli cells and Leydig cells. CYP11A1, CYP17A1 and CYP19A1 mRNA have been detected in mouse Leydig tumor cells (MA-10) and rat Leydig tumor cells (R2C) (Heneweer et al., 2004; Mellon and Vaisse, 1989; Payne, 1990; Rao et al., 2002). The expression of CYP21A1 is adrenal-specific and is involved in the hydroxylation of progesterone at the C21 position, a vital step in the biosynthesis of glucocorticoids and mineralocorticoids (Payne and Hales, 2004).

Table 1.2: Total CYP content in various human and rat organs (taken from Hrycay and Bandiera, 2008).

Organ	Human (nmol/mg microsomal protein)	Rat (nmol/mg microsomal protein)
Liver	0.30-0.60	0.8-1.1
Adrenal	0.23-0.54	0.5
Small Intestine	0.03-0.21	0.02-0.13
Kidney	0.03	0.05-0.2
Lung	0.01	0.035-0.05
Brain	0.10	0.025-0.05
Testis	0.005	0.07-0.12
Skin	<0.001	0.05
Mammary	<0.001	0.001-0.003

Table 1.3: Expression of human CYP enzymes in various organs (compiled from Hrycay and Bandiera, 2008; Jiang et al., 1998; Lee et al., 2007; Leung et al., 2009; Payne and Hales, 2004; Seng et al., 1991).

Organ	CYP genes expressed (mRNA or protein)
Liver	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP3A7, CYP4A11
Small Intestine	CYP1A1, CYP2C9, CYP2C19, CYP2D6, CYP2J2, CYP3A4, CYP3A5
Nasal Mucosa	CYP2A6, CYP2A13, CYP2B6, CYP2C, CYP2J2, CYP3A
Trachea	CYP2A6, CYP2A13, CYP2B6, CYP2C, CYP2J2, CYP2S1, CYP3A, CYP4X1
Lung	CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C18, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, CYP4B1
Stomach	CYP1A1, CYP1A2, CYP2C, CYP2J2, CYP2S1, CYP3A4
Colon	CYP1A1, CYP1A2, CYP1B1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, CYP4F12
Kidney	CYP1B1, CYP2A6, CYP2B6, CYP2E1, CYP2R1, CYP2S1, CYP3A5, CYP4A11, CYP4F2, CYP4F12
Skin	CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2S1, CYP3A4, CYP3A5, CYP4A11
Brain	CYP1A1, CYP2B6, CYP2D6, CYP2E1, CYP2U1
Mammary	CYP1A1, CYP1B1, CYP2C, CYP2D6, CYP2S1, CYP3A4, CYP3A5, CYP4Z1
Placenta	CYP1A1, CYP2E1, CYP2F1, CYP2S1, CYP3A4, CYP3A5, CYP4B1
Adrenal gland	CYP11A1, CYP11B1, CYP17A1, CYP21A1
Testis	CYP1A1, CYP1B1, CYP2A1, CYP2E1, CYP11A1, CYP17A1, CYP19A1
Ovary	CYP11A1, CYP17A1, CYP19A1

1.1.5. Orphan CYP Enzymes

At present, 13 human CYP enzymes are termed as ‘orphans’ because detailed information related to their catalytic activity and function is unknown (Guengerich et al., 2005; Stark and Guengerich, 2007). Although the mRNA expression profile and putative substrates for some orphan CYP enzymes have been studied, information about most of the orphans is very limited. The majority of orphan CYP enzymes are found in families 1-4 (Stark and Guengerich, 2007). The amino acid sequences of human orphan CYP enzymes and their orthologs in rats, mice and eukaryotes share a high degree of sequence identity (Stark and Guengerich, 2007). The orphan CYP enzyme group consists of CYP2A7, CYP2S1, CYP2U1, CYP2W1, CYP3A43, CYP4A22, CYP4F11, CYP4F22, CYP4V2, CYP4X1, CYP4Z1, CYP20A1, and CYP27C1 (Guengerich et al., 2005; Stark and Guengerich, 2007). Expression of orphan CYP enzymes appears to be predominantly extrahepatic. The expression of CYP2W1 protein is tumor-specific in humans and is found mainly in colon and lung cancerous tissues (Karlgren et al., 2006). Human CYP4V2 is an eye-specific orphan CYP enzyme (Stark and Guengerich, 2007). CYP4Z1 protein is overexpressed in human breast cancer tissues (Rieger et al., 2004).

The primary reason for classifying certain CYP enzymes as ‘orphan’ is the lack of defined functionality of these relatively recently identified CYP enzymes. Various studies suggested potential biological roles for these orphan CYP enzymes based on the mRNA and/or protein expression profiles, which can be misleading due to the ubiquitous expression pattern of these CYP enzymes in humans (Stark and Guengerich, 2007). Another weakness of the currently published studies on orphan CYP enzymes is dependence on recombinant CYP enzymes as the source of protein samples for functionality screening. For example, to identify the potential substrate(s) of CYP2U1, a widely studied orphan CYP enzyme, functionality studies were performed with recombinant proteins expressed in bacteria (e.g. *Escherichia coli*) and yeast (e.g. *Saccharomyces cerevisiae*) rather than purified protein samples from rat, mouse or human

tissues (Karlgren et al., 2005). The metabolism of substrates by recombinant proteins is strong supportive evidence that the CYP enzyme in question will be capable of catalyzing the same reaction in vivo, although the catalytic efficiency of recombinant proteins may differ from that of microsomal samples and the substrate of a recombinant CYP enzyme may not be a physiological substrate.

CYP2S1 is considered to be an orphan CYP enzyme. It is predominantly expressed in extrahepatic tissues, mainly in lung (Rylander et al., 2001). Recombinant human CYP2S1 has been expressed in *Escherichia coli* and *Saccharomyces cerevisiae* to determine its catalytic activity. Retinoic acid and naphthalene are metabolized by recombinant human CYP2S1 (Bui, 2006; Karlgren et al., 2005; Smith et al., 2003). However, it is not known if retinoic acid is a physiological substrate of human CYP2S1.

Occasionally, CYP1B1 is also categorized as an orphan CYP enzyme (Stark et al., 2008). In humans and rodents, CYP1B1 expression in liver is low or undetectable, whereas CYP1B1 is expressed at readily measurable levels in steroidogenic tissues such as testis and adrenal (Bhattacharyya et al., 1995). The majority of metabolism studies involving CYP1B1 were carried out using recombinant CYP1B1. A definitive physiological function of CYP1B1 is not yet elucidated.

The chromosomal location, tissue distribution and putative substrates of human orphan CYP enzymes are summarized in Table 1.4.

Table 1.4: The chromosomal location, tissue distribution and putative substrates of human orphan CYP enzymes (modified from Stark and Guengerich, 2007).

Enzyme	Location	Expression	Putative Substrates
CYP1B1	2p21	Adrenal gland, testis, tumor tissues from prostate, mammary gland, ovary	Estradiol, DMBA, retinoic acid
CYP2A7	19q13.2	Liver, mammary gland, thyroid	n.d.
CYP2S1	19q13.1	Skin, GI-tract, urinary tract, and respiratory organ tumors (lung and cervix squamous cell carcinomas)	Retinoic acid, naphthalene
CYP2U1	4q25	Thymus, heart, spleen, and prostate	Arachidonic acid, other polyunsaturated fatty acids
CYP2W1	7p22.3	Cancer tissues from colon and lung	Arachidonic acid, polycyclic hydrocarbons, testosterone
CYP3A43	7q21	Liver	Testosterone?
CYP4A22	1p33	Liver	n.d.
CYP4F11	19p13.1-2	Kidney, liver, heart	Eicosanoids and xenobiotics (erythromycin, benzamphetamine, imipramine)
CYP4F22	19p13.1-2	Skin	Fatty acids?
CYP4X1	1p33	Brain, trachea, aorta	Fatty acids?
CYP4V2	4q35	Retina, eye tissues	Fatty acids?
CYP4Z1	1p33	Mammary tissues, breast carcinomas, kidney and liver	n.d.
CYP20A1	2..33.2	Liver?	n.d.
CYP27C1	-	Liver, kidney, pancreas	n.d.

Abbreviations: n.d.- Not determined; DMBA, 7,12-dimethylbenzanthracene.

1.2. Induction and Suppression of CYP Expression

The expression of CYP enzymes in humans and rodents is regulated by various factors including age, hormones (e.g. pituitary hormones, sex hormones), pregnancy, dietary components, nutritional status, and pathophysiological conditions. Altered CYP expression has been observed in humans after exposure to therapeutic agents (e.g. rifampicin, tamoxifen) and environmental toxicants (e.g. cigarette smoke) (Chang et al., 2003; Ickenstein and Bandiera, 2002; Urquhart et al., 2007). Endogenous compounds (e.g. bile acids) are also involved in the modulation of CYP expression (Chiang, 2002). Differential exposure to xenobiotics or endogenous compounds may lead to variability in CYP-mediated metabolism, leading to altered pharmacokinetic and toxicological profiles of therapeutic agents.

Induction of CYP enzymes is defined as an increase in protein expression after treatment of an animal or cells in culture with a xenobiotic or an endogenous compound. Increased protein expression is accompanied by increased enzymatic activity. The induction of CYP enzymes was first identified when altered pharmacological responses were observed after treatment of rats with phenobarbital or other xenobiotics (Conney et al., 1960; Remmer and Merker, 1963). For example, rats treated repeatedly with phenobarbital became tolerant to the hypnotic effect of the drug because CYP protein levels and consequently the metabolism of phenobarbital increased (Conney et al., 1960; Remmer and Merker, 1963). Thus, induction of CYP enzyme expression can cause a reduction in clinical response by augmenting the clearance of therapeutic agents, which explains some cases of drug-drug or drug-food interactions. CYP induction can be beneficial when the CYP enzyme of interest is involved in the detoxification of toxic xenobiotics, which will accumulate unless converted to water soluble metabolites (Lewis, 1996). This confers the cell with the ability to survive in a potentially toxic environment. Occasionally, an increase in CYP protein levels can lead to increased reactive metabolite formation leading to

increased toxic effects including neoplasm (Guengerich, 2008; Lewis, 2001).

Induction of CYP1 family enzymes by environmental pollutants and carcinogens, known as PAHs, has been widely studied in rats, mice and humans (Denison and Nagy, 2003; Whitlock, 1999; Whitlock et al., 1997). Increased CYP protein expression leads to bioactivation of potential carcinogens (e.g. BaP), which increases the risk of carcinogenesis. In addition, an increase in CYP protein levels can cause increased metabolism of endogenous substrates (e.g. steroid hormones, fatty acids and eicosanoids), which may disturb the homeostasis of important physiological pathways (Elbekai and El-Kadi, 2006; Yager and Liehr, 1996; Zimniak, 1993).

CYP enzyme expression can also be suppressed or downregulated after exposure to endogenous compounds, inflammatory mediators and xenobiotics including therapeutic agents and PAHs. Tamoxifen, an orally active selective estrogen receptor modulator, suppresses hepatic CYP2A1 expression in female rats (Ickenstein and Bandiera, 2002). Rat CYP2C11, a male specific enzyme, was downregulated after treatment of rats with 3-MC (Riddick et al., 2003). The suppression of CYP enzyme expression by xenobiotics has been linked to inhibition of hormonal pathways involved in the constitutive expression of these enzymes (Ickenstein and Bandiera, 2002; Riddick et al., 2003).

Downregulation of CYP enzymes may be a physiological response to a change in a homeostatic process or a disease condition. Endogenous substances such as sex hormones and bile acids have the ability to decrease CYP protein levels in hepatic and gonadal tissues (Chiang, 2002; Nozu et al., 1981a; Nozu et al., 1981c). The suppressive effect of sex hormones on expression of certain CYP enzymes is one of the mechanisms for the sex-dependent expression of CYP enzymes. Estrogen is known to suppress the expression of CYP17A1, a key enzyme in steroidogenesis in the adrenal gland, testis and ovary (Payne, 1990; Payne and Youngblood, 1995). The expression of CYP17A1 in mouse and rat Leydig cells is suppressed by testosterone

(Georgiou et al., 1987; Hales et al., 1987). Similarly, bile acids decrease hepatic CYP7A1 expression in humans and mice. CYP7A1 catalyzes the rate-limiting step in the biosynthesis of bile acids from cholesterol (Chiang, 2002).

Disease conditions such as diabetes, obesity, rheumatoid arthritis, multiple sclerosis, cholestasis, and viral infections (e.g. hepatitis, influenza) can suppress the expression of CYP enzymes and drug biotransformation (Morgan, 1997; Renton, 2004). During inflammatory and viral disease conditions, the circulating levels of inflammatory mediators (e.g. tumor necrosis factor α , interleukins, and interferons) are increased significantly (Aukrust et al., 1994; Drachenberg et al., 1999; Nawata et al., 1989; Waehre et al., 2004), leading to a decrease in total hepatic CYP content as well as decreased hepatic CYP1A, CYP2B, CYP2C and CYP3A levels (Christen et al., 2001; Hopkins, 2003; Renton, 2001; Sharief and Hentges, 1991).

Induction and suppression of CYP enzyme expression are controlled mainly at the transcription level (Handschin and Meyer, 2003; Honkakoski and Negishi, 2000). Increased CYP expression is typically mediated through a receptor-linked pathway that activates the transcriptional activity of one or more CYP genes. The suppression of CYP enzyme expression is mediated by several distinct pathways including destabilization of RNA transcripts, activation of gene corepressors, alteration of histone acetylation (i.e. incorporation of acetyl group into lysine residues of histone proteins that are the main components of chromatin), and protein degradation (Hales et al., 1987; Morales et al., 2003; Riddick et al., 2004).

In comparison with hepatic CYP enzymes, regulation of CYP enzymes in extrahepatic tissues has not been well studied. Expression of CYP1 family enzymes is regulated chiefly through two main pathways, namely PAH-mediated regulation and hormonal regulation. The PAH-mediated pathway is the predominant mechanism of expression of CYP1 enzymes in lung, kidney, small intestine, stomach and placenta (Pavek and Dvorak, 2008), whereas hormonal

regulation is active in the regulation of CYP1B1 and steroidogenic CYP enzymes in adrenal gland, testis, ovary, prostate, mammary, and uterus (Leung et al., 2009; Murray et al., 2001; Payne, 1990).

1.2.1. Receptor-Mediated Regulation of CYP Expression

Receptors play a key role in constitutive and inducible CYP expression in humans and rodents. The receptors involved in the regulation of CYP enzymes are localized in cytosol and act primarily in the nucleus, where the receptor-ligand complex binds to the CYP regulatory regions to increase the gene transcription leading to altered protein levels and activities (Honkakoski and Negishi, 2000; Riddick et al., 2003; Schmidt and Bradfield, 1996). Inducers of CYP enzymes are structurally diverse and bind to receptors with different specificity and potency to initiate the transcription process. Regulation of CYP expression is controlled by at least five different receptors, specifically, aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptors (PPARs), and glucocorticoid receptor (Lewis, 2001). The specific involvement of these receptors in CYP regulation has been elucidated using receptor-ligand binding assay and advanced molecular biology techniques including knock-out mice and gene reporter assays. A review of the literature suggests that AhR is chiefly involved in the regulation of CYP1 enzymes, whereas PXR and CAR are involved in the regulation of CYP2 and CYP3 family enzymes (Lewis, 2001). Expression of CYP4 family enzyme(s) is regulated by PPAR α (Lewis, 2001). The activators and the target CYP genes of four major xenobiotic receptors are summarized in Table 1.5. Regulation of CYP expression by AhR ligands is one of the main foci of this thesis and thus, AhR will be discussed in detail in the following section.

Table 1.5: Ligands and xenobiotic activators of human CYP genes (taken from Hrycay and Bandiera, 2008).

Receptors	Xenobiotic activators/ligands	Regulated CYP genes
AhR ^a	guanabenz, omeprazole, PCBs ^a , polycyclic aromatic hydrocarbons (<i>e.g.</i> BaP, 3-MC), TCDD, TCDF, thiabendazole	CYP1A1, CYP1A2, CYP1B1, (CYP2S1?)
CAR	CITCO, hyperforin, methoxychlor, phenobarbital, phenytoin, rifampicin	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4
PPAR α	bezafibrate, clofibrate, fenofibrate, fenoprofen, flufenamic acid, gemfibrozil, herbicides, ibuprofen, indomethacin, phthalates, Wy-14,643	CYP4A11
PXR	amprenavir, avasimibe, bosentan, carbamazepine, chlorpyrifos, CITCO, clotrimazole, cypermethrin, dexamethasone, DDT, dieldrin, efavirenz, endosulfan, endrin, etoposide, glibenclamide, hyperforin, lovastatin, mifepristone, methoxychlor, nelfinavir, nifedipine, nonylphenol, paclitaxel, phenobarbital, phenytoin, rifabutin, rifampicin, ritonavir, simvastatin, tamoxifen, TCPOBOP, topiramate, triclosan, troglitazone	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4

^a, Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; DDT, dichlorodiphenyltrichloroethane; BaP, benzo[a]pyrene; 3-MC, 3-methylcholanthrene; PCBs, polychlorinated biphenyls; PPAR α , peroxisome proliferator-activated receptor α ; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCPOBOP, 1,4-bis[(3,5-dichloropyridyloxy)]benzene; Wy-14,643, [4-chloro-6-(2,3-xylidino)pyrimidinylthio]acetic acid.

1.2.1.1. Aryl Hydrocarbon Receptor (AhR)

The AhR is a cytosolic ligand-activated transcription factor that mediates CYP1 enzyme induction (Schmidt and Bradfield, 1996). Ligands for AhR include PAHs (e.g. 3-MC, BaP), TCDD, polychlorinated biphenyls (PCBs), dietary components (e.g. flavonoids), and therapeutic agents (e.g. omeprazole, thiabendazole). PAHs including 3-MC and BaP are planar highly lipophilic ligands of AhR that bind to the receptor and activate its transcriptional activity (Denison and Nagy, 2003). Omeprazole is one of the few therapeutic agents known to induce CYP enzymes in an AhR-dependent manner (Dzeletovic et al., 1997). Tryptophan-derived products such as tryptamine, indirubin and indole acetic acid have been identified as low affinity endogenous ligands of AhR (Denison and Nagy, 2003). Flavonoids are naturally occurring xenobiotic AhR ligands. Flavonoids are dietary components that are widely distributed in vegetables, fruits and beverages (Denison and Nagy, 2003). AhR regulates both the constitutive and inducible expression of CYP1A1, CYP1A2 and CYP1B1 enzymes in liver and extrahepatic tissues such as lung, kidney, stomach, small intestine, and placenta (Pavek and Dvorak, 2008; Riddick et al., 2003; Shimada et al., 2002; Shimada et al., 2003; Whitlock, 1999).

In some species (e.g. fish, birds), two subtypes of AhR are expressed, while in mammals there is only one type of AhR (Hahn et al., 2006; Yasui et al., 2007). AhR is expressed at high levels in lung, liver, placenta, and thymus in rats (Carver et al., 1994). The transcriptional regulation of CYP1 enzymes by AhR is mediated through a heterodimerizing partner known as aryl hydrocarbon receptor nuclear translocator (ARNT) (Riddick et al., 2003; Schmidt and Bradfield, 1996). ARNT is expressed in lung, liver, placenta, thymus, and kidney and follows an expression pattern similar to AhR in rats (Carver et al., 1994).

The mechanism of AhR-mediated regulation of CYP1 enzymes has been extensively studied. In the absence of its ligands, AhR is localized in the cytoplasm and is associated with chaperone proteins, such as heat shock proteins (HSP90), and AhR-interacting proteins (Carlson

and Perdew, 2002). Upon ligand binding, AhR protein undergoes a conformational change, which initiates translocation of the ligand-bound complex of AhR and HSP proteins into the nucleus. In the nucleus, the ligand-bound AhR protein dissociates from its HSP90 chaperone and forms a heterodimer with ARNT (Schmidt and Bradfield, 1996). The AhR-ARNT heterodimer-ligand complex binds to the DNA regulatory element of the CYP gene, resulting in activation and subsequent increase in transcription of CYP genes (Carlson and Perdew, 2002; Schmidt and Bradfield, 1996) (Figure 1.5). This results in increased expression of CYP proteins and a corresponding increase in the biotransformation of xenobiotics including procarcinogens and drugs (Nebert and Dalton, 2006; Riddick et al., 2003). The DNA regulatory elements, known as and xenobiotic response elements (XREs), dioxin response elements, or Ah-responsive elements, of CYP genes play a key role in the mechanism of AhR-mediated induction.

AhR ligands cannot induce CYP1 enzymes in mutant mouse hepatoma cells having impaired XRE function (Whitlock et al., 1997). The XREs are composed of a nucleotide sequence, 5'-TNGCGTG-3', and base pairs of flanking DNA, which act as an enhancer region of CYP genes (Whitlock et al., 1997). The 5'-CGTG-3' nucleotide sequence is essential for the AhR-ARNT-dependent induction of CYP1 enzymes (Whitlock et al., 1997). The promoter region that is upstream of the transcription start site is another important regulatory element in the CYP1A1 nucleotide sequence (Jones and Whitlock, 1990). Transfection studies with mutations of promoter region sequences reveal that TATA (i.e. 5'-TATAAA-3' DNA sequence found in the promoter region of genes considered as transcription factor or histone binding site) sites are absolutely required for the induction of CYP1A1 by AhR ligands (Whitlock et al., 1997). The promoter region of the CYP1A1 gene remains latent in the absence of AhR ligands, and is thus, unable to facilitate AhR-mediated induction of CYP1A1 expression (Whitlock et al., 1997).

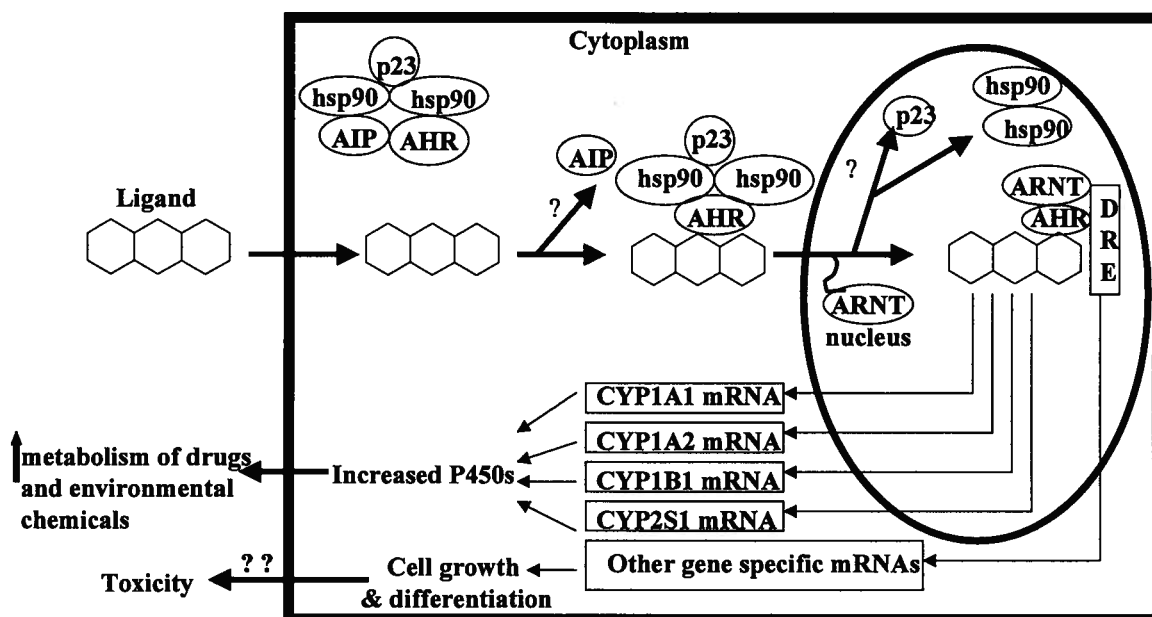


Figure 1.5: A working model for regulation of CYP enzymes by AhR (taken from Riddick et al., 2003). Abbreviations: DRE, Dioxin responsive elements; AHR, Aryl hydrocarbon receptors; ARNT, Aryl hydrocarbon receptor nuclear translocator; AIP, Aryl hydrocarbon receptor-interacting protein; hsp90, Heat Shock Protein 90.

Although the AhR-mediated induction mechanism is well understood, recent studies indicate that events other than the formation of AhR-ARNT-XRE complex can influence the increase in transcription of CYP1 enzymes after exposure to AhR ligands. Phosphorylation of AhR by kinase-mediated pathways (e.g. protein kinase C (PKC), mitogen-activated protein kinase (MAPK)) may be an alternative mechanism of AhR translocation to nucleus (Chen and Tukey, 1996; Tan et al., 2004). Co-treatment of human hepatoma cells with TCDD and phorbol-12-myristate-13-acetate (a potent PKC activator) increased CYP1A1 expression by 2-3 times more than treatment with TCDD only (Chen and Tukey, 1996). PKC inhibitors block the TCDD-mediated induction of CYP1A1 in human hepatoma cells (Machemer and Tukey, 2005). However, information in this area is limited and further studies are needed to completely understand the involvement of phosphorylation pathways in AhR-mediated regulation.

The AhR repressor is another key component in AhR-mediated regulation of CYP enzymes. Although the precise function of the AhR repressor protein remains to be elucidated, at present it is considered that AhR repressor is responsible for the negative regulation of AhR function in rats, mice, and humans (Bernshausen et al., 2005; Nishihashi et al., 2006; Yamamoto et al., 2004). AhR repressor competes with AhR to form a dimerization complex with ARNT (AhR repressor-ARNT) and binds to XRE by competing with AhR-ARNT dimer (Bernshausen et al., 2005; Tsuchiya et al., 2003b). AhR repressor is expressed in human lung, liver, testis, ovary, spleen, pancreas, and small intestine (Yamamoto et al., 2004). Gonadal tissues (testis and ovary) have the greatest levels of AhR repressor expression. AhR repressor expression is inducible by PAHs in rats and mice. No correlation has been found between induction of AhR repressor by PAHs and the effect of AhR agonists on CYP1A1 induction (Bernshausen et al., 2005).

1.2.2. Hormonal Regulation of CYP Expression

Several hepatic CYP enzymes are expressed in a sexually dimorphic and age-dependent manner in rats (Kato and Yamazoe, 1992). In general, female rats have 10% to 30% less total CYP as compared to male rats (Mugford and Kedderis, 1998). In particular, enzymes belonging to the CYP2 and CYP3 families exhibit sex- and age-dependent expression (Waxman et al., 1995). Growth hormone (GH) and sex steroids (e.g. testosterone, estrogen) regulate the sexually dimorphic and age-dependent expression of hepatic CYP enzymes (Pampori and Shapiro, 1999; Wang and Strobel, 1997; Waxman et al., 1995).

Reports suggest that CYP enzymes (e.g. CYP1B1, CYP2C11, CYP2E1, CYP11A1, CYP17, CYP19, and CYP21) in extrahepatic tissues such as adrenal gland, testis, ovary, kidney and lymphoid tissues are primarily regulated by pituitary hormones and sex hormones (Chen et al., 1999; Imaoka et al., 1992; Leung et al., 2009; Payne and Hales, 2004; Thangavel et al., 2007). However, there is no report of hormonal regulation of CYP enzymes in lung. Steroidogenic tissues (e.g. adrenal gland, testis and ovary) are exposed to local as well as circulating sex hormones (e.g. testosterone, estrogen, and progesterone). Furthermore, these tissues are primary targets of anterior pituitary hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, and adrenocorticotrophic hormone (ACTH). Examples of CYP enzymes regulated by hormones in hepatic and extrahepatic tissues are summarized in Table 1.6.

1.2.2.1. Effect of Pituitary Hormones

The role of GH in the regulation of CYP enzymes in liver has been extensively studied. The sex-specific expression of several hepatic CYP enzymes (e.g. CYP2C11, CYP2C12) is related to the sex-dependent GH secretory profiles, i.e. episodic in males and continuous in females (Pampori and Shapiro, 1999). The episodic male-type GH secretion induces CYP2C7

Table 1.6: Examples of hormonal regulation of CYP enzymes in various tissues in rats, mice and humans.

Hormone	Tissues	Target CYP Enzymes[*]
Growth hormone	Liver	CYP2A1 ^{b,c} (1,2), CYP2B1 ^{b,d} (3), CYP2C11 ^{a,d} (4), CYP2C12 ^c (5), CYP3A2 ^b (6)
	Kidney	CYP2E1 ^a (7)
	Spleen	CYP2C11 ^b (8)
	Thymus	CYP2C11 ^b (8)
	Bone marrow	CYP2C11 ^a (8)
Luteinizing hormone	Testis	CYP1B1 ^a (9), CYP11A1 ^a (10), CYP17A1 ^a (11), CYP19A1 ^a (12)
	Ovary	CYP19A1 ^d (13)
Follicle stimulating hormone	Testis	CYP1B1 ^a (9)
	Ovary	CYP19A1 ^c (14)
Prolactin	Testis	CYP1B1 ^a (9)
Adrenocorticotrophic hormone	Adrenal	CYP1B1 ^c (15), CYP11A1 ^a (16), CYP17A1 ^a (16), CYP21A1 ^a (16)
Estradiol	Liver	CYP2A1 ^c (2), CYP2C11 ^b (17), CYP2C12 ^a (17), CYP2E1 ^d (2)
	Testis	CYP1B1 ^b (2), CYP17A1 ^b (18)
Testosterone	Liver	CYP2A1 ^b (2), CYP2C11 ^a (19), CYP2C12 ^b (17), CYP3A2 ^a (20)
	Kidney	CYP2C11 ^a (7), CYP2E1 ^a (21), CYP2J5 ^a (22)
	Testis	CYP17A1 ^b (11)

*Data for CYP1, CYP2, and CYP3 enzymes were compiled from rat tissues. Data for steroidogenic CYP enzymes were compiled from mouse and human tissues. See section 1.2.2 for details.

^a Induces in male tissues;

^b Suppresses in male tissues;

^c Induces in female tissues;

^d Suppresses in female tissues

References: (1) Pampori and Shapiro, 1996; (2) Waxman et al., 1989a; (3) Yamazoe et al., 1987; (4) Waxman et al., 1991; (5) Legraverend et al., 1992; (6) Kawai et al., 2000; (7) Chen et al., 1997; (8) Thangavel et al., 2007; (9) Leung et al., 2009; (10) Mellon and Vaisse, 1989; (11) Payne, 1990; (12) Genissel et al., 2001; (13) Morales et al., 2003; (14) Kwintkiewicz et al., 2007; (15) Brake and Jefcoate, 1995; (16) Sewer and Waterman, 2003; (17) Waxman et al., 1985; (18) Nozu et al., 1981c; (19) Anderson et al., 1998; (20) Ribeiro and Lechner, 1992; (21) Henderson and Wolf, 1991; (22) Ma et al., 2004.

and CYP2C11 expression (Waxman et al., 1991; Westin et al., 1990) and suppresses CYP2A1, CYP2B1, CYP2B2, CYP2E1, and CYP3A2 expression in rat liver (Kawai et al., 2000; Waxman et al., 1989b; Yamazoe et al., 1987). Continuous female-type GH secretion induces CYP2A1, CYP2C7, and CYP2C12 expression (Pampori and Shapiro, 1996; Westin et al., 1990) and suppresses CYP2B1, CYP2B2, CYP2C11, and CYP2E1 expression in rat liver (Pampori and Shapiro, 1996; Waxman et al., 1989b; Yamazoe et al., 1987). GH is also involved in regulating CYP2C11, CYP2E1 and CYP4A2 in kidney, thymus, spleen and bone marrow (Chen et al., 1999; Imaoka et al., 1992; Thangavel et al., 2007). The regulation of CYP expression by GH in liver and extrahepatic tissues is not the focus of this thesis and has been reviewed in the cited references (Waxman and Chang, 2005; Waxman and O'Connor, 2006).

There are no reports of regulation of CYP expression in liver, lung, kidney and lymphoid by other pituitary hormones such as LH, FSH, prolactin and ACTH. However, expression of CYP1B1, CYP11A1, CYP17A1 and CYP19A1 in steroidogenic tissues is influenced by LH, FSH, prolactin and ACTH (Payne, 1990; Peter and Dubuis, 2000; Stocco et al., 2005). The pulsatile secretion pattern of gonadotropin releasing hormone (GnRH), a decapeptide formed in the hypothalamus, controls the pulsatile release of LH, FSH, ACTH and prolactin from the anterior pituitary (Sisk and Foster, 2004; Walker and Cheng, 2005), which subsequently act on testis, ovary and adrenal gland. Testis is a primary site of steroidogenesis and hormonal regulation of testicular CYP1B1 expression is a main focus of this thesis.

LH, FSH and prolactin are the major pituitary hormones involved in the positive regulation of steroidogenic CYP expression in testis. LH and prolactin primarily act on Leydig cells, whereas Sertoli cells are the main target cells of FSH in testis. Hypophysectomy (removal of the pituitary gland) suppresses testicular CYP11A1 and CYP17A1 protein expression in rats and administration of LH in hypophysectomized rats completely restores their expression (Dombrowicz et al., 1996). Similarly, testicular CYP17A1 activity (i.e. 17-hydroxylation), which

was decreased in hypophysectomized rats, is restored following treatment with LH (Takeyama et al., 1986). CYP17A1 transcript is expressed at a very low or undetectable levels in mouse Leydig MA-10 cells. CYP17A1 expression was increased 2- to 3-fold after exposure to LH in MA-10 cells with stimulation of steroidogenesis (e.g. testosterone formation). CYP11A1 expression was increased 1.6- to 3-fold after treatment of MA-10 cells and rat and mouse primary Leydig cells with LH or 3',5'-cyclic adenosine monophosphate (cAMP) analogs (Anakwe and Payne, 1987; Anderson and Mendelson, 1985; Mellon and Vaisse, 1989). Although the LH-elicited increase in CYP11A1 and CYP17A1 in Leydig cells is cAMP-dependent, the LH-mediated regulation of CYP11A1 and CYP17A1 is not exerted via the cAMP response element binding protein (CREB), as is usually the case for other cAMP-regulated genes (e.g. steroidogenic acute regulatory protein) in Leydig cells (Payne and Hales, 2004; Payne and Youngblood, 1995). Steroidogenic factor-1 (SF-1), a transcription factor widely present in steroidogenic tissues, is required for CYP11A1 and CYP17A1 expression in Leydig cells (Payne and Hales, 2004; Payne and Youngblood, 1995; Peter and Dubuis, 2000). CYP19A1 expression was increased 2-fold by treatment with LH (50 ng/ml) in primary rat Leydig cells and cAMP analogs (e.g. dibutyl-cAMP) were able to mimic the effects of LH treatment in Leydig cells (Genissel et al., 2001). Phosphorylation of transcription factors, namely, CREB and SF-1, by protein kinase A (PKA) pathways is essential for the expression of CYP19A1 (Carlone and Richards, 1997a).

FSH is another important pituitary hormone involved in the regulation of CYP expression in testis, mainly in Sertoli cells. Testicular CYP17A1 mRNA levels are increased 19-fold after exposure of mice to FSH (10 IU/mouse) (Sadate-Ngatchou et al., 2004). CYP19A1 mRNA and protein expression are low or undetectable in rat Sertoli cells and FSH (1 IU/ml) induces CYP19A1 mRNA and protein expression in rats (McDonald et al., 2006). Similar to LH, the inductive effects of FSH on CYP expression in testis cells is mediated through stimulation of

adenylyl cyclase activity which leads to an increase in cAMP formation and activation of the PKA pathway. Subsequently, PKA phosphorylates transcription factors (e.g. CREB, SF-1) that are involved in the regulation of CYP expression in Sertoli cells (Walker and Cheng, 2005).

Prolactin is involved in the regulation of testicular CYP expression in humans and rodents. In contrast to LH and FSH, available information on the effect of prolactin on testicular CYP regulation is limited. Prolactin restored testicular CYP11A1 and CYP17A1 protein expression in hypophysectomized rat testis and the inductive effect of prolactin is approximately 40% less than that seen after treatment with LH (De Buck et al., 2005; Dombrowicz et al., 1992; Dombrowicz et al., 1996). It has been proposed that prolactin could influence CYP expression through an indirect mechanism in Leydig cells; potentially by increasing LH receptor density in Leydig cells (Maran et al., 2001; Zipf et al., 1978). Recently, it was reported that prolactin increased LH secretion by directly acting on the anterior pituitary (Henderson et al., 2008), which may explain the inductive effect of prolactin on testicular CYP expression *in vivo*.

1.2.2.2. Effect of Sex Steroids

Sex hormones (e.g. testosterone and estrogen) are involved in the regulation of CYP expression in liver and extrahepatic tissues such as kidney, testis, ovary, and mammary. Estrogen and androgens can influence CYP expression either through a direct effect on their transcription or by altering the GH secretion pattern (Carlone and Richards, 1997a; Chowen et al., 2004; Chowen et al., 1996; Hales et al., 1987; Payne, 1990). Estradiol and testosterone also indirectly regulate CYP expression through negative feedback control of the hypothalamus-pituitary axis (Akingbemi et al., 2003; Delbes et al., 2005; Fukuzawa et al., 2004; Wersinger et al., 1999). Secretion of LH from the anterior pituitary leads to stimulation of CYP17A1-mediated testosterone formation in Leydig cells (Fukuzawa et al., 2004; Payne, 1990). Subsequently, estradiol is synthesized from testosterone via the aromatization reaction catalyzed by CYP19A1

(Abney, 1999). Elevated levels of sex steroids block the hypothalamus-pituitary axis, leading to decreased secretion of anterior pituitary hormones such as LH and FSH (Sisk and Foster, 2004). Similarly, exogenously administered androgens and estradiol can block the secretion of LH and FSH from pituitary (Dufy-Barbe et al., 1975; Masuyama et al., 2001; Walczewska et al., 1999). Thus, decreased levels of serum LH and FSH lead to less exposure of gonads, liver and other organs to these hormones. A simplified outline of feedback inhibition of pituitary hormone secretion by sex steroids is shown in Figure 1.6.

Testosterone and estradiol are involved in the regulation of hepatic CYP expression in rodents. Administration of testosterone in female rats induces hepatic CYP2C11 protein and suppresses CYP2C12 protein expression, whereas treatment of male rats with estradiol induces hepatic CYP2C12 and suppresses CYP2C11 protein expression (Anderson et al., 1998; Bandiera and Dworschak, 1992; Chang and Bellward, 1996; Waxman et al., 1985; Waxman et al., 1989a). Estradiol also induces hepatic CYP2A1 and suppresses hepatic CYP2E1 in female rats (Waxman et al., 1989a). Hepatic CYP3A2 levels are positively regulated by testosterone in adult male rats, whereas estradiol had no effect on CYP3A2 (Ribeiro and Lechner, 1992; Waxman et al., 1988).

Estradiol and testosterone are also active in the regulation of CYP expression in testis and kidney. Renal CYP2C11 protein levels, which were decreased in hypophysectomized rats, were restored to constitutive levels after treatment with testosterone (Chen et al., 1997). Testosterone positively regulates expression of several renal CYP enzymes from CYP2A, CYP2C, CYP2E, and CYP2J subfamilies in male mice (Henderson et al., 1990; Henderson and Wolf, 1991). Testosterone and estradiol have been found to regulate CYP4A expression in rat kidney and have been implicated in the sex-dependent control of renal pressure (Hercule et al., 2003; Imaoka et al., 1992; Wang et al., 2003).

In general, estradiol benzoate has a suppressive effect on CYP expression and activity in testis (Akingbemi et al., 2003; Nozu et al., 1981a; Nozu et al., 1981b; Nozu et al., 1981c),

whereas testosterone exerts differential effects on steroidogenic CYP expression and activity (Payne, 1990; Payne and Hales, 2004; Payne and Youngblood, 1995). Total CYP content of testis was decreased after treatment of rats with estradiol (20 $\mu\text{g}/\text{rat}$) (Nozu et al., 1981b). CYP17A1 mRNA expression and 17-hydroxylation activity were increased 2.4-fold in Leydig cells obtained from estrogen receptor- α gene knockout mice (Akingbemi et al., 2003). Nozu et al. (1981b) reported that CYP17A1 activity is inhibited by estradiol in rat Leydig cells. LH or cAMP analogs increase estrogen formation leading to impaired steroidogenesis in Leydig cells (Cigorruga et al., 1980; Nozu et al., 1981a; Nozu et al., 1981b). The mechanism of estrogen-mediated suppression of CYP17A1 enzyme expression is not yet clear. In contrast, CYP19A1 mRNA levels are increased by 20% after treatment of rat Leydig cells with estradiol (Bourguiba et al., 2003). There are contrasting effects of estradiol on testicular CYP11A1 expression in neonatal and adult mice. Akingbemi et al. (2003) showed that CYP11A1 mRNA expression did not change in estrogen receptor- α gene knockout adult mice, whereas Delbes et al. (2005) reported an approximately 2-fold increase in CYP11A1 mRNA levels in estrogen receptor- α gene knockout neonatal mice.

Elevated testosterone levels, formed during LH- or cAMP-mediated stimulation of steroidogenesis, suppresses CYP17A1 mRNA levels and activity in mouse and rat primary Leydig cells (Georgiou et al., 1987; Hales et al., 1987; Rani and Payne, 1986) and in adult rat testis (O'Shaughnessy and Payne, 1982). Testosterone-mediated degradation of CYP17A1 protein and androgen receptor activities have been implicated in the downregulation of CYP17A1 expression in Leydig cells (Hales et al., 1987). In contrast, testosterone (400 ng/ml) increased CYP19A1 mRNA levels by 30% in primary rat Leydig cells (Genissel et al., 2001). It has been proposed the inductive effect of testosterone on CYP19A1 mRNA expression may occur due to a 2-fold increase in half-life of the CYP19A1 mRNA transcript (Bourguiba et al., 2003).

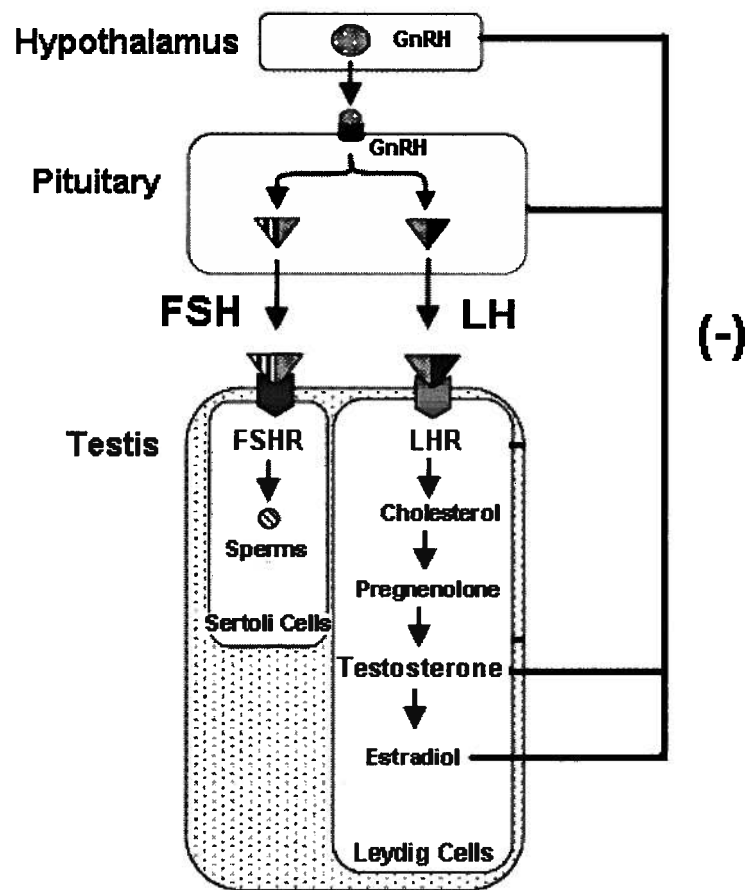


Figure 1.6: Feedback inhibition of hypothalamus-pituitary axis by sex steroids (modified from Fukuzawa et al., 2004). Abbreviations: GnRH- gonadotropin releasing hormone; LH- luteinizing hormone; FSH- follicle-stimulating hormone; FSHR- follicle-stimulating hormone receptor; LHR- luteinizing hormone receptor.

1.3. CYP1 Family Enzymes

Enzymes belonging to the CYP1 family (i.e. CYP1A1, CYP1A2, and CYP1B1) are expressed in mammalian liver and extrahepatic tissues such as lung, kidney, gastrointestinal tract, testis and adrenal (Bieche et al., 2007; Ding and Kaminsky, 2003; Pavek and Dvorak, 2008). CYP1 enzymes are expressed constitutively at a low level in hepatic and extrahepatic tissues but are inducible. AhR agonists such as 3-MC, BaP, and TCDD induce CYP1 expression in various tissues including liver, lung, kidney, placenta, skin, and gastrointestinal tract (Hukkanen et al., 2002; Pavek and Dvorak, 2008; Schmidt and Bradfield, 1996; Shimada et al., 2002; Shimada et al., 2003; Walker et al., 1999; Whitlock, 1999).

CYP1 enzymes identified in birds, fish, rats, mice, and humans exhibit a high degree of amino acid sequence identity (Mitsuo et al., 2001). Enzymes in the CYP1A subfamily are designated as CYP1A1 and CYP1A2 in mammals. In non-mammalian species, the CYP1A subfamily is represented by CYP1A4 and CYP1A5 in birds and CYP1A1 and CYP1A3 in fish. The percentage identity between amino acid sequences of CYP1 enzymes in various species is summarized in Table 1.7.

The catalytic activity of CYP1 enzymes is also well conserved among species (Lewis, 1996). CYP1 enzymes are active in the metabolism of PAHs and aromatic amines, leading to bioactivation of potential procarcinogens. For example, CYP1 enzymes are involved in the bioactivation of 7,12-dimethylbenzanthracene (DMBA) and BaP, which have been implicated in the chemical carcinogenesis of breast, skin, lung and liver in rodents (Buters et al., 2003; Conney, 1982; Guengerich, 2003; Shou et al., 1996). CYP1 enzymes catalyze the formation of reactive intermediates such as epoxides and the formation of monohydroxy and dihydroxy metabolites from DMBA (Figure 1.7) and BaP (Figure 1.8). DMBA-3,4-diol and DMBA-8,9-diol are the major metabolites of DMBA among approximately thirty DMBA metabolites formed by rat liver microsomes (Yang and Dower, 1975). The bioactivated metabolite of PAHs is more

carcinogenic than the parent compound. BaP-7,8-diol-9,10-epoxide is a major metabolite of BaP and is much more carcinogenic and potent than BaP (Conney, 1982; Kapitulnik et al., 1977; Wislocki et al., 1976). Examples of other pro-toxic compounds bioactivated by CYP1 enzymes include chrysene, benz[a]anthracene and dibenzo[a,l]pyrene (Shimada, 2006; Shimada and Fujii-Kuriyama, 2004). CYP1 enzymes are also involved in the metabolism of estradiol to potential genotoxic metabolites such as 4-hydroxyestradiol (Yager and Liehr, 1996). It has been suggested that CYP1 enzymes may be involved in hormonal and chemical carcinogenesis in rodents and humans (Nebert and Dalton, 2006; Nebert et al., 2004; Shimada, 2006; Yager and Liehr, 1996).

Table 1.7: The percentage identity between amino acids sequences of CYP1A1, CYP1A2 and CYP1B1 enzymes in fish, birds, rats and humans (taken from Mitsuo et al., 2001).

	CYP1A1				CYP1A2				CYP1A4				CYP1B1	
	Eel	Trout	Plaice	Scup	Butterfly	Toadfish	Human	Rat	Human	Rat	Chicken	Human	Rat	Human
Novel Eel CYP1A	78	77	73	75	72	74	54	56	53	50	55	56	35	36
Eel CYP1A1		80	76	79	76	74	54	56	52	49	56	57	35	37
Rainbow trout CYP1A1			79	83	78	77	56	56	52	50	55	55	35	36
Plaice CYP1A1				84	84	81	55	56	52	50	53	55	35	35
Scup CYP1A1					83	80	56	57	54	51	55	57	36	36
Butterfly fish CYP1A1						80	55	56	51	49	52	54	33	33
Toadfish CYP1A1							54	55	50	49	55	55	34	34
Human CYP1A1								78	70	66	59	61	36	37
Rat CYP1A1									66	68	60	62	35	35
Human CYP1A2										73	55	61	35	36
Rat CYP1A2											53	57	36	36
Chicken CYP1A4												78	33	35
Chicken CYP1A5													35	36
Human CYP1B1														80

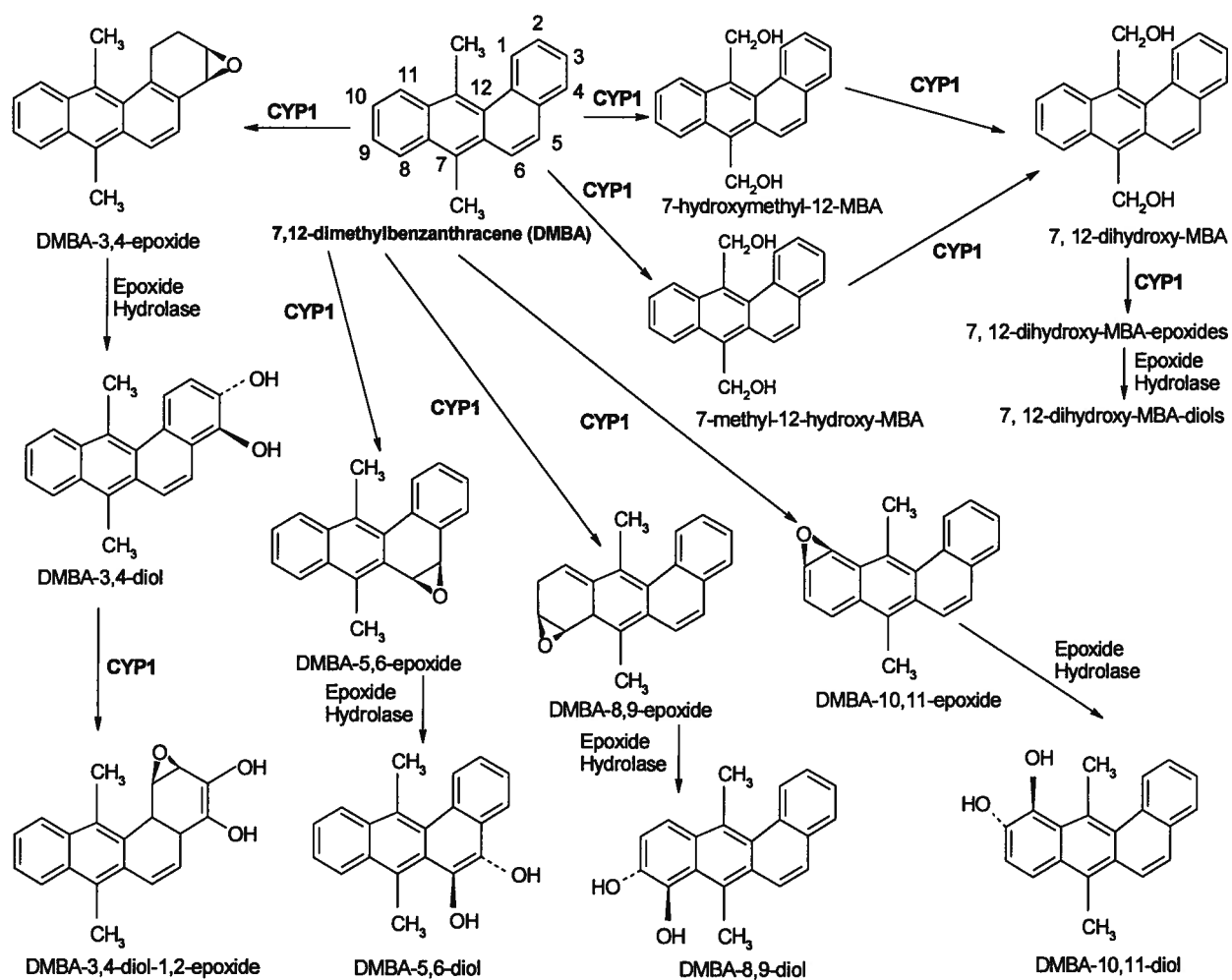


Figure 1.7: Metabolism of 7,12-dimethylbenzanthracene (DMBA) to its monohydroxy and dihydroxy metabolites by CYP1 enzymes. In CYP1-mediated DMBA metabolism, other metabolites with different stereoisomeric structures can also be formed and are not shown in the figure. Abbreviations- 7-hydroxymethyl-12-MBA: 7-hydroxymethyl-12-methylbenzanthracene; 7-methyl- 12-hydroxy-12-MBA: 7-methyl-12-hydroxy-methylbenzanthracene; 7,12-dihydroxy-MBA-epoxides: 7,12-dihydroxy-methylbenzanthracene-epoxides; 7,12-dihydroxy-MBA-diols: 7,12-dihydroxy-methylbenzanthracene-diols.

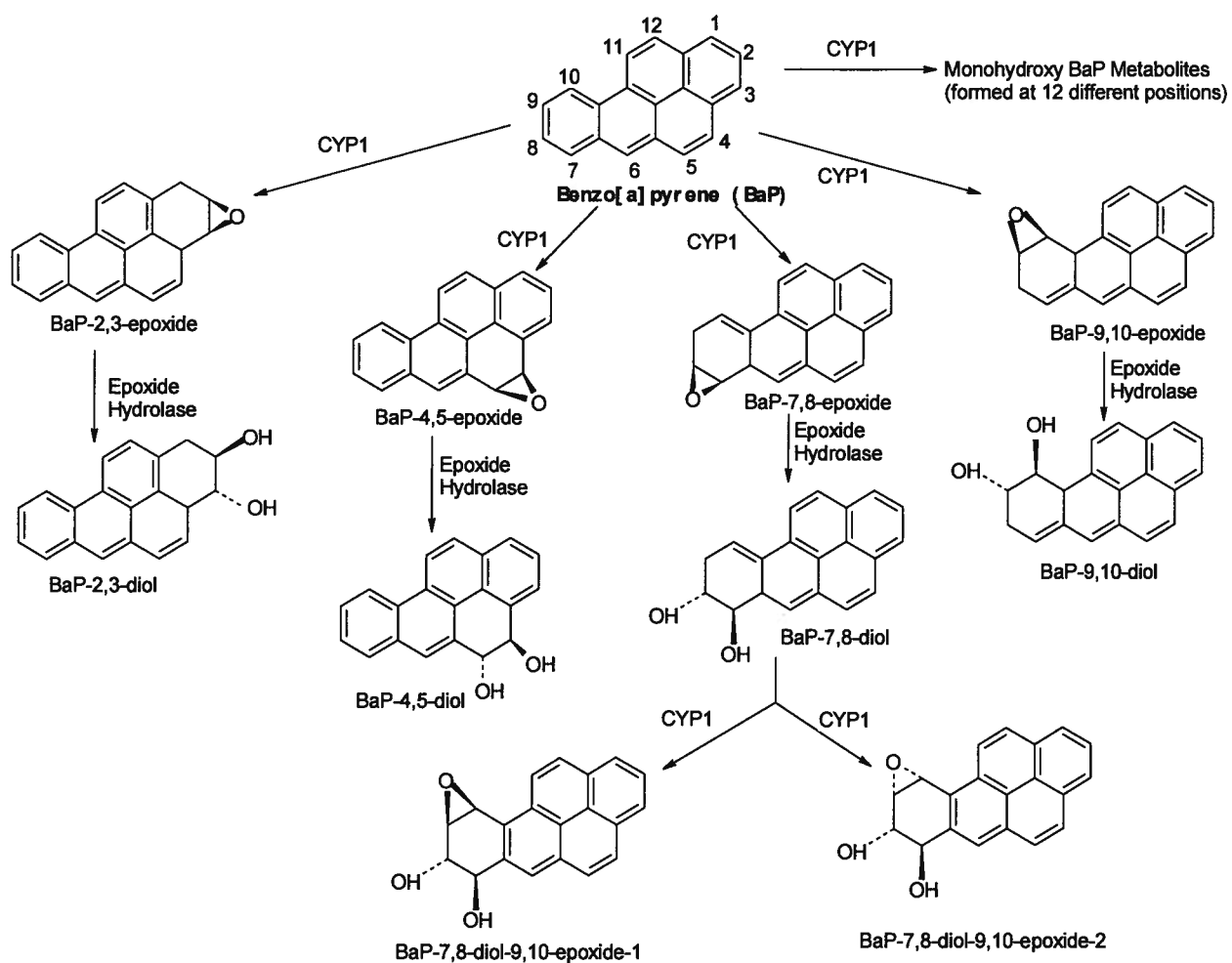


Figure 1.8: Metabolism of benzo[a]pyrene (BaP) to its reactive intermediate, BaP epoxides, and dihydrodiol metabolites by CYP1 and epoxide hydrolase enzymes. In CYP1-mediated BaP metabolism, other metabolites with different stereoisomeric structures can also be formed and are not shown in the figure.

1.3.1. CYP1A1 and CYP1A2

CYP1A2 accounts for approximately 8% to 14% of the total hepatic CYP protein in humans (Lewis, 2001). Basal CYP1A1 levels are undetectable in human hepatic and extrahepatic tissues such as lung, gastrointestinal tissues, and skin (Ding and Kaminsky, 2003; Pavek and Dvorak, 2008). In contrast, CYP1A2 protein is expressed at readily detectable levels in human liver (Chang et al., 2003; Walker et al., 1999) and the expression is mostly liver-specific. Human and rodent hepatic and pulmonary CYP1A1 expression is induced after exposure to cigarette smoke (Anttila et al., 1991; Willey et al., 1997; Zevin and Benowitz, 1999). The hepatic and extrahepatic expression of CYP1A1 and CYP1A2 enzymes is transcriptionally regulated by the same type of compounds, namely AhR agonists. CYP1A2 is less inducible after treatment with AhR agonists than CYP1A1 in rats and mice (Walker et al., 1999). In comparison to liver, CYP1A1 and CYP1A2 protein levels were less inducible in extrahepatic tissues after exposure to PAHs. In rats, the magnitude of CYP1A1 enzyme induction in liver was significantly greater than in lung and kidney (Pavek and Dvorak, 2008; Walker et al., 1999; Whitlock, 1999). Placental tissues express AhR and ARNT at high levels and CYP1A1 mRNA is induced 10-fold by TCDD in the placenta or placental trophoblast cell lines (Hakkola et al., 1997). CYP1A1 mRNA and protein expression were induced 6-fold by AhR agonists such as benz[a]anthracene and β -naphthoflavone in human epidermal keratinocytes (Khan et al., 1992).

In humans, very few therapeutic compounds are metabolized by CYP1A enzymes. CYP1A2 catalyzes the *O*-demethylation of naproxen, tricyclic antidepressants, and methylxanthines (e.g. theophylline and caffeine) and *N*-hydroxylation of acetaminophen (Parkinson, 2001). CYP1A enzymes are involved in the bioactivation of PAH (e.g. BaP, DMBA) and aromatic amine (e.g. 4-aminobiphenyl, 2-naphthylamine, and 2-acetylaminofluorene) procarcinogens, which are environmental and industrial toxicants (Guengerich, 2003; Guengerich and Shimada, 1991; Nebert et al., 2004; Shimada and Fujii-Kuriyama, 2004). CYP1A1 is predominant in

bioactivation of PAHs, whereas CYP1A2 preferentially metabolizes aromatic amines (Guengerich and Shimada, 1991; Nebert et al., 2004). Other substrates of CYP1A enzymes include flavonoids, resorufins and certain PCBs such as PCB 77 (Denison and Nagy, 2003; Matsusue et al., 1996).

1.3.2. CYP1B1

CYP1B1 is expressed in humans, rats, mice, fish and other vertebrates (Christou et al., 1995; Hakkola et al., 1997; Leaver and George, 2000; Murray et al., 1997; Savas et al., 1994). The amino acid sequence of human CYP1B1 is approximately 40% identical with the sequences of CYP1A1 and CYP1A2. The human CYP1B1 gene structure exhibits several differences compared to the structure of the CYP1A1 and CYP1A2 genes including the presence of three exons instead of seven exons and its localization on human chromosome 2 rather than chromosome 15 (Wo et al., 1997). The CYP1B1 gene is unique because it has both AhR responsive and cAMP responsive elements in its nucleotide sequence (Zheng et al., 2003). The CYP1B1 gene promoter region in humans and rodents is composed primarily of a TATA-like sequence and two Sp1 (a transcription factor) binding sites (Wo et al., 1997). The cAMP-responsive elements such as CREB and SF-1 are localized in the far upstream enhancer region of the CYP1B1 gene (Zheng et al., 2003; Zheng and Jefcoate, 2005).

CYP1B1 expression is mostly extrahepatic in rats, mice, and humans (Bhattacharyya et al., 1995; Shimada et al., 2003). Although CYP1B1 mRNA has been identified in normal hepatic and extrahepatic tissues including lung, kidney, mammary, testis, ovary, uterus, brain and eye in humans, there are conflicting reports about the presence of immunoreactive CYP1B1 protein in most of the tissues. CYP1B1 protein is not constitutively expressed in lung or liver but is induced in rats by treatment with TCDD, 3-MC or BaP (Shimada et al., 2002; Shimada et al., 2003; Walker et al., 1999). Hepatic CYP1B1 mRNA expression was reported to be induced 5-

fold in people who smoked compared to non-smokers (Chang et al., 2003). Similarly, hepatic CYP1B1 protein levels were found to be approximately 2- to 3-fold higher in smokers than non-smokers (Kim et al., 2004). Induction of CYP1B1 expression by AhR agonists is tissue-specific in rats and mice. CYP1B1 enzyme is induced to a greater extent in liver than in extrahepatic tissues, but the expression of hepatic CYP1B1 enzyme is less responsive to treatment with PAHs than CYP1A1 and CYP1A2 enzymes (Shimada et al., 2003; Walker et al., 1999). Studies with AhR knockout mice show that the inducible expression of CYP1B1 in liver, lung, and kidney is AhR-dependent and is transcriptionally regulated (Shimada et al., 2002; Shimada et al., 2003). The mechanism of AhR-mediated induction of CYP1B1 follows the classical model described in Figure 1.5.

CYP1B1 protein is constitutively expressed at high levels in some steroidogenic tissues such as adrenal and testis (Leung et al., 2009; Otto et al., 1992) but at relatively low levels in breast, ovary, and prostate (Murray et al., 2001; Otto et al., 1992; Sissung et al., 2006). There are conflicting reports about regulation of testicular CYP1B1 expression by AhR agonists. Mandal et al. (2001) and Zheng et al. (2003) showed that CYP1B1 mRNA is inducible by TCDD in mouse Leydig tumor cells (MA-10, a type of testis cell). However, in the same study, Mandal et al. (2001) reported that CYP1B1 mRNA expression is not induced by TCDD in adult rat testis. Treatment of adult male rats with BaP failed to induce testicular CYP1B1 protein (Kawai et al., unpublished data).

CYP1B1 protein expression is developmentally regulated in rat adrenal and testis (Brake et al., 1999; Leung et al., 2009). Testicular CYP1B1 mRNA and protein are expressed in 22-day old rats and the levels increased near the onset of puberty (34 days of age). The expression of CYP1B1 protein is highest in the adult rats (Leung et al., 2009). Testicular CYP1B1 protein levels are decreased by 69% in hypophysectomized adult rats (Figure 1.9), which indicates that pituitary hormones are involved in regulation of constitutive testicular CYP1B1 expression.

Pituitary hormones such as LH, FSH and prolactin induced testicular CYP1B1 expression in hypophysectomized rats (Leung et al., 2009). LH elicited the greatest increase in testicular CYP1B1 protein expression in hypophysectomized rats and prolactin potentiated the inductive effect of LH on testicular CYP1B1 protein expression in hypophysectomized rats (Leung et al., 2009). Testicular CYP1B1 expression was restored to 50% to 80% of that in intact rats by treatment of hypophysectomized rats with LH alone or LH plus prolactin (Leung et al., 2009) (Figure 1.9).

The mechanism of LH-mediated regulation of CYP1B1 expression is not yet elucidated. In general, LH exerts its regulatory role on steroidogenic pathways through the LH receptor (Dufau, 1998). LH binds to luteinizing hormone receptor, a G-protein coupled receptor, and mediates its physiological activities via various kinase- and phospholipase-mediated pathways (Dufau, 1998; Jo et al., 2005; Stocco et al., 2005) (Figure 1.10). PKA, PKC and MAPK activities are stimulated by LH (Stocco et al., 2005). Kinase pathways phosphorylate various transcription factors, leading to regulation of mRNA and protein expression of target proteins such as CYP17A1 and CYP19A1. Of the kinase pathways modulated by LH, the PKA-mediated pathway is the most active signaling mechanism in testis (Clark et al., 2001; Dufau, 1998). CREB and SF-1 act as transcriptional regulators of steroidogenic CYP enzymes such as CYP19A1 (Carlone and Richards, 1997a; Carlone and Richards, 1997b).

In contrast to LH, GH decreased testicular CYP1B1 protein levels by 63% after intermittent administration in hypophysectomized rats (Leung et al., 2009). Treatment of adult hypophysectomized rats with 3,5,3'-triiodothyronine (T3), but not thyroxine (T4), increased testicular CYP1B1 protein levels by 3-fold (Leung et al., 2009). CYP1B1 protein expression and activity were increased 4-fold in rat adrenal and cultured adrenocortical cells after treatment with ACTH (Brake and Jefcoate, 1995; Otto et al., 1991). CYP1B1 protein expression and activity, which were decreased in hypophysectomized rats, were restored to constitutive levels

by ACTH (Otto et al., 1991). cAMP analogs (e.g. 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP) induced CYP1B1 mRNA by 9-fold in primary rat adrenal cells (Zheng et al., 2003). CYP1B1 protein levels were induced 4-fold by 8-Br-cAMP and forskolin (a potent stimulant of cAMP formation) in rat adrenocortical cells (Brake and Jefcoate, 1995). It has been suggested that ACTH-elicited increase in CYP1B1 expression may be due to activation of cAMP-dependent regulatory pathways (Brake and Jefcoate, 1995).

Testicular CYP1B1 expression is also regulated by sex steroids. Estradiol benzoate suppressed testicular CYP1B1 protein levels by approximately 90% in adult intact rats (Leung et al., 2009) (Figure 1.11). It has been proposed that blockade of anterior pituitary hormone secretion by inhibition of hypothalamus-pituitary axis may contribute to the estradiol-mediated suppression of CYP1B1 expression in testis (Leung et al., 2009). In contrast, testicular CYP1B1 protein levels were increased 2.5-fold in hypophysectomized adult rats after administration of testosterone (Leung et al., 2009).

In contrast to testis, estradiol exerted an inductive effect on CYP1B1 expression in mammary tissues. CYP1B1 protein levels were increased by 50% to 75% in rat mammary epithelial cells after treatment with estradiol (0.2 μ M) (Christou et al., 1995). Similarly, CYP1B1 mRNA expression is upregulated 3-fold by estradiol in MCF-7 human breast cancer cells (Tsuchiya et al., 2004). In addition, estradiol potentiated TCDD-mediated induction of CYP1B1 mRNA levels in MCF-7 cells (Spink et al., 2003). It has been suggested that induction of CYP1B1 expression by estradiol in MCF-7 cells is regulated by estrogen receptor through an estrogen receptor response element (identified by a computer-assisted homology analysis) in the CYP1B1 gene (Tsuchiya et al., 2004). Progesterone (1.5 μ M) and cortisol (1.5 μ M) suppressed constitutive and PAH-induced CYP1B1 protein levels and activities by 80% in rat mammary epithelial cells (Christou et al., 1995).

Due to the low constitutive levels of CYP1B1 protein in rodents and humans, the majority of metabolism studies with CYP1B1 were carried out using recombinant protein systems. CYP1B1 has been shown to be active with both endogenous and xenobiotic substrates (Murray et al., 2001; Sissung et al., 2006). Recombinant human CYP1B1 is involved in the metabolism of estradiol to its potentially carcinogenic hydroxylated metabolites such as 4-hydroxyestradiol (Hayes et al., 1996; Shimada et al., 1998). Melatonin, a neurohormone, is metabolized to 6-hydroxymelatonin by CYP1B1 in humans (Ma et al., 2005). Recombinant human and mouse CYP1B1 is also involved in the biotransformation of retinoids and arachidonic acid (Choudhary et al., 2004; Savas et al., 1997). There has been considerable interest in the potential role of CYP1B1 in the development of chemical and hormonal carcinogenesis in humans and rodents. CYP1B1 is involved in the bioactivation of potential PAH procarcinogens such as DMBA and BaP in rats and mice (Buters et al., 2003; Kim et al., 1998; Shimada et al., 1996a). DMBA, a procarcinogen, is metabolized to its dihydroxy metabolites (e.g. DMBA-3,4-diol and DMBA-5,6-diol) by mouse, rat and human CYP1B1 (Choudhary et al., 2004; Pottenger and Jefcoate, 1990; Savas et al., 1997). Estradiol and DMBA are substrates for multiple CYP enzymes including CYP1A1, CYP1A2, CYP1B1, and CYP3A4. The catalytic efficiency (V_{max}/K_m) of recombinant human CYP1B1 enzyme (V_{max}/K_m , $1.2 \times 10^8 \text{ sec}^{-1} \cdot \text{M}^{-1}$) in the formation of 4-hydroxyestradiol from estradiol is approximately 63-fold and 750-fold higher than recombinant human CYP1A2 (V_{max}/K_m , $1.9 \times 10^6 \text{ sec}^{-1} \cdot \text{M}^{-1}$) and CYP3A4 (V_{max}/K_m , $1.6 \times 10^5 \text{ sec}^{-1} \cdot \text{M}^{-1}$), respectively (Jefcoate et al., 2000). Recombinant mouse CYP1B1 is 11-fold more active and 5-fold less active than recombinant mouse CYP1A1 in the formation of DMBA-3,4-diol and DMB-8,9-diol, respectively (Savas et al., 1997).

CYP1B1 protein is overexpressed in tumors obtained from human brain, lung, breast, gastrointestinal tract, and testis (McKay et al., 1995; Murray et al., 1997; Spivack et al., 2001). Interestingly, CYP1B1 protein levels are very low or undetectable in normal human tissues

(Murray et al., 2001). Recent studies have suggested that mutation of the human CYP1B1 gene can lead to development of congenital glaucoma, a clinical condition that occurs due to increased fluid pressure inside the eye at the time of birth or during the first few years of life (Alward, 2003; Melki et al., 2004; Vincent et al., 2002). CYP1B1-null mice experience abnormalities in their ocular structure, which are identical to the congenital glaucoma in humans (Libby et al., 2003). The exact mechanism of this effect is not clear. Similarly, a functional role for CYP1B1 in kidney has also been detected because CYP1B1-null mice exhibited a higher frequency (approximately 64%) of development of progressive glomerulonephritis and histiocytic sarcoma (Ward et al., 2004).

In summary, constitutive and inducible CYP1B1 expression is tissue-specific and is regulated primarily by two different mechanisms in a distinctly tissue-specific manner. CYP1B1 regulation is under the control of AhR in liver, lung, kidney and gastrointestinal tissues. In contrast, hormonal regulation is the predominant pathway in steroidogenic tissues such as adrenal, testis and ovary. There is no marker reaction available for CYP1B1 and very little or no metabolic activity is observed with microsomal samples from untreated or normal tissues. The biological role of CYP1B1 is largely unknown. The putative role of CYP1B1 in modulating the development and progression of chemical-induced cancer in certain tissues (e.g. ovary, bone marrow, skin) has been studied in rodents; however, further studies are required to fully understand the mechanism.

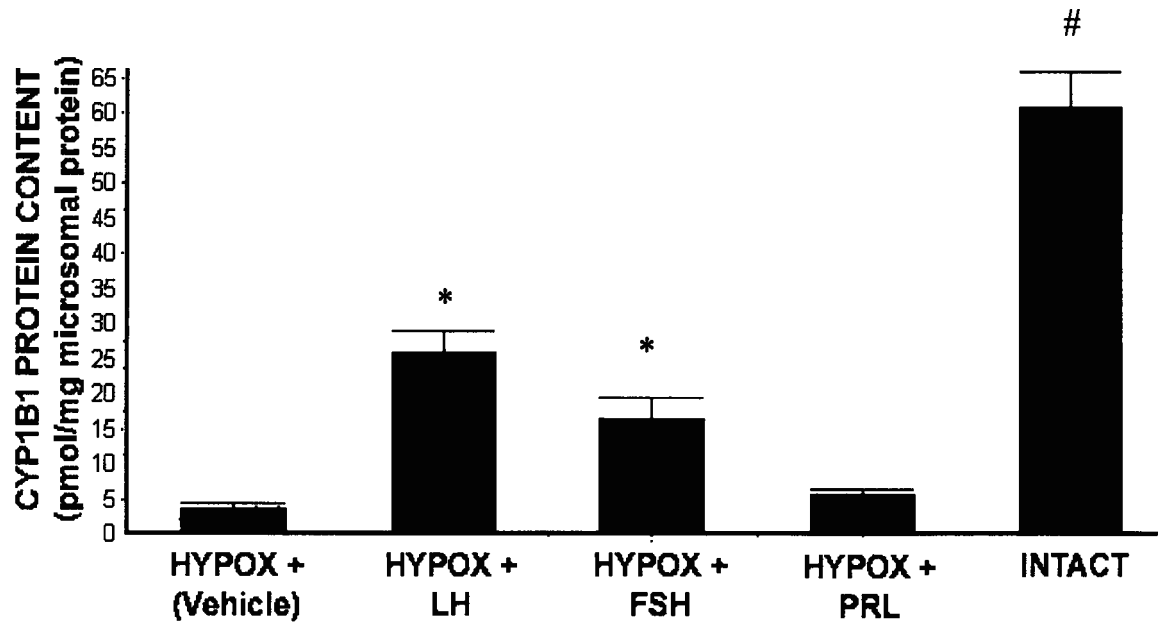


Figure 1.9: Effect of various pituitary hormones on testicular CYP1B1 protein expression in hypophysectomized (HYPOX) adult rats (taken from Leung et al., 2009). Data are expressed as mean \pm S.E.M. for 6 individual rats per treatment group. * Significantly different from the Hypox + Vehicle treated group with a *P* value < 0.05 . # Significantly different from all other groups with a *P* value < 0.05 . Abbreviations: LH, luteinizing hormone; FSH, follicle stimulating hormone; PRL, prolactin.

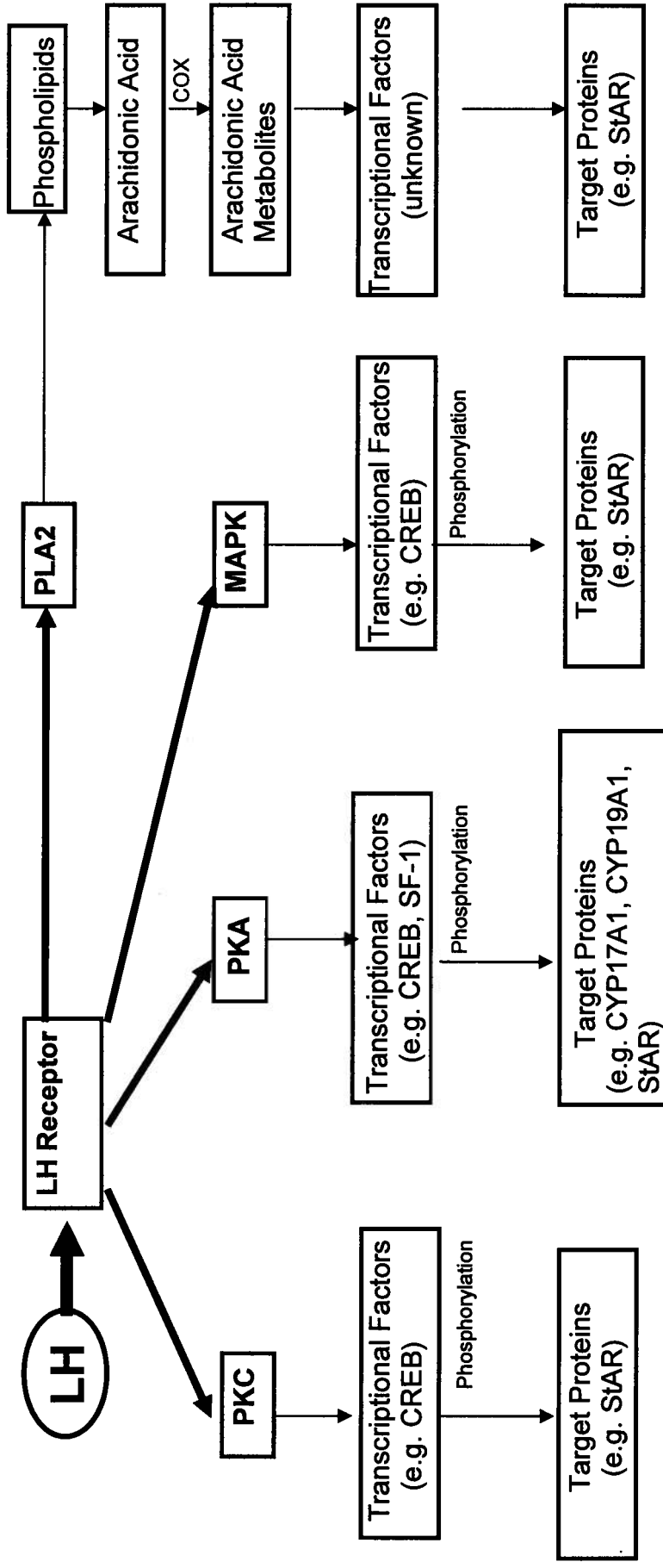


Figure 1.10: Signaling pathways regulated by luteinizing hormone (LH) in Leydig cells. LH binds to LH receptor (a G-protein coupled receptor) and mediates its effects by several kinase pathways and phospholipase-based pathways. In this diagram, pathways are simplified to summarize the regulatory pathways modulated by LH in Leydig cells. Abbreviations: PKC, protein kinase C; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; PLA2, phospholipase A2; CREB, cAMP response element binding protein ; SF-1, steroidogenic factor-1; COX, cyclooxygenase; StAR, steroidogenic acute regulatory protein.

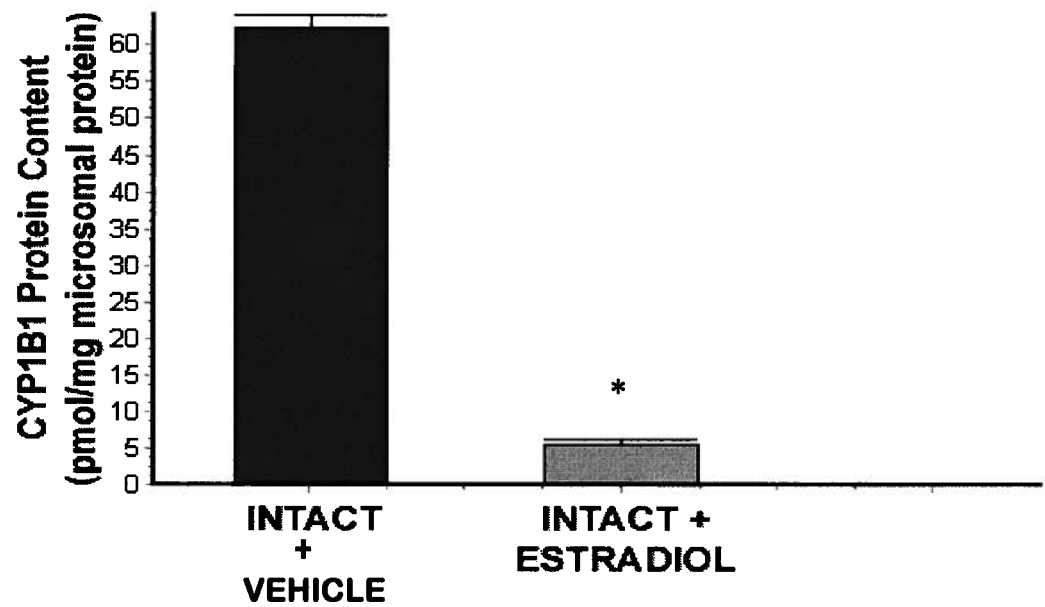


Figure 1.11: Effect of estradiol benzoate on testicular CYP1B1 protein expression in intact adult rats (taken from Leung et al., 2009). Data are expressed as mean \pm S.E.M. for 5 individual rats per treatment group. *Significantly different from the intact group with a *P* value <0.01 .

1.4. CYP2 Family Enzymes

About one third of human CYP enzymes identified belong to the CYP2 family. CYP2 enzymes constitute approximately 20% to 40% of total CYP content in human liver (Lewis, 2001). Several orphan CYP enzymes including CYP2A7, CYP2S1, CYP2U1, and CYP2W1 are part of the CYP2 family (Stark and Guengerich, 2007).

CYP2 enzymes are present in liver and extrahepatic tissues including lung, small intestine, kidney, stomach, brain, thymus, heart, mammary, and prostate in humans (Choudhary et al., 2005). CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 are expressed in human liver (Lewis, 2001). CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2D6, CYP2E1, CYP2F1, CYP2J2, and CYP2S1 mRNA and protein are expressed in human lung tissue (Ding and Kaminsky, 2003; Hukkanen et al., 2002; Pavek and Dvorak, 2008). CYP2B1, CYP2C11, CYP2C23, CYP2J2, and CYP2E1 proteins have been detected in rat kidney (Liu et al., 2003; Orellana et al., 2002; Zhao et al., 2003). Enzymes belonging to the CYP2B, CYP2C, CYP2D, CYP2E, and CYP2J subfamilies have been detected in rat small intestine (Kaminsky and Zhang, 2003; Obach et al., 2001). CYP2B, CYP2D, CYP2E and CYP2U enzymes are expressed in human, rat, and mouse brain tissue (Meyer et al., 2007). A summary of CYP enzymes detected in various extrahepatic tissues is given in Table 1.3.

CYP2 family enzymes show diverse substrate specificities and are involved in the oxidation of approximately 50% of currently marketed drugs (Lewis, 2001). CYP2 family enzymes are involved in the activation of several mutagens and procarcinogens. For example, CYP2A6 and CYP2A13 metabolically activate nitrosamines (Crespi et al., 1990) and nicotine (Nakajima et al., 2006) in lung. CYP2E1 in lung is involved in the oxidation of ethanol, benzene and 1,1-dichloroethylene (Hukkanen et al., 2002; Sheets et al., 2004; Simmonds et al., 2004a; Simmonds et al., 2004b). CYP2C and CYP2J enzymes in kidney have been implicated in renal blood pressure control mechanisms via arachidonic acid metabolism (Zhao et al., 2003). The small

intestine is an important site for first-pass metabolism of orally ingested xenobiotics. CYP2C and CYP2D subfamily enzymes metabolize various therapeutic and endogenous substances in rat enterocytes (Kaminsky and Zhang, 2003; Obach et al., 2001). CYP2J4 has an active role in arachidonic acid and *all-trans*- and 9-*cis*-retinal metabolism in rat small intestine (Kaminsky and Zhang, 2003; Zhang et al., 1998).

CYP enzymes belonging to the CYP2 family are inducible. CYP2A, CYP2B and CYP2C subfamily enzymes are regulated by CAR agonists or activators (e.g. phenobarbital) and PXR agonists (e.g. dexamethasone) in human liver (Lewis, 2001) (Table 1.5). CYP2A subfamily enzymes such as CYP2A3 and CYP2A5 are induced 3- to 5-fold by AhR agonists in mouse lung (Arpiainen et al., 2005; Nagata et al., 1987). Renal CYP2B10 protein levels were increased after treatment of mice with dexamethasone (Jarukamjorn et al., 2001). CYP2B1 expression is induced 6-fold by phenobarbital in rat small intestine (Traber et al., 1990). Ethanol induces CYP2E1 expression in rat liver and small intestine (Roberts et al., 1994). CYP2B1 protein expression and activity were induced by nicotine in rat brain (Anandatheerthavarada et al., 1993).

1.4.1 CYP2S1

Human CYP2S1 was first identified by Rylander et al. (2001) through nucleotide database searches (Basic Local Alignment Search Tool for Nucleotides; BLASTN) while mouse CYP2S1 was cloned by Rivera et al. (2002). Human and mouse CYP2S1 cDNAs are 81% identical in nucleotide sequence. Human and mouse CYP2S1 enzymes share 78% amino acid sequence identity (Rivera et al., 2002). Human CYP2S1 is located on the CYP2 gene cluster on chromosome 19q, which also contains other CYP2 genes such as CYP2A6, CYP2A13, CYP2B6 and CYP2F1 (Hoffman et al., 2001). The human CYP2S1 gene is 13 kb long and has the characteristic CYP2 family gene structure with 9 exons (Rivera et al., 2002; Rylander et al.,

2001). CYP2S1 shares other common features of the CYP2 family such as a N-terminal hydrophobic stretch, followed by a proline-rich region and a conserved cysteine in the C-terminal region (Negishi et al., 1996; Yamazaki et al., 1993). The amino acid sequence of human CYP2S1 is 49% identical to the amino acid sequences of human CYP2A6 and CYP2A13. In comparison, mouse CYP2S1 exhibits 47% to 50% amino acid sequence identity with mouse CYP2A3, CYP2B10, CYP2F2 and CYP2G1 proteins (Rivera et al., 2002).

Human CYP2S1 mRNA is expressed mainly in extrahepatic tissues such as lung, small intestine and spleen (Rivera et al., 2002; Rylander et al., 2001). CYP2S1 mRNA and protein are localized in the epithelial cell lining of the human respiratory tract (nasal cavity, bronchi, bronchioli), gastrointestinal tract (esophagus, stomach, liver, duodenum, rectum), urinary system (kidney, urinary bladder), reproductive system (uterine cervix, testis, ovary, placenta), lymphoid tissues (bone marrow, spleen, thymus, leukocytes) and skin (Bieche et al., 2007; Choudhary et al., 2005; Du et al., 2005; Saarikoski et al., 2005b). Analysis of human fetal tissues indicates that CYP2S1 mRNA is expressed in lung, kidney, spleen, and thymus (Choudhary et al., 2005). Mouse CYP2S1 mRNA has been detected in lung, liver, kidney, brain, testis, spleen, heart, and skeletal muscles (Choudhary et al., 2005; Rivera et al., 2002). Mouse CYP2S1 mRNA is expressed during different stages at 7-, 11-, 15-, and 17-days of gestation of fetal development (Choudhary et al., 2003). The expression of CYP2S1 mRNA at early stages of life suggests that CYP2S1 may be involved in embryonic development (Saarikoski et al., 2005a).

CYP2S1 mRNA is expressed at 4-fold higher levels in psoriatic human skin samples compared to normal skin samples (Smith et al., 2003). Coal tar (a rich source of PAHs), ultraviolet (UV) radiation (290-400 nm), and psoralen-UV radiation were reported to increase cutaneous CYP2S1 mRNA expression in human psoriatic skin by 2- to 5-fold. Human CYP2S1 mRNA levels were induced 2-fold in skin after topical treatment with *all-trans*-retinoic acid. Similarly, retinoic acid increased CYP2S1 mRNA expression by 3-fold in human A549

pulmonary cells with a concomitant increase (125-fold) in luciferase activity of the human CYP2S1 promoter region (Rowland et al., 2007), which indicates that CYP2S1 expression is transcriptionally regulated. Cutaneous CYP2S1 expression is induced by topical application of dinitrochlorobenzene, a chemical used in photography processing, compared to saline in mice (Saarikoski et al., 2005a).

The human CYP2S1 gene exhibits polymorphism in the coding region. Recently, nine allelic variants of human CYP2S1 were identified in a Finnish population and nine allelic variants were identified in a Korean population (Jang, 2006; Saarikoski et al., 2004). Also, constitutive CYP2S1 mRNA expression demonstrates interindividual variation (up to 5-fold) in human skin. Induction of cutaneous CYP2S1 mRNA expression by UV radiation and retinoic acid showed distinct interindividual differences (up to 2-fold) (Smith et al., 2003). These observations suggest that CYP2S1 may metabolize its potential substrates differentially in normal as well as xenobiotic exposed skin tissues.

Human CYP2S1 mRNA levels were approximately 2-fold higher in bronchiolar samples (broncho-alveolar lavage cells) from people who smoke compared to non-smokers (Thum et al., 2006). However, there was no difference in CYP2S1 mRNA and protein expression in placental samples from women who were smokers versus non-smokers (Saarikoski et al., 2005a). Human CYP2S1 mRNA levels are increased 2-fold by TCDD (100 nM) in human A549 lung cancer cells (Rivera et al., 2002). Treatment with TCDD (30 µg/kg) also increased CYP2S1 mRNA levels in mouse lung by 2-fold (Rivera et al., 2002). Similarly, CYP2S1 mRNA is induced 10-fold by TCDD (100 nM) in Hepa-1 mouse hepatoma cells (Rivera et al., 2002). Moreover, CYP2S1 mRNA expression is induced by TCDD in MCF10A human breast epithelial cells (Thomas et al., 2006). These results indicate that expression of human and mouse CYP2S1 is inducible.

CYP2S1 has molecular characteristics of both CYP1 and CYP2 family enzymes. It has been proposed that mouse CYP2S1 expression is regulated by the AhR. In vitro studies with Hepa-1 mutant mouse hepatoma cell lines showed that AhR, ARNT and XREs are required for induction and transcriptional activation of the mouse CYP2S1 gene by AhR agonists (e.g. TCDD), which is similar to the situation with CYP1 enzymes (Rivera et al., 2002). Smith et al. (2003) analyzed the nucleotide sequence of the CYP2S1 gene promoter to identify regulatory elements that might influence CYP2S1 expression and inducibility. Multiple transcription factor-binding sites such as XREs, which regulate CYP expression under the influence of PAHs, have been found in the promoter region of the human CYP2S1 gene. These XRE sequences (5'-GCGTGCAC-3') are identical to those of CYP enzymes inducible by PAHs (CYP1A1, CYP1A2 and CYP1B1) (Smith et al., 2003). Smith et al. (2003) also reported the presence of retinoic acid receptor response elements in the CYP2S1 promoter region. *All-trans*-retinoic acid binds and activates the retinoic acid receptor, which forms a heterodimer with retinoid X receptor and subsequently binds to retinoic acid response elements, leading to transcription of target genes. Analysis of the CYP2S1 promoter sequence revealed the presence of multiple copies of the activator protein-1 transcription factor-binding site, which mediates induction of cutaneous gene transcription in response to various extracellular stimuli including UV radiation (Smith et al., 2003).

There are few reports of CYP2S1-catalyzed reactions, and there is no known catalytic marker reaction for CYP2S1. To date, the few metabolism studies involving CYP2S1 that have been carried out used recombinant CYP2S1. Heterologously-expressed human CYP2S1 has been shown to metabolize *all-trans*-retinoic acid to 4-hydroxy-retinoic acid and 5,6-epoxy-retinoic acid (Bui, 2006; Smith et al., 2003). Recombinant human CYP2S1 also metabolized naphthalene, an environmental and occupational toxicant, to its reactive intermediates (e.g. naphthalene oxide) (Bui, 2006; Karlgren et al., 2005). Heterologously-expressed human CYP2S1 catalyzed the formation of several hydroxylated metabolites (e.g. aflatoxin B1-8,9-dihydrodiol,

4-hydroxyafatoxin B1-8,9-diol, 9-hydroxyafatoxin B1-8,9-diol) of aflatoxin B1, a naturally occurring mycotoxin (Bui et al., 2006; Wang et al., 2005). However, there are conflicting reports regarding the metabolism of potent tobacco procarcinogens (e.g. BaP, nicotine) by recombinant CYP2S1. Bui et al. (2006) reported that recombinant human CYP2S1 expressed in *Escherichia coli* is capable of metabolizing BaP. In contrast, Wang et al. (2005) reported that CYP2S1 protein did not metabolize the prototypical lung carcinogen, nicotine, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). In addition, BaP-mediated toxicity was not increased after incubating BaP with human CYP2S1 protein expressed in Chinese hamster ovary cells (Wang et al., 2005). Dickmann et al. (2006) reported that human CYP2S1 catalyzed the *O*-dealkylation of methoxyresorufin with a K_m of 5.5 μ M and V_{max} of 0.17 nmol/nmol CYP/min. Natural product compounds such as flavonoids, trans-stilbenes, and biogenic amines inhibited CYP2S1 activity (Dickmann et al., 2006). The biological role of CYP2S1 has not yet been established. Higher expression of CYP2S1 protein in tumors of epithelial origin (Downie et al., 2005; Kumarakulasingham et al., 2005; Saarikoski et al., 2005b) and metabolism of small PAHs (Karlgren et al., 2005) have led to the speculation that CYP2S1 may play a role, analogous to CYP1 enzymes, in chemically-induced carcinogenesis. Furthermore, the role of CYP2S1 in treatment of skin disorders (e.g. psoriasis) is of considerable interest (Smith et al., 2003).

A summary of CYP2S1 studies in humans and mice is presented in Table 1.8.

Table 1.8: Literature review of CYP2S1 studies in humans and mice (compiled from references cited in the text).

Study	Species	mRNA/protein	Reference
1. Identification of CYP2S1	Human	mRNA, Protein ^a	Rylander et al. (2001), Kumarakulasingham et al. (2005)
2. Induction of CYP2S1 by TCDD	Mouse	mRNA	Rivera et al. (2002)
3. Expression of CYP2S1 in fetal tissues	Mouse	mRNA	Choudhary et al. (2003)
4. Induction of cutaneous CYP2S1 by coal-tar and retinoic acid; higher level in psoriatic skin; metabolism of retinoic acid	Human	mRNA, Protein ^b	Smith et al. (2003)
5. Genetic polymorphism	Human	Gene sequence	Saarikoski et al. (2004)
6. No metabolism of cigarette smoke carcinogens and nicotine	Human	hrCYP2S1 ^c	Wang et al. (2005)
7. Localization of CYP2S1 by IHC ^b and ISH ^d	Human	mRNA, Protein ^{b,c}	Saarikoski et al. (2005)
8. Metabolism of naphthalene	Human	hrCYP2S1 ^d	Karlgren et al. (2005)
9. Higher CYP2S1 in colorectal and ovarian cancer	Human	Protein ^b	Downie et al. (2005) Kumarakulasingham et al. (2005)
10. CYP2S1 in keratinocyte cells	Human	mRNA	Du et al. (2005)
11. Identification of a novel promoter element for induction of CYP2S1 by both dioxin and hypoxia	Mouse, Hepa cells	mRNA, protein ^a	Rivera et al. (2007)

^a Western blot; ^b Immunohistochemistry; ^c Human recombinant CYP2S1 protein; ^d In situ hybridization.

1.5. Similarities Between CYP1B1 and CYP2S1 Expression, Regulation and Function

Several similarities exist between the expression pattern and regulation of CYP1B1 and CYP2S1 as well as metabolic activities of these enzymes.

- Expression of CYP1B1 and CYP2S1 mRNA and protein is predominantly extrahepatic. Both the enzymes demonstrate tissue-specific expression in rats. CYP1B1 protein levels are very low or undetectable in most normal human tissues, whereas it is overexpressed in breast, prostate, ovary, and colon tumors (Murray et al., 2001). Similarly, CYP2S1 protein is expressed more strongly in colorectal and ovarian cancer than in normal tissues (Downie et al., 2005; Kumarakulasingham et al., 2005; Saarikoski et al., 2005b).
- CYP1B1 and CYP2S1 mRNA transcripts have been detected in all the fetal stages of mouse (Choudhary et al., 2003), which indicates that both enzymes may be involved in embryonic development and metabolism of endogenous substrates. Human CYP1B1 and CYP2S1 might metabolize retinoids (Choudhary et al., 2004; Smith et al., 2003), which play a critical role in embryonic development and also in promyelocytic leukemia and skin disorders (Marill et al., 2003).
- Human, mouse and rat CYP1B1 mRNA and protein are induced by AhR agonists in liver and extrahepatic tissues including lung, kidney and mammary tissues (Murray et al., 2001). Similarly, mouse CYP2S1 mRNA is induced by TCDD (an AhR agonist) in liver, lung and mammary tissues and/or cells (Rivera et al., 2002; Rylander et al., 2001; Thomas et al., 2006). Likewise, cutaneous expression of both CYP1B1 and CYP2S1 is induced by retinoic acid and UV radiation (Katiyar et al., 2000; Smith et al., 2003).
- CYP1B1 and possibly CYP2S1 are involved in the metabolism of PAHs and environmental toxicants (Saarikoski et al., 2005a; Shimada and Fujii-Kuriyama, 2004). However, most metabolism studies of these enzymes have been carried out with recombinant proteins and a true

physiological substrate is yet to be established. The biological roles of CYP1B1 and CYP2S1 are currently unknown. Thus, CYP1B1 and CYP2S1 may be categorized as orphan enzymes.

1.6. Rationale

CYP1B1

Results from Jefcoate's laboratory (Bhattacharyya et al., 1995; Brake and Jefcoate, 1995; Christou et al., 1995) and studies with AhR-knockout mice (Shimada et al., 2002; Shimada et al., 2003) led to the paradigm that CYP1B1 expression is regulated primarily by two different pathways: 1) AhR-mediated regulation; and 2) hormonal regulation. AhR-mediated CYP1B1 regulation is postulated to be predominant in liver, lung, kidney and mammary tissues. Hormonal regulation is postulated to be active mainly in steroidogenic tissues such as the adrenal gland, testis and ovary. CYP1B1 protein is basally expressed at a higher level in the adrenal gland and testis than in liver and other extrahepatic tissues (Shimada et al., 2002; Shimada et al., 2003). To explain how this occurs, it is important to understand the effects of hormones on CYP1B1 regulation. Previously in our laboratory, it was established that testicular CYP1B1 is regulated by anterior pituitary hormones, specifically by LH, in rats (Leung et al., 2009). The mechanism of LH-mediated regulation of testicular CYP1B1 expression is not yet identified. It is known that LH stimulates cAMP formation and that cAMP increases protein kinase activity in Leydig cells (Cooke et al., 1976; Hansson et al., 2000) (Figure 1.12). CYP1B1 mRNA levels are increased approximately 50% by synthetic cAMP analogs in mouse Leydig tumor cells (Zheng et al., 2003; Zheng and Jefcoate, 2005). It has been suggested that a PKA-mediated phosphorylation pathway is involved in the regulation of CYP1B1 in Leydig cells (Zheng et al., 2003; Zheng and Jefcoate, 2005). Hence, we hypothesize that regulation of testicular CYP1B1 expression by LH occurs primarily through cAMP-mediated protein kinase pathways.

A wide array of therapeutic agents interfere with plasma LH levels in the body. In rats, flutamide (an antiandrogen), ICI 182,780 (an anti-estrogen), estrogen receptor modulators (e.g. raloxifen) or naloxone (an opioid antagonist) augment serum LH levels (Akingbemi et al., 2003;

Andrews et al., 2001; O'Connor et al., 2002), while melatonin decreases LH levels (Vanecek, 1998; Yilmaz et al., 2000). Estradiol-mediated desensitization of steroidogenesis is an established phenomenon in Leydig cells (Cigorrage et al., 1980; Nozu et al., 1981a; Nozu et al., 1981b). Also, estrogen negatively regulates LH levels in mammals. In estrogen receptor- α gene knockout mice, serum LH levels were higher than in wild type mice (1.31 Vs 0.45 ng/ml). Similarly in ICI 182,780-treated mice, serum LH levels were increased relative to untreated mice (1.15 Vs 0.45 ng/ml) (Akingbemi et al., 2003). A review of the literature suggests that suppression of rat testicular CYP1B1 protein expression by estradiol may be an indirect effect via interference with LH-regulated pathways. It is also possible that estrogen receptors (α and β) are directly involved in the downregulation of testicular CYP1B1 expression by estradiol.

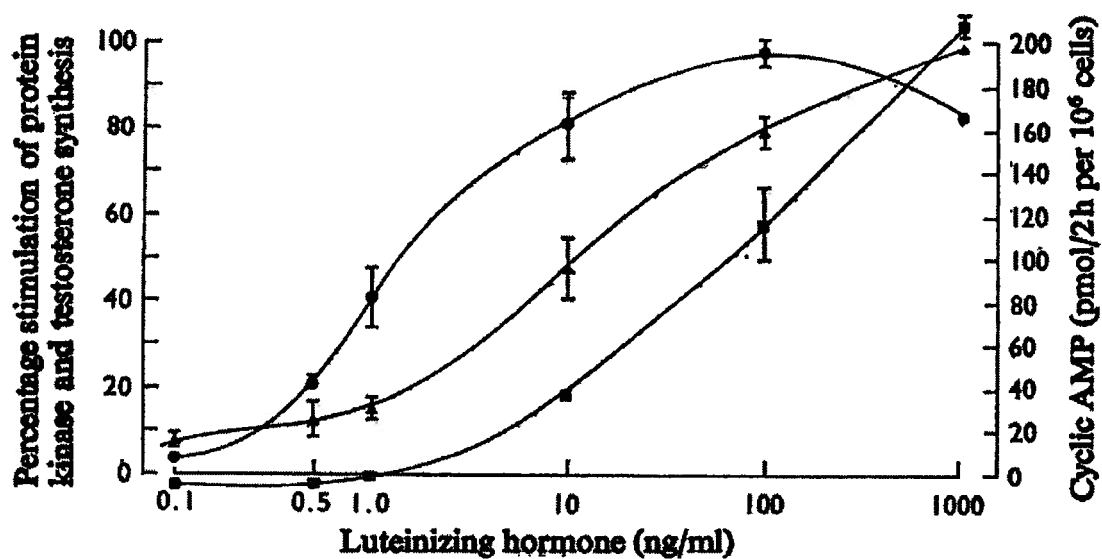


Figure 1.12: Luteinizing hormone-mediated increase in cAMP formation, protein kinase activation and testosterone formation in adult rat Leydig cell culture (taken from Cooke et al., 1976). Results are presented as means \pm S.E.M. For testosterone (●), protein kinase (▲), cAMP (■).

CYP2S1

Human CYP2S1 is designated as an orphan CYP enzyme (Guengerich et al., 2005). Although CYP2S1 mRNA and protein have been detected in humans and mice, to my knowledge, no information is available about expression of CYP2S1 mRNA and protein in rats. The information on CYP2S1 protein expression in humans and mice is limited and the tissue distribution pattern of CYP2S1 protein is not available. Hence, I set out to study the tissue distribution of CYP2S1 mRNA and protein in rats.

It has been observed that expression of several CYP enzymes from family 2 is sex-dependent and regulated by GH in rats (Pampori and Shapiro, 1999). CYP genes localized on human chromosome 19 (such as CYP2 and CYP4F) are sexually dimorphic (Ingelman-Sundberg, 2004; Kalsotra et al., 2002; Waxman et al., 1995). Due to the close molecular resemblance of the CYP2S1 gene with those of other CYP2 enzymes, I postulated that the expression of CYP2S1 in male and female rats is different. Rivera et al. (2002) reported that TCDD-mediated CYP2S1 expression is AhR, ARNT, and XRE dependent in mouse hepatoma cells (Figure 1.13). Multiple XRE sequences similar to sequences present in CYP1 family enzymes were identified in the CYP2S1 promoter region (Smith et al., 2003). The presence of XREs in the CYP2S1 promoter region, identical to those of AhR agonist-inducible CYP enzymes, led to the postulate that CYP2S1 expression is regulated by AhR agonists (Smith et al., 2003). Hence, I propose to determine if expression of CYP2S1 is induced by AhR agonists in rats.

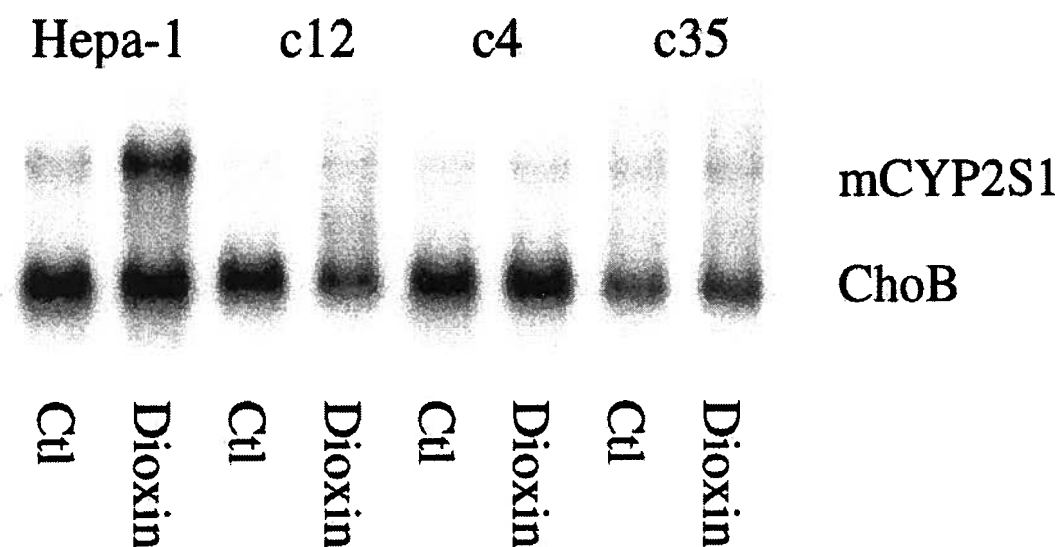


Figure 1.13: Induction of CYP2S1 mRNA by dioxin (TCDD) in wild type and mutant mouse Hepa-1 cells (taken from Rivera et al., 2002). Abbreviation: Hepa-1, wild type mouse hepatoma cells; c12, AhR deficient hepatoma cells; c4, ARNT deficient hepatoma cells, c35, hepatoma cells with defective AhR that is impaired in xenobiotic response element binding activity; ChoB- Chinese hamster ovary B, a constitutively expressed gene; Ctl- control.

1.7. Hypotheses

1. LH up-regulates testicular CYP1B1 expression by a PKA-mediated pathway.
2. Estradiol suppresses testicular CYP1B1 expression by a direct effect via the estrogen receptor α and β .
3. Testicular CYP1B1 expression is inducible by TCDD (an AhR agonist) in vivo and in vitro.
4. CYP2S1 is expressed constitutively in rats in a sex- and age-dependent manner and is inducible by AhR agonists.

1.8. Specific Objectives

1. To study the regulation of testicular CYP1B1 expression by LH via the PKA pathway in vitro (mouse MA-10 and rat R2C Leydig tumor cells).
2. To study the effects of estradiol, ICI 182,780 (a competitive antagonist of estrogen receptor) and anastrozole (an inhibitor of aromatase enzyme that converts androgens into estrogens), testosterone and flutamide (an anti-androgen) treatment on testicular CYP1B1 expression in vitro (mouse MA-10 and rat R2C Leydig tumor cells).
3. To assess testicular CYP1B1 expression after treatment with TCDD in rat (in vivo) and Leydig tumor cells (in vitro).
4. To study the tissue distribution of CYP2S1 in liver, lung, kidney, stomach, small intestine, and skin tissues in rat.
5. To characterize the sex-specific expression of CYP2S1 in rat.
6. To measure CYP2S1 induction in liver, lung, and kidney by AhR agonists (such as 3-MC, BaP and TCDD) in rat.

2. MATERIALS AND METHODS

2.1. Chemicals

LH (from sheep pituitary), FSH (from porcine pituitary), prolactin (from sheep pituitary), 8-Br-cAMP, 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP), N-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), 8-(4-chlorophenylthio)-cAMP, Rp isomer (Rp-CPT-cAMP), flutamide, estradiol benzoate, testosterone propionate, DMBA, BaP, corn oil, NADPH tetrasodium salt, chloroform, isoamyl alcohol, isopropanol, diethylpyrocarbonate (DEPC), formaldehyde, glycine, 5-bromo-4-chloro-3-indolyl phosphate (BCIP; disodium salt), potassium chloride (KCl), dextran, triton-X 100, benzamidine, β -glycerolphosphate, ethylene glycol bis-(2-aminoethyl ether) tetraacetic acid (EGTA), 4-morpholinepropanesulfonic acid (MOPS), leupeptin, aprotinin, ethylene diamine tetraacetic acid (EDTA), phenylmethanesulphonylfluoride (PMSF), sodium vanadate, Tween 20 (polyoxyethylene sorbitan monolaurate), and *o*-phenylenediamine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Acrylamide 99.9%, N,N'-methylene-bis-acrylamide (Bis), sodium dodecyl sulphate (SDS), 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), tris-(hydroxymethyl)aminoethane (Tris base), *p*-nitro-blue tetrazolium chloride (NBT), bovine serum albumin (globulin and fatty acid free, fraction V), high-performance liquid chromatography (HPLC)-grade methanol, HPLC-grade ethyl acetate, HPLC-grade acetone, bromophenol blue, sodium chloride, sodium phosphate, potassium dihydrogen phosphate, sodium carbonate, sodium bicarbonate, sucrose, dimethyl sulfoxide (DMSO), and concentrated hydrochloric acid (HCl) were obtained from Fisher Scientific (Vancouver, BC, Canada).

Agarose, TriZolTM, SuperscriptTM II reverse transcriptase, deoxyribonuclease I, oligo(dT)₁₂₋₁₈ primer, dithiothreitol, Platinum *Taq* DNA polymerase, and 2'-deoxynucleoside 5'-

triphosphate (dNTP) mix were purchased from Invitrogen Canada (Burlington, ON, Canada).

Forward and reverse primers for rat CYP1A1, rat CYP2S1, rat β -actin, mouse CYP1B1, mouse LH receptor, and mouse β -actin were synthesized at the Nucleic Acid and Protein Service Unit, University of British Columbia (Vancouver, BC, Canada).

3-MC and TCDD were obtained from Eastman Kodak Company (Rochester, NY, USA) and Wellington Laboratories (Guelph, ON, Canada), respectively.

7,12-DMBA-trans-3,4-dihydrodiol (DMBA-3,4-diol) and 7,12-DMBA-trans-5,6-dihydrodiol (DMBA-5,6-diol) were purchased from Midwest Research Institute (NCI Chemical Repository, Kansas, MO, USA).

Keyhole limpet hemocyanin (KLH) and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) were purchased from Pierce (Rockford, IL).

ICI 182,780 (fulvestrant) and anastrozole were purchased from Tocris Cookson (Ballwin, MO, USA) and Toronto Research Chemicals (North York, ON, Canada), respectively.

Go 6983 (3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione) was purchased from Calbiochem (San Diego, CA, USA).

Nitrocellulose membrane was obtained from the Pall Corporation (Pensacola, FL, USA).

Dithiothreitol was purchased from Diagnostic Chemicals Limited (Charlottetown, PEI, Canada).

Testosterone (epi isomer) was obtained from Steraloids Inc. (Newport, RI, USA).

Concentrated sulphuric acid and sodium fluoride were purchased from BDH Inc. (Toronto, ON, Canada).

Protein molecular weight ladder (precision plus proteinTM KaleidoscopeTM prestained standards) was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

2.2. Animals

Adult male and female Sprague-Dawley (SD) rats (8 weeks old; 240 to 300 g) were obtained from the Animal Care Center of the University of British Columbia (Vancouver, British Columbia, Canada). Rats were allowed to acclimatize in the animal facility of the Faculty of Pharmaceutical Sciences for four days. Rats were housed in polycarbonate cages with free access to water and a commercial rat diet (Rodent Laboratory Diet, No. 5001, PMI Feeds Inc., Richmond, IN). Animal quarters were maintained at a temperature of 20–23°C with a 12-h photoperiod.

Adult male mice (FVB/NJ strain) were housed at the British Columbia Cancer Agency (Vancouver, BC, Canada).

Adult female New Zealand rabbits, which were used for antibody production, obtained from the Animal Care Center of the University of British Columbia (Vancouver, British Columbia, Canada), were housed in the South Campus Animal Care Center (University of British Columbia, BC, Canada).

Animals were cared for and treated in accordance with the principles and guidelines of the Canadian Council on Animal Care.

2.3. Animal Treatment and Tissue collection

Adult female rats were treated with 3-MC, dissolved in corn oil, (25 mg/kg b.w./day, n=4) or with an equal volume of corn oil (2.25 ml/kg b.w./day, n=4), by daily intraperitoneal injections, for three consecutive days and were killed by decapitation 24 h after the last treatment. Liver, lungs, kidneys, stomach, jejunum, duodenum, ileum, spleen, skin (from the ventral surface) and adrenal glands were excised from each female rat, as well as from a group of untreated adult male rats (n=4), and quickly frozen in liquid nitrogen for subsequent mRNA analysis.

Adult male rats were treated with TCDD, dissolved in vehicle (1 part dioxane plus 6 parts corn oil, v/v), at dosages of 1, 5, 10, 50 or 100 µg TCDD/kg b.w. (n=3 rats per dosage) or with an equal volume of vehicle (2.5 ml/kg b.w., n=3), by a single intraperitoneal injection. Body weight and food consumption were monitored during the treatment period. Rats were killed by decapitation three days after treatment and liver, lungs and kidneys were immediately excised. One portion of lung, liver and kidney tissues from each rat was quickly frozen in liquid nitrogen and subsequently stored at -80°C for mRNA analysis at a later date. The remaining portions of lung, liver and kidney tissues from rats in each treatment group were pooled and were used for preparation of microsomes.

A second group of adult male rats was treated with BaP. Rats were administered BaP, dissolved in corn oil, by oral gavage at dosages of 50, 100, and 200 mg/kg (n=3 per dosage), once daily, for three consecutive days. Control rats (n=3) received an equivalent volume (3 ml/kg/day) of vehicle. All rats were killed by decapitation one day after the last dose. The lung and liver tissues were excised quickly from each rat and pooled for microsome preparation.

A third group of adult male rats was treated with 3-MC, dissolved in corn oil, at a dosage of 25 mg/kg/day (n=5) or with an equal volume of corn oil (3.3 ml/kg/day, n=5), by intraperitoneal injection, for three consecutive days, and were killed by decapitation 24 h after the last treatment. Lung and liver tissues were excised quickly from each rat and pooled for microsome preparation.

Untreated adult male mice (FVB/NJ) (n=4) were killed by cervical dislocation and lungs were immediately removed and pooled for microsome preparation.

2.4. Cell Culture

Two Leydig cell lines, mouse Leydig tumor cells (MA-10) and rat Leydig tumor cells (R2C), were used for CYP1B1 mRNA and protein studies, respectively. MA-10 cells were a

generous gift from Dr. Mario Ascoli (University of Iowa, USA) and were cultured in RPMI 1640 growth medium (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 15% heat inactivated horse serum, 20 mM HEPES, 50 µg/ml gentamycin and 2 mM glutamine (Invitrogen Life Technologies, Burlington, ON, Canada). R2C cells (passage number 37) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown in Ham's F-10 nutrient medium (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 15% horse serum and 2.5% (vol/vol) fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen Life Technologies, Burlington, ON, Canada). Both cell lines were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C.

An image of mouse Leydig MA-10 cells, as observed under a microscope (200x), is shown in Figure 2.1. As reported by Ascoli and Puett (1978), the MA-10 tumor cells are a clonal cell line derived from a transplantable Leydig cell tumor that originated spontaneously in C57BL/6 mice (Ascoli and Puett, 1978). The MA-10 cell line is a permanent cell line that retains many of the characteristics of normal Leydig cells, except that MA-10 cells do not have measurable CYP17A1 activity and thus do not produce testosterone (Payne, 1990). Treatment of MA-10 cells with LH or cAMP analogues leads to induction of CYP17A1 expression and activity and to a simultaneous increase in testosterone and progesterone production (Anakwe and Payne, 1987; Malaska and Payne, 1984). A review of the literature indicates that treatment with LH, hCG, and FSH (or cAMP analogs) results in activation of the PKA pathway which stimulates steroidogenesis by >500-fold in MA-10 cells (Anakwe and Payne, 1987; Ascoli and Puett, 1978; Malaska and Payne, 1984; Rao et al., 2002; Rao et al., 2003).

The R2C tumor cells are derived from Leydig cells of testis from a two-month old rat (Wfu strain). R2C cells are highly active in steroidogenesis relative to MA-10 cells and synthesize large amounts of testosterone and progesterone (Rao et al., 2003). R2C cells are less

responsive to treatment with cAMP analogues than MA-10 cells and have constitutively active PKA pathways (Heneweer et al., 2004; Rao et al., 2003). An image of rat Leydig R2C cells was not available for inclusion in the thesis.

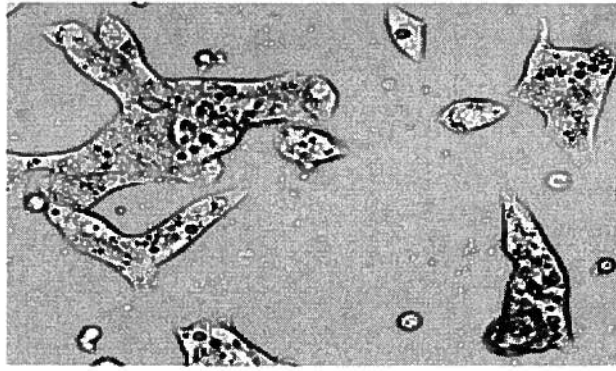


Figure 2.1: Photograph of mouse Leydig MA-10 cells adhered to the culture flask surface as observed under the microscope (200x).

(taken from http://homepages.cae.wisc.edu/~bme200/microencapsulation_fall05/)

2.5. Cell Culture Treatments

MA-10 or R2C cells were suspended in a cold-storage medium (growth medium plus 10% DMSO) in plastic vials and kept in a liquid nitrogen tank at -190°C. For each experiment, a vial containing frozen cells was removed from the liquid nitrogen tank and was placed in ice for 1 min. Subsequently, the vial was thawed and subjected to centrifugation at 1000 rpm for 5 min at 25°C. The supernatant containing DMSO and growth medium was removed and the cells were resuspended in freshly prepared growth medium. MA-10 cells were grown in T25 flasks by incubating approximately one million cells/flask and R2C cells were grown in T75 flasks by incubating approximately two million cells/flask, for six days in a humidified atmosphere of 5% CO₂ and 95% air, at 37°C. When the cells reached 75-80% confluence (typically a week after incubating the cells in growth medium), they were exposed to each of the following treatments in serum-free medium:

- LH dissolved in water at 0.01-0.2 IU/ml for 12 h,
- or FSH dissolved in water at 0.05 IU/ml for 12 h,
- or prolactin dissolved in water at 0.05 IU/ml for 12 h,
- or 8-Br-cAMP (PKA activator) dissolved in DMSO or water at 0.25-2 mM for 6 h,
- or H89 (a PKA inhibitor) dissolved in DMSO at 1-100 µM for 1 h,
- or Rp-CPT-cAMP (a specific PKA inhibitor) dissolved in water at 1-150 µM for 1 h,
- or 8-Br-cGMP (a protein kinase G (PKG) activator) dissolved in water at 0.05-10 mM for 6 h,
- or Go 6983 (a specific PKC inhibitor) dissolved in DMSO at 1-200 µM for 1 h,
- or estradiol benzoate dissolved in DMSO at 10-500 nM for 24 h,
- or ICI 182,780 (a competitive antagonist of estrogen receptor α) dissolved in DMSO at 0.5-100 µM for 24 h,

- or anastrozole dissolved in DMSO at 1000 nM for 24 h,
- or testosterone propionate dissolved in DMSO at 1000 nM for 24 h,
- or flutamide dissolved in DMSO at 10 μ M for 24 h,
- or TCDD dissolved in DMSO at 1-1000 nM for 24 h.

Co-treatment of cells involved exposure to:

- LH plus FSH, for 12 h,
- or LH plus prolactin, for 12 h,
- or LH plus FSH plus prolactin, for 12 h,
- or LH plus H89, preincubation with H89 for 1 h, then LH for 12 h,
- or 8-Br-cAMP plus H89, preincubation with H89 for 1 h, then 8-Br-cAMP for 6 h,
- or LH plus Rp-CPT-cAMP, preincubation with Rp-CPT-cAMP for 1 h, then LH for 12 h,
- or LH plus Go 6983, preincubation with Go 6983 for 1 h, then LH for 12 h,
- or estradiol benzoate plus ICI 182,780, for 24 h,
- or estradiol benzoate plus LH, for 24 h,

Concentration-response and time-course studies were carried out for the treatments. Each of the treatments was repeated three to five times on different days.

2.6. Preparation of Microsomes from Tissues and Cell Culture

Microsomes from tissues were used for CYP1B1 and CYP2S1 protein studies whereas microsomes from cell cultures were used for CYP1B1 protein studies only. Microsomal fractions were prepared by differential ultracentrifugation of tissues that were freshly excised from rats or mice. Immediately after decapitation, tissues were removed, minced and placed in ice-cold 0.05

M Tris buffer (pH 7.4) containing 1.15% KCl. Tissues were homogenized using a Potter-Elvehjem glass mortar with motor-driven pestle (Talboys Engineering Corp., Emerson, NJ, USA). The homogenate was spun in a centrifugal field of 9,000 g for 20 min at 5°C using a Beckman J2-21 centrifuge (Beckman Instrument, Palo Alto, CA, USA). The supernatant obtained was filtered through four layers of cheesecloth and centrifuged at 105,000 g for 60 min at 5°C using a Beckman LE-80 ultracentrifuge. The resulting pellet was resuspended in ice-cold 10 mM EDTA containing 1.15% KCl (pH 7.4) using a homogenizer. The suspension was centrifuged at 105,000 g for 60 min at 5°C. Glycogen was removed from the final microsomal pellet and the pellet was then suspended in 0.25 M sucrose and stored at -80°C (Lu and Levin, 1972). Total protein concentration in each microsomal sample was determined by the method of Lowry et al. (1951), using bovine serum albumin as the protein standard. Protein concentrations were measured in duplicate at 750 nm using a Shimadzu UV-160 UV-visible recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Microsomal fractions of rat Leydig R2C cells were also prepared. Culture medium was removed from the T75 flasks and R2C cells were incubated for 20 min, on ice, with 5 ml of ice-cold 0.5 M Tris buffer (pH 7.4) containing 1.15% KCl. Cells were scraped into pre-chilled microcentrifuge tubes and microsomes were prepared from cell homogenates by differential ultracentrifugation as described above.

2.7. Recombinant Proteins and Antibodies

Recombinant rat CYP1A1, CYP1A2 and CYP1B1 proteins were obtained from BD Gentest Corporation (Woburn, MA, USA). Recombinant mouse CYP1B1 protein was a generous gift from Dr. John Schenkman (University of Connecticut Health Center, CT, USA).

Polyclonal anti-rat CYP1A serum, which recognizes both rat CYP1A1 and rat CYP1A2,

was previously prepared in Dr. Bandiera's laboratory from rabbits immunized with purified rat CYP1A2 as described (Chang et al., 2003). Several different antibodies against CYP1B1 were obtained from various sources. Rabbit anti-rat CYP1B1 serum was obtained from BD Gentest Corporation (Woburn, MA, USA). Alpha Diagnostics International (San Antonio, TX, USA) was contracted to prepare an antibody against mouse CYP1B1 using a synthetic 14-amino acid peptide corresponding to an internal sequence (CNFKANQNESSNMS; 491-504) of mouse CYP1B1 protein as the antigen of immunization (Tang et al., 1999). Anti-mouse CYP1B1 serum was obtained from two New Zealand rabbits and was sent to us by Alpha Diagnostics International. Anti-human CYP1B1 IgG was previously prepared in Dr. Bandiera's laboratory from rabbits immunized with a synthetic 16-amino acid peptide corresponding to amino acids 284–299 (CESLRPGAAPRDMMDA) (Chang et al., 2003). Rabbit anti-human CYP1B1 serum was purchased from BD Gentest Corporation (Woburn, MA, USA). Goat anti-human CYP1B1 serum was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

The secondary antibodies, specifically, alkaline phosphatase-conjugated goat [Fab']₂ anti-rabbit IgG, alkaline phosphatase-conjugated swine [Fab']₂ anti-goat IgG and horseradish peroxidase-conjugated goat F(ab')₂ anti-rabbit IgG, were purchased from Biosource Inc. (Camarillo, CA, USA). Alkaline phosphatase-conjugated rabbit [Fab']₂ anti-goat IgG and horseradish peroxidase-conjugated anti-goat IgG were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.8. Preparation of CYP2S1 Anti-serum

Antibody against rat CYP2S1 was not commercially available. Antibody against CYP2S1 was needed to detect and quantify CYP2S1 protein expression in rats. Hence, we generated two antibodies to rat CYP2S1 using the anti-peptide approach, which is an established

method for the generation of antibodies. Two peptide sequences were selected based on comparative analysis of the deduced amino acid sequences of rat, mouse and human CYP2S1 with those of other CYP2 enzymes. One peptide corresponded to an internal sequence (CLKMAQEKQDPGTEF; amino acids 272-286) and a second peptide corresponded to the C-terminus sequence (CDFQLRVWPTGDQSR; amino acids 486-499). The selection of the peptide sequences of rat CYP2S1 was based on regions that had relatively high hydrophilic character and contained sequences that were specific to CYP2S1. Peptides were synthesized at the Nucleic Acid and Protein Service Unit (University of British Columbia, Vancouver, BC, Canada) and were conjugated to keyhole limpet hemocyanin (KLH) through the sulfhydryl group of the terminal cysteine residue using Sulfo-SMCC as the conjugating agent. The final antigen concentration was determined using the Lowry protein assay (Lowry et al., 1951). Two adult female New Zealand rabbits were injected with each synthetic peptide conjugate according to the following protocol. Peptide-conjugate (200 µg) emulsified in Freund's complete adjuvant (600 µl) was administered subcutaneously to each rabbit. Four weeks later, each rabbit received a boost consisting of 50 µg of peptide-conjugate emulsified in Freund's incomplete adjuvant (600 µl) administered by intramuscular injection. The same boost procedure was repeated four weeks later. After the second boost, each rabbit received an intravenous injection in the ear vein with 50 µg of peptide-conjugate dissolved in phosphate-buffered saline (200 µl) (PBS, 137 mM sodium chloride, 2.6 mM potassium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium dihydrogen phosphate; pH 7.4). The intravenous injections were repeated on a monthly basis. Blood samples (approximately 30 ml) were collected from each rabbit 7 days after each intravenous injection. The blood was allowed to clot at 37°C and serum was collected by centrifugation and stored at -20°C.

2.9. Noncompetitive Enzyme-linked Immunosorbent Assay (ELISA)

The reactivity and specificity of anti-CYP2S1 serum was determined by noncompetitive enzyme-linked immunosorbent assay (ELISA). ELISA was performed using 96-well microtest plates (Life Technologies, Burlington, ON, Canada). Wells were incubated overnight with solutions (150 µl/well) of unconjugated C-terminus peptide sequence, purified rat CYP enzymes, or other antigens at protein concentrations of 0.2 or 1 µg/ml, prepared in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.5. Uncoated wells served as blanks. Anti-rat CYP2S1 serum and preimmune serum were used at a dilution of 1:100. Horseradish peroxidase-conjugated goat F(ab')₂ anti-rabbit IgG was used as secondary antibody at a dilution of 1:3000. The substrate solution (2.2 mM *o*-phenylenediamine in 0.01% H₂O₂) was allowed to react for 2.5 min before addition of 40 µl of 4 M H₂SO₄ to stop the reaction. Absorbance was measured at 490 nm on a microplate reader (Bio-Tek instruments, Winooski, VT, USA).

2.10. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Microsomal proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using a Hoefer SE 600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The discontinuous SDS-polyacrylamide gel consisted of a 3.0% acrylamide stacking gel (0.75 mm thick, 1 cm long, 0.125 M Tris-HCl, pH 6.8) and a 7.5% acrylamide separating gel (0.75 mm thick, 12.5 cm long, 0.375 M Tris-HCl, pH 8.8). The final concentrations of reagents in the stacking gel were 3% acrylamide : Bis (22.2%:0.6% w/w), 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.08% (w/v) of ammonium persulphate, and 0.05% (v/v) TEMED. The final concentrations of reagents in the separating gel were 7.5% acrylamide : Bis (22.2%:0.6% w/w), 0.375 Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.042% (w/v) ammonium persulphate, and 0.03%

(v/v) TEMED. The electrophoresis buffer contained 0.1 M Tris base, 0.767 M glycine and 0.4% (w/v) SDS. Microsomes were diluted in sample dilution buffer containing 0.062 M Tris-HCl (pH 6.8), 1% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) glycerol, and 5% (v/v) mercaptoethanol and were boiled for 2 min. Denatured microsomal proteins were subjected to electrophoresis, with constant cooling, using a constant current setting of 11.5 mA/gel for approximately 1 h to allow migration of the dye front through the stacking gel and a setting of 23 mA/gel for approximately 2 h or until the dye front reached the bottom of the separating gel.

2.11. Immunoblot Analysis

Immunoblot analysis was used to determine the reactivity and specificity of anti-CYP2S1 serum and to measure levels of CYP1A1, CYP1A2, CYP1B1 and CYP2S1 protein in microsomes from tissues and cell culture. Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes according to Towbin et al. (1979) using a Hoefer Transphor Apparatus (model TE 52) at a setting of 0.4 A for 2 h at 4°C (Towbin et al., 1979). Nitrocellulose membranes were incubated individually with the following antibodies at 37°C for 2 h with shaking:

- rabbit polyclonal anti-rat CYP1A serum (1:500 dilution),
- rabbit anti-rat CYP1B1 serum (1:500 dilution),
- rabbit anti-mouse rabbit CYP1B1 serum (1:200 dilution),
- rabbit anti-rat CYP2S1 sera (1:200 dilution),
- rabbit anti-human CYP1B1 IgG (200 µg/ml),
- rabbit anti-human CYP1B1 serum (1:50 dilution),
- or goat anti-human CYP1B1 serum (1:50 dilution)

After washing three times with wash buffer (wash buffer= 137 mM sodium chloride, 2.6 mM

potassium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium dihydrogen phosphates, 0.2 mM EDTA, 0.05% Tween 20; pH 7.4), the membranes were incubated with alkaline phosphatase-conjugated goat [Fab']₂ anti-rabbit secondary antibody or alkaline phosphatase-conjugated rabbit [Fab']₂ anti-goat secondary antibody at a dilution of 1:3000 dilution or alkaline phosphatase-conjugated swine [Fab']₂ anti-goat secondary antibody at a dilution of 1:1000 dilution at 37°C for 2 h with shaking. The nitrocellulose membranes were then washed with wash buffer and the substrate solution containing 0.03% NBT and 0.015% BCIP in 0.1M Tris-HCl buffer, pH 9.5 (containing with 0.5 mM MgCl₂) was added to the membranes under subdued light to visualize the protein bands. The reaction was stopped by immersing the membranes in distilled water. In some instances, horseradish peroxidase-conjugated goat F(ab')₂ anti-rabbit IgG or horseradish peroxidase-conjugated anti-goat IgG was used as secondary antibody and the enhanced chemiluminescence detection method was employed to detect immune complexes.

Staining intensities of CYP protein bands on blots were quantified using a pdi 420oe scanning densitometer (pdi Inc., Huntington Station, NY, USA) connected to a personal computer and using pdi Quantity One 3.0 software. A defined amount of an appropriate purified rat CYP protein was loaded onto each gel as an internal standard. Intensities of the protein bands were calculated as optical density multiplied by the stained area, a measure known as contour quantity (OD X mm²). The amount of immunoreactive protein in each stained band was determined from the ratio of the contour quantity (OD X mm²) value of the stained band to that of the internal standard band. CYP1A1, CYP1A2 and CYP1B1 protein levels (pmol/mg total protein) in microsomal samples were derived from values of the contour quantity ratio using calibration curves. Calibration curves of purified CYP protein were prepared by loading various amounts of purified rat CYP standards onto gels followed by immunoblotting and densitometric

analysis as described. To generate a calibration curve, the ratio of the contour quantity values of purified CYP proteins and the internal standard was plotted against the amount (pmol) of protein loaded onto the gel. Recombinant or purified rat CYP2S1 protein was not commercially available for generation of calibration curves and quantification of CYP2S1 bands. Hence, CYP2S1 protein levels in microsomes are expressed as the contour quantity (i.e. intensity) of the stained band relative to the contour quantity of the reference band (i.e. CYP2S1 band obtained from 50 µg lung microsomal protein prepared from vehicle-treated male rats).

2.12. Isolation and Quantification of Total RNA

Total RNA was isolated from tissue samples using TrizolTM reagent following the instructions of the manufacturer (Invitrogen Canada, Burlington, ON, Canada). The RNA pellet was dissolved in 10 mM Tris buffer, pH 8 (containing 1 mM EDTA). The purity and concentration of each RNA preparation were determined (using GenespecTM software) by the absorbance ratio at 260 nm and 280 nm. RNA preparations with a ratio ≥ 1.9 were used for subsequent experiments. The integrity of the RNA preparations was evaluated by electrophoresis on a 1.7% agarose gel containing 0.66 M formaldehyde.

2.13. Multiplex Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to determine the CYP1B1, CYP2S1 and LH receptor mRNA levels after treatment of rats or Leydig cells with test hormones or chemicals. Complementary deoxyribonucleic acid (cDNA) was prepared by incubating 2 µg of total RNA with 0.5 µg oligo(dT)₁₂₋₁₈ primer and DEPC-treated water at 65°C for 10 min. Then, 1 µl of 10 mM dNTP mix, 1 µl of 0.1 M DTT, 2 units of deoxyribonuclease I, and 2 µl of 10× PCR buffer were added to each tube and the mixture was incubated at 37°C for 30 min. Reverse transcription was

carried out by adding 200 units of SuperscriptTM II reverse transcriptase. The reaction mixture was incubated at 42°C for 20 min, and the cDNA that was obtained was used immediately or stored at -20°C until needed.

For the CYP1B1 study, the following oligonucleotide sequences (primers) were synthesized by the Nucleic Acid and Protein Service Unit, University of British Columbia (Vancouver, BC, Canada) for use in PCR: 5'-GGCGTTCGGTCACTACTCTG-3' (forward) and 5'-AGGTTGGGCTGGTCACTCAT-3' (reverse) to amplify a 737-bp region of mouse CYP1B1 gene (Shimada et al., 2002); 5'-GTGGGCCGCTCTAGGCACCAA-3' (forward) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (reverse) to amplify a 525-bp region of mouse β -Actin gene (Zhang et al., 2004); and 5'-CTCACCTATCTCCCTGTCAAAGT-3' (forward) and 5'-ATGGACTCATTATTCATCCCTTG-3' (reverse) to amplify a 365-bp region of mouse LH receptor gene (Fukuzawa et al., 2004).

For the CYP2S1 study, the following primers were designed using Primer3 (<http://frodo.wi.mit.edu/>), DNAssist and BLAST search programs to amplify a 387-bp region of the rat CYP2S1 gene (accession number NM_001107495.1): 5'-CCAGGCCTACGAGATGTTCT-3' (forward) and 5'-TCGCTGAGACTTGGAGTCCT-3' (reverse). The following primers were selected, as described previously (Walker et al., 1999), to amplify a 246-bp region of the rat β -actin gene: 5'-CAGCCTTCCTTCCTGGGTATG-3' (forward) and 5'-TAGAGCCACCAATCCACACAG-3' (reverse); and to amplify a 341-bp region of the rat CYP1A1 gene: 5'-CCATGACCAGGAAGTATGGG-3' (forward) and 5'-TCTGGTGAGCATCCAGGACA-3' (reverse). Primers were synthesized by the Nucleic Acid and Protein Service Unit, University of British Columbia (Vancouver, BC, Canada).

PCR reactions were optimized for various components such as cycle number and cDNA, Taq polymerase, primer, magnesium chloride and dNTP concentration. CYP1B1 and β -actin

(internal control) or LH receptor and β -actin (internal control) cDNAs were co-amplified using platinum Taq polymerase enzyme (2 units per reaction). For all the genes, the amplification reaction was initiated by heating at 94°C for 2 min. This was followed by 25 cycles (for β -actin), 38 cycles (for LH receptor), or 42 cycles (for CYP1B1) using the following thermocycling conditions: 60 sec of denaturation at 94°C, 30 sec for annealing at 60°C, and 1 min for LH receptor or 2 min for CYP1B1 of extension at 72°C.

CYP2S1 and β -actin (internal control) or CYP1A1 and β -actin (internal control) cDNAs were co-amplified using platinum Taq polymerase enzyme (1 unit). The PCR amplification was initiated by heating at 94°C for 60 sec. This was followed by 22 cycles (for β -actin), 28 cycles (for CYP1A1), or 35 cycles (for CYP2S1) using the following thermocycling conditions: 30 sec of denaturation at 94°C, 30 sec of annealing at 54°C, and 30 sec (for CYP1A1) or 60 sec (for CYP2S1) of extension at 75°C.

After thermocycling, final PCR products were subjected to electrophoresis using 1.7% agarose gels. The agarose gels were photographed and the integrated optical densities of the ethidium bromide-stained bands were quantified using LabWorkTM software (UVP Inc, Cambridge, UK).

2.14 Optimization of PCR Reactions

PCR reaction conditions for the co-amplification of CYP1B1 and β -actin, LH receptor and β -actin, CYP2S1 and β -actin, or CYP1A1 and β -actin were varied to determine the optimal conditions for each reaction. The PCR reaction conditions included cycle number, Taq polymerase concentration, cDNA concentration, primer concentration, dNTPs concentration and magnesium chloride concentration were determined. The optimized conditions for PCR reactions are summarized in Table 2.1.

The cDNA obtained from total RNA was amplified with specific primers using various thermal cycles (Table 2.2) in block cycler PCR reactions for individual genes (CYP1B1 or β -actin). Integrated optical density of the amplified cDNA was measured using densitometric analysis of the ethidium bromide-stained bands on agarose gels. Linearity of integrated optical density of amplified cDNA of the CYP1B1 or β -actin gene was analyzed using a range of cycle numbers (Figure 2.2). The optimal compatible PCR cycle number for co-amplification of CYP1B1 and β -actin mRNA in multiplex reactions was determined by the primer-dropping method (Wong et al., 1994) (Figure 2.3). The CYP1B1 primer was amplified using a constant number of cycles and β -actin primer was added to different reaction tubes at different cycle numbers as indicated in the figure legends. It was found that CYP1B1 and β -actin genes were optimally co-amplified using 42 and 25 thermal cycles, respectively, under the reaction conditions used.

The linearity ranges of thermal cycles for CYP2S1 and β -actin gene amplification were determined using individual PCR reactions (Figure 2.4). The optimal compatible PCR cycle number for co-amplification of CYP2S1 and β -actin mRNA in multiplex reactions was determined using the primer-dropping method (Figure 2.5). It was found that CYP2S1 and β -actin genes were optimally co-amplified using 35 and 22 thermal cycles, respectively, under the reaction conditions used.

Table 2.1: Optimal parameters determined for multiplex PCR reactions of CYP1A1, CYP1B1, LH receptor (LHR), or CYP2S1 with β -actin.

Parameters	CYP1B1^a	LHR^a	CYP2S1^b	CYP1A1^b
Cycle number	42	38	35	28
Platinum Taq polymerase (U)	2	2	1	1
Forward primer (μ M)	0.2	4	0.2	0.2
Reverse primer (μ M)	0.2	4	0.2	0.2
Magnesium chloride (mM)	2	2	2	2
dNTPs (μ M)	400	400	400	400

^a Mouse β -actin forward and reverse primers were used at final concentration of 0.2 μ M each. Co-amplification of β -actin gene was carried out using 25 thermal cycles.

^b Rat β -actin forward and reverse primers were used at final concentration of 0.2 μ M each. Co-amplification of β -actin gene was carried out using 22 thermal cycles.

Table 2.2: Nucleotide sequences of primer pairs and temperatures used in block cyclor RT-PCR reactions and PCR product size (in base-pair) obtained from ethidium bromide-stained agarose gel carried out after PCR reaction.

Gene	Primer sequences (5'-3')	Direction ^a	Annealing Temp (°C)	Extension Temp (°C)	PCR Product (bp)
CYP1B1	GGCGTTCGGTCACTACTCTG AGGTTGGGCTGGTCACTCAT	F R	60	72	737
LHR	CTCACCTATCTCCCTGTCAAAGT ATGGACTCATTATTCATCCCTTG	F R	60	72	365
β-Actin (Mouse)	GTGGGCCGCTCTAGGCACCAA CTCTTTGATGTCACGCACGATTTC	F R	60	72	525
CYP2S1	CCAGGCCTACGAGATGTTCT TCGCTGAGACTTGGAGTCCT	F R	54	75	387
CYP1A1	CCATGACCAGGAAGTATGGG TCTGGTGAGCATCCAGGACA	F R	54	75	341
β-Actin (Rat)	CAGCCTTCCTTCCTGGGTATG TAGAGCCACCAATCCACACAG	F R	54	75	246

^a F- Forward; R- Reverse

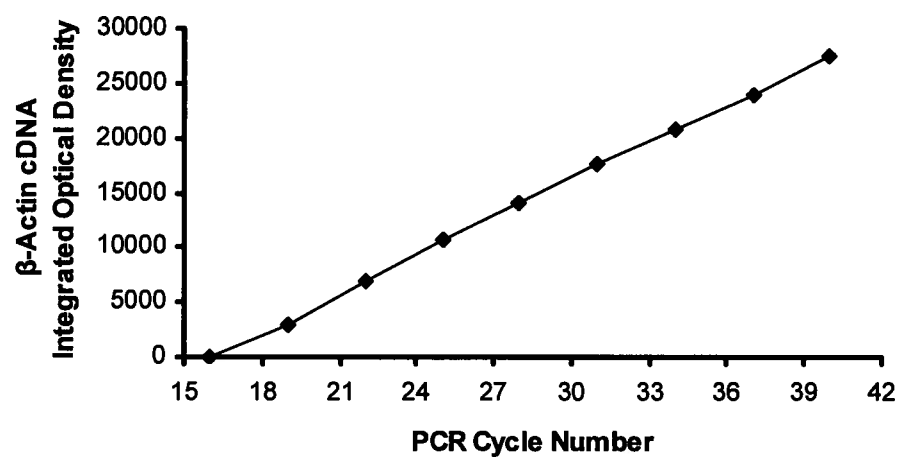
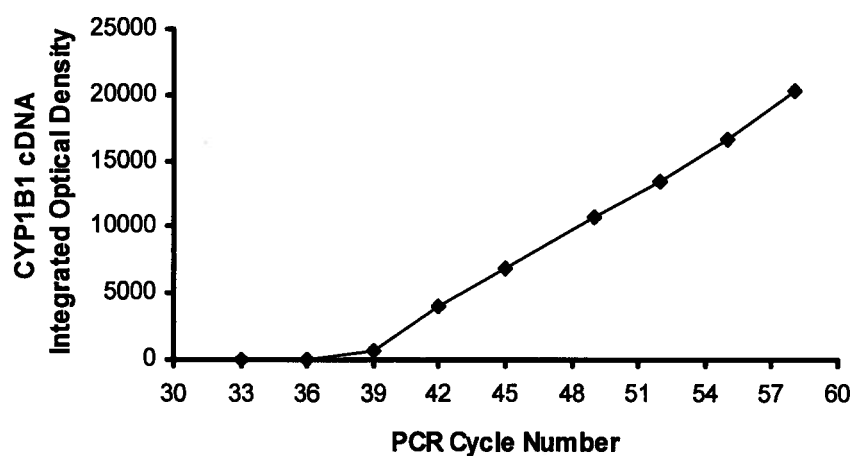


Figure 2.2: Relationship between PCR cycle number and CYP1B1 and β -actin cDNA amplification in individual PCR reactions. Total RNA samples were obtained from untreated mouse Leydig MA-10 cells. Integrated optical densities were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR reaction.

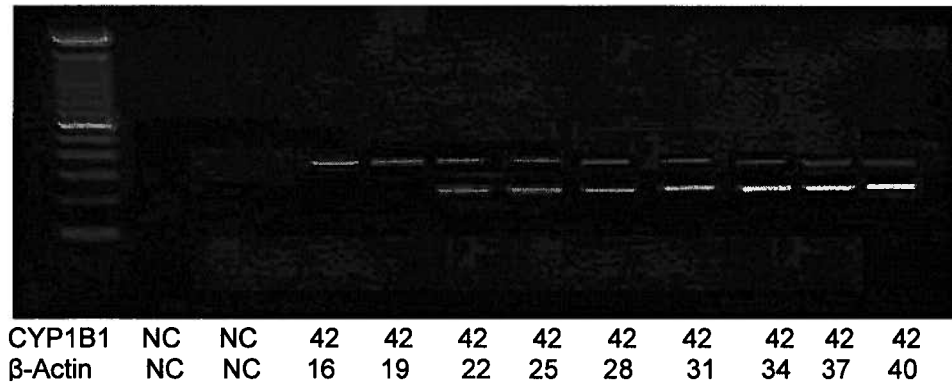
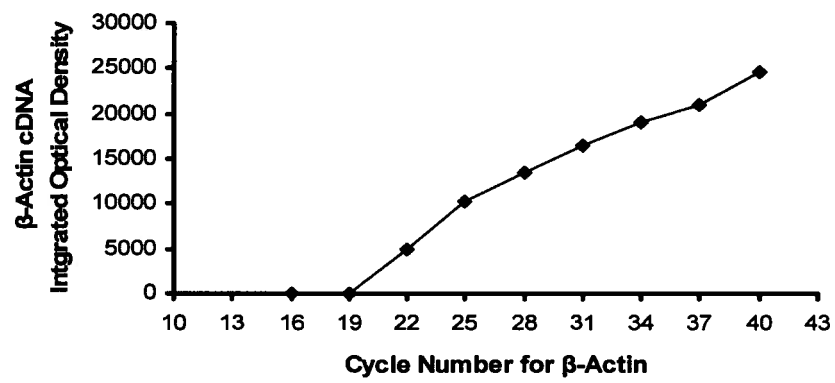
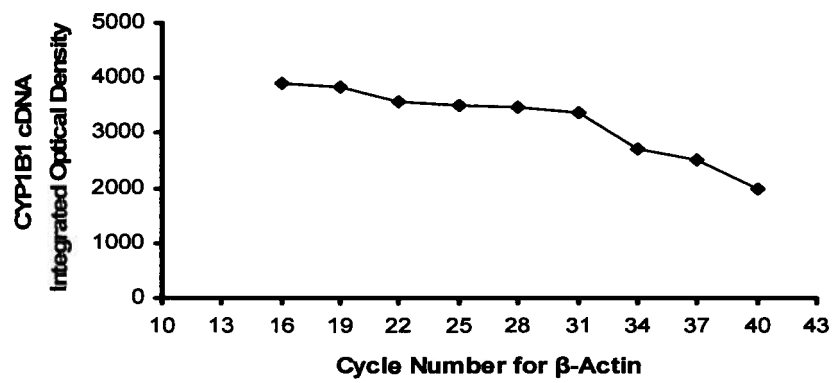
A**B****C**

Figure 2.3: Determination of optimal PCR cycle numbers for multiplex reactions of CYP1B1 and β -actin cDNA by the primer-dropping method. (A) Ethidium bromide-stained agarose gel after co-amplification of CYP1B1 and β -actin cDNA using 42 cycles for CYP1B1 primer and different number of cycles for β -actin primer. (B) Integrated optical density of β -actin cDNA (different number of thermocycles) after co-amplification with CYP1B1 cDNA (constant 42 thermocycles). (C) Integrated optical density of CYP1B1 cDNA (constant 42 thermocycles) after co-amplification with β -actin cDNA (different number of thermocycles). Total RNA samples were obtained from untreated mouse Leydig MA-10 cells. Integrated optical densities were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR. NC- Negative control.

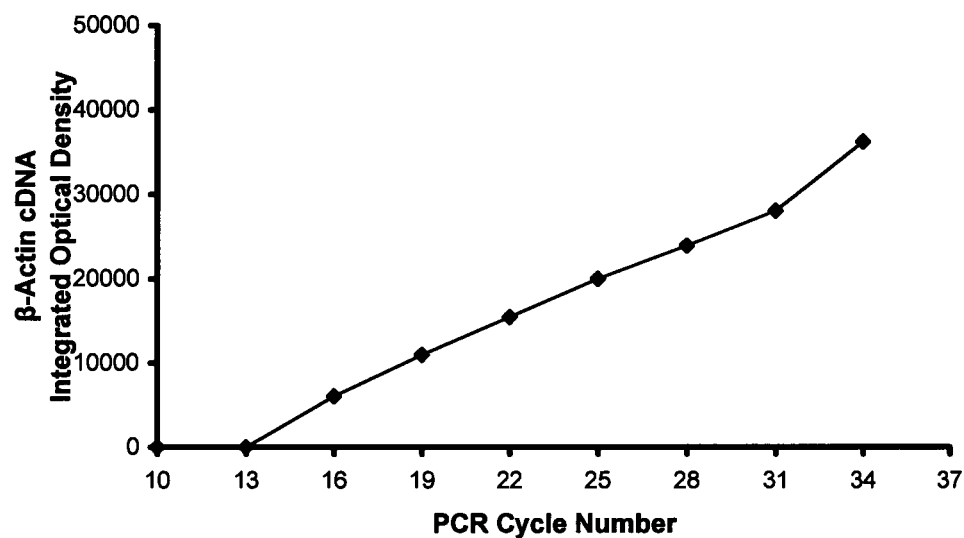
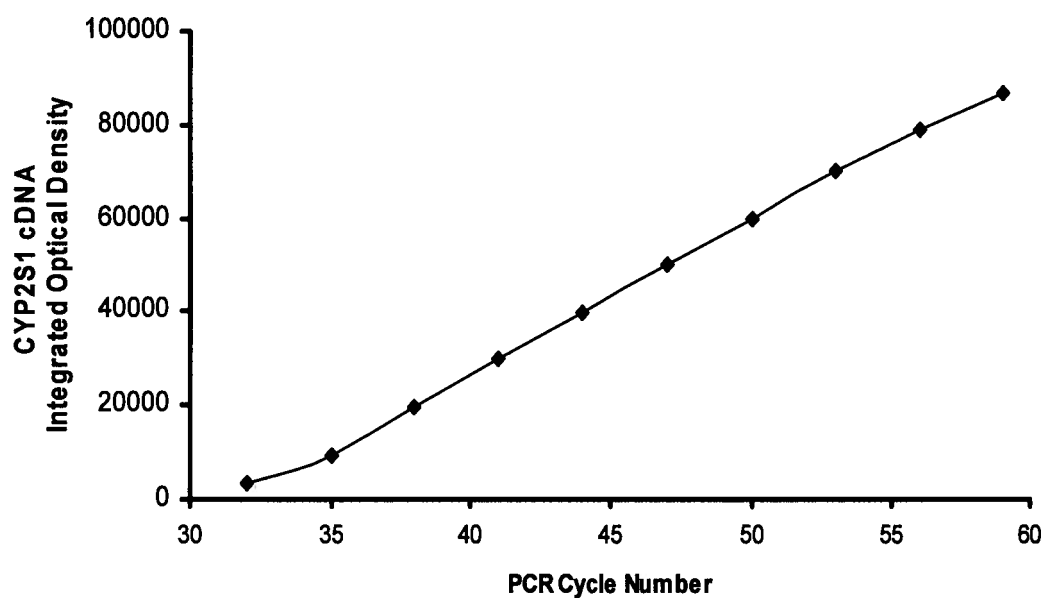


Figure 2.4: Relationship between PCR cycle number and CYP2S1 and β -actin cDNA amplification in individual PCR reactions. Total RNA samples were obtained from untreated adult male rat lung tissues. Integrated optical densities were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR reaction.

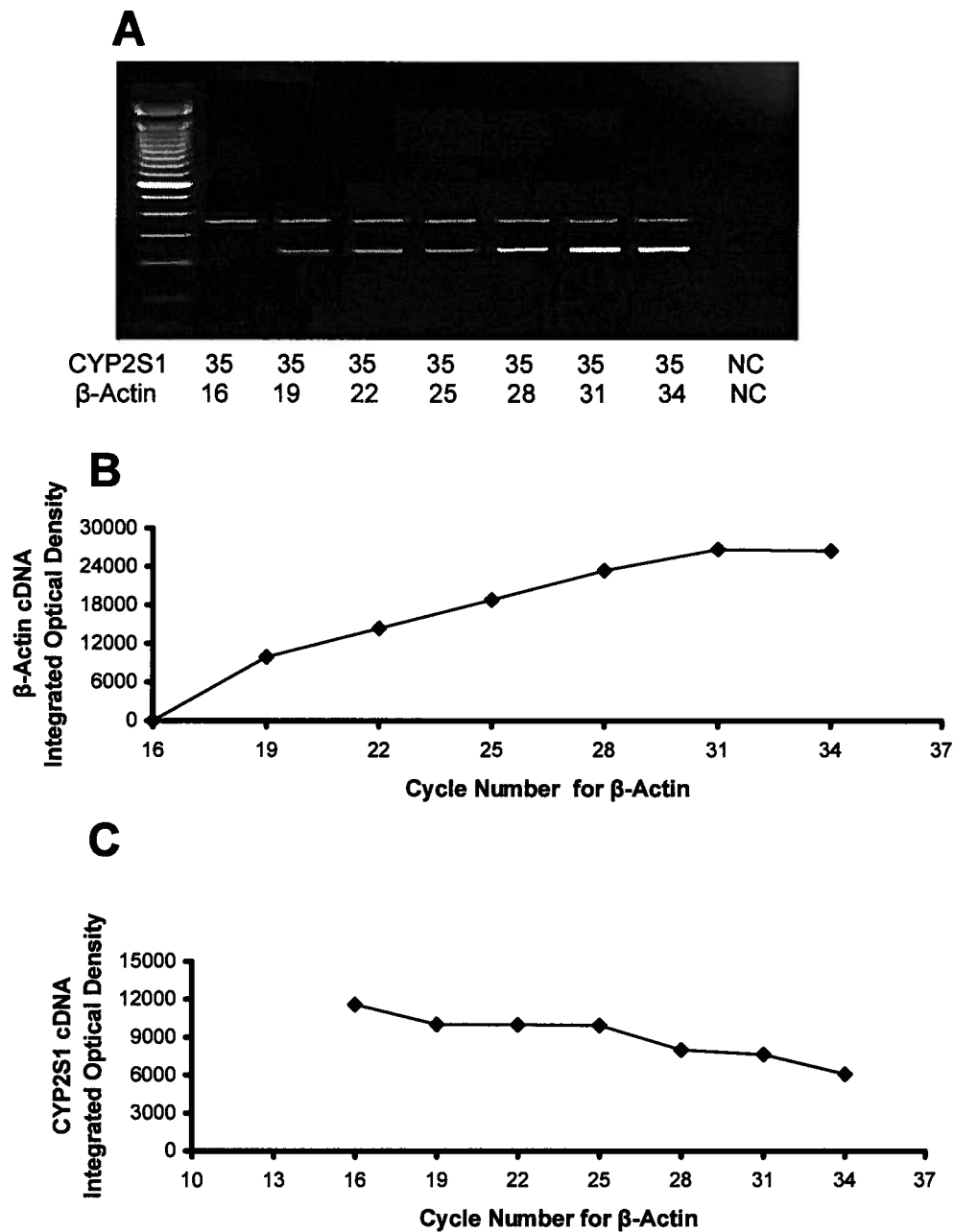


Figure 2.5: Determination of optimal PCR cycle numbers for multiplex reactions of CYP2S1 and β -actin cDNA by the primer-dropping method. (A) Ethidium bromide-stained agarose gel after co-amplification of CYP2S1 and β -actin cDNA using 35 cycles for CYP2S1 primer and different number of cycles for β -actin primer. (B) Integrated optical density of β -actin cDNA (different number of thermocycles) after co-amplification with CYP2S1 cDNA (constant 35 thermocycles). (C) Integrated optical density of CYP2S1 cDNA (constant 35 thermocycles) after co-amplification with β -actin cDNA (different number of thermocycles). Total RNA samples were obtained from untreated adult male rat lung tissues. Integrated optical densities were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR. NC- Negative control (no cDNA).

2.15. Protein Kinase A (PKA) Activity Assay

PKA activity was measured in cytosolic fraction of Leydig cells to determine if LH and estradiol modulate PKA activity in the regulation of testicular CYP11B1 expression in MA-10 and R2C cells. MA-10 cells or R2C cells were cultured in T25 flasks and were exposed to vehicle (DMSO or water) or various test chemicals or hormones for various periods of time as mentioned in the figure legends. Culture medium was removed from the flasks at the end of the treatment period and cells were washed with 5 ml of ice-cold PBS (137 mM sodium chloride, 2.6 mM potassium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium dihydrogen phosphate, 0.2 mM EDTA; pH 7.4) and were then placed on ice. One ml of lysis buffer [lysis buffer= 20 mM MOPS, 50mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40 (NP40), 1 mM dithiothreitol, 1 mM benzamidine, 1 mM PMSF, 10 μ g/mL leupeptin and 10 μ g/mL aprotinin] was added to each flask. After a 10-min incubation on ice, the cells were scraped off and transferred into pre-chilled microcentrifuge tubes and sonicated briefly to lyse the cells completely. Cell lysates were centrifuged for 15 min at 13,000 rpm, and the resulting supernatant containing the cytosolic fraction was transferred to clean microcentrifuge tubes. Protein concentration was determined using the Lowry assay (Lowry et al., 1951). PKA activity was measured using a sensitive non-radioactive assay kit according to the manufacturer's instructions (Stressgen Biotechnologies, San Diego, CA, USA). The assay was optimized for cytosolic protein concentration and incubation time. The assay was based on a solid phase enzyme-linked immunosorbent assay (solid phase ELISA) that uses a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. A peroxidase conjugated secondary antibody, subsequently added to each well, was bound to the phosphospecific antibody. The assay was developed with tetramethylbenzidine substrate solution and a color develops in proportion to PKA

phosphotransferase activity. The absorbance was measured spectrophotometrically at 450 nm on a microplate reader. Relative PKA activity was calculated as the difference between the PKA activity of cell lysates and the blank, which contained all the components of the assay except for cell lysate, and divided by the amount of total protein (μg) used in the assay.

2.16. MTT Assay (Chemical Name: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

An MTT-based assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used to assess cell viability after treatment with various test chemicals or hormones. MA-10 cells or R2C cells were grown in 96-well plates and were treated with various test chemicals or hormones for various treatment periods. Triton-X 100 (1%) was used as a positive control and dextran (5%) was used as a negative control in the MTT assay. At the end of the treatment period, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h to allow MTT reduction by mitochondrial dehydrogenase in viable cells. The culture medium and MTT solution were removed from each well. MTT-formazan crystals which remained behind in the wells were dissolved with 100 μl of solvent (0.1 N HCl in anhydrous isopropanol). The absorbance was measured spectrophotometrically at 540 nm using a microplate reader. The absorbance value served as an estimation of cell viability in the wells. Cell viability of vehicle-treated cells was set at 100% and viability of cells treated with test chemicals or hormones was expressed as percentage of vehicle-treated cells. The absorbance values of vehicle-treated cells in the MTT assay was between 0.8 to 1 for all experiments.

2.17. DMBA Metabolism Assay

DMBA undergoes oxidation by CYP1A1 and CYP1B1 enzymes to form dihydroxy

metabolites (Choudhary et al., 2004; Savas et al., 1997) (Figure 2.6). A review of the literature indicates that CYP1B1 can catalyze the formation of DMBA-3,4-diol and DMBA-5,6-diol from DMBA (Choudhary et al., 2004; Pottenger and Jefcoate, 1990; Savas et al., 1997). To measure the CYP1B1-mediated catalytic activity in microsomal samples, the formation of DMBA-3,4-diol (molecular weight: 290) and DMBA-5,6-diol (molecular weight: 290) from DMBA (molecular weight: 256.34) was investigated using a liquid chromatography/mass spectrometry (LC/MS)-based assay as reported by Savas et al. (1997) and Choudhary et al. (2004). Microsomes (0.5 mg protein/reaction tube) or recombinant human CYP1B1 protein (50 pmol/reaction tube) was incubated with DMBA (dissolved in ethanol, 200-500 μ M) in reaction mixtures containing 50 mM of potassium phosphate buffer, pH 7.4, 3 mM MgCl₂, in a final volume of 1 ml. After initial preincubation at 37°C for 5 min, the reaction was initiated by adding 10 μ l of NADPH (final conc. 0.5 mM NADPH/ml) and was allowed to proceed for 60 min. The reaction was terminated with 4 ml of ethyl acetate/acetone (2:1) containing 1 mM dithiothreitol. A constant amount (0.1 μ M) of internal standard (testosterone, epi isomer; molecular weight: 288.4) was added to each sample. Tubes were vortex-mixed for 1 min and centrifuged at 2000 g for 5 min at room temperature to separate the organic and aqueous phases. The top organic layer containing unreacted DMBA and its hydroxylated metabolites was transferred to a clean test tube and the aqueous phase was re-extracted with a second 4-ml aliquot of ethyl acetate/acetone (2:1). The final aqueous phase was discarded and the organic phases were pooled and evaporated to dryness under a gentle stream of nitrogen. The residue obtained was reconstituted in 200 μ l of methanol with vortex-mixing and filtered through a 3-mm, 0.45 μ m syringe filter.

A 20 μ l aliquot of each reconstituted filtered sample was analyzed by LC/MS (Waters Ultra Performance Liquid Chromatograph System consisting of a Binary Solvent Manager and

Sample Manager, Waters, Milford, MA) using a Waters AcquityTM ultraperformance liquid chromatography C18 (2.1 x 100 mm, 1.7 μ m) column to measure the formation of DMBA-3,4-diol and DMBA-5,6-diol. The mobile phase consisted of 50% methanol (0-1 min), followed by a gradient of 50-100% methanol (1-10 min), and then isocratic elution (100% methanol; 10-45 min) (Choudhary et al., 2004). The flow rate was maintained at 0.3 ml/min and total run time was 13 min/sample. The LC eluant was introduced into a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters), which was operated in single ion recording mode using atmospheric electrospray positive ionization (Chou and Yang, 1978), with bath gas flow and nebulizer gas flow rates of 250 and 20 l/h, respectively, a source temperature of 120°C, and capillary and cone voltages of 3.5 kV and 20 V, respectively (Chou and Yang, 1978). MASSLYNX version 4.1 software (Waters) was used for data acquisition.

DMBA-3,4-diol and DMBA-5,6-diol metabolite standards were resolved by the LC conditions used, as shown on the LC/MS chromatogram (Figure 2.7). DMBA-3,4-diol and DMBA-5,6-diol eluted between 4.3-6.8 min and were monitored at m/z 290. Under these conditions, the internal standard, epi-testosterone (molecular weight 288.4), was identified and resolved on the column and typically eluted at 6.7 min and was monitored at m/z 290. Unreacted DMBA (molecular weight 256.34) eluted at 7 min and was monitored at m/z 256. DMBA-3,4-diol and DMBA-5,6-diol metabolite standards were detected as positive ions, which indicates that the ions were mainly formed by protonation (i.e. by generation of $[M-H]^+$ ions).

Calibration curves for the metabolite standards (DMBA-3,4-diol and DMBA-5,6-diol) were prepared by directly injecting varying amounts of metabolite standards (25, 50, 100 and 200 pmol of DMBA-3,4-diol and DMBA-5,6-diol dissolved in ethanol) into the column. Representative calibration curves for DMBA-3,4-diol are shown in Figure 2.8. An internal standard was not used in constructing the calibration curves shown in Figure 2.8.

Metabolite standard mixtures containing DMBA-3,4-diol and DMBA-5,6-diol at concentrations of 0.1, 1, 10, and 100 μ M were prepared. The metabolite mixture (10 μ l) was added to the reaction mixture containing all the assay components as described above except substrate (DMBA) solution. The internal standard, epi-testosterone, was also added to each tube. The extraction of authentic metabolite standards and epi-testosterone from the reaction mixture and the LC/MS analysis steps were the same as described above. DMBA-3,4-diol and DMBA-5,6-diol metabolites were detected in the LC/MS chromatogram, but the signal intensity was very low, when metabolite standard mixtures containing DMBA-3,4-diol and DMBA-5,6-diol were added to the reaction mixture at a concentration of 1000 pmol/ml of reaction mixture. No signal was detected when metabolite standard mixtures containing DMBA-3,4-diol and DMBA-5,6-diol were added to the reaction mixture at concentrations of 1, 10, and 100 pmol/ml of reaction mixture. Extraction efficiency, accuracy, sensitivity, precision and reproducibility of this assay were not determined.

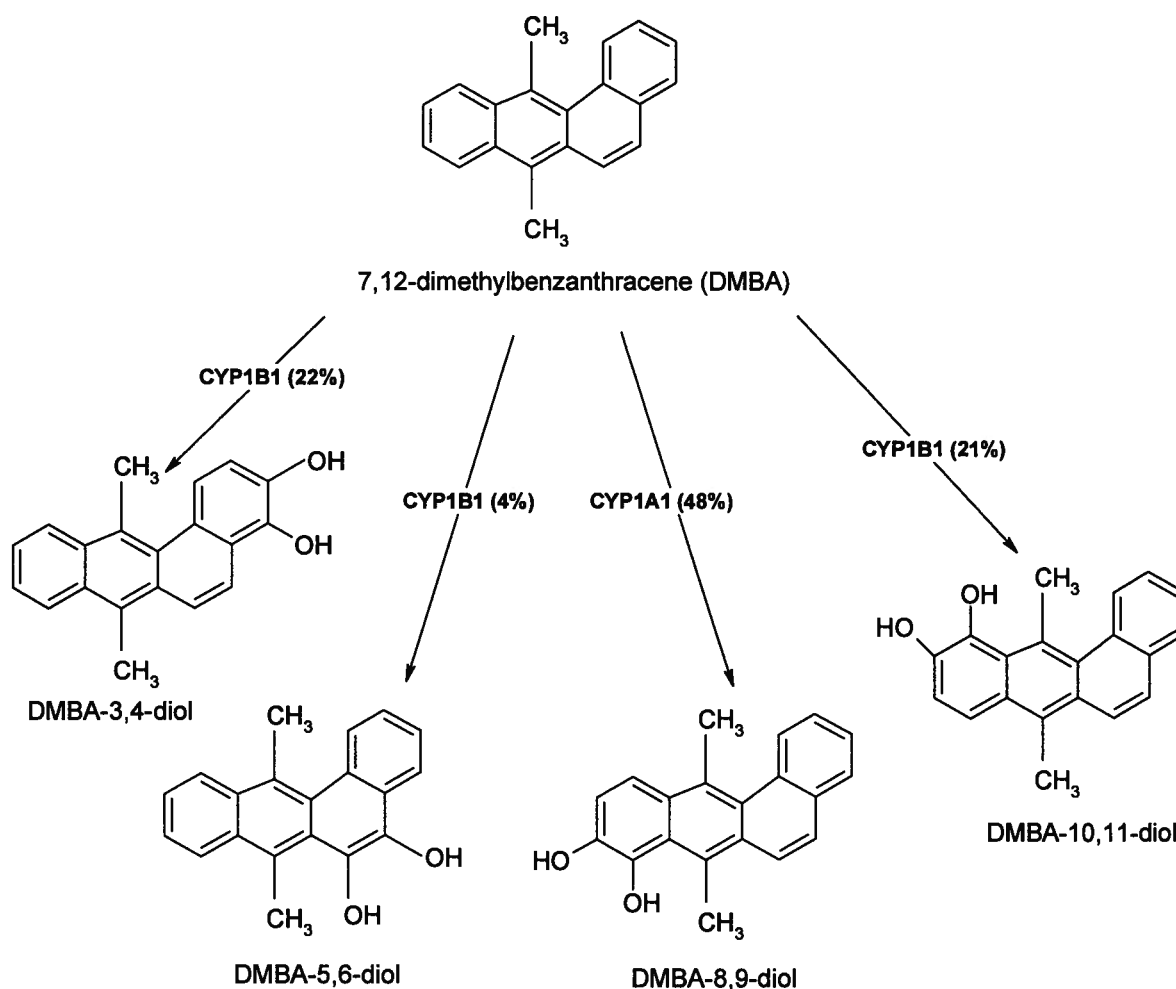


Figure 2.6: Regioselective metabolism of 7,12-dimethylbenzanthracene (DMBA) to its dihydroxy metabolites by CYP1A1 and CYP1B1 enzymes in rat, mouse and human. The numbers in parentheses represent the percentage of the particular metabolite in comparison to total DMBA metabolites formed by individual recombinant mouse CYP enzymes (Savas et al., 1997).

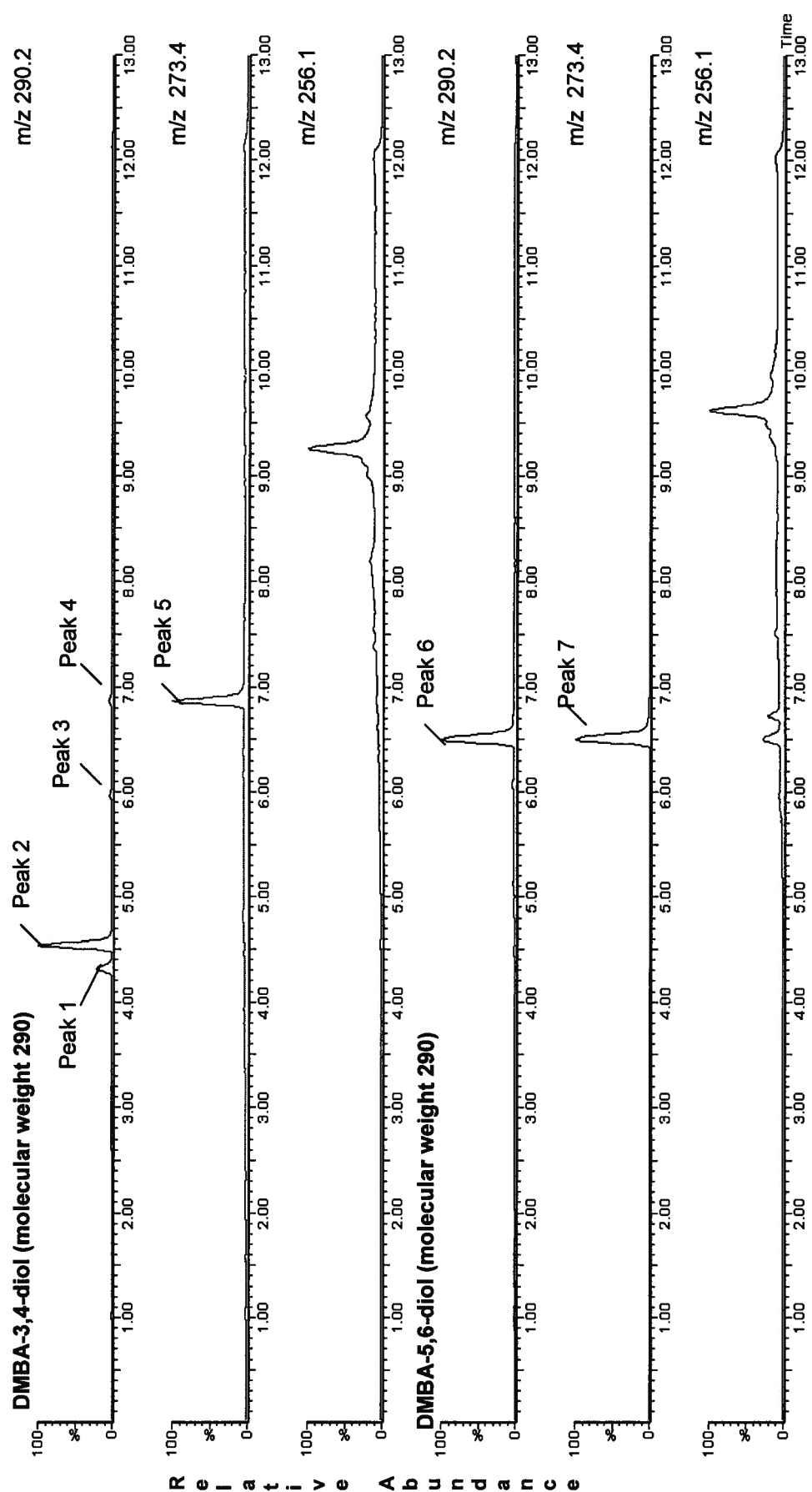


Figure 2.7: Representative LC/MS chromatogram showing separation of authentic metabolite standards of DMBA, DMBA-3,4-diol (molecular weight 290) and DMBA-5,6-diol (molecular weight 290). Peaks 1, 2, 3 and 4 (*m/z* 290) represent different stereoisomeric forms of DMBA-3,4-diol authentic metabolite standard, and peak 6 (*m/z* 290) represents DMBA-5,6-diol authentic metabolite standard. Peak 5 and peak 7 are ions (with a *m/z* equivalent to monohydroxy metabolites of DMBA i.e. 273) that are formed during in-source fragmentation of DMBA-3,4-diol and DMA-5,6-diol, respectively.

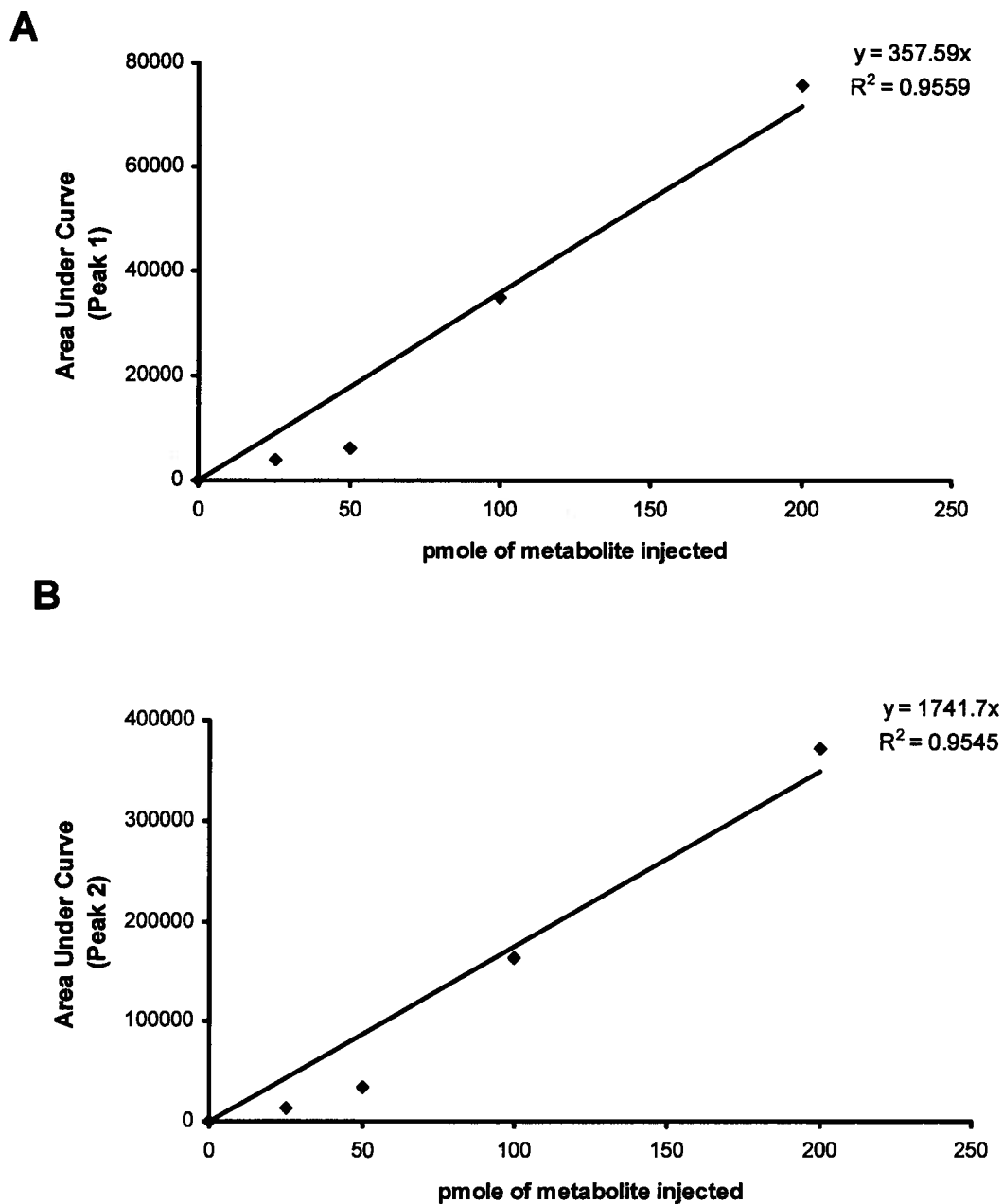


Figure 2.8: Relationship between amount of DMBA-3,4-diol authentic metabolite standard injected into the LC/MS and area under the curve obtained after resolving the metabolites on the column. Plot A shows the area under the curve obtained for peak 1 (m/z ratio 290) and plot B shows the area under the curve obtained for peak 2 (m/z ratio 290).

2.18. Data Analysis

Parametric tests were applied when data passed the normality and equal variance test, while nonparametric tests were used when the data failed to pass the normality test and equal variance test (SigmaStat™ Statistical Software, Version 3.1, SPSS Inc., Chicago, IL, USA). Differences between mean values of two treatment groups were assessed by the Student's t-test (parametric) or Mann-Whitney test (nonparametric unpaired t-test). In cases where there were more than two groups, the data were analyzed by one-way analysis of variance (parametric) or Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul's multiple comparison test (SigmaStat™ Statistical Software, Version 3.1, SPSS Inc., Chicago, IL, USA). The level of significance was set *a priori* at $P < 0.05$. ED₅₀ values were determined by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS

PART 1: TESTICULAR CYP1B1 EXPRESSION

Section A-Regulation of CYP1B1 Expression in Mouse Leydig MA-10 Cells

3.1. Effect of Pituitary Hormones on CYP1B1 mRNA Expression

To determine the effect of pituitary hormones on CYP1B1 expression in mouse Leydig MA-10 cells, CYP1B1 mRNA levels were measured in MA-10 cells treated with LH, FSH and prolactin, either alone or in combination, for 12 h. Treatment with LH at a concentration of 0.05 IU/ml increased CYP1B1 mRNA levels by 3.9-fold relative to vehicle-treated cells. Treatment with FSH (0.05 IU/ml) or prolactin (0.05 IU/ml) increased CYP1B1 mRNA levels by 1.5-fold or less (Figure 3.1). Treatment of MA-10 cells with LH plus FSH, LH plus prolactin, or LH plus FSH plus prolactin increased CYP1B1 mRNA levels in MA-10 cells to the same extent as treatment with LH alone.

The results suggest that LH has the greatest effect, among the pituitary hormones examined, on CYP1B1 mRNA expression in mouse Leydig cells.

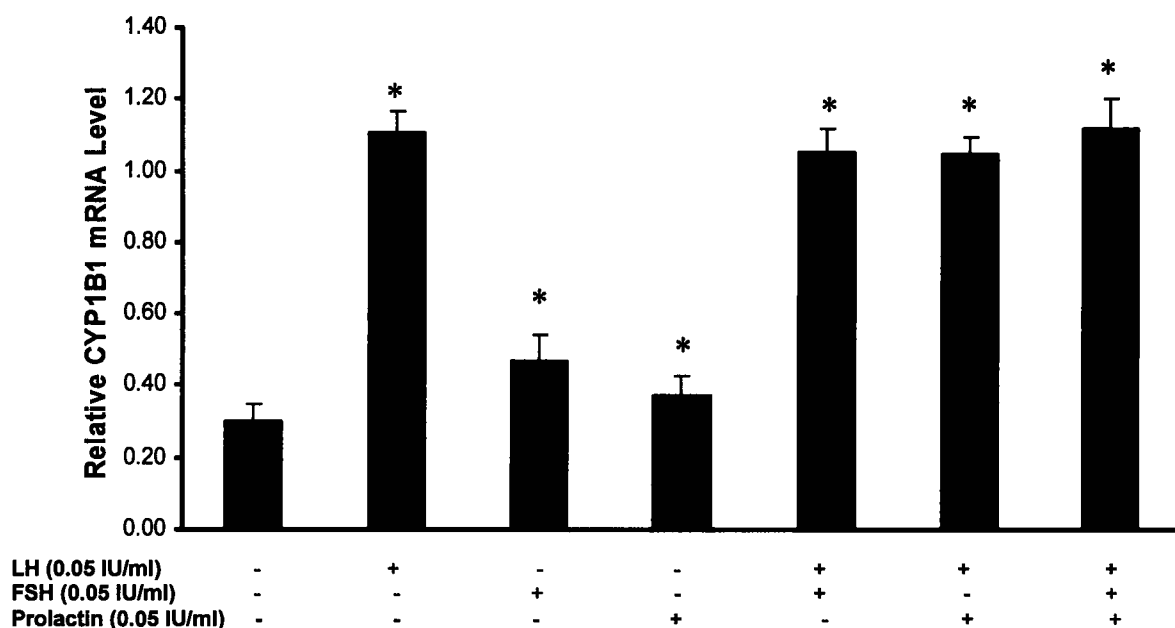


Figure 3.1: Effect of treatment with various pituitary hormones on CYP1B1 mRNA expression in mouse Leydig MA-10 cells. Cells were treated with LH, FSH, prolactin, either alone or in combination, for 12 h. The concentrations indicated are the final concentrations of hormones in the T25 culture flask. Relative CYP1B1 mRNA levels were determined using RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

3.2. Regulation of CYP1B1 mRNA Expression by LH

3.2.1. Effect of Varying Concentrations of LH on CYP1B1 mRNA and LH Receptor mRNA Expression

To more fully characterize the effect of LH, CYP1B1 mRNA expression in MA-10 cells was measured following treatment with varying concentrations of LH. In this study, cells were treated with LH at a range of concentrations between 0.01 and 0.2 IU/ml for 12 h. RT-PCR analysis showed that CYP1B1 mRNA exhibited a concentration-dependent increase with increasing concentrations of LH (Figure 3.2A). Induction of CYP1B1 mRNA levels was observed at all LH concentrations tested. CYP1B1 mRNA levels were increased by 4.2 fold relative to vehicle-treated cells after treatment with LH at a concentration of 0.2 IU/ml. It appears that the inductive effect of LH on CYP1B1 mRNA expression reached a plateau starting at a concentration of 0.05 IU/ml.

In Leydig cells, LH exerts its regulatory effects by binding to the LH receptor (Dufau, 1998). Hence, LH receptor mRNA levels were measured to investigate if the LH-elicited increase in CYP1B1 expression is a consequence of increased LH receptor expression. MA-10 cells were treated with varying concentrations of LH (0.01-0.2 IU/ml) for 12 h and LH receptor mRNA levels were measured. In contrast to CYP1B1 mRNA, LH receptor mRNA levels were decreased in a concentration-dependent manner with increasing concentrations of LH. Suppression of LH receptor mRNA levels was observed at all LH concentrations tested. LH receptor mRNA levels were decreased by approximately 60% compared to vehicle-treated cells after treatment with LH (0.1 IU/ml) (Figure 3.2B). It appears that the suppressive effect of LH on LH receptor expression reached a plateau starting at a concentration of 0.05 IU/ml.

A time course study of the effect of LH on CYP1B1 mRNA and LH receptor mRNA expression in MA-10 cells was also performed. The inductive effect of LH (0.05 IU/ml) on

CYP1B1 mRNA levels and the suppressive effect of LH (0.05 IU/ml) on LH receptor mRNA levels were found to be time-dependent. The LH-mediated effect on CYP1B1 mRNA and LH receptor mRNA levels was observed as early as 3 h after treatment and was greatest after 12 h of treatment (Figure 3.3). At the 24 h-time-point, CYP1B1 mRNA levels were decreased compared to levels at the 12 h-time-point. Similarly, LH receptor mRNA levels were increased at 24 h time point compared to levels at the 12 h-time-point.

3.2.2. Effect of Varying Concentrations of LH on PKA Activity

In Leydig cells, LH activates the PKA pathway via stimulation of cAMP formation (Cooke et al., 1976). The effect of varying the concentration of exogenous LH (0.01-0.2 IU/ml) on relative PKA activity in MA-10 cells was characterized. Relative PKA activity was increased in a concentration-dependent manner with increasing concentrations of LH (Figure 3.4). Increased PKA activity was observed at all LH concentrations used in this study. Relative PKA activity was increased by 2.7-fold compared to vehicle-treated cells in MA-10 cells, after treatment with LH at a concentration of 0.2 IU/ml (Figure 3.4).

3.2.3. Effect of Varying Concentrations of LH on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with various concentrations of LH (0.01-0.2 IU/ml) on cell viability. According to the results of the MTT-based assay, the viability of MA-10 cells did not change after treatment with LH at the concentrations used in this study (Figure 3.5).

The results of treatment with varying LH concentrations and for different periods of time suggest that there was a correlation between increased CYP1B1 mRNA expression and stimulation of PKA activity by LH in MA-10 cells. In contrast, LH receptor mRNA levels were decreased by LH.

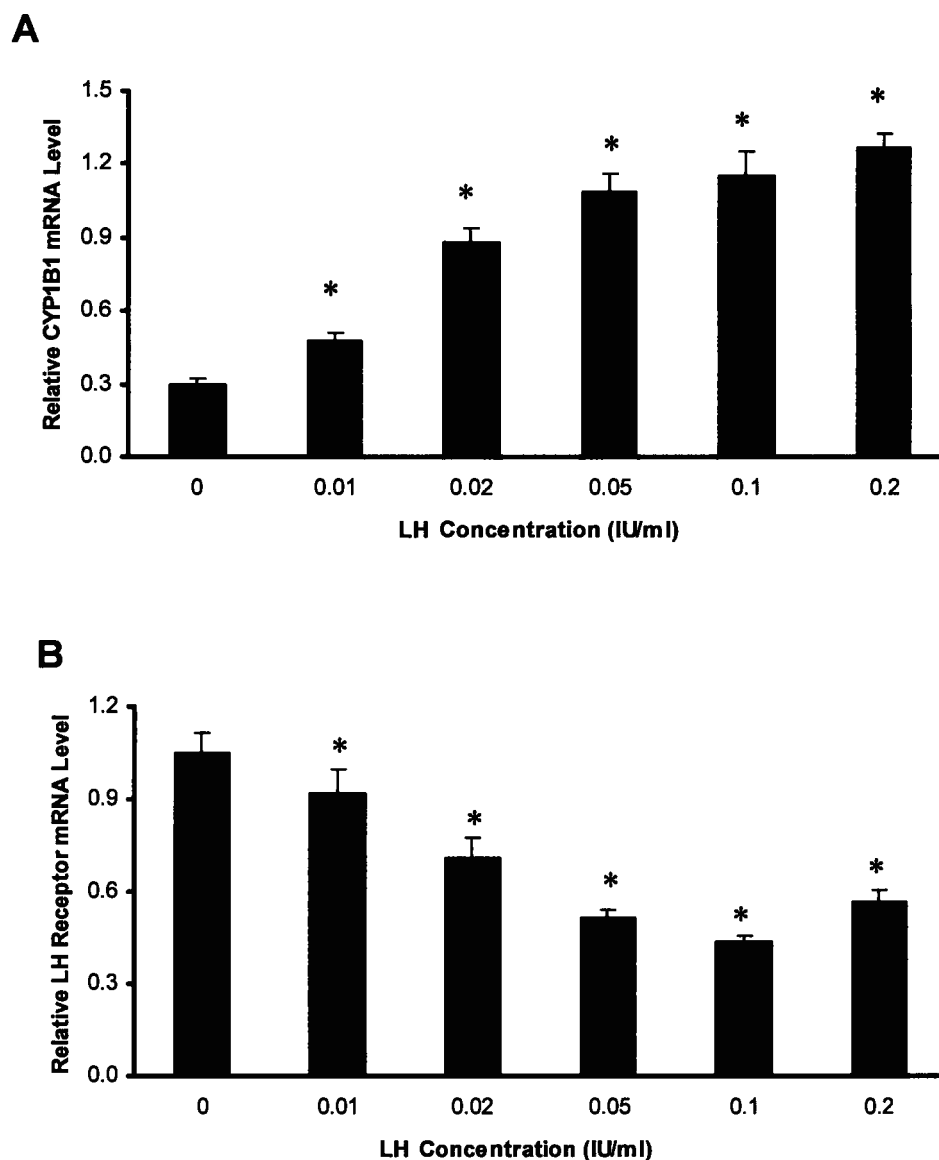


Figure 3.2: Effect of treatment with varying concentrations of LH (from sheep pituitary) on (A) CYP1B1 mRNA or (B) LH receptor mRNA in MA-10 cells. Cells were treated with LH (at a final concentration range of 0.01-0.2 IU/ml in the T25 culture flask) or with water for 12 h. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

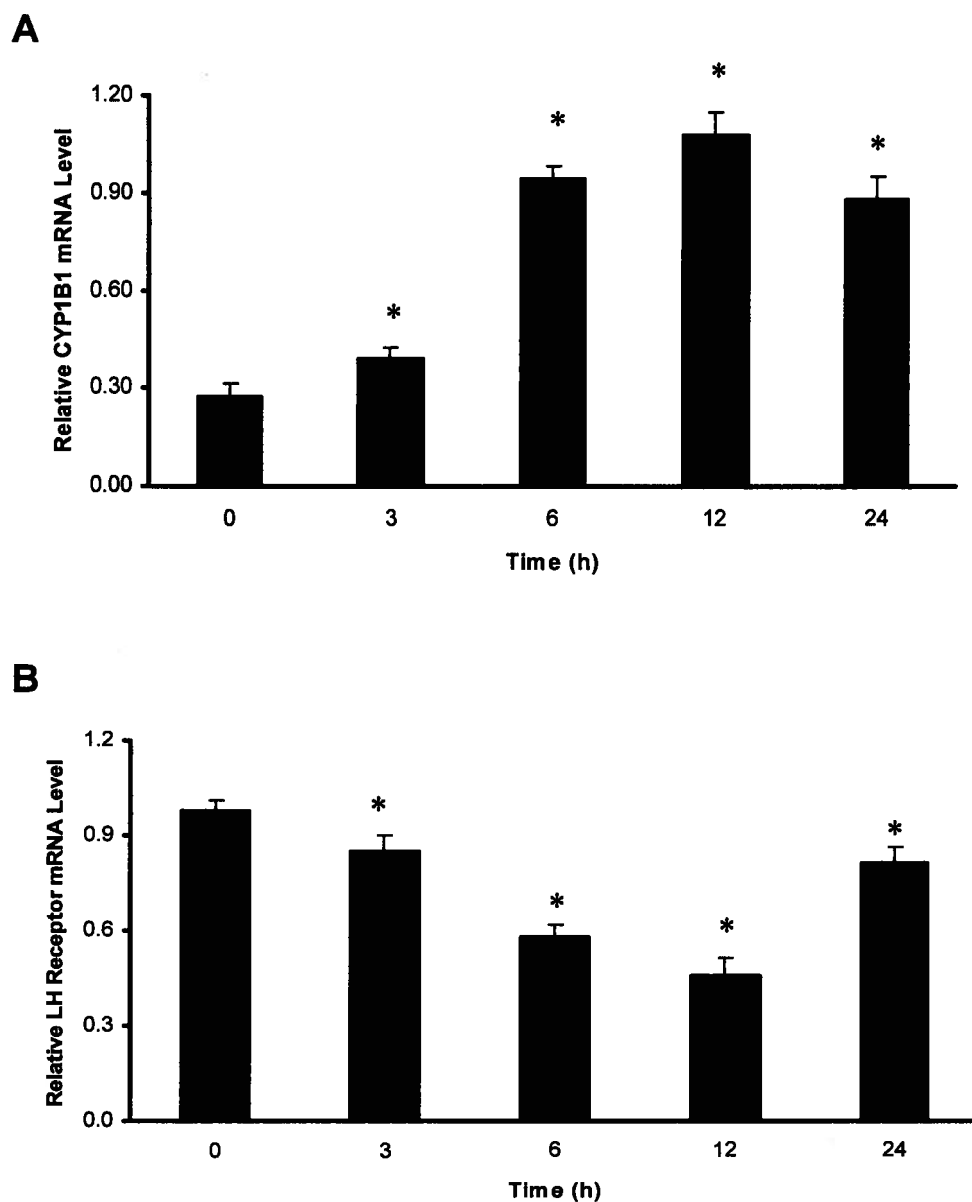


Figure 3.3: Effect of LH treatment for different periods of time on (A) CYP1B1 mRNA or (B) LH receptor mRNA expression in MA-10 cells. Cells were treated with 0.05 IU/ml LH (final concentration in the T25 culture flask) for different periods of time. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (zero- time-point) cells with a P value < 0.05 .

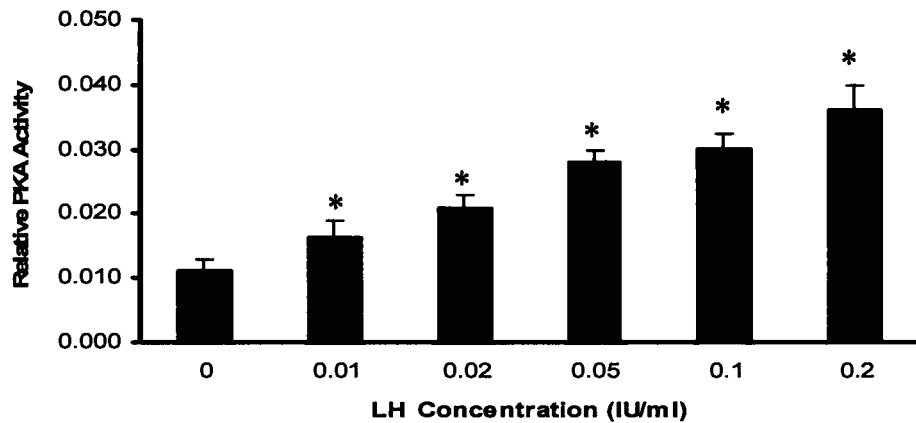


Figure 3.4: Effect of treatment with varying concentrations of LH on relative PKA activity. Cells were treated with LH (at a final concentration range of 0.01-0.2 IU/ml in the T25 culture flask) or with water for 12 h. Relative PKA activity was determined using a nonradiometric ELISA assay. Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

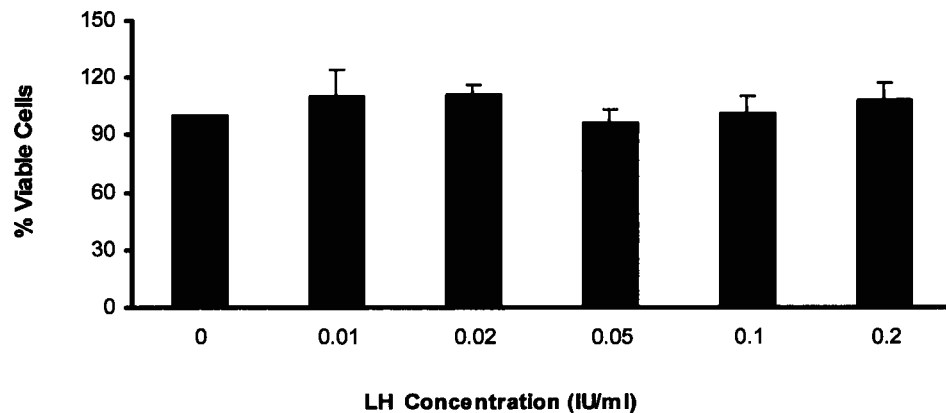


Figure 3.5: Effect of treatment with varying concentrations of LH on cell viability in MA-10 cells. Cells were treated with LH (at a final concentration range of 0.01-0.2 IU/ml in the culture plate well) or with water for 12 h. The MTT assay was carried out according to the procedure described in "Materials and Methods". Viability of LH-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

3.3. Involvement of the PKA Pathway in CYP1B1 mRNA Expression

3.3.1. Effect of 8-Br cAMP (a PKA activator) on CYP1B1 and LH receptor mRNA Expression

To explore the role of the PKA pathway in the regulation of CYP1B1 expression in MA-10 cells, a PKA activator was used. 8-Br-cAMP is a synthetic analog of cAMP (endogenous second messenger) and is known to activate PKA pathways in Leydig cells (Clark et al., 2001). MA-10 cells were treated with varying concentrations of 8-Br-cAMP (0.25-2 mM) for 6 h and CYP1B1 mRNA levels were measured. RT-PCR analysis showed that CYP1B1 mRNA levels increased in a concentration-dependent manner with increasing concentrations of 8-Br-cAMP (Figure 3.6A). Induction of CYP1B1 mRNA levels was observed at all concentrations of 8-Br-cAMP used in this study. CYP1B1 mRNA levels were increased by 4.3 fold relative to vehicle-treated cells after treatment with 8-Br-cAMP at a concentration of 2 mM. In contrast, LH receptor mRNA levels were decreased in a concentration-dependent manner with increasing concentrations of 8-Br-cAMP. Suppression of LH receptor mRNA levels was observed at all concentrations of 8-Br-cAMP used in this study. LH receptor mRNA levels were decreased by approximately 50% compared to vehicle-treated cells after treatment with 8-Br-cAMP at a concentration of 2 mM (Figure 3.6B).

A time course study of the effect of 8-Br-cAMP on CYP1B1 mRNA and LH receptor mRNA expression in MA-10 cells was performed. The inductive effect of 8-Br-cAMP (1 mM) on CYP1B1 mRNA levels and the suppressive effect of 8-Br-cAMP (1 mM) on LH receptor mRNA levels were found to be time-dependent (Figure 3.7). The 8-Br-cAMP-mediated increase in CYP1B1 mRNA levels was observed as early as 3 h after treatment and was greatest after 12 h of treatment. The suppressive effect of 8-Br-cAMP on LH receptor mRNA levels was observed starting at the 6 h-time-point (Figure 3.7). At the 24 h-time-point, CYP1B1 mRNA

levels were decreased compared to levels at the 12 h-time-point. Similarly, LH receptor mRNA levels increased at the 24 h-time-point compared to levels at 12 h-time-point.

3.3.2. Effect of 8-Br cAMP (a PKA activator) on PKA Activity

Relative PKA activity was measured after treatment with varying concentrations of 8-Br-cAMP (0.25-2 mM) to determine if the increase in CYP1B1 mRNA expression was a consequence of stimulation of PKA activity. Relative PKA activity was increased in a dose-dependent manner with increasing concentrations of 8-Br-cAMP (Figure 3.8). Increased PKA activity was observed at all 8-Br-cAMP concentrations used in this study. Relative PKA activity was increased by 4.4-fold compared to vehicle-treated cells after treatment with 8-Br-cAMP (2 mM).

3.3.3. Effect of 8-Br cAMP (a PKA activator) on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with various concentrations of 8-Br-cAMP (0.25-2 mM) on cell viability. According to the results of the MTT-based assay, the viability of MA-10 cells did not change after treatment with 8-Br-cAMP at the concentrations used in this study (Figure 3.9).

The results of treatment of MA-10 cells with 8-Br-cAMP, a PKA activator, show that CYP1B1 mRNA levels and PKA activity were increased by 8-Br-cAMP. The increase in CYP1B1 mRNA expression may be a consequence of PKA activation. In contrast, LH receptor mRNA levels were decreased by 8-Br-cAMP and it appears that the 8-Br-cAMP-elicited increase in CYP1B1 mRNA expression was not related to increased LH receptor expression.

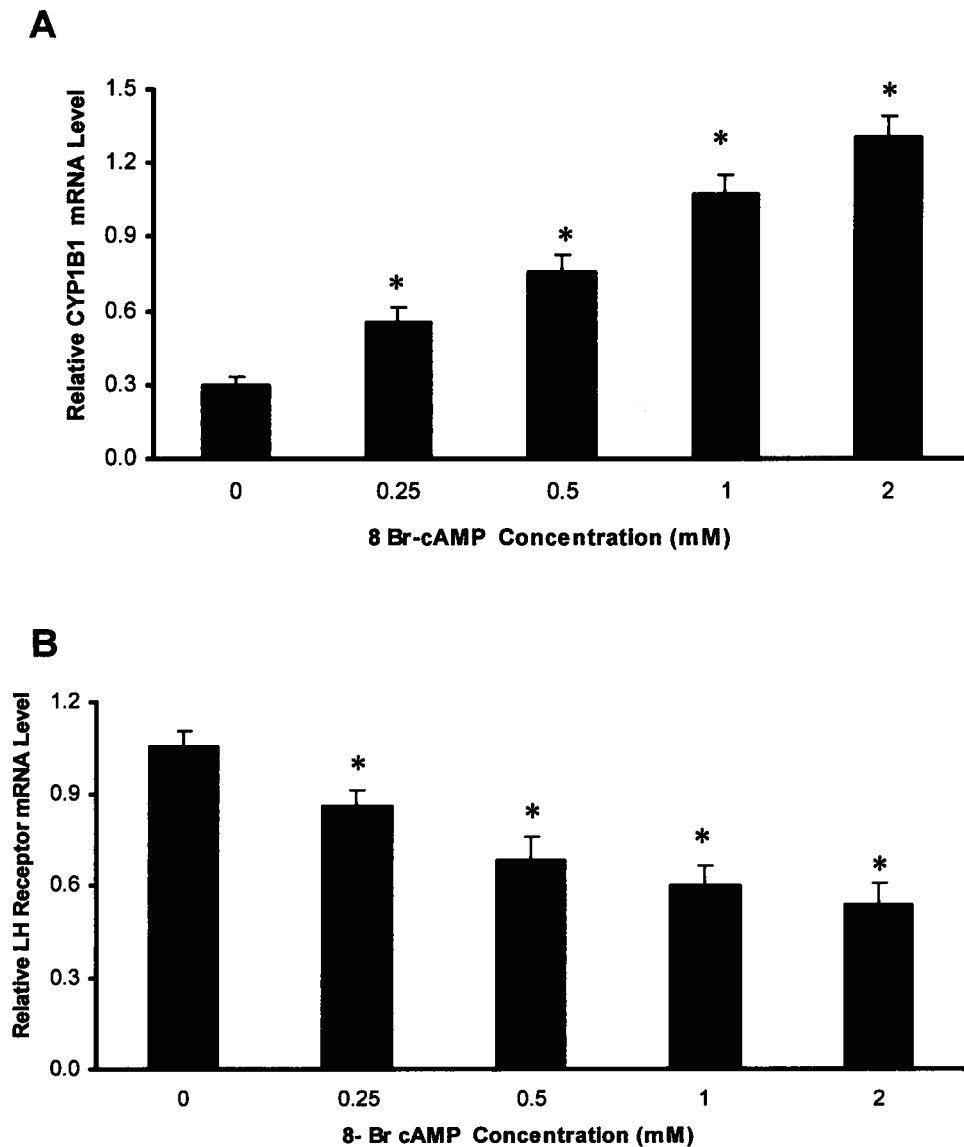


Figure 3.6: Effect of treatment with varying concentrations of 8-Br-cAMP (a PKA activator) on (A) CYP1B1 mRNA and (B) LH receptor mRNA expression in MA-10 cells. Cells were treated with 8-Br-cAMP (at a final concentration range of 0.25-2 mM in the T25 culture flask) or with DMSO for 6 h. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

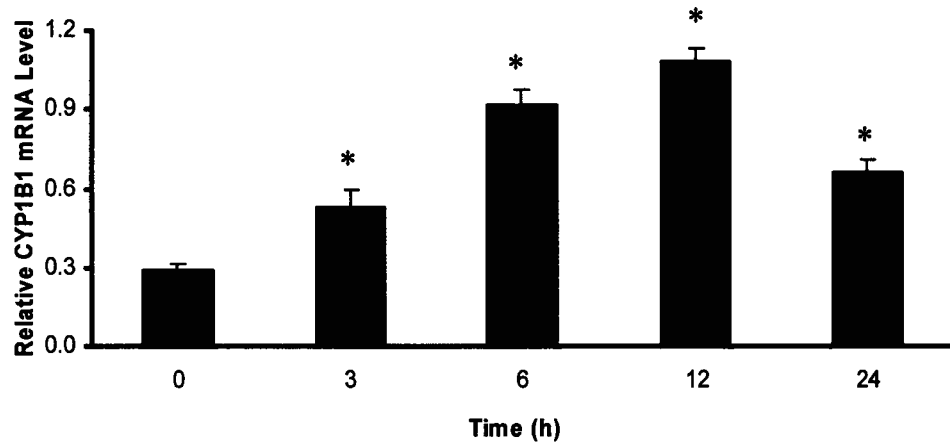
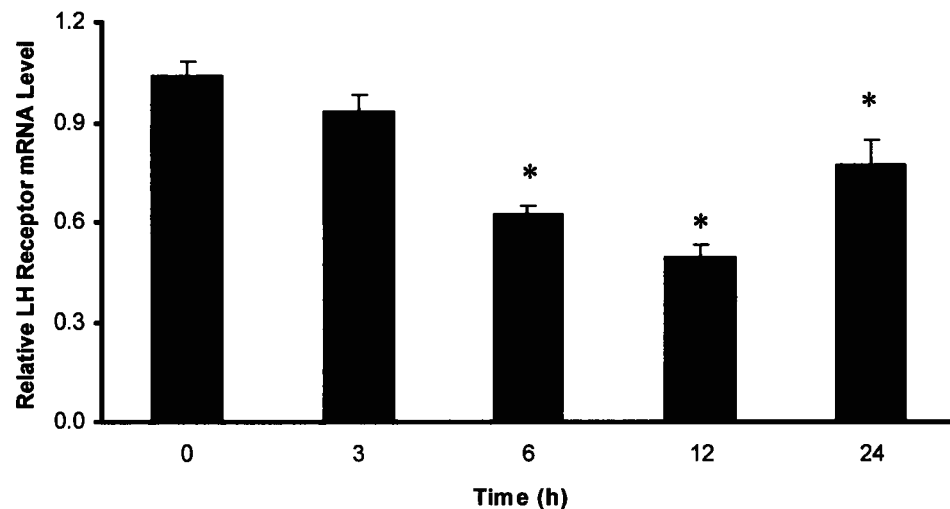
A**B**

Figure 3.7: Effect of 8-Br-cAMP treatment for different periods of time on (A) CYP1B1 mRNA or (B) LH receptor mRNA expression in MA-10 cells. Cells were treated with 1 mM 8-Br-cAMP (final concentration in the T25 flask) for different periods of time. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (zero-time-point) cells with a P value < 0.05 .

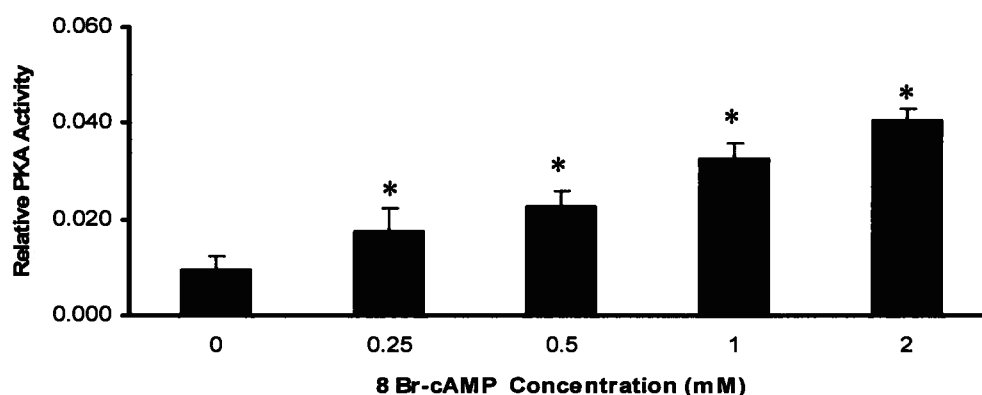


Figure 3.8: Effect of treatment with varying concentrations of 8-Br-cAMP (a PKA activator) on relative PKA activity in MA-10 cells. Cells were treated with 8-Br-cAMP (at a final concentration range of 0.25-2 mM in the T25 culture flask) or with DMSO for 6 h. Relative PKA activity was determined using a nonradiometric ELISA assay. Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *,Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

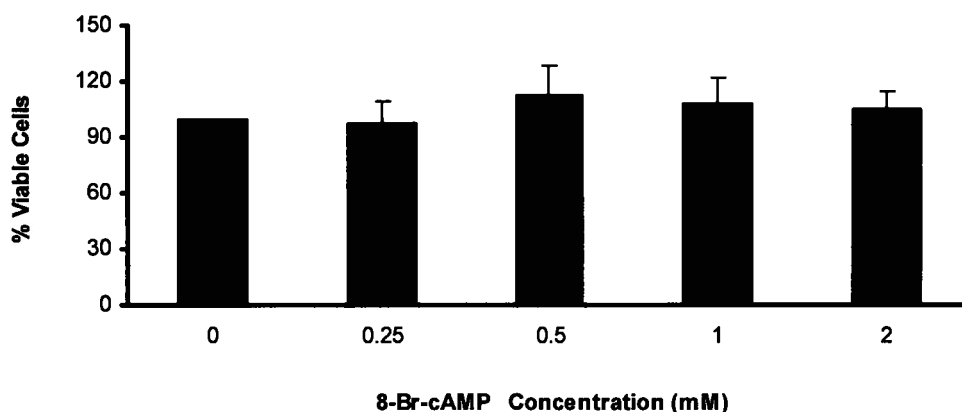


Figure 3.9: Effect of treatment with varying concentrations of 8-Br-cAMP on cell viability in MA-10 cells. Cells were treated with 8-Br-cAMP (at a final concentration range of 0.25-2 mM in the culture plate well) or with DMSO for 6 h. The MTT assay was carried out according to the procedure described in "Materials and Methods". Viability of 8-Br-cAMP-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

3.3.4. Effect of Treatment with H89 (a relatively non-specific PKA inhibitor)

To further explore the role of the PKA pathway in the regulation of CYP1B1 mRNA expression in MA-10 cells, a PKA inhibitor was used. H89 is a non-selective PKA inhibitor that can also block PKC, PKG, and other kinase pathways (Chijiwa et al., 1990; Lochner and Moolman, 2006). MA-10 cells were treated with varying concentrations of H89 (1-100 μ M) for 1 h and CYP1B1 mRNA levels were measured. RT-PCR analysis showed that CYP1B1 mRNA levels decreased in a dose-dependent manner with increasing concentrations of H89 (Figure 3.10A). CYP1B1 mRNA levels were decreased at all concentrations of H89 tested. CYP1B1 mRNA levels were approximately 65% lower, relative to vehicle-treated cells, after treatment with H89 at a concentration of 100 μ M (Figure 3.10A).

A time course study of the effect of H89 (25 μ M) on CYP1B1 mRNA expression in MA-10 cells was also performed. The suppressive effect of H89 treatment on CYP1B1 mRNA levels was found to be time-dependent (Figure 3.10B). The suppressive effect of H89 on CYP1B1 mRNA levels was observed as early as 30 min after treatment (Figure 3.10B).

The effect of varying the concentrations of H89 on relative PKA activity in MA-10 cells was characterized. Relative PKA activity also decreased in a dose-dependent manner with increasing concentrations of H89 (Figure 3.11). Decreased PKA activity was observed at all concentrations of H89 tested. Relative PKA activity was approximately 75% lower, compared to vehicle-treated cells, after treatment with H89 at a concentration of 100 μ M (Figure 3.11).

The MTT-based assay was carried out to assess the effect of treatment with various concentrations of H89 (1-100 μ M) on cell viability. According to the results of the MTT-based assay, the viability of MA-10 cells did not change after treatment with H89 at the concentrations used in this study (Figure 3.12).

8-Br-cAMP is a PKA activator and there is no report of its effect on other kinase pathways. To determine if H89, a PKA inhibitor, can block the increase in CYP1B1 mRNA levels after treatment with a PKA activator (8-Br-cAMP), MA-10 cells were co-treated with 8-Br-cAMP plus H89. Cells were preincubated with H89 (25 μ M) for 1 h, followed by treatment with 8-Br-cAMP (0.25-2 mM) for 6 h. As a positive control, cells were treated with 8-Br cAMP (1 mM) without H89 preincubation. It was observed that H89 attenuated the 8-Br-cAMP-elicited increases in CYP1B1 mRNA levels and PKA activity (Figure 3.13). CYP1B1 mRNA levels and relative PKA activity, which were decreased after treatment with H89 (see 2nd bar), were restored to near basal levels following treatment with increasing concentrations of 8-Br-cAMP.

To determine if the increase in CYP1B1 mRNA levels following treatment with LH is mediated through the PKA pathway, MA-10 cells were co-treated with LH plus H89 (PKA inhibitor). Cells were preincubated with H89 (25 μ M) for 1 h, followed by treatment with LH (0.01-0.2 IU/ml) for 12 h. As a positive control, cells were treated with LH (0.05 IU/ml) without H89 preincubation. It was observed that H89 attenuated the LH-elicited increases in CYP1B1 mRNA levels and PKA activity (Figure 3.14). CYP1B1 mRNA levels and relative PKA activity, which were decreased after treatment with H89 (see 2nd bar), were restored to near basal levels following treatment with increasing concentrations of LH.

The results of treatment of MA-10 cells with H89, a non-selective PKA inhibitor, suggest that a PKA-mediated pathway may be involved in the constitutive CYP1B1 mRNA expression in Leydig cells. The results of co-treatment of MA-10 cells with 8-Br-cAMP (a PKA activator) plus H89 indicate that the increased CYP1B1 mRNA levels after treatment with 8-Br-cAMP was a consequence of PKA activation in MA-10 cells. Similarly, the results of co-treatment with LH plus H89 suggest that a PKA pathway may play an important role in the LH-elicited increase in CYP1B1 mRNA levels.

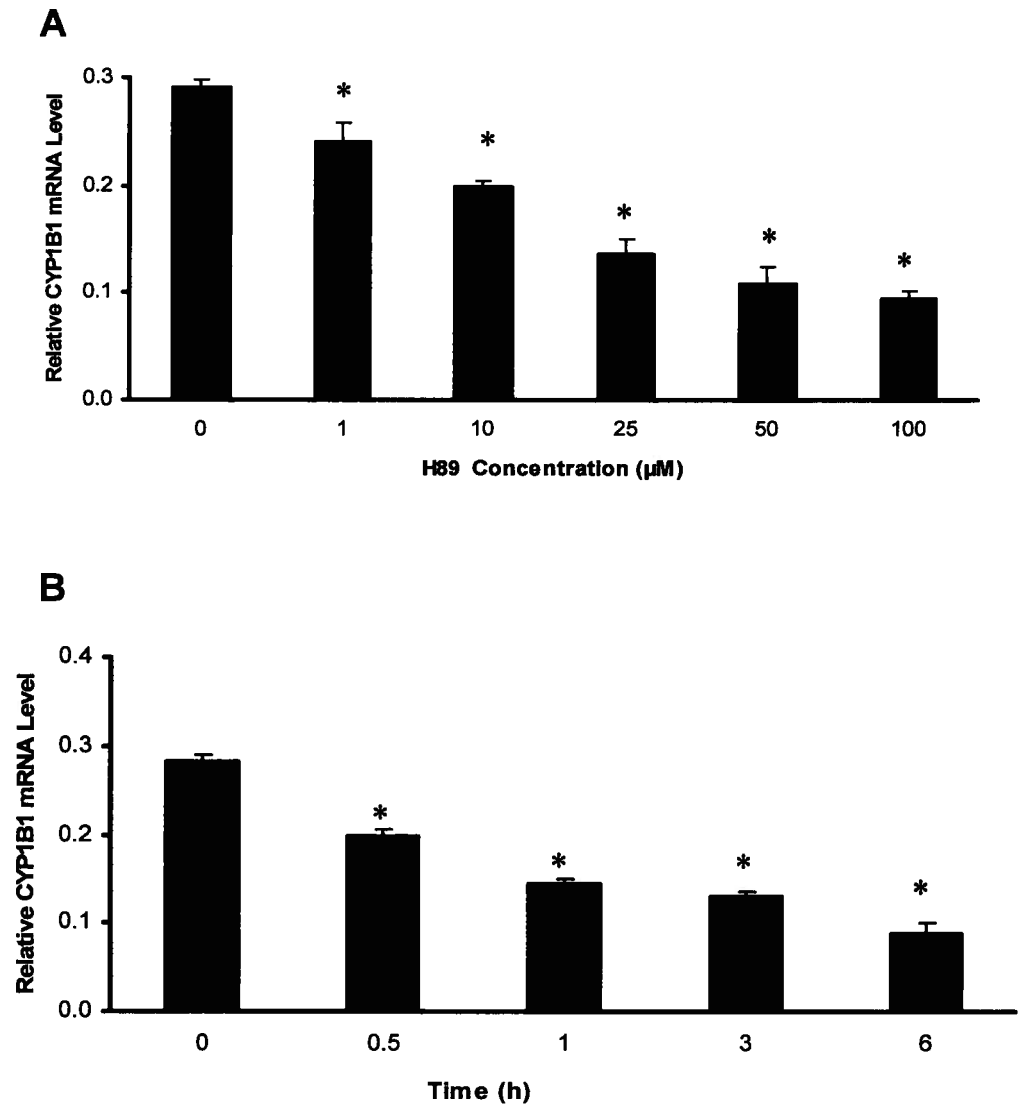


Figure 3.10: Effect of treatment with varying concentrations of H89 (a PKA inhibitor) on CYP1B1 mRNA expression in MA-10 cells. In the concentration response study (A), cells were treated with H89 (at a final concentration range of 1-100μM in the T25 culture flask) or with DMSO for 1 h. In the time course study (B), cells were treated with 25 μM H89 (final concentration in the T25 flask) for different periods of time. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA band to that of the β-actin mRNA band (reference gene). Values shown are the mean ± SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (zero-time-point or vehicle-treated) cells with a *P* value < 0.05.

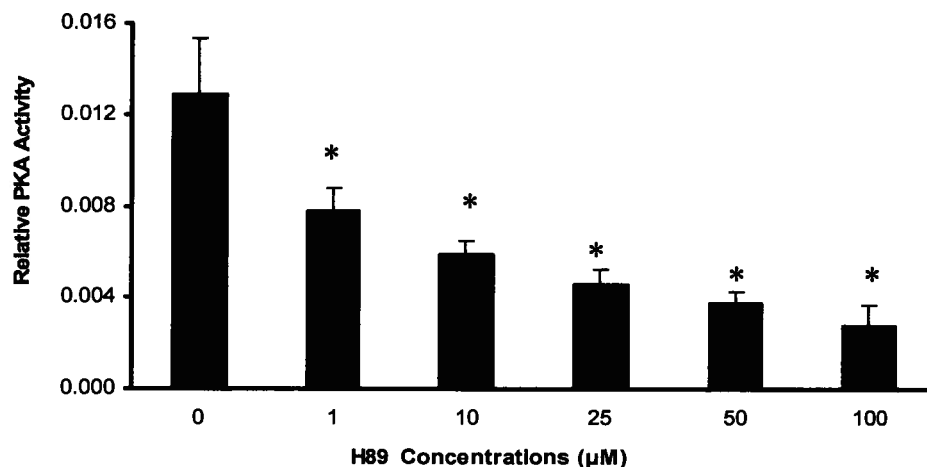


Figure 3.11: Effect of treatment with varying concentrations of H89 on relative PKA activity in MA-10 cells after treatment with H89 (a PKA inhibitor). Cells were treated with H89 (at a final concentration range of 1-100 μ M in the T25 culture flask) or with DMSO for 1 h. Relative PKA activity was determined using a nonradiometric ELISA assay. Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05.

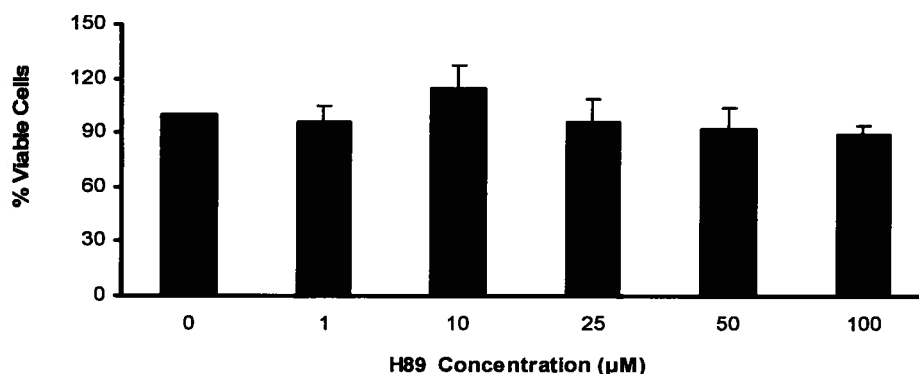


Figure 3.12: Effect of treatment with varying concentrations of H89 on cell viability in MA-10 cells. Cells were treated with H89 (at a final concentration range of 1-100 μ M in the culture plate well) or with DMSO for 1 h. The MTT assay was carried out according to the procedure described in "Materials and Methods". Viability of H89-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

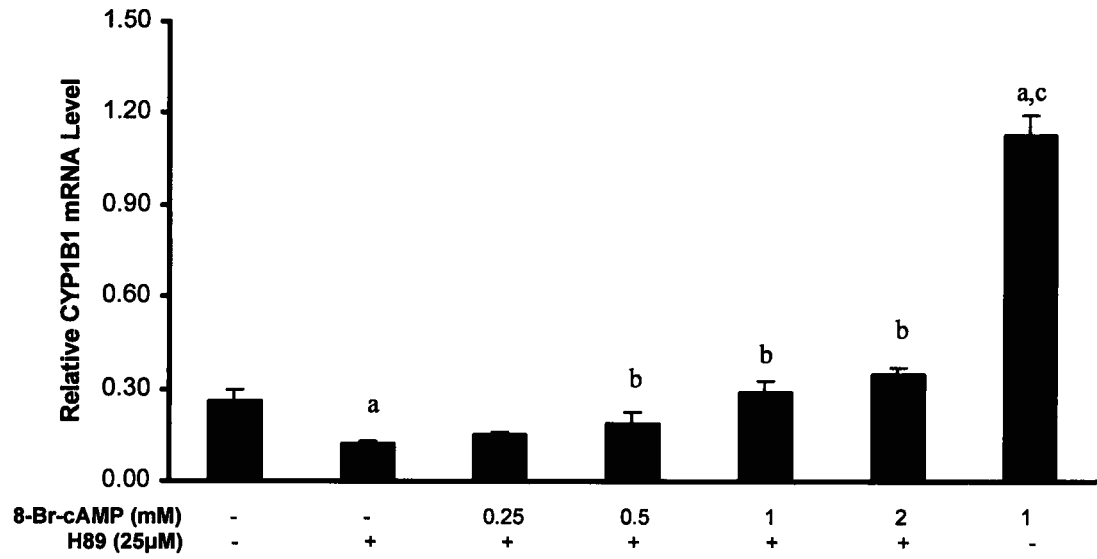
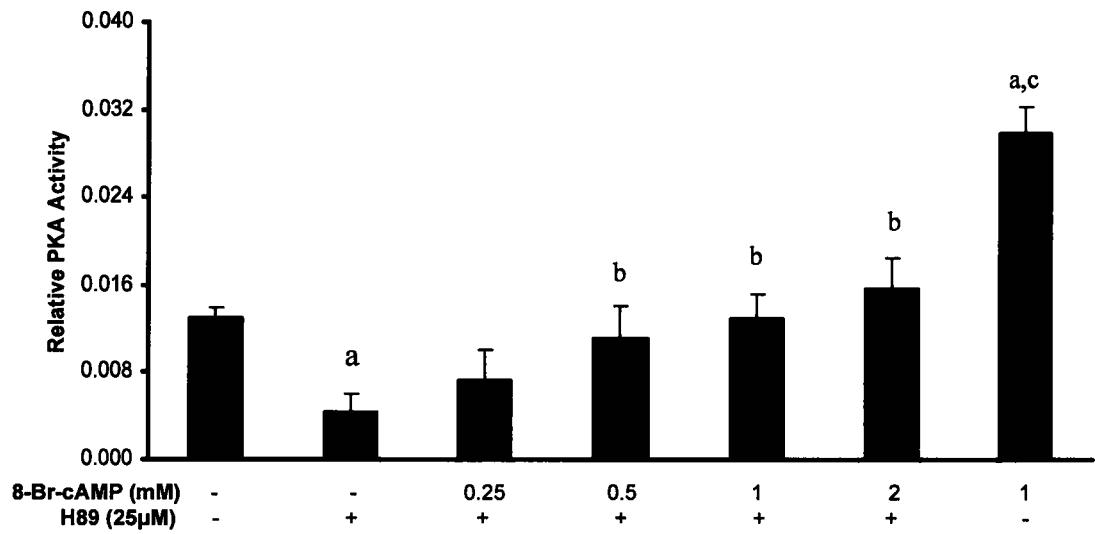
A**B**

Figure 3.13: Effect of co-treatment with H89 (PKA inhibitor) and 8-Br-cAMP on (A) CYP1B1 mRNA expression and (B) relative PKA activity in MA-10 cells. Cells were preincubated with H89 for 1 h and then 8-Br-cAMP was added to the culture medium for 6 h. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels (A). Relative PKA activity was determined using a nonradiometric ELISA assay (B). Values shown are the mean \pm SD of five separate experiments performed on different days. CYP1B1 mRNA levels and PKA activity in cells treated with H89 alone (2nd bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). CYP1B1 mRNA levels and PKA activity in cells treated with H89 alone (2nd bar from the left) were compared with that of cells treated with H89 plus varying concentrations of 8-Br-cAMP (3rd bar, 4th bar, 5th bar and 6th bar from the left) using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. CYP1B1 mRNA levels in cells treated with 8-Br-cAMP alone (7th bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). PKA activity in cells treated with 8-Br-cAMP alone (7th bar from the left) was compared with that of cells treated with vehicle (1st bar from the left) using Mann-Whitney test (nonparametric unpaired t-test). CYP1B1 mRNA levels and PKA activity in cells treated with 8-Br-cAMP alone (7th bar from the left) were compared with that of cells treated with H89 plus 8-Br-cAMP (5th bar from the left) using unpaired student's t-test (parametric). a, Significantly different from vehicle-treated cells (1st bar from the left) with a P value < 0.05 . b, Significantly different from cells that were treated with H89 but not 8-Br-cAMP (2nd bar from the left) with a P value < 0.05 . c, Significantly different from cells that were treated with H89 plus 8-Br-cAMP (5th bar from the left) with a P value < 0.05 .

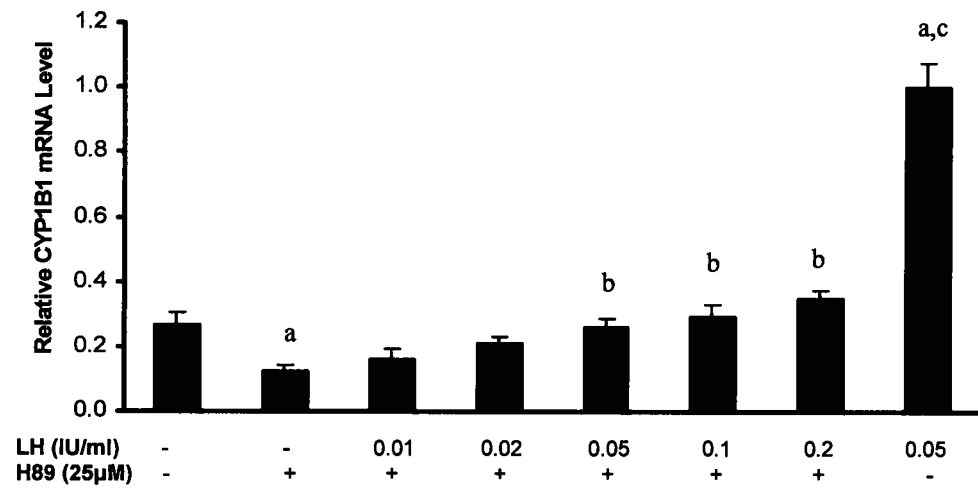
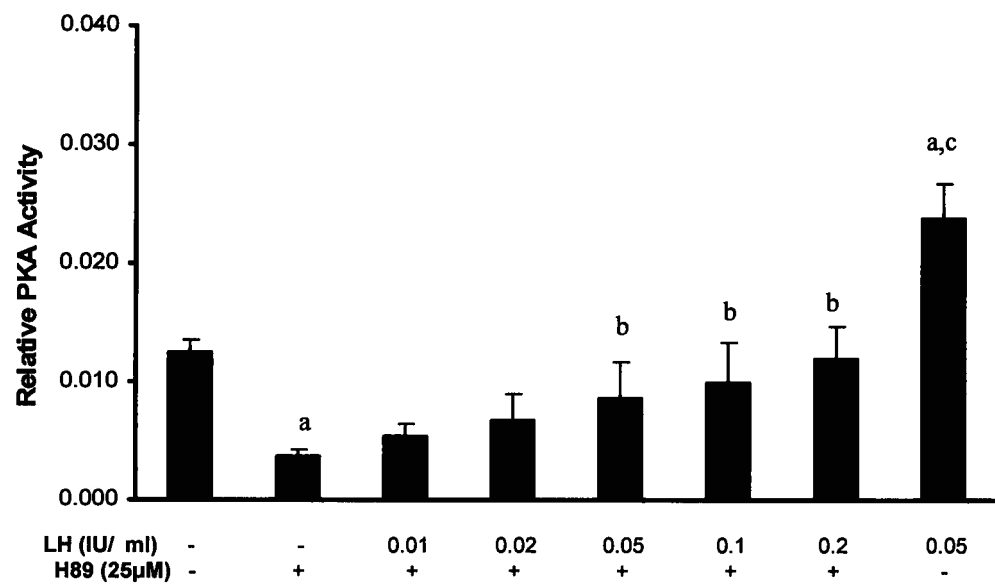
A**B**

Figure 3.14: Effect of co-treatment with H89 (PKA inhibitor) and LH on (A) CYP1B1 mRNA expression and (B) PKA activity in MA-10 cells. Cells were preincubated with H89 for 1 h and then LH was added to the culture medium for 12 h. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels (A). Relative PKA activity was determined using a nonradiometric ELISA assay (B). Values shown are the mean \pm SD of five separate experiments performed on different days. CYP1B1 mRNA levels and PKA activity in cells treated with H89 alone (2nd bar from the left) or with LH alone (8th bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). CYP1B1 mRNA levels and PKA activity in cells treated with H89 alone (2nd bar from the left) were compared with that of cells treated with H89 plus varying concentrations of LH (3rd bar, 4th bar, 5th bar, 6th bar and 7th bar from the left) using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. CYP1B1 mRNA levels and PKA activity in cells treated with LH alone (8th bar from the left) were compared with that of cells treated with H89 plus LH (5th bar from the left) using unpaired student's t-test (parametric). a, Significantly different from vehicle-treated cells (1st bar from the left) with a P value < 0.05 . b, Significantly different from cells that were treated with H89 but not LH (2nd bar from the left) with a P value < 0.05 . c, Significantly different from cells that were treated with H89 plus LH (5th bar from the left) with a P value < 0.05 .

3.3.5. Effect of Treatment with Rp-CPT-cAMP (a specific PKA inhibitor)

Rp-CPT-cAMP is a more specific PKA inhibitor than H89 and does not inhibit other kinase enzymes (Lochner and Moolman, 2006). Rp-CPT-cAMP was used to verify the involvement of the PKA pathway in CYP1B1 mRNA expression. MA-10 cells were treated with varying concentrations of Rp-CPT-cAMP (1-150 μ M) for 1 h and CYP1B1 mRNA levels were measured. RT-PCR analysis showed that CYP1B1 mRNA levels decreased in a concentration-dependent manner with increasing concentrations of Rp-CPT-cAMP (Figure 3.15A). The results are similar to those observed with H89 treatment (previous section). CYP1B1 mRNA levels were approximately 70% lower, relative to vehicle-treated cells, after treatment with Rp-CPT-cAMP at a concentration of 150 μ M (Figure 3.15A).

A time course study of the effect of Rp-CPT-cAMP (25 μ M) on CYP1B1 mRNA expression in MA-10 cells was also performed. As was observed with H89 treatment, the suppressive effect of Rp-CPT-cAMP treatment on CYP1B1 mRNA levels was time-dependent and was observed as early as 30 min after treatment (Figure 3.15B).

The effect of varying the concentration of Rp-CPT-cAMP on relative PKA activity in MA-10 cells was characterized. Relative PKA activity decreased in a concentration-dependent manner with increasing concentrations of Rp-CPT-cAMP (Figure 3.16). Relative PKA activity was approximately 90% lower, compared to vehicle-treated cells, after treatment with Rp-CPT-cAMP at a concentration of 150 μ M (Figure 3.16).

The MTT-based assay was carried out to assess the effect of treatment with Rp-CPT-cAMP (1-150 μ M) on cell viability. The viability of MA-10 cells did not change after treatment with Rp-CPT-cAMP at the concentrations used in this study (Figure 3.17).

To further explore if PKA is the major kinase responsible for the increase in CYP1B1 mRNA levels after treatment with LH, MA-10 cells were co-treated with Rp-CPT-cAMP plus

LH. Cells were preincubated with Rp-CPT-cAMP (25 μ M) for 1 h, followed by treatment with LH (0.01-0.2 IU/ml) for 12 h. As a positive control, cells were treated with LH (0.05 IU/ml) without Rp-CPT-cAMP preincubation. It was observed that Rp-CPT-cAMP attenuated the LH-elicited increase in CYP1B1 mRNA levels and relative PKA activity (Figure 3.18). CYP1B1 mRNA levels and relative PKA activity, which were decreased after treatment with Rp-CPT-cAMP (see 2nd bar), were restored to near basal levels following treatment with increasing concentrations of LH (Figure 3.18).

The results of treatment of MA-10 cells with Rp-CPT-cAMP, a specific PKA inhibitor, suggest that a PKA-mediated pathway was involved in the constitutive expression of CYP1B1 mRNA. The results of co-treatment of MA-10 cells with LH plus Rp-CPT-cAMP indicate that LH-elicited increase in CYP1B1 mRNA levels was mediated through a PKA pathway.

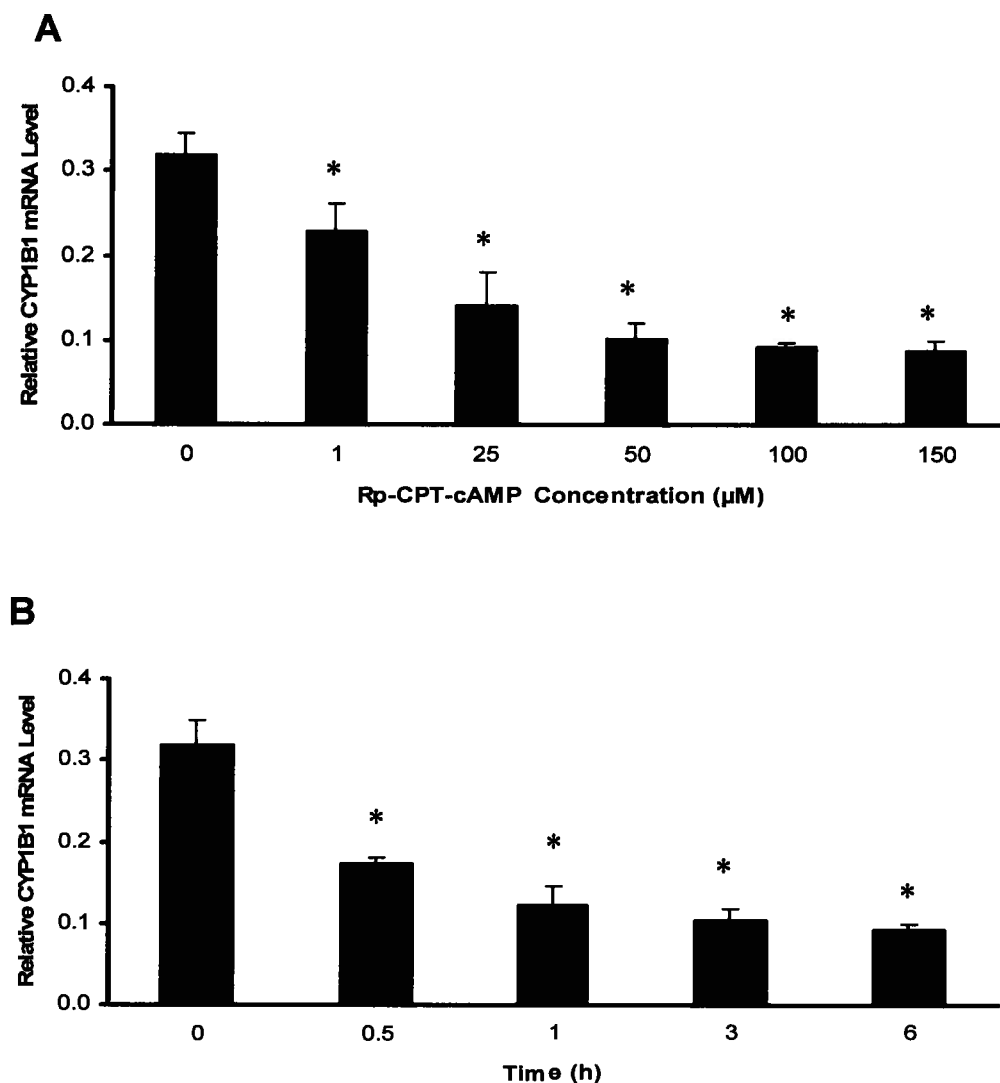


Figure 3.15: Effect of treatment with varying concentrations of Rp-CPT-cAMP (a specific PKA inhibitor) on CYP1B1 mRNA expression in MA-10 cells. In the concentration response study (A), cells were treated with Rp-CPT-cAMP (at a final concentration range of 1-150 μM in the T25 culture flask) or with water for 1 h. In the time course study (B), cells were treated with 25 μM Rp-CPT-cAMP (final concentration in the T25 flask) for different periods of time. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated or zero-time-point) cells with a P value < 0.05 .

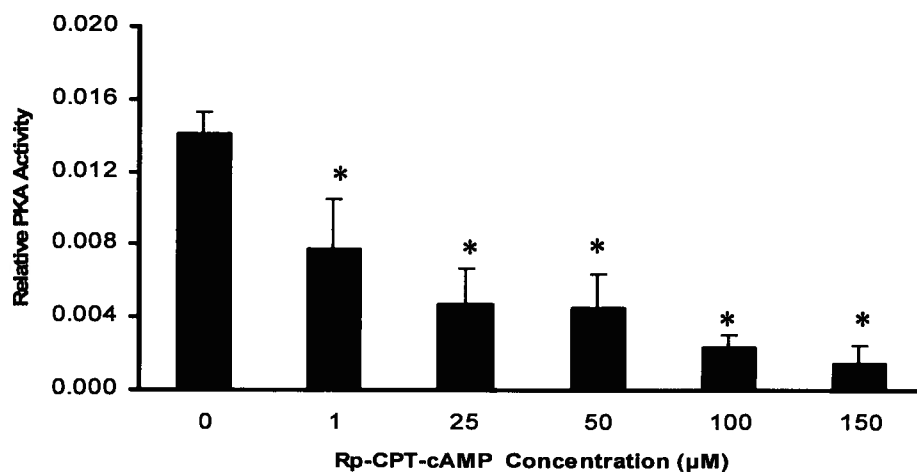


Figure 3.16: Effect of treatment with varying concentrations of Rp-CPT-cAMP (a specific PKA inhibitor) on relative PKA activity in MA-10 cells. Cells were treated with Rp-CPT-cAMP (at a final concentration range of 1-150 μM in the T25 culture flask) or with water for 1 h. Relative PKA activity was determined using a nonradiometric ELISA assay. Values shown are the mean \pm SD of five separate experiments performed on different days. Data were analyzed using Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a P value < 0.05 .

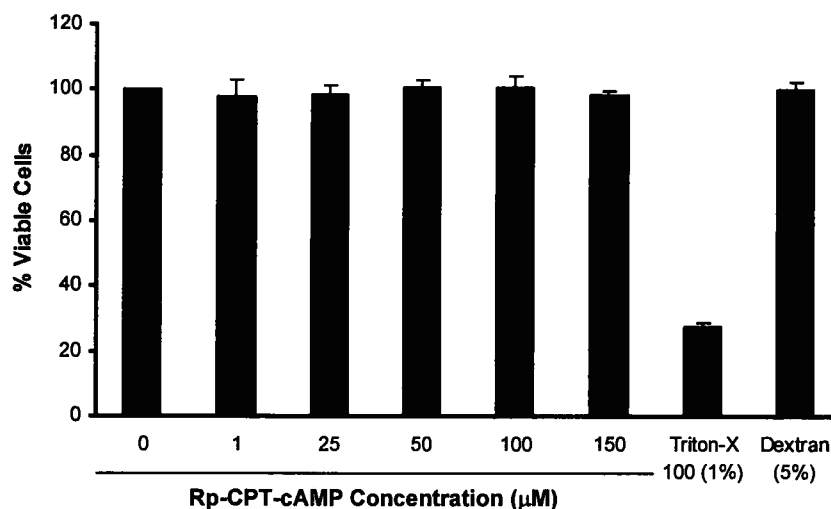


Figure 3.17: Effect of treatment with varying concentrations of Rp-CPT-cAMP on cell viability in MA-10 cells. Cells were treated with Rp-CPT-cAMP (at a final concentration range of 1-150 μM in the culture plate well) or with water for 1 h. The MTT assay was carried out according to the procedure described in "Materials and Methods". Viability of Rp-CPT-cAMP-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

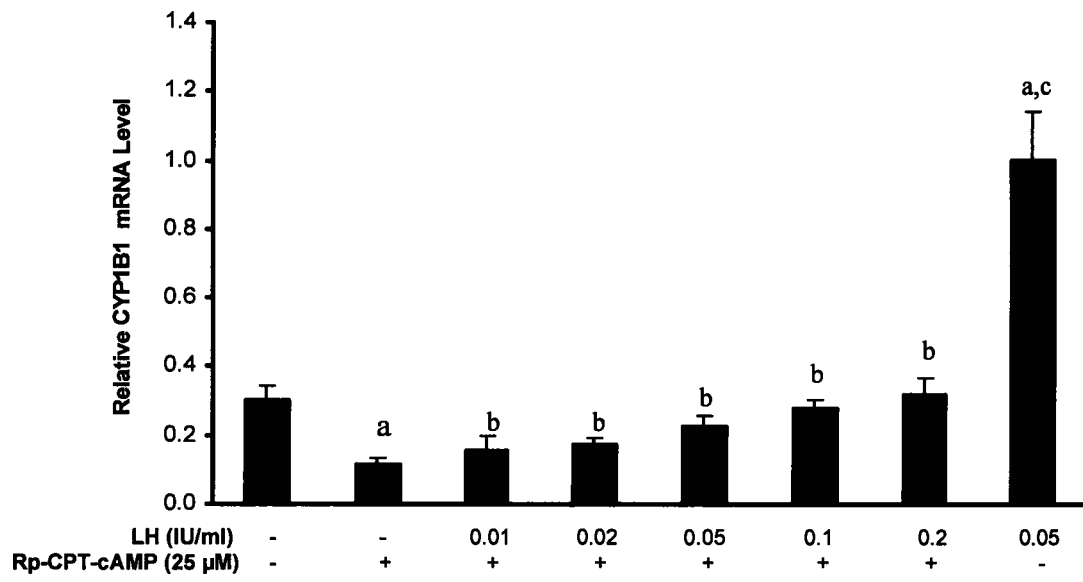
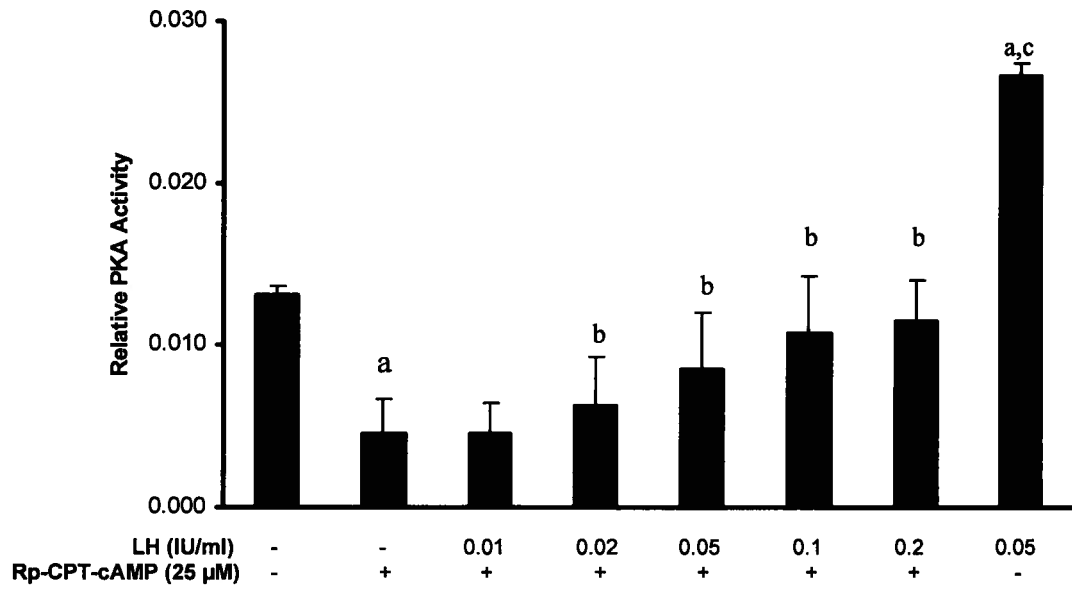
A**B**

Figure 3.18: Effect of co-treatment with Rp-CPT-cAMP (a specific PKA inhibitor) and LH on (A) CYP1B1 mRNA expression and (B) relative PKA activity in MA-10 cells. Cells were preincubated with Rp-CPT-cAMP for 1 h and then LH was added to the culture medium for 12 h. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels (A). Relative PKA activity was determined using a nonradiometric ELISA assay (B). Values shown are the mean \pm SD of five separate experiments performed on different days. CYP1B1 mRNA levels in cells treated with Rp-CPT-cAMP alone (2nd bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). PKA activity in cells treated with Rp-CPT-cAMP alone (2nd bar from the left) was compared with that of cells treated with vehicle (1st bar from the left) using Mann-Whitney test (nonparametric unpaired t-test). CYP1B1 mRNA levels in cells treated with Rp-CPT-cAMP alone (2nd bar from the left) were compared with that of cells treated with Rp-CPT-cAMP plus varying concentrations of LH (3rd bar, 4th bar, 5th bar, 6th bar and 7th bar from the left) using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. PKA activity in cells treated with Rp-CPT-cAMP alone (2nd bar from the left) were compared with that of cells treated with Rp-CPT-cAMP plus varying concentrations of LH (3rd bar, 4th bar, 5th bar, 6th bar and 7th bar from the left) using Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul's multiple comparison test. CYP1B1 mRNA levels and PKA activity in cells treated with LH alone (8th bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). CYP1B1 mRNA levels in cells treated with LH alone (8th bar from the left) were compared with that of cells treated with Rp-CPT-cAMP plus LH (5th bar from the left) using unpaired student's t-test (parametric). PKA activity in cells treated with LH alone (8th bar from the left) were compared with that of cells treated with Rp-CPT-cAMP plus LH (5th bar from the left) using Mann-Whitney test (nonparametric unpaired t-test). a, Significantly different from vehicle-treated cells (1st bar from the left) with a *P* value < 0.05. b, Significantly different from cells that were treated with Rp-CPT-cAMP but not LH (2nd bar from the left) with a *P* value < 0.05. c, Significantly different from cells that were treated with Rp-CPT-cAMP plus LH (5th bar) with a *P* value < 0.05.

3.4. Involvement of PKG and PKC Pathways in CYP1B1 mRNA Expression

3.4.1. Effect of 8-Br-cGMP (a PKG activator) on CYP1B1 mRNA Expression

PKG is involved in the regulation of steroidogenesis in testis and regulatory pathways mediated by PKG are active in Leydig cells (Andric et al., 2007). 8-Br-cGMP is an activator of PKG (Andric et al., 2007) and was used in this study to determine if a PKG-mediated pathway was involved in the regulation of CYP1B1 mRNA expression in Leydig cells. MA-10 cells were treated with varying concentrations of 8-Br-cGMP (0.05-10 mM) for 6 h. RT-PCR analysis showed that treatment with 8-Br-cGMP, at the concentrations used in this study, had no effect on CYP1B1 mRNA levels in MA-10 cells (Figure 3.19A), suggesting that the PKG pathway plays little or no role in the regulation of CYP1B1 expression.

A time course study of the effect of 8-Br-cGMP (1 mM) on CYP1B1 mRNA expression in MA-10 cells was also performed. Treatment with 8-Br-cGMP (1 mM) had no effect on CYP1B1 mRNA levels in MA-10 cells up to 24 h after treatment (Figure 3.19B)

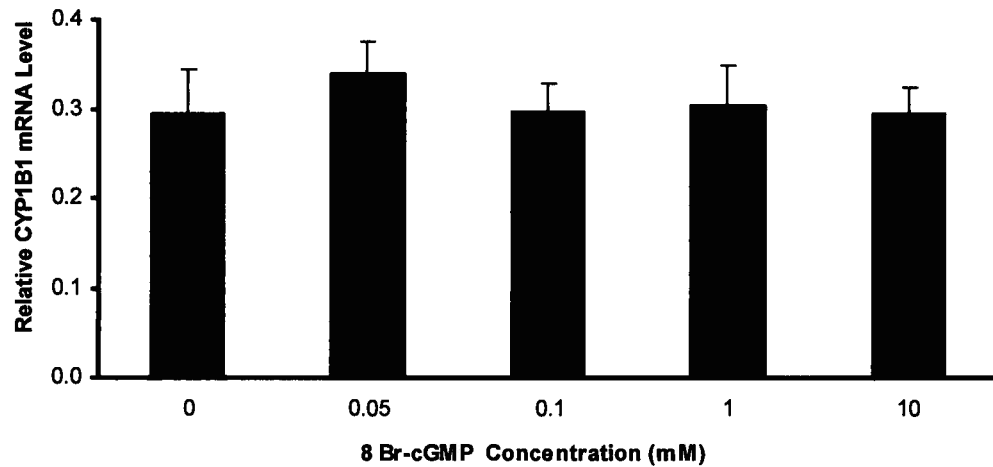
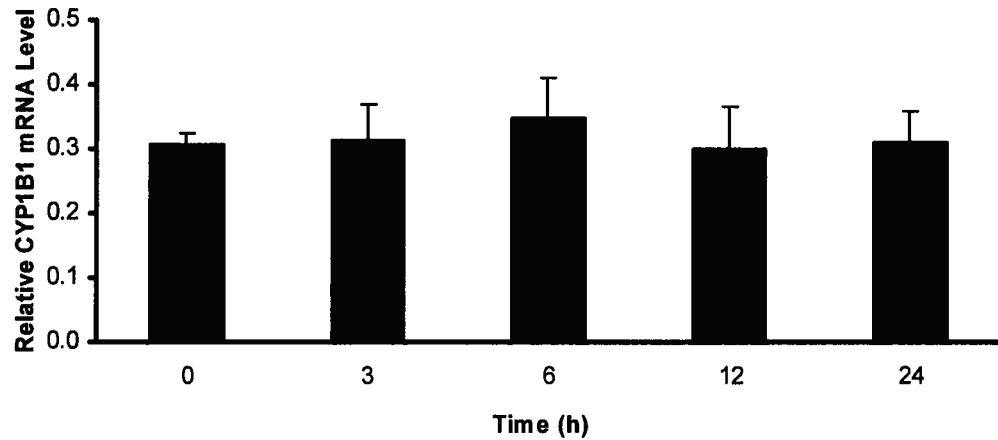
A**B**

Figure 3.19: Effect of treatment with varying concentrations of 8-Br-cGMP (a PKG activator) on CYP1B1 mRNA expression in MA-10 cells. In the concentration response study (A), cells were treated with 8-Br-cGMP (at a final concentration range of 0.05-10 mM in the T25 culture flask) or with water for 6 h. In the time course study (B), cells were treated with 1 mM 8-Br-cGMP (final concentration in the T25 flask) for different periods of time. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days.

3.4.2. Effect of Go 6983 (a specific PKC inhibitor) on CYP1B1 mRNA Expression

PKC pathways are involved in the regulation of steroidogenic enzymes in Leydig cells (Jo et al., 2005). PKC pathways are activated by LH in Leydig cells (Jo et al., 2005). In addition to PKA, H89 can also act as a PKC inhibitor. H89-elicited suppression of constitutive CYP1B1 mRNA expression in MA-10 cells may be partly due to inhibition of PKC-mediated pathways. In this study, Go 6983, a specific PKC inhibitor, was used to investigate the putative role of PKC in constitutive CYP1B1 mRNA expression. MA-10 cells were treated with varying concentrations of Go 6983 (1-200 μ M) for 1 h and CYP1B1 mRNA levels were measured. RT-PCR analysis showed that treatment with Go 6983, at the concentrations used in this study, did not affect constitutive CYP1B1 mRNA levels in MA-10 cells (Figure 3.20A).

A time course study of the effect of Go 6983 (25 μ M) on CYP1B1 mRNA expression in MA-10 cells was also performed. Treatment with Go 6983 (25 μ M) had no effect on CYP1B1 mRNA levels in MA-10 cells up to 48 h after treatment (Figure 3.20B)

MA-10 cells were also co-treated with Go 6983 plus LH to determine if PKC is involved in the process leading to induction of CYP1B1 mRNA after LH treatment. Cells were preincubated with Go 6983 (100 μ M) for 1 h, followed by treatment with LH at various concentrations (0.01-0.2 IU/ml) for 12 h. As a positive control, cells were treated with LH (0.05 IU/ml) without Go 6983 preincubation. Co-treatment of MA-10 cells with Go 6983 plus LH did not attenuate the LH-elicited increase in CYP1B1 mRNA levels (Figure 3.21).

The results of treatment of MA-10 cells with a PKG activator and a PKC inhibitor suggest that PKG- and PKC-mediated pathways have little or no role in the regulation of CYP1B1 mRNA expression. The results from co-treatment of MA-10 cells with LH plus Go 6983 (PKC inhibitor) suggest that PKC was not involved in the LH-elicited increase in CYP1B1 mRNA levels in Leydig cells.

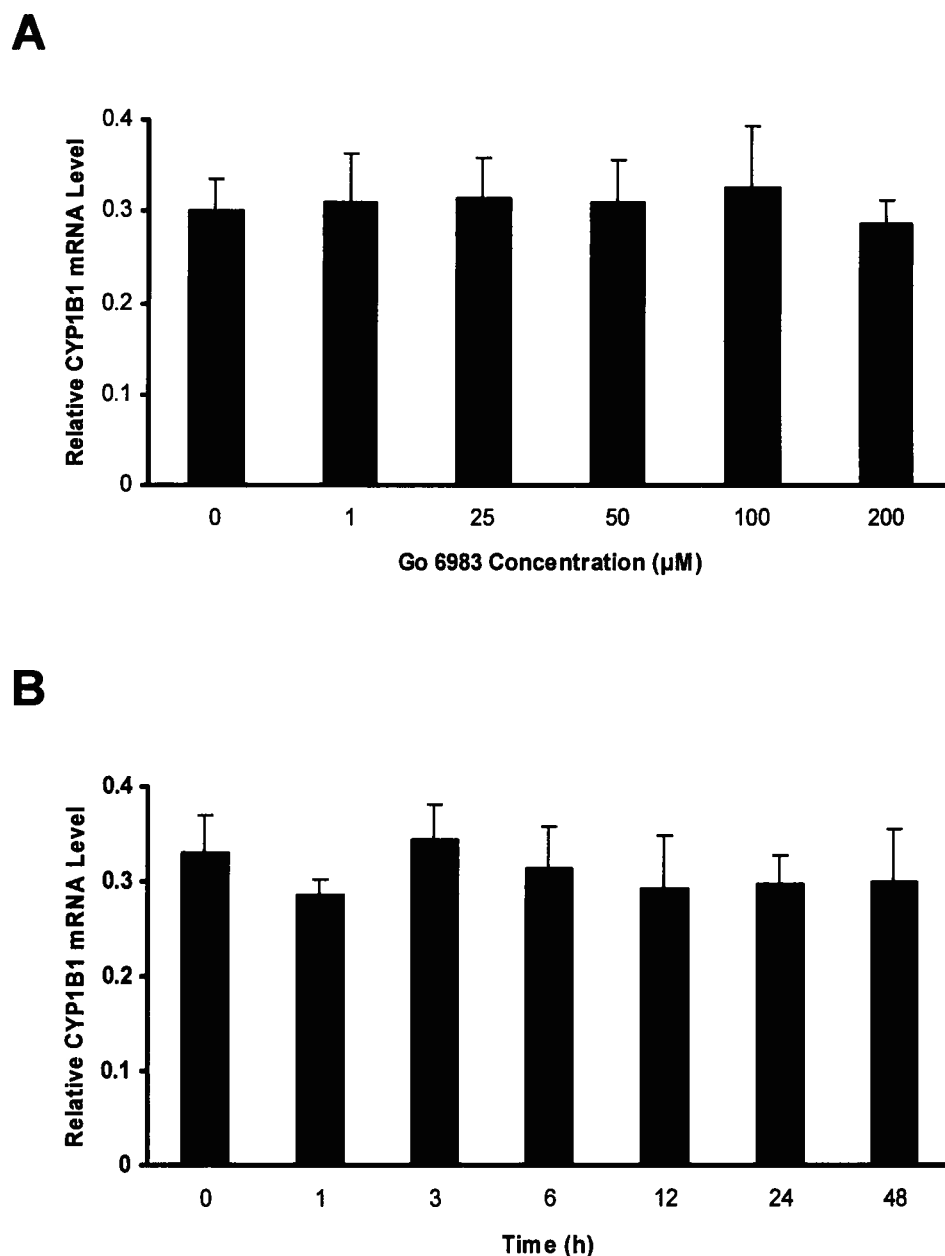


Figure 3.20: Effect of treatment with varying concentrations of Go 6983 (a specific PKC inhibitor) on CYP1B1 mRNA expression in MA-10 cells. In the concentration response study (A), cells were treated with Go 6983 (at a final concentration range of 1-200 μ M in the T25 culture flask) or with DMSO for 1 h. In the time course study (B), cells were treated with 25 μ M Go 6983 (final concentration in the T25 flask) for different periods of time. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days.

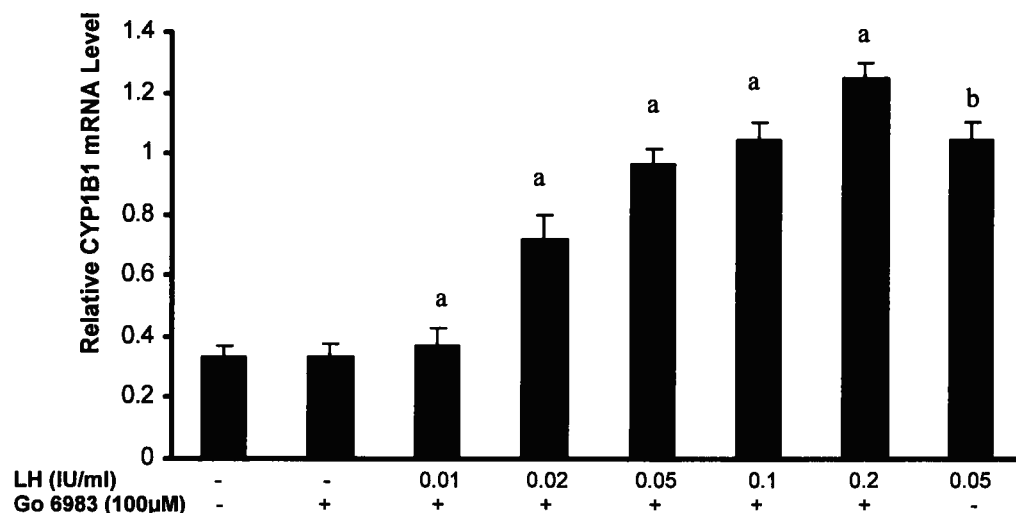


Figure 3.21: Effect of co-treatment with Go 6983 (PKC inhibitor) and LH on CYP1B1 mRNA expression in MA-10 cells. Cells were preincubated with Go 6983 for 1 h and then LH was added to the culture medium for 12 h. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Values shown are the mean \pm SD of three separate experiments performed on different days. CYP1B1 mRNA levels in cells treated with Go 6983 alone (2nd bar from the left) were compared with that of cells treated with H89 plus varying concentrations of LH (3rd bar, 4th bar, 5th bar, 6th bar and 7th bar from the left) using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. CYP1B1 mRNA levels in cells treated with LH alone (8th bar from the left) were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). a, Significantly different from cells that were treated with Go 6983 but not LH (2nd bar from the left) with a P value < 0.05 . b, Significantly different from vehicle-treated cells (1st bar from the left) with a P value < 0.05 .

3.5. Regulation of CYP1B1 mRNA Expression by Estradiol Benzoate

It was previously shown in our laboratory that testicular CYP1B1 protein levels in adult rats were decreased in rats treated with estradiol benzoate (1.5 mg/kg for 6 days) (Leung et al., 2009). Results from in vivo and in vitro studies involving CYP17A1 and CYP11A1 suggest that the suppressive effect of estradiol on CYP expression may be indirect and mediated through pathways regulated by LH (Akingbemi et al., 2003; Cigorrage et al., 1980). Estradiol benzoate was used in this study to determine if CYP1B1 expression in MA-10 cells was suppressed by estradiol and if this effect was mediated through estrogen receptor and/or through pathway(s) modulated by LH.

3.5.1. Effect of Estradiol Benzoate on CYP1B1 mRNA and LH receptor mRNA Expression

To characterize the effect of estradiol benzoate on CYP1B1 expression, dose-response and time-course experiments were performed and CYP1B1 mRNA levels were measured in MA-10 cells. Mouse Leydig MA-10 cells were treated with varying concentrations of estradiol benzoate (10-500 nM) for 24 h. RT-PCR analysis showed that CYP1B1 mRNA levels exhibited a concentration-dependent decrease with increasing concentrations of estradiol benzoate (Figure 3.22A). CYP1B1 mRNA levels were approximately 75% lower, compared to vehicle-treated cells, following treatment with estradiol benzoate at a concentration of 500 nM.

LH receptor mRNA expression was studied to determine if downregulation of LH receptor expression is responsible for the suppressive effect of estradiol benzoate on CYP1B1 mRNA levels. RT-PCR analysis showed that treatment with estradiol benzoate, at the concentrations used in this study, did not affect LH receptor mRNA levels in MA-10 cells (Figure 3.22B).

The suppressive effect of estradiol benzoate (500 nM) on CYP1B1 mRNA levels was time-dependent (Figure 3.23A). Decreased CYP1B1 mRNA levels were observed at all time

points tested and were greatest after 72 h of treatment (Figure 3.23A). Treatment of MA-10 cells with estradiol benzoate (500 nM) had no effect on LH receptor mRNA levels cells up to 72 h after treatment (Figure 3.23B).

3.5.2. Effect of Estradiol Benzoate on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with various concentrations of estradiol benzoate (10-500 nM) on cell viability. According to the results of the MTT-based assay, the viability of MA-10 cells did not change after treatment with estradiol benzoate at the concentrations used in this study (Figure 3.24).

The results of treatment with estradiol benzoate suggest that CYP1B1 mRNA expression in MA-10 cells was suppressed by estradiol benzoate. In contrast, treatment with estradiol benzoate had no effect on LH receptor mRNA expression. The decrease in CYP1B1 mRNA expression after estradiol benzoate treatment does not appear to be related to LH receptor expression.

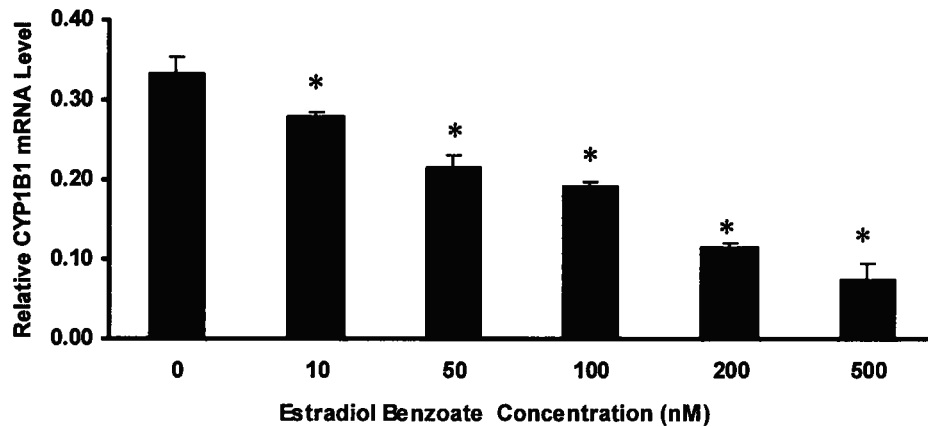
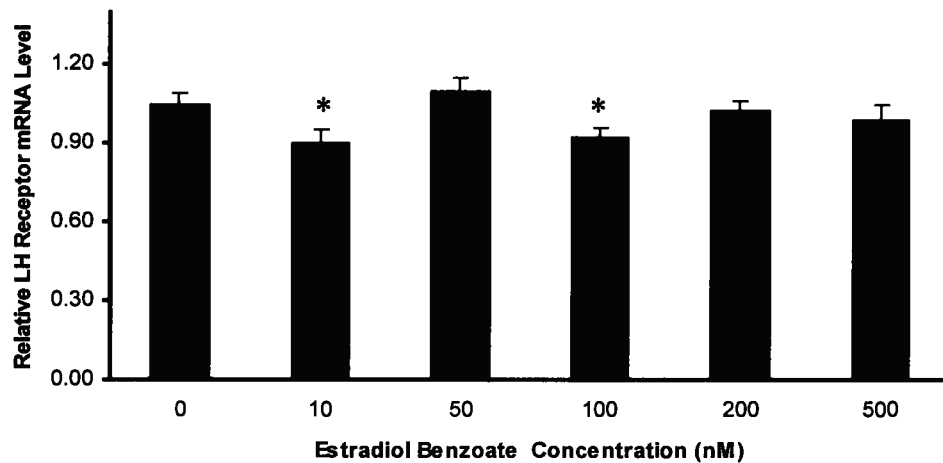
A**B**

Figure 3.22: Effect of treatment with varying concentrations of estradiol benzoate on (A) CYP1B1 mRNA expression and (B) LH receptor mRNA expression in MA-10 cells. Cells were treated with estradiol (at a final concentration range of 10-500 nM in the T25 culture flask) or with DMSO for 24 h. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) cells with a *P* value < 0.05.

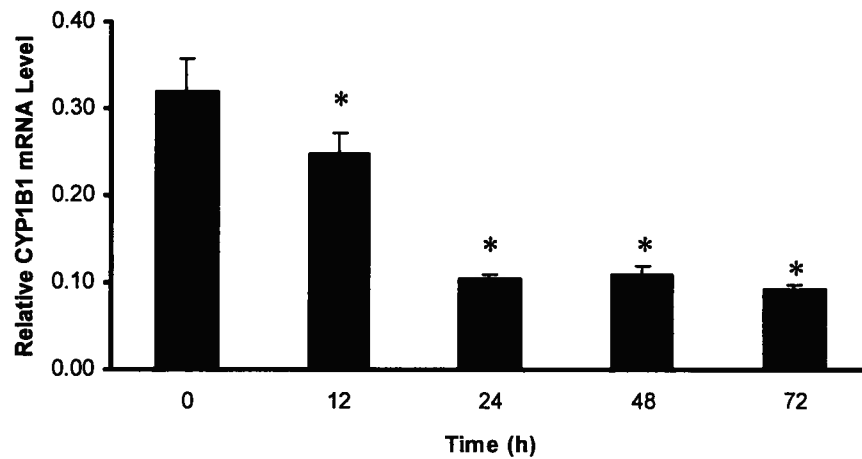
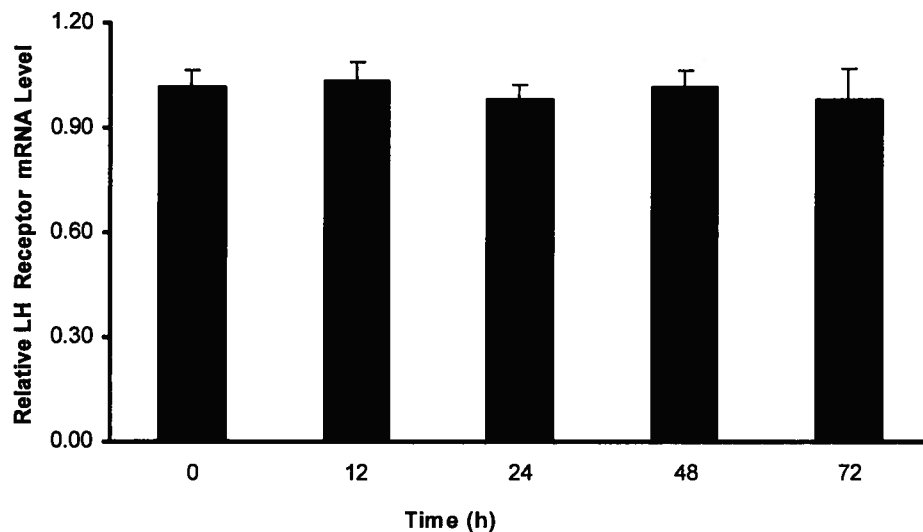
A**B**

Figure 3.23: Effect of estradiol benzoate treatment for different periods of time on (A) CYP1B1 mRNA expression or (B) LH receptor mRNA expression in MA-10 cells. Cells were exposed to 500 nM estradiol benzoate (final concentration in the T25 culture flask) for different periods of time. Relative CYP1B1 mRNA and LH receptor mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 or LH receptor mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (zero-time-point) cells with a P value < 0.05 .

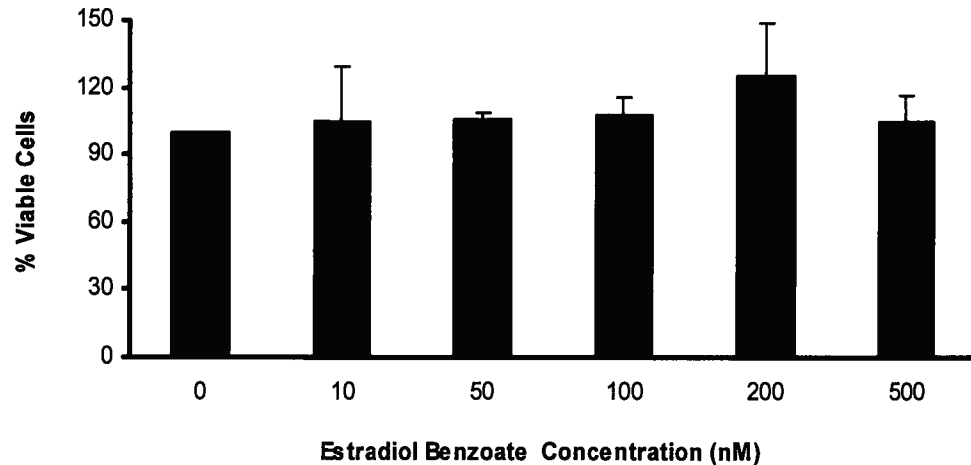


Figure 3.24: Effect of treatment with varying concentrations of estradiol benzoate on cell viability in MA-10 cells. Cells were treated with estradiol (at a final concentration range of 10-500 nM in the culture plate well) or with DMSO for 24 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of estradiol benzoate-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

3.5.3. Effect of ICI 182,780 on CYP1B1 mRNA Expression

ICI 182,780 (also known as fulvestrant) is a competitive steroidal antagonist of estrogen receptor α and estrogen receptor β (Howell et al., 2000). MA-10 cells were treated with varying concentrations of ICI 182,780 (0.5-100 μ M) for 24 h to determine if estrogen receptors (α and β) were involved in regulating constitutive expression of CYP1B1 mRNA. RT-PCR analysis showed that treatment with ICI 182,780, at the concentrations used in this study, had no effect on CYP1B1 mRNA levels (Figure 3.25A).

A time course study of the effect of ICI 182,780 (100 μ M) on CYP1B1 mRNA expression in MA-10 cells was also performed. Treatment with ICI 182,780 (100 μ M) did not affect CYP1B1 mRNA levels in MA-10 cells up to 24 h after treatment (Figure 3.25B).

MA-10 cells were co-treated with estradiol benzoate (100 nM) and ICI 182,780 (10-1000 μ M) to determine if the suppressive effect of estradiol benzoate on CYP1B1 mRNA levels was mediated through the estrogen receptor (α and β). Cells were co-treated with estradiol benzoate (100 nM) and ICI 182,780 (10-1000 μ M) for 24 h. RT-PCR analysis showed that ICI 182,780 did not attenuate the suppressive effect of estradiol benzoate on CYP1B1 mRNA levels (Figure 3.26).

The results of treatment of MA-10 cells with ICI 182,780, a competitive steroidal antagonist of estrogen receptor (α and β), and co-treatment of cells with ICI 182,780 plus estradiol benzoate indicate that estrogen receptors α and β play little or no role in CYP1B1 expression in Leydig cells.

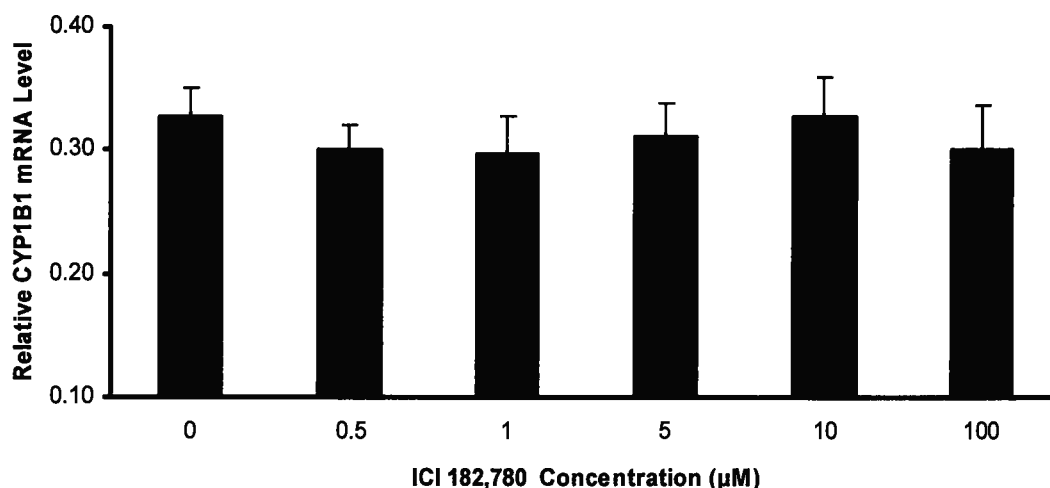
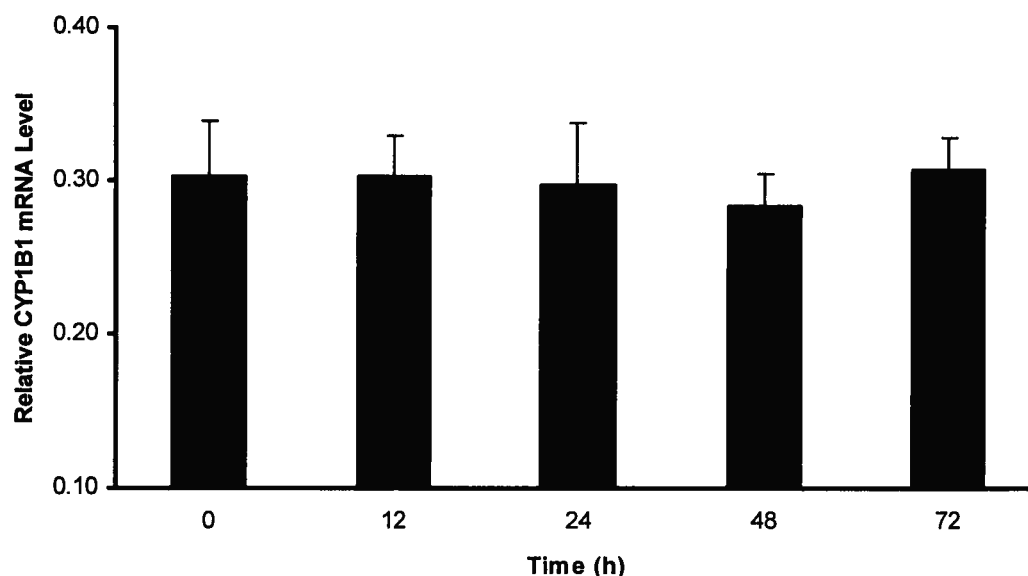
A**B**

Figure 3.25: Effect of treatment with ICI 182,780 (a competitive steroidal antagonist of estrogen receptor α and β) on CYP1B1 mRNA expression in MA-10 cells. In the concentration response study (A), cells were treated with ICI 182,780 (at a final concentration range of 0.5-100 μ M in the T25 culture flask) or with DMSO for 24 h. In the time course study (B), cells were treated with 100 μ M ICI 182,780 (final concentration in the T25 culture flask) for different periods of time. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the mean ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days.

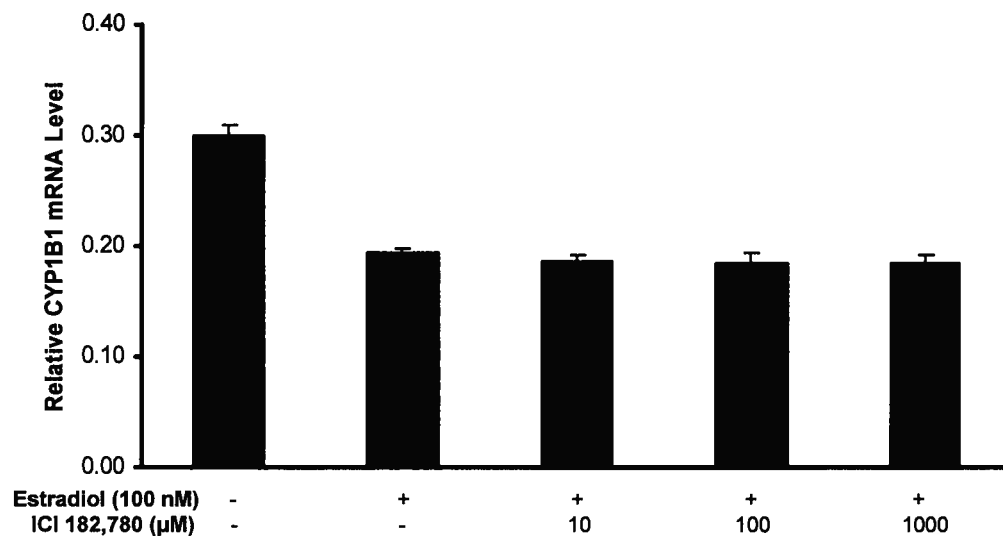


Figure 3.26: Effect of co-treatment (for 24 h) with estradiol benzoate and ICI 182,780 on CYP1B1 mRNA expression in MA-10 cells. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the mean ratio of the intensity of the CYP1B1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three separate experiments performed on different days.

3.5.4. Effect of Co-treatment with Estradiol Benzoate and LH on CYP1B1 mRNA Expression and Relative PKA Activity

MA-10 cells were co-treated with varying concentrations of estradiol benzoate (10-500 nM) plus LH (0.2 IU/ml) for 24 h to determine if the suppressive effect of estradiol benzoate on CYP1B1 expression was due to inhibition of the LH-mediated PKA pathway in Leydig cells. As a positive control, cells were treated with estradiol benzoate (500 nM) alone. RT-PCR analysis showed that estradiol benzoate did not attenuate the LH-elicited increase in CYP1B1 mRNA levels in MA-10 cells (Figure 3.27A). Similarly, estradiol benzoate did not suppress the LH-elicited increase in PKA activity (Figure 3.27B).

The results of co-treatment of MA-10 cells with LH plus estradiol benzoate suggest that the estradiol benzoate-elicited decrease in CYP1B1 expression was not due to suppression or modulation of the LH-mediated PKA pathway.

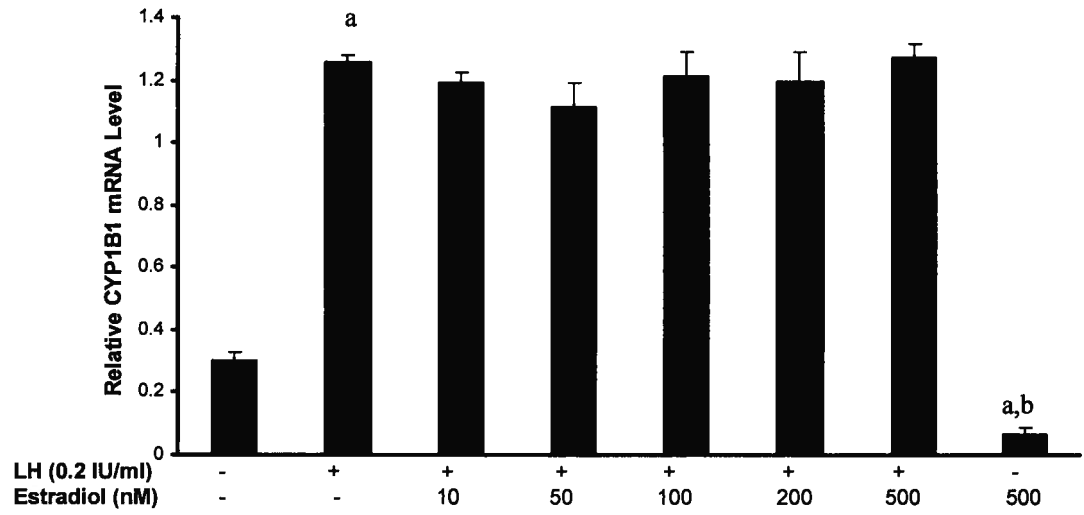
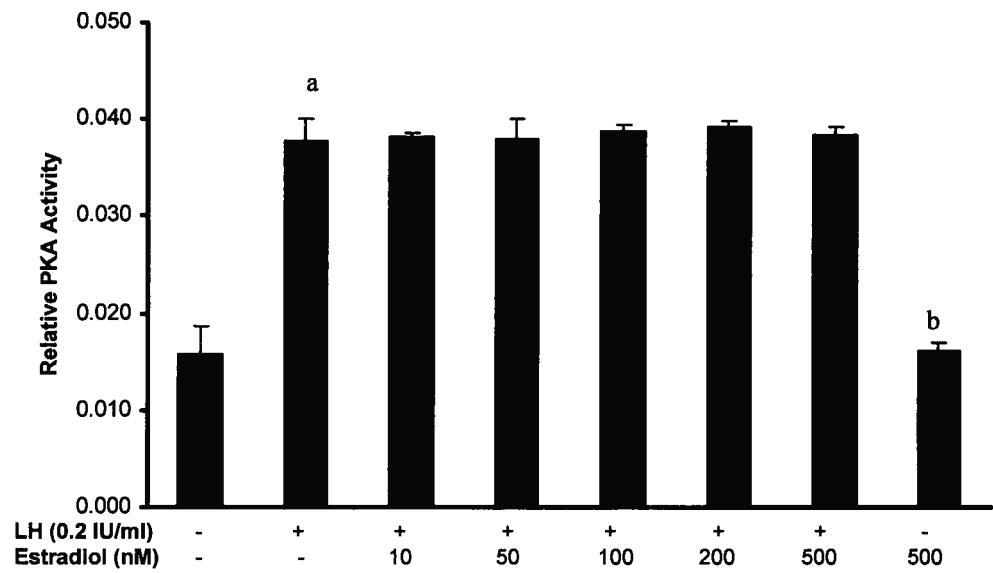
A**B**

Figure 3.27: Effect of co-treatment with LH and estradiol benzoate on (A) CYP1B1 mRNA expression and (B) relative PKA activity in MA-10 cells. MA-10 cells were co-treated with estradiol benzoate (10-500 nM) plus LH (0.2 IU/ml) for 24 h. Relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels (A). Relative PKA activity was determined using a nonradiometric ELISA assay (B). Values shown are the mean \pm SD of five separate experiments performed on different days. CYP1B1 mRNA levels and PKA activity in cells treated with LH alone (2nd bar from the left) or estradiol benzoate alone were compared with that of cells treated with vehicle (1st bar from the left) using unpaired student's t-test (parametric). CYP1B1 mRNA levels and PKA activity in cells treated with estradiol benzoate alone (8th bar from the left) were compared with that of cells treated with LH plus estradiol benzoate (7th bar from the left) using unpaired student's t-test (parametric). a, Significantly different from vehicle-treated cells (1st bar from the left) with a *P* value < 0.05. b, Significantly different from cells that were treated with LH plus estradiol benzoate (7th bar from the left) with a *P* value < 0.05.

3.6. CYP1B1 Protein Expression in MA-10 Cells

To detect CYP1B1 protein in microsomes prepared from MA-10 cells, immunoblot analysis was attempted with five different antibodies. Rabbit anti-mouse CYP1B1 serum prepared by Alpha Diagnostics International (San Antonio, TX, USA) was unable to detect recombinant mouse CYP1B1 protein standards (0.75-0.1 pmol) and CYP1B1 protein in mouse testicular microsomes and MA-10 microsomal samples. Commercially available anti-human CYP1B1 serum from Santa Cruz Biotechnology Inc., anti-human CYP1B1 serum from BD Gentest Inc., and anti-rat CYP1B1 serum from BD Gentest Inc., did not react with recombinant mouse CYP1B1 protein. Anti-human CYP1B1 serum, which was previously prepared in our laboratory using a 16-amino acid peptide as the antigen of immunization, was able to detect recombinant mouse CYP1B1 protein standards (0.25-0.75 pmol) (Figure 3.28). No CYP1B1 protein band was observed, however, in microsomal samples prepared from mouse testis or Leydig MA-10 cells. Possible explanations for the failure of this antibody preparation to detect CYP1B1 in mouse testis and Leydig cells are a low level of CYP1B1 protein expression in mouse testis and in MA-10 cells and/or a low titer (i.e. weak immunoreactivity) of the CYP1B1 antibody.

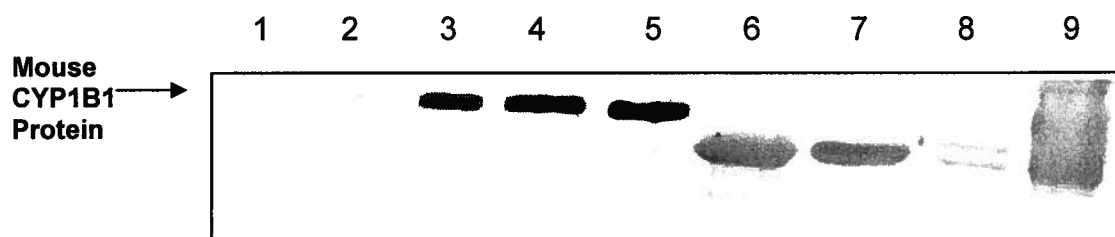


Figure 3.28: Immunoblot of CYP1B1 protein in microsomes from mouse tissues and mouse Leydig MA-10 cells. Microsomal protein samples were subjected to SDS-PAGE and immunoblots were developed using rabbit anti-human CYP1B1 IgG (200 µg/ml) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. Lane 1 contains 0.1 pmol recombinant mouse CYP1B1 protein; lane 2 contains 0.25 pmol recombinant mouse CYP1B1 protein; lane 3 contains 0.375 pmol recombinant mouse CYP1B1 protein; lane 4 contains 0.5 pmol recombinant mouse CYP1B1 protein; lane 5 contains 0.75 pmol recombinant mouse protein, lane 6 contains mouse liver microsomes (20 µg); lane 7 contains mouse kidney microsomes (20 µg); lane 8 contains mouse testis microsomes (60 µg); lane 9 contains microsomes from mouse Leydig MA-10 cells (40 µg).

Section B- Regulation of CYP1B1 Expression in Rat Leydig R2C Cells

Rat R2C cells were used to investigate the hormonal regulation of CYP1B1 protein expression as it was not possible to study CYP1B1 expression in MA-10 cells at the protein level. In addition, earlier studies of hormonal regulation of CYP1B1 were carried out in SD rats. Hence, rat Leydig cells may be a more relevant in vitro model for the in vivo studies. Rat Leydig R2C cells were used in the present study to investigate possible mechanism(s) of LH- and estradiol-mediated regulation of CYP1B1 protein expression.

3.7. Regulation of CYP1B1 Protein Expression by LH

3.7.1. Effect of LH on CYP1B1 Protein Expression

Rat R2C cells were treated with varying concentrations of LH (0.01-0.2 IU/ml) for 12 h and CYP1B1 protein expression was measured by immunoblot analysis using rabbit anti-rat CYP1B1 serum. Immunoblot analysis indicated that LH at concentrations of 0.05 and 0.2 IU/ml increased CYP1B1 protein levels, relative to vehicle-treated cells, in R2C cells. The lowest concentration of LH used in this study (0.01 IU/ml) had no effect on CYP1B1 protein levels (Figure 3.29A and 3.29B).

3.7.2. Effect of LH on PKA Activity

The effect of varying concentrations of LH (0.01-0.2 IU/ml) on relative PKA activity in R2C cells was also characterized. LH treatment, at the concentrations used in this study, had no effect on relative PKA activity (Figure 3.29C).

3.7.3. Effect of LH on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with LH (0.01-0.2 IU/ml) on cell viability. According to the results of the MTT-based assay, the viability of R2C

cells did not change after treatment with LH at the concentrations used in this study (Figure 3.30).

The results of treatment of R2C cells with LH suggest that CYP1B1 protein expression in R2C cells is regulated by LH, whereas PKA activity was not stimulated by LH. It was observed that basal PKA activity in R2C cells was approximately 2-fold higher than in MA-10 cells.

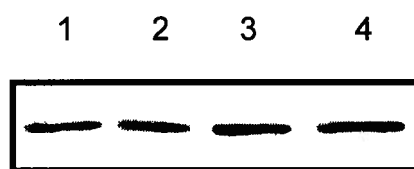
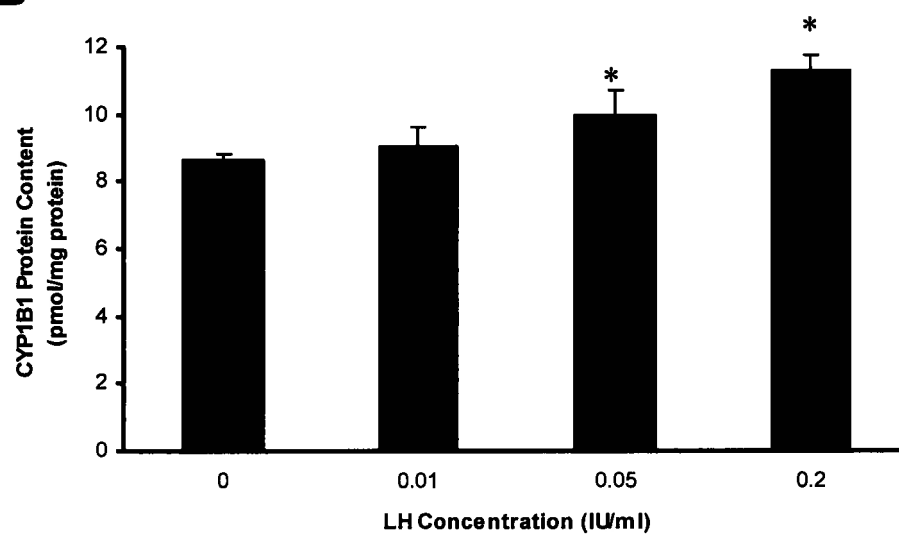
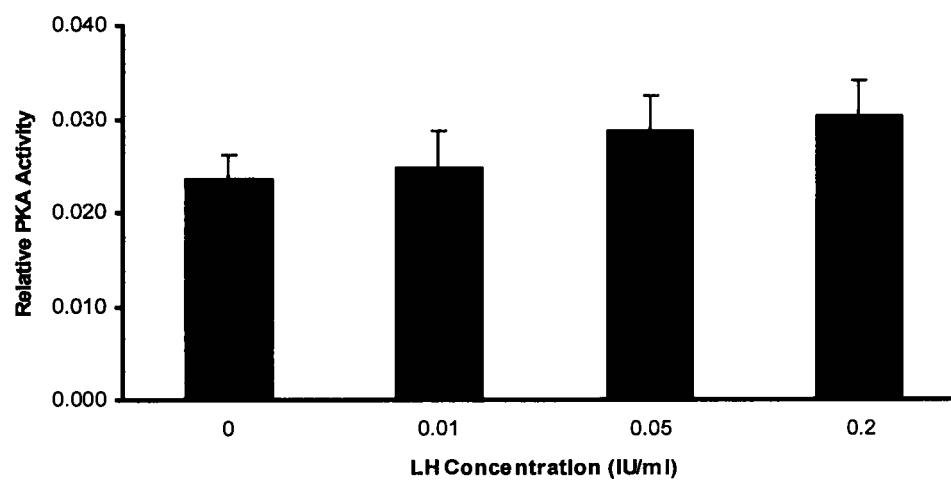
A**B****C**

Figure 3.29: Effect of treatment with varying concentrations of LH (from sheep pituitary) on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with LH. (C) Relative PKA activity in R2C cells after LH treatment. Cells were treated with LH (at a final concentration range of 0.01-0.2 IU/ml in the T75 culture flask) for 12 h. For panel A, 20 µg total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with water (vehicle), lane 2 contains cells treated with 0.01 IU/ml LH, lane 3 contains cells treated with 0.05 IU/ml LH, lane 4 contains cells treated with 0.2 IU/ml LH. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days. CYP1B1 protein data from LH-treatment study were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul’s multiple comparison test. Relative PKA activity data from LH-treatment study were analyzed using Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul’s multiple comparison test. *, Significantly different from the vehicle-treated cells with a *P* value < 0.05.

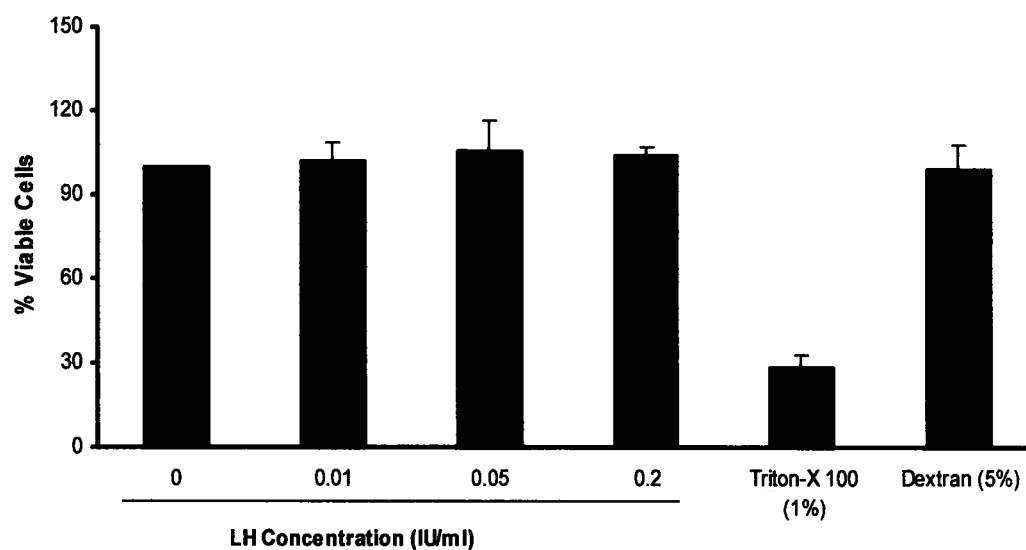


Figure 3.30: Effect of treatment with varying concentrations of LH on cell viability in R2C cells. Cells were treated with LH (at a final concentration range of 0.01-0.2 IU/ml in the culture plate well) or with water for 12 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of LH-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of four separate experiments performed on different days.

3.8. Involvement of the PKA Pathway in CYP1B1 Protein Expression

3.8.1. Effect of 8-Br-cAMP (a PKA activator) on CYP1B1 Protein Expression

To explore the role of the PKA pathway in the regulation of CYP1B1 protein expression in R2C cells, a PKA activator was used. R2C cells were treated with 8-Br-cAMP (0.25 and 2 mM) for 12 h and CYP1B1 protein levels were measured by immunoblot analysis using rabbit anti-rat CYP1B1 serum. Immunoblot analysis showed that 8-Br-cAMP, at a concentration of 2 mM, increased CYP1B1 protein levels, relative to vehicle-treated cells, in R2C cells (Figure 3.31A and 3.31B). Treatment of R2C cells with 8-Br-cAMP at a lower concentration of 0.25 mM had no effect on CYP1B1 protein levels.

3.8.2. Effect of 8-Br-cAMP on PKA Activity

The effect of treatment with 8-Br-cAMP (0.25 and 2 mM) on relative PKA activity in R2C cells was also characterized. Relative PKA activity was increased 1.4-fold, compared to vehicle-treated cells, after treatment with 8-Br-cAMP at a concentration of 2 mM (Figure 3.31C). Treatment of R2C cells with 8-Br-cAMP at a lower concentration of 0.25 mM did not affect relative PKA activity.

3.8.3. Effect of 8-Br-cAMP on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with 8-Br-cAMP (0.25 and 2 mM) on cell viability. The viability of R2C cells did not change after treatment with 8-Br-cAMP at the concentrations used in this study (Figure 3.32).

The results of treatment with 8-Br-cAMP, a PKA activator, suggest that CYP1B1 protein expression and PKA activity in R2C cells were increased by 8-Br-cAMP and stimulation of PKA activity may be responsible for the 8-Br-cAMP-elicited increase in CYP1B1 protein levels.

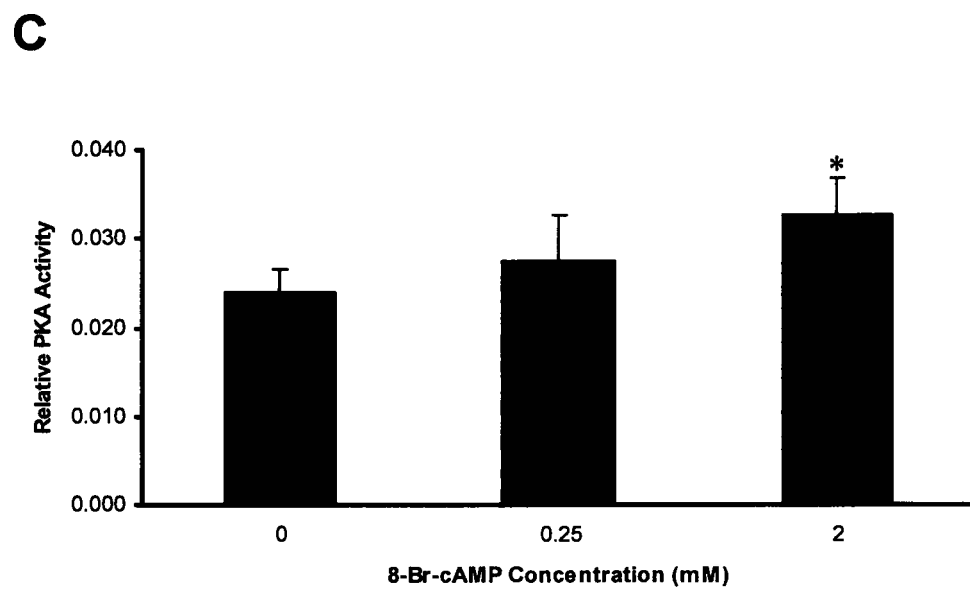
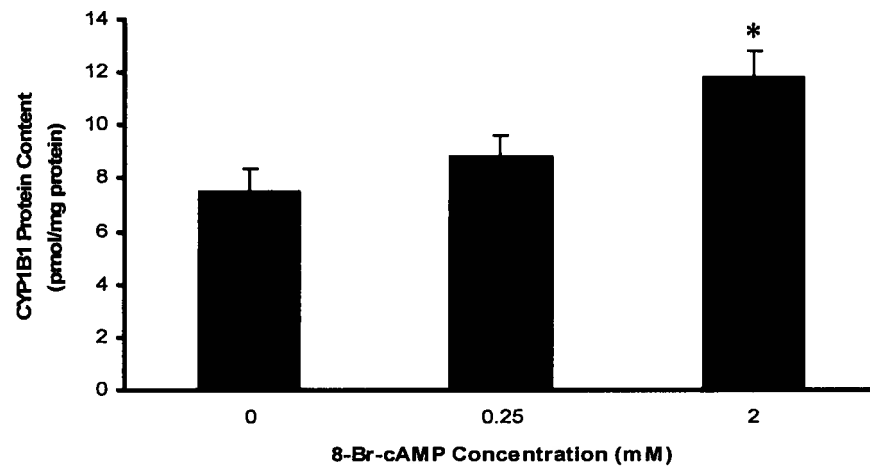
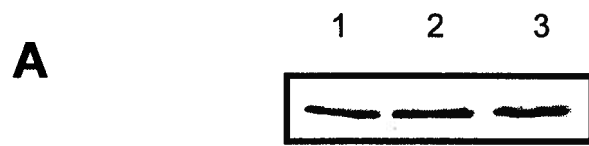


Figure 3.31: Effect of treatment with 8-Br-cAMP (a PKA activator) on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with 8-Br-cAMP. (C) Relative PKA activity in R2C cells after 8-Br-cAMP treatment. Cells were treated with 8-Br-cAMP (at final concentrations of 0.25 and 2 mM in the T75 culture flask) or with water for 12 h. For panel A, 20 µg total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with water (vehicle), lane 2 contains cells treated with 0.25 mM 8-Br-cAMP, lane 3 contains cells treated with 2 mM 8-Br-cAMP. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul’s multiple comparison test. *, Significantly different from the vehicle-treated cells with a *P* value < 0.05.

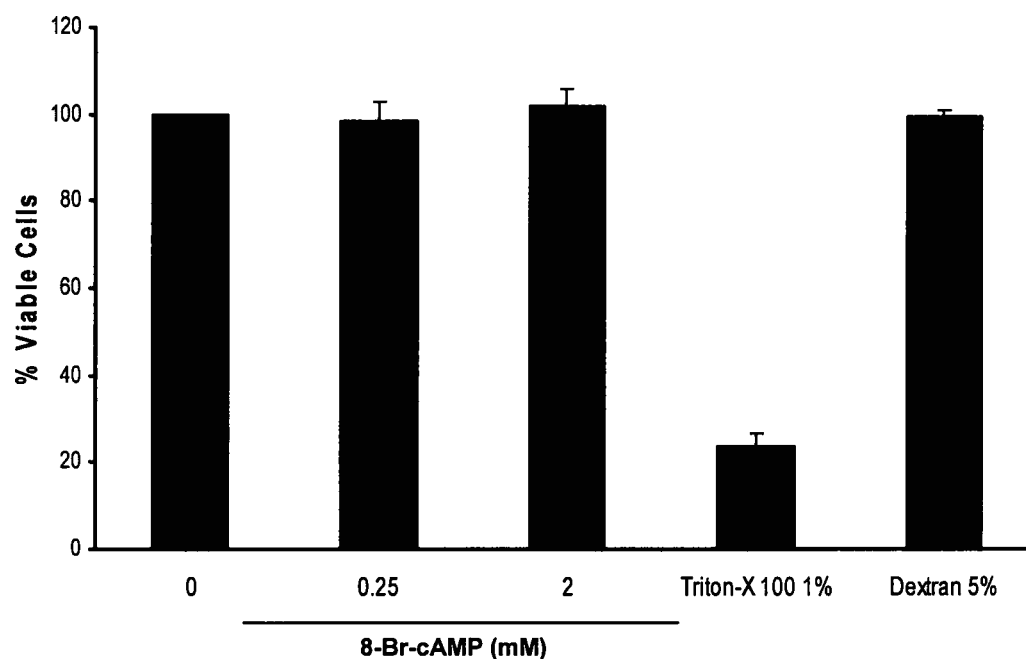


Figure 3.32: Effect of treatment with 8-Br-cAMP on cell viability in R2C cells. Cells were treated with 8-Br-cAMP (at a final concentrations of 0.25 mM and 2 mM in the culture plate well) or with water for 12 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of 8-Br-cAMP-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of four separate experiments performed on different days.

3.8.4. Effect of Treatment with Rp-CPT-cAMP (a specific PKA inhibitor)

To further explore the role of the PKA pathway in the regulation of constitutive and LH-mediated CYP1B1 protein expression in R2C cells, a specific PKA inhibitor (Rp-CPT-cAMP) was used. R2C cells were treated with varying concentrations of Rp-CPT-cAMP (1-150 μ M) for 6 h and CYP1B1 protein levels were measured by immunoblot analysis using rabbit anti-rat-CYP1B1 serum. Immunoblot analysis showed that Rp-CPT-cAMP at concentrations of 50 and 150 μ M decreased basal CYP1B1 protein levels, relative to vehicle-treated cells, in R2C cells. CYP1B1 protein levels were approximately 50% lower, relative to vehicle-treated cells, after treatment with Rp-CPT-cAMP at a concentration of 150 μ M (Figure 3.33A and 3.33B). Treatment of R2C cells with Rp-CPT-cAMP at the lowest concentration of 1 μ M had no effect on CYP1B1 protein levels.

The effect of varying the concentration of Rp-CPT-cAMP on relative PKA activity in R2C cells was also characterized. Relative PKA activity was decreased in a concentration-dependent manner with increasing concentrations of Rp-CPT-cAMP (Figure 3.33C). Decreased PKA activity was observed at all concentrations of Rp-CPT-cAMP tested. Relative PKA activity was approximately 65% lower, compared to vehicle-treated cells, after treatment with Rp-CPT-cAMP at a concentration of 150 μ M (Figure 3.33C).

The MTT-based assay was carried out to assess the effect of treatment with various concentration of Rp-CPT-cAMP (1-150 μ M) on cell viability. According to the results of the MTT-based assay, the viability of R2C cells did not change after treatment with Rp-CPT-cAMP at the concentrations used in this study (Figure 3.34).

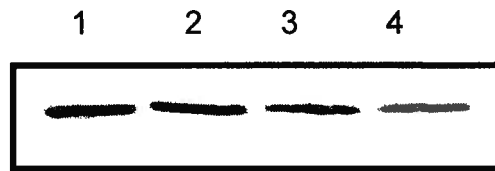
To further explore if Rp-CPT-cAMP, a specific PKA inhibitor, can block the increase in CYP1B1 protein levels after treatment with 8-Br-cAMP (PKA activator), R2C cells were co-treated with 8-Br-cAMP plus Rp-CPT-cAMP. Cells were preincubated with Rp-CPT-cAMP (25

μM) for 1 h, followed by treatment with 8-Br-cAMP (2 mM) for 12 h. As a positive control, cells were treated with 8-Br-cAMP (2 mM) without Rp-CPT-cAMP preincubation. It was observed that Rp-CPT-cAMP partially blocked the 8-Br-cAMP-elicited increase in CYP1B1 protein expression and PKA activity (Figure 3.35A and 3.35B).

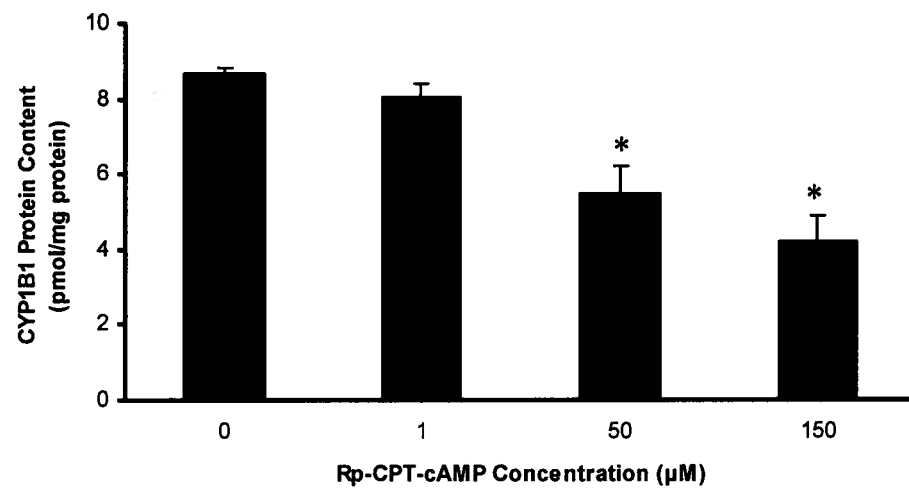
R2C cells were co-treated with Rp-CPT-cAMP plus LH to investigate if PKA was responsible for the LH-elicited increase in CYP1B1 protein expression in R2C cells. Cells were preincubated with Rp-CPT-cAMP (25 μM) for 1 h, followed by treatment with LH (0.2 IU/ml) for 12 h. It was observed that treatment with Rp-CPT-cAMP (25 μM) attenuated the LH-elicited increase in CYP1B1 protein expression and PKA activity (Figure 3.35C).

The results of treatment of R2C cells with Rp-CPT-cAMP, a specific PKA inhibitor, suggest that PKA pathway was involved in the constitutive expression of CYP1B1 protein. The results of co-treatment of R2C cells with 8-Br-cAMP (a PKA activator) plus Rp-CPT-cAMP indicate that the increase in CYP1B1 protein levels after treatment with 8-Br-cAMP was a consequence of PKA activation in R2C cells. Similarly, the results of co-treatment of R2C cells with LH plus Rp-CPT-cAMP indicate that LH-elicited increase in CYP1B1 protein levels was mediated through a PKA pathway.

A



B



C

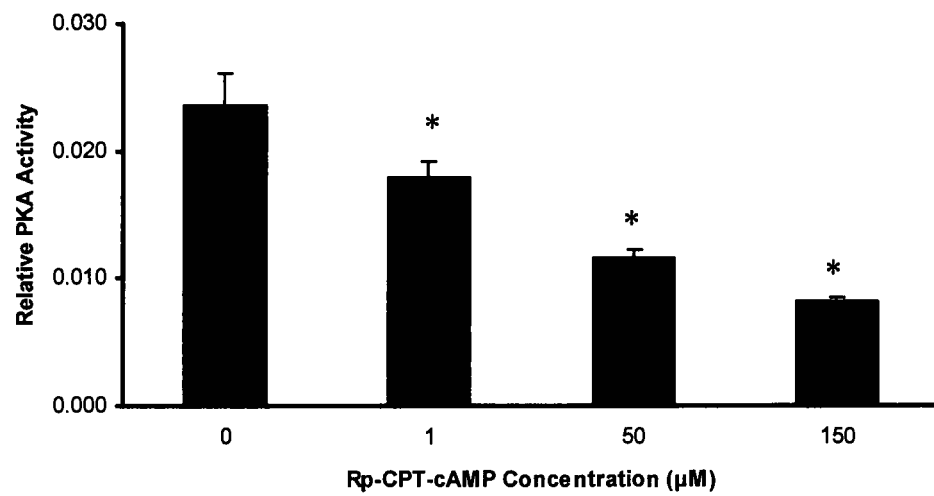


Figure 3.33: Effect of treatment with varying concentrations of Rp-CPT-cAMP (a specific PKA inhibitor) on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with Rp-CPT-cAMP. (C) Relative PKA activity in R2C cells after Rp-CPT-cAMP treatment. Cells were treated with Rp-CPT-cAMP (at a final concentration range of 1-150 μ M in the T75 culture flask) for 6 h. For panel A, 20 μ g total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with water (vehicle), lane 2 contains cells treated with 1 μ M Rp-CPT-cAMP, lane 3 contains cells treated with 50 μ M Rp-CPT-cAMP, lane 4 contains cells treated with 150 μ M Rp-CPT-cAMP. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul’s multiple comparison test. *, Significantly different from the vehicle-treated cells with a *P* value < 0.05.

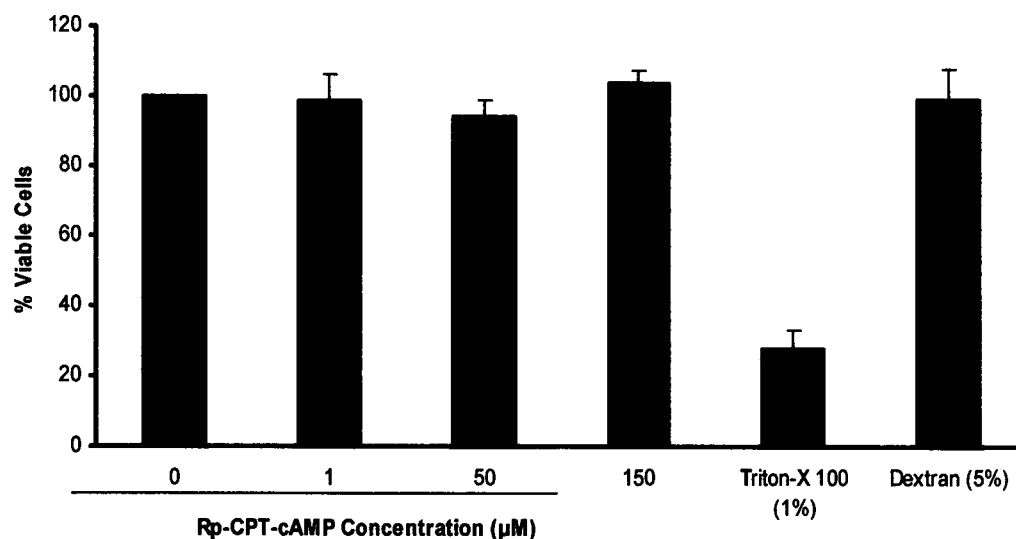


Figure 3.34: Effect of treatment with varying concentrations of Rp-CPT-cAMP on cell viability in R2C cells. Cells were treated with Rp-CPT-cAMP (at a final concentration range of 1-150 μM in the culture plate well) or with water for 6 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of Rp-CPT-cAMP-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of four separate experiments performed on different days.

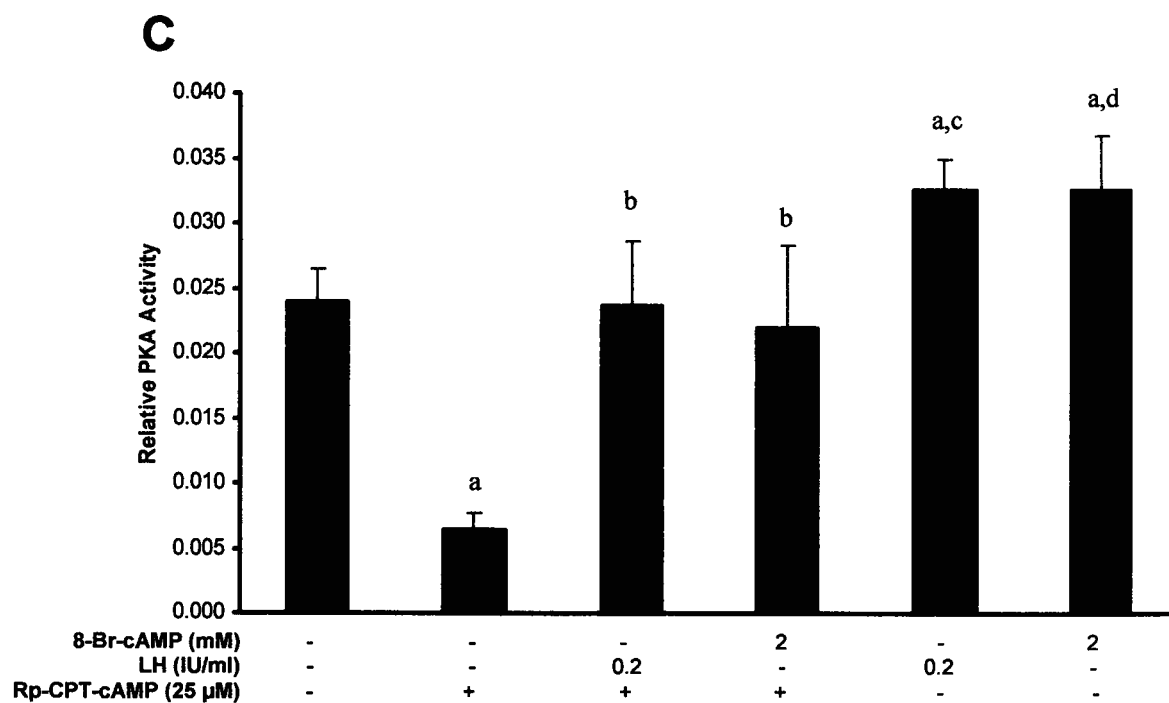
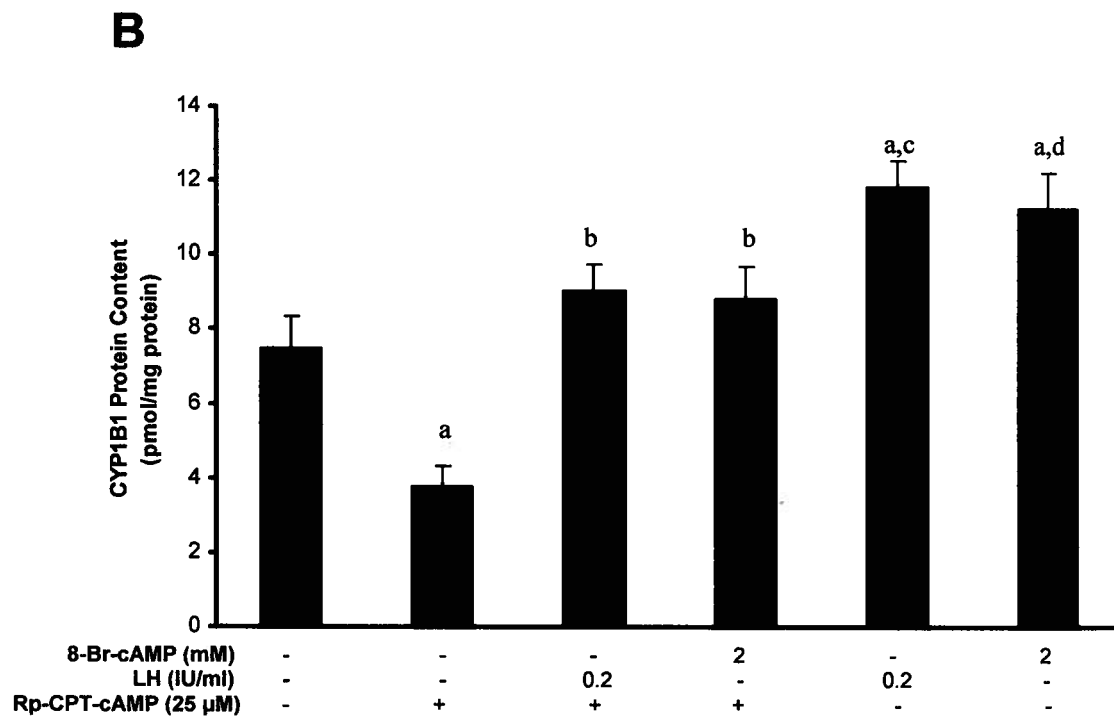
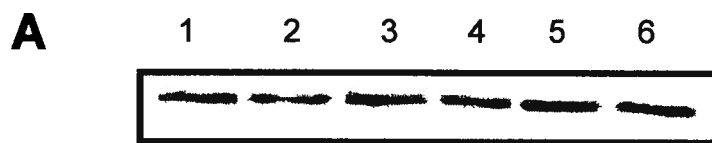


Figure 3.35: Effect of co-treatment with Rp-CPT-cAMP (a specific PKA inhibitor) plus LH or Rp-CPT-cAMP plus 8-Br-cAMP on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after co-treatment of R2C cells. (C) Relative PKA activity in R2C cells. Cells were preincubated with Rp-CPT-cAMP for 1 h and then LH or 8-Br-cAMP (dissolved in water) was added to the culture medium for 12 h. For panel A, 20 μ g total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with water (vehicle), lane 2 contains cells treated with 25 μ M Rp-CPT-cAMP, lane 3 contains cells co-treated with 25 μ M Rp-CPT-cAMP and 0.2 IU/ml LH, lane 4 contains cells co-treated with 25 μ M Rp-CPT-cAMP and 2 mM 8-Br-cAMP, lane 5 contains cells treated with 0.2 IU/ml LH, lane 6 contains 2 mM 8-Br-cAMP. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days. Data were analyzed using unpaired student’s t-test (parametric). a, Significantly different from vehicle-treated cells (1st bar from the left) with a *P* value < 0.05. b, Significantly different from cells that were treated with Rp-CPT-cAMP but not LH or 8-Br-cAMP (2nd bar from the left) with a *P* value < 0.05. c, Significantly different from cells that were treated with Rp-CPT-cAMP plus LH (3rd bar from the left) with a *P* value < 0.05. d, Significantly different from cells that were treated with Rp-CPT-cAMP plus 8-Br-cAMP (4th bar from the left) with a *P* value < 0.05.

3.9. Involvement of the PKC Pathway in CYP1B1 Protein Expression

3.9.1. Effect of Treatment with Go 6983 (a specific PKC inhibitor)

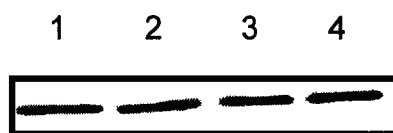
Go 6983, a specific PKC inhibitor, was used to investigate the putative role of PKC-mediated pathways in CYP1B1 protein expression in R2C cells. To determine if the PKC enzyme is involved in constitutive CYP1B1 protein expression, R2C cells were treated with varying concentrations of Go 6983 (1-200 μ M) for 12 h and CYP1B1 protein levels were measured by immunoblot analysis using rabbit anti-rat-CYP1B1 serum. Immunoblot analysis showed that treatment with Go 6983, at the concentrations used in this study, did not affect constitutive CYP1B1 protein expression in R2C cells (Figure 3.36A and 3.36B).

The effect of varying concentrations of Go 6983 (1-200 μ M) on relative PKA activity in R2C cells was also characterized. Treatment with Go 6983, at the concentrations used in this study, did not affect relative PKA activity in R2C cells (Figure 3.36C).

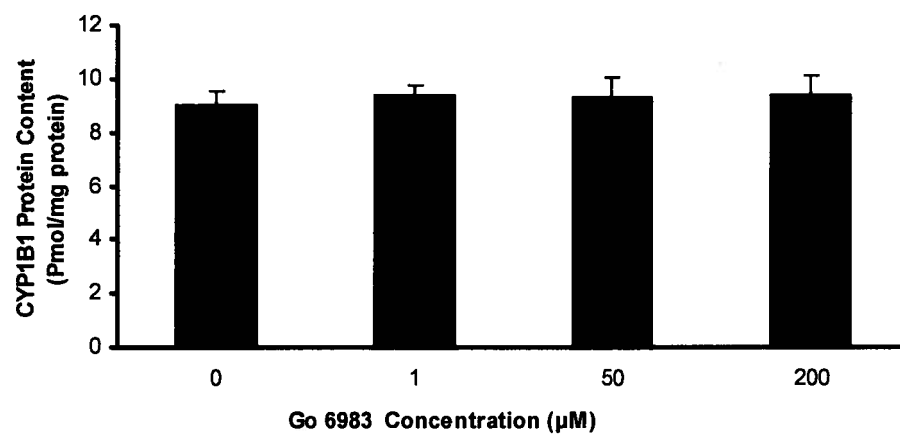
The MTT-based assay was carried out to assess the effect of treatment with varying concentrations of Go 6983 (1-200 μ M) on cell viability. The viability of R2C cells did not change after treatment with Go 6983 at the concentrations used in this study (Figure 3.37).

The results of treatment of R2C cells with Go 6983, a PKC inhibitor, suggest that PKC-mediated pathways have little or no role in the regulation of CYP1B1 protein expression.

A



B



C

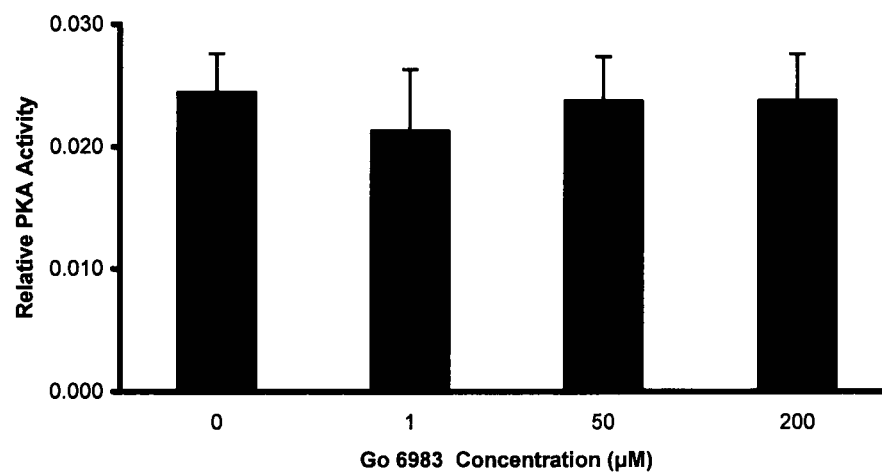


Figure 3.36: Effect of treatment with varying concentrations of Go 6983 (a specific PKC inhibitor) on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with Go 6983. (C) Relative PKA activity in R2C cells after Go 6983 treatment. Cells were treated with Go 6983 (at a final concentration range of 1-200 μ M in the T75 culture flask) or with DMSO for 12 h. For panel A, 20 μ g total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with DMSO (vehicle), lane 2 contains cells treated with 1 μ M Go 6983, lane 3 contains cells treated with 50 μ M Go 6983, lane 4 contains cells treated with 200 μ M Go 6983. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days.

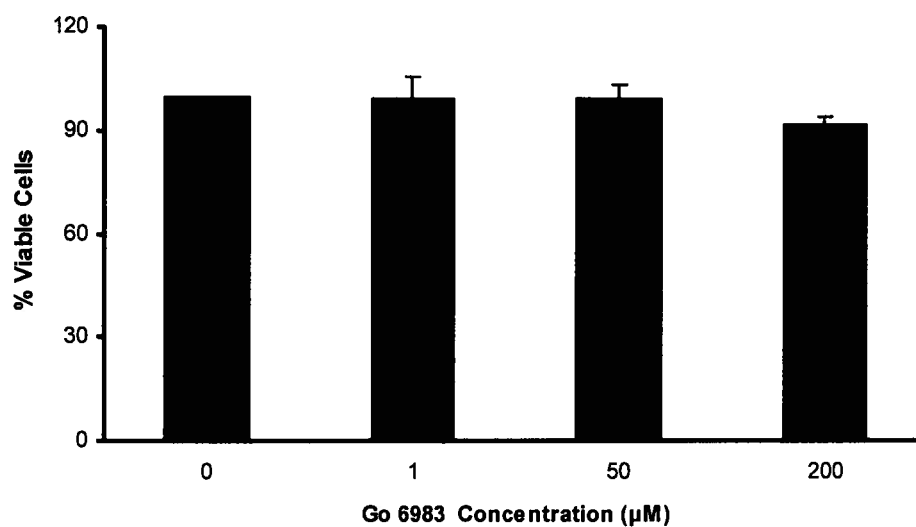


Figure 3.37: Effect of treatment with varying concentrations of Go 6983 on cell viability in R2C cells. Cells were treated with Go 6983 (at a final concentration range of 1-200 µM in the culture plate well) or with DMSO for 12 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of Go 6983-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of four separate experiments performed on different days.

3.10. Regulation of CYP1B1 Protein Expression by Estradiol Benzoate

3.10.1. Effect of Sex Hormones, Their Receptor Antagonists and Aromatase Inhibitor on CYP1B1 Protein Expression

To determine the role of sex hormones and therapeutic agents (related to biosynthesis and/or mechanism of action of sex hormones) in CYP1B1 protein expression, estradiol benzoate, ICI 182,780, anastrozole (a chemical inhibitor of aromatase enzyme that converts androgens into estrogens), testosterone propionate and flutamide (an anti-androgen) were used. R2C cells were treated with estradiol benzoate (1000 nM), ICI 182,780 (100 μ M), anastrozole (1000 nM), testosterone propionate (1000 nM), and flutamide (10 μ M) for 24 h and CYP1B1 protein levels were measured by immunoblot analysis using rabbit anti-rat-CYP1B1 serum. Immunoblot analysis showed that CYP1B1 protein levels did not change after treatment with estradiol benzoate, ICI 182,780, anastrozole, testosterone propionate, and flutamide at the concentration used in this study (Figure 3.38A and 3.38B).

3.10.2. Effect of Sex Hormones, Their Receptor Antagonists and Aromatase Inhibitor on PKA Activity

The effect of sex hormones and therapeutic agents on relative PKA activity in R2C cells was also characterized. Treatment with estradiol benzoate, ICI 182,780, anastrozole, testosterone propionate, and flutamide did not affect relative PKA activity at the concentration used in this study (Figure 3.38C).

3.10.3. Effect of Sex Hormones, Their Receptor Antagonists and Aromatase Inhibitor on Cell Viability

The MTT-based assay was carried out to assess the effect of treatment with sex hormones and therapeutic agents on cell viability. The viability of R2C cells did not change after treatment with estradiol benzoate, ICI 182,780, anastrozole, testosterone propionate, and flutamide at the concentration used in this study (Figure 3.39).

The results of treatment of R2C cells with sex hormones, their receptor antagonists and aromatase inhibitor suggest that estradiol or testosterone have little or no role in the constitutive expression of CYP1B1 protein in Leydig cells.

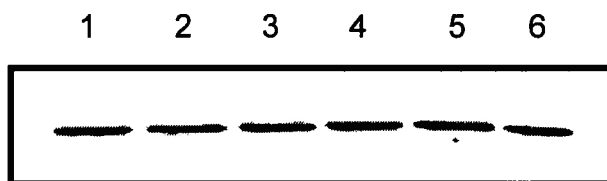
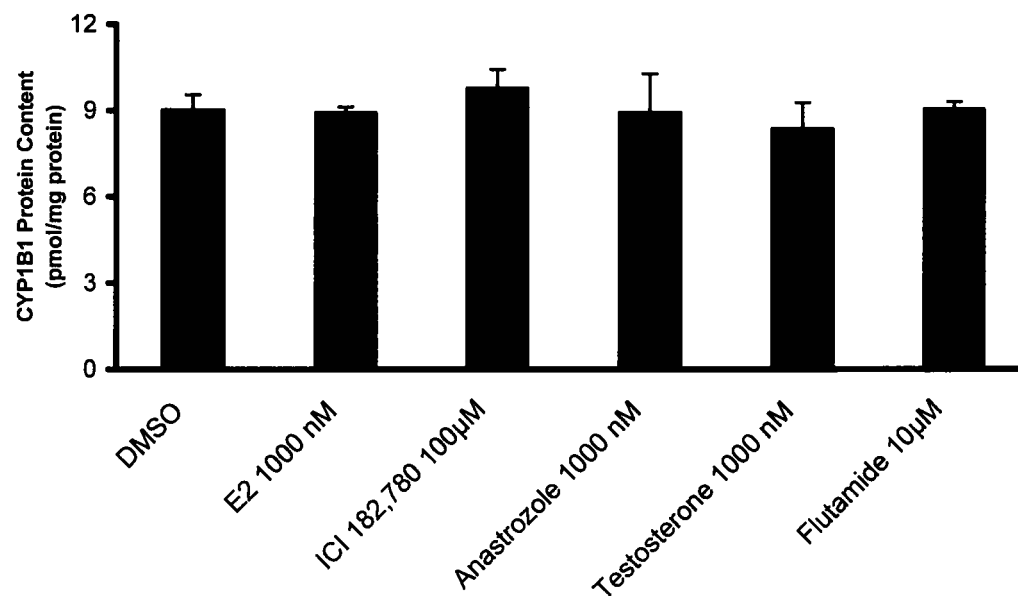
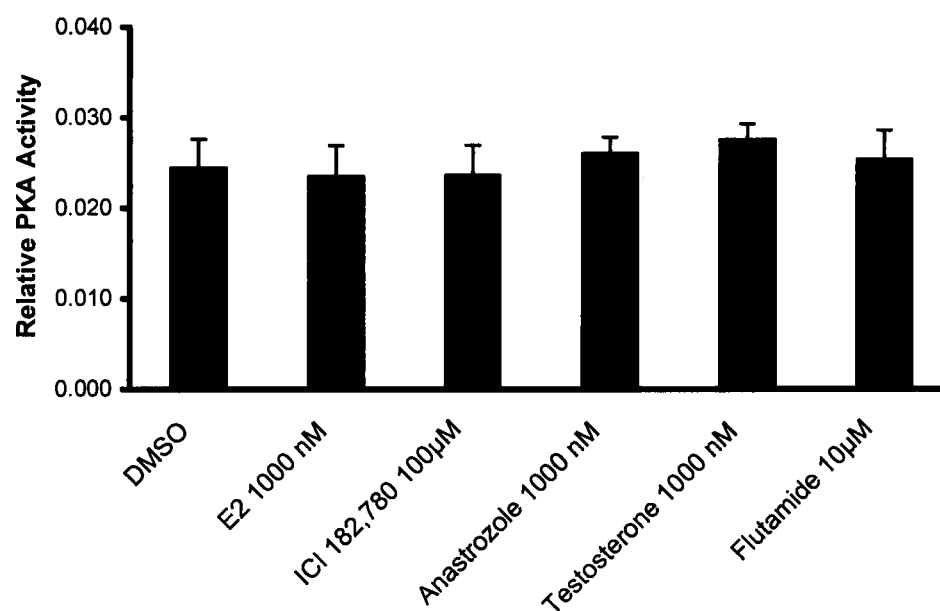
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Figure 3.38: Effect of treatment with sex hormones (estradiol benzoate and testosterone propionate), their receptor antagonists (ICI 182,780 and flutamide) or aromatase inhibitor (anastrozole) on CYP1B1 protein expression and PKA activity. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with sex hormones and therapeutic agents. (C) Relative PKA activity in R2C cells. Cells were treated with sex hormones and therapeutic agents (at a final concentration in the T75 culture flask as indicated) or with DMSO for 24 h. For panel A, 20 μ g total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with DMSO (vehicle), lane 2 contains cells treated with 1000 nM estradiol benzoate, lane 3 contains cells treated with 100 μ M ICI 182,780, lane 4 contains cells treated with 1000 nM anastrozole, lane 5 contains cells treated with 1000 nM testosterone, lane 6 contains cells treated with 10 μ M flutamide. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. For panel C, relative PKA activity was determined in the cytosolic fraction of cell lysates using a nonradiometric ELISA assay. Results are expressed as mean \pm SD of four separate experiments performed on different days.

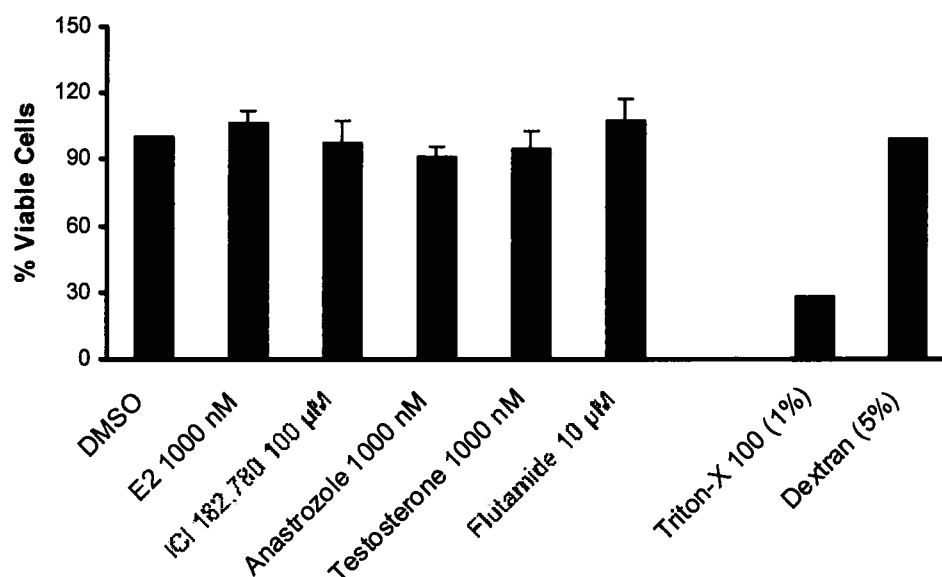


Figure 3.39: Effect of treatment with sex hormones (estradiol benzoate and testosterone propionate), their receptor antagonists (ICI 182,780 and flutamide) or aromatase inhibitor (anastrozole) on cell viability in R2C cells. Cells were treated with sex hormones and therapeutic agents (at a final concentration in the culture plate well as indicated) or with DMSO for 24 h. The MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of chemical- and hormone-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of four separate experiments performed on different days.

Section C- Effect of a Potent AhR Agonist on Testicular CYP1B1 Expression

in vivo and in vitro

CYP1B1 mRNA and protein expression are regulated by AhR agonists in liver, lung and kidney (Shimada et al., 2002; Shimada et al., 2003; Walker et al., 1999). However, there are conflicting reports regarding regulation of CYP1B1 expression by AhR agonists in testis. TCDD, the most potent AhR agonist, was used in this study to investigate the role of the AhR in CYP1B1 mRNA and protein expression in testis and Leydig cell lines.

3.11. Effect of TCDD Treatment on Testicular CYP1B1 Protein Expression in Rats

To determine the role of AhR activation on testicular CYP1B1 protein expression, adult SD rats were treated with varying dosages of TCDD (1-100 µg/kg). Immunoblot analysis was carried out to measure CYP1B1 protein levels in testis microsomes prepared from TCDD-treated rats. CYP1B1 protein is expressed at a high level (84 ± 4 pmol/mg protein; $n = 3$) in testis microsomes from vehicle-treated rats. Although testicular CYP1B1 protein levels were increased at TCDD dosages of 10 and 50 µg/kg, there was no increase in CYP1B1 protein levels at 100 µg/kg dosage (Figure 3.40). Moreover, the increase in CYP1B1 protein levels was limited to <1.5-fold, which is minimal relative to CYP1B1 protein induction observed in liver after TCDD treatment (Figure 3.57).

The results of treatment of adult SD rats with TCDD (at the dosages used in this study) suggest that CYP1B1 protein expression is not regulated by the most potent AhR agonist (TCDD).

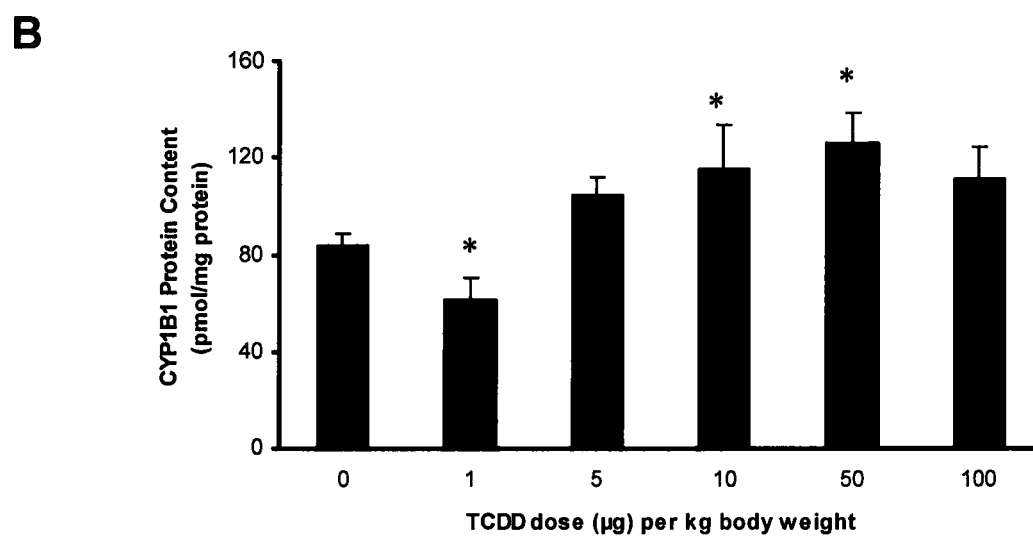
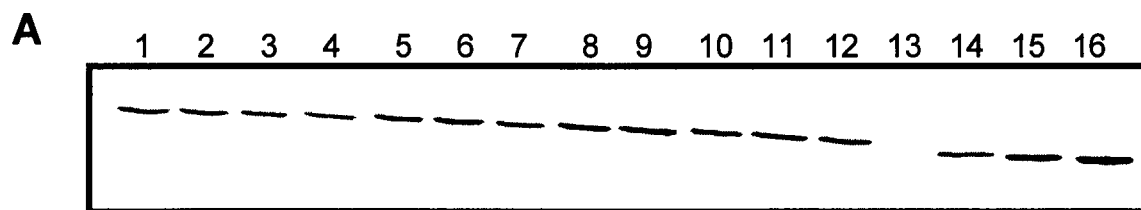


Figure 3.40: Effect of treatment with varying dosages of TCDD on testicular CYP1B1 protein expression in adult SD rats. (A) A representative immunoblot of testicular microsomal samples prepared from TCDD-treated rats. (B) Testicular microsomal CYP1B1 protein levels after treatment with TCDD. Testicular microsomes were prepared from rats (n=3) treated with a single intraperitoneal injection of vehicle (1 part dioxane: 6 parts corn oil) or 1, 5, 10, 50, 100 µg TCDD/kg. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, 2.5 µg total protein was loaded for each microsome sample per lane; lane 1 and 2 contain testis (from vehicle-treated rats), lane 3 and 4 contain testis (from rats treated with 1 µg TCDD/kg), lane 5 and 6 contain testis (from rats treated with 5 µg TCDD/kg), lane 7 and 8 contain testis (from rats treated with 10 µg TCDD/kg), lane 9 and 10 contain testis (from rats treated with 50 µg TCDD/kg), lane 11 and 12 contain testis (from rats treated with 100 µg TCDD/kg), lanes 13-16 contain recombinant rat CYP1B1 protein standards at 0.05, 0.1, 0.2, and 0.4 pmol/lane. For panel B, CYP1B1 protein concentration was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Results are expressed as mean \pm SD of three rats per treatment group. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. Data were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul’s multiple comparison test. *, Significantly different from the vehicle treated cells with a *P* value < 0.05.

3.12. Effect of Treatment with TCDD on CYP1B1 Protein Expression in R2C Cells

To determine if the lack of induction of CYP1B1 protein expression after TCDD treatment in vivo is a result of inability of TCDD to cross the blood-testis-barrier, rat Leydig R2C cells were exposed to varying concentrations of TCDD (10-1000 nM) for 24 h. CYP1B1 protein levels were measured by immunoblot analysis using rabbit anti-rat CYP1B1 serum. Treatment with TCDD, at the concentrations used in this study, had no effect on CYP1B1 protein levels (Figure 3.41A and 3.41B).

3.13. Effect of Treatment with TCDD on Cell Viability in R2C Cells

The MTT-based assay was carried out to assess the effect of treatment with varying concentrations of TCDD (10-1000 nM) on cell viability. The viability of R2C cells did not change after treatment with TCDD at the concentrations used in this study (Figure 3.41C).

3.14. Effect of Treatment with TCDD on CYP1B1 mRNA Expression in MA-10 Cells

Mouse MA-10 cells were also treated with varying concentrations of TCDD (1-1000 nM) for 12 h and CYP1B1 mRNA levels were measured. RT-PCR analysis showed that treatment with TCDD, at the concentrations used in this study, did not affect CYP1B1 mRNA levels (Figure 3.42A).

3.15. Effect of Treatment with TCDD on Cell Viability in MA-10 Cells

The MTT-based assay was carried out to assess the effect of treatment with various concentrations of TCDD (1-1000 nM) on cell viability. The viability of MA-10 cells did not change after treatment with TCDD at the concentrations used in this study (Figure 3.42B).

The results of treatment of rat Leydig R2C cells and mouse Leydig MA-10 cells with TCDD suggest that CYP1B1 mRNA and protein expression are not regulated by the AhR-mediated pathway.

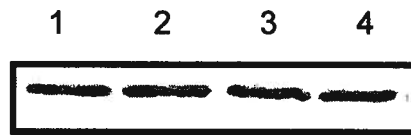
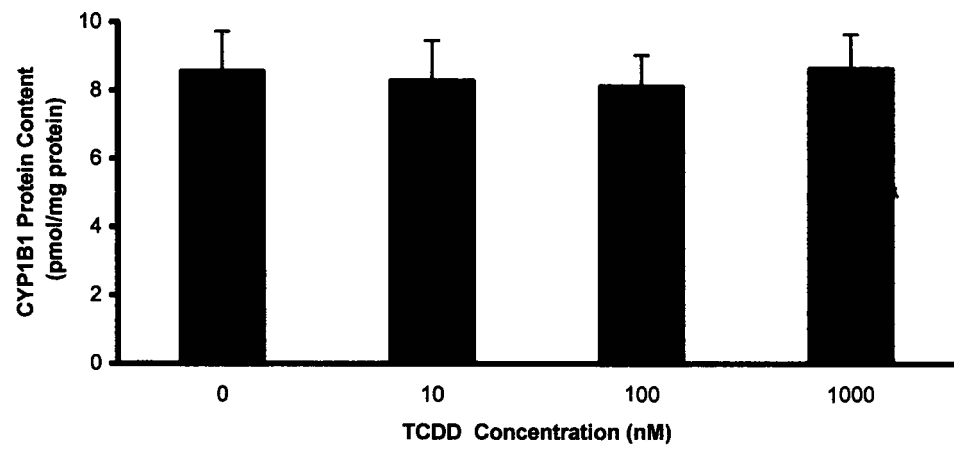
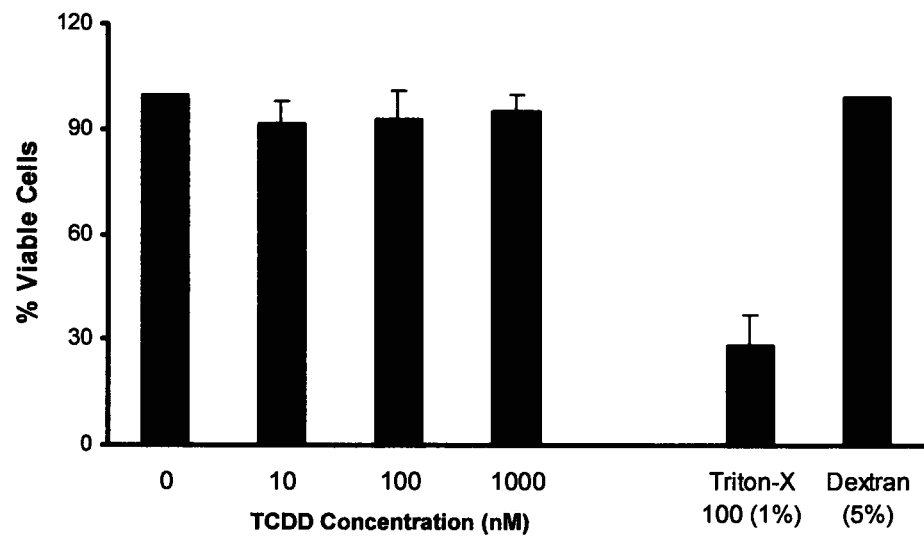
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Figure 3.41: Effect of treatment with varying concentrations of TCDD on CYP1B1 protein expression and cell viability. (A) A representative immunoblot of microsomal samples from R2C Leydig cells. (B) CYP1B1 protein levels after treatment of R2C cells with TCDD. (C) cell viability in rat Leydig R2C cells. Cells were treated with TCDD (at a final concentration range of 10-1000 nM in the T75 culture flask for CYP1B1 protein study or in the culture plate well for MTT assay) or with DMSO for 24 h. For panel A, 20 μ g total protein was loaded for each microsome sample per lane; lane 1 contains Leydig cells treated with DMSO (vehicle), lane 2 contains cells treated with 10 nM TCDD, lane 3 contains cells treated with 100 nM TCDD, lane 4 contains cells treated with 1000 nM TCDD. Immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel B, CYP1B1 protein concentration in microsomes was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein. Results are expressed as mean \pm SD of four separate experiments performed on different days. For panel C, the MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of TCDD-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

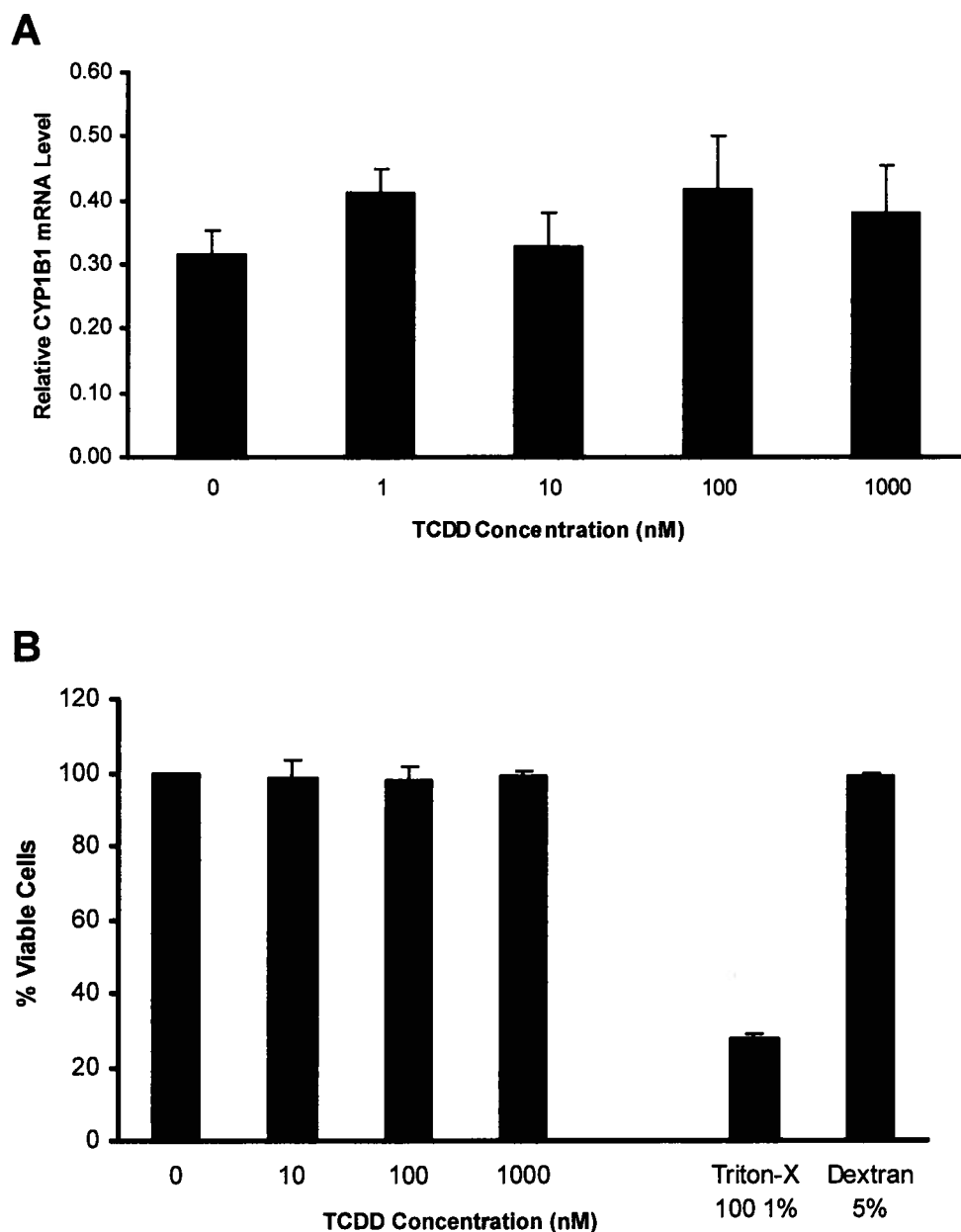


Figure 3.42: Effect of treatment with varying concentrations of TCDD on (A) CYP1B1 mRNA expression and (B) cell viability in mouse Leydig MA-10 cells. Cells were treated with TCDD (at a final concentration range of 1-1000 nM in the T25 culture flask) or with DMSO for 12 h. For panel A, relative CYP1B1 mRNA levels were determined by RT-PCR followed by densitometric analysis of ethidium bromide-stained agarose gels. Data are expressed as the ratio of the intensity of the CYP1B1 band to that of the β -actin mRNA band (reference gene). For panel B, the MTT assay was carried out according to the procedure described in “Materials and Methods”. Viability of TCDD-treated cells is expressed as a percentage of the viability of vehicle-treated cells, which was set at 100%. Values shown are the mean \pm SD of three separate experiments performed on different days.

3.16. DMBA Metabolism Assay

CYP1B1 catalyzes the formation of dihydroxy metabolites of DMBA such as DMBA-3,4-diol and DMBA-5,6-diol (Choudhary et al., 2004; Savas et al., 1997). Hence, formation of DMBA-3,4-diol and DMBA-5,6-diol from DMBA can be used as a marker assay for CYP1B1. An LC/MS-based DMBA metabolism assay was developed to measure CYP1B1 catalytic activity in microsomal samples.

Hepatic microsomal samples prepared from rats treated with 3-MC (25 mg/kg/day for three consecutive days), testicular microsomal samples prepared from TCDD-treated rats (single intraperitoneal injection with 100 µg TCDD/kg), and recombinant human CYP1B1 protein were tested for DMBA metabolism. Peaks with retention times and m/z similar to those of DMBA-3,4-diol and DMBA-5,6-diol metabolite standards were not detected after incubation of DMBA with liver microsomes prepared from 3-MC treated rats (containing high levels of CYP1A1 and CYP1A2 proteins), testis microsomes prepared from TCDD-treated rats (containing a high level of CYP1B1 protein) and recombinant human CYP1B1 protein for 60 min. However, several new peaks (M1, M2, M3, M4 and M5), which had different retention times than those of the DMBA-3,4-diol and DMBA-5,6-diol metabolite standards, were observed after incubation of DMBA with liver microsomes prepared from 3-MC-treated rats (Figure 3.43A). The new peaks were not present in chromatograms obtained using control reaction mixture (i.e. a zero minute incubation period) or reaction mixtures containing hepatic microsomes prepared from control rats. The identity of the new peaks could not be established and the peaks were not observed after incubation of DMBA with testis microsomes prepared from rats treated with TCDD (containing a high level of CYP1B1 protein) (Figure 3.43B). Thus, formation of these new peaks does not appear to be useful for measuring CYP1B1 catalytic activity. In summary, the catalytic activity of CYP1B1 was not measured successfully. An inefficient extraction of the metabolites (DMBA-

3,4-diol and DMBA-5,6-diol) from the reaction mixture is the most likely reason for the lack of detection of DMBA-3,4-diol and DMBA-5,6-diol peaks in the LC/MS chromatogram.

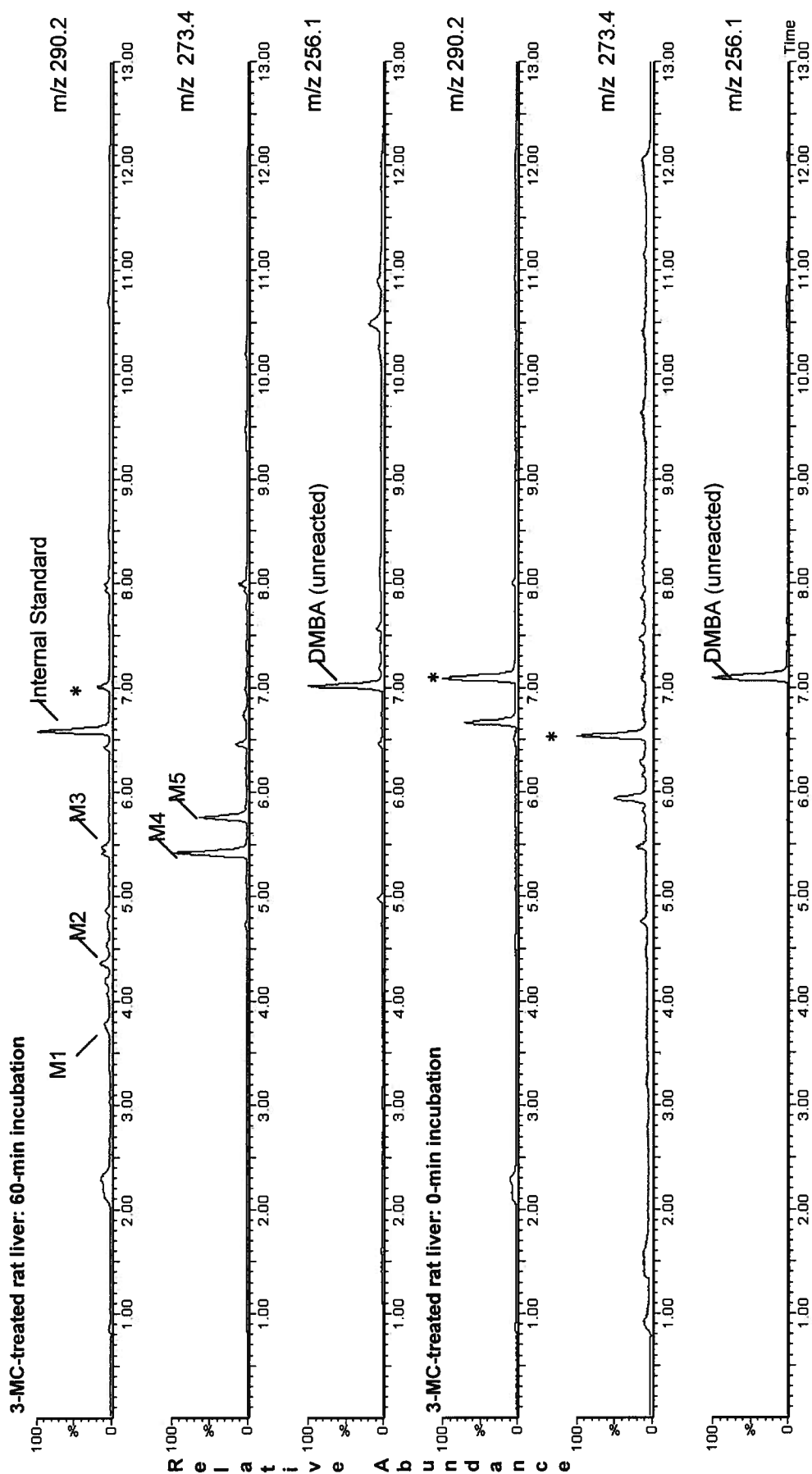


Figure 3.43A: Representative LC/MS chromatogram showing metabolites from a standard mixture after incubation of DMBA with microsomes from 3-MC-treated rat liver for 0- and 60-min. The internal standard was epi-testosterone. Peaks M1, M2, M3, M4 and M5 are metabolites that did not correspond to DMBA-3,4-diol and DMBA-5,6-diol metabolite standards. *, peaks that were present in both 0-min incubation and 60-min incubation mixtures.

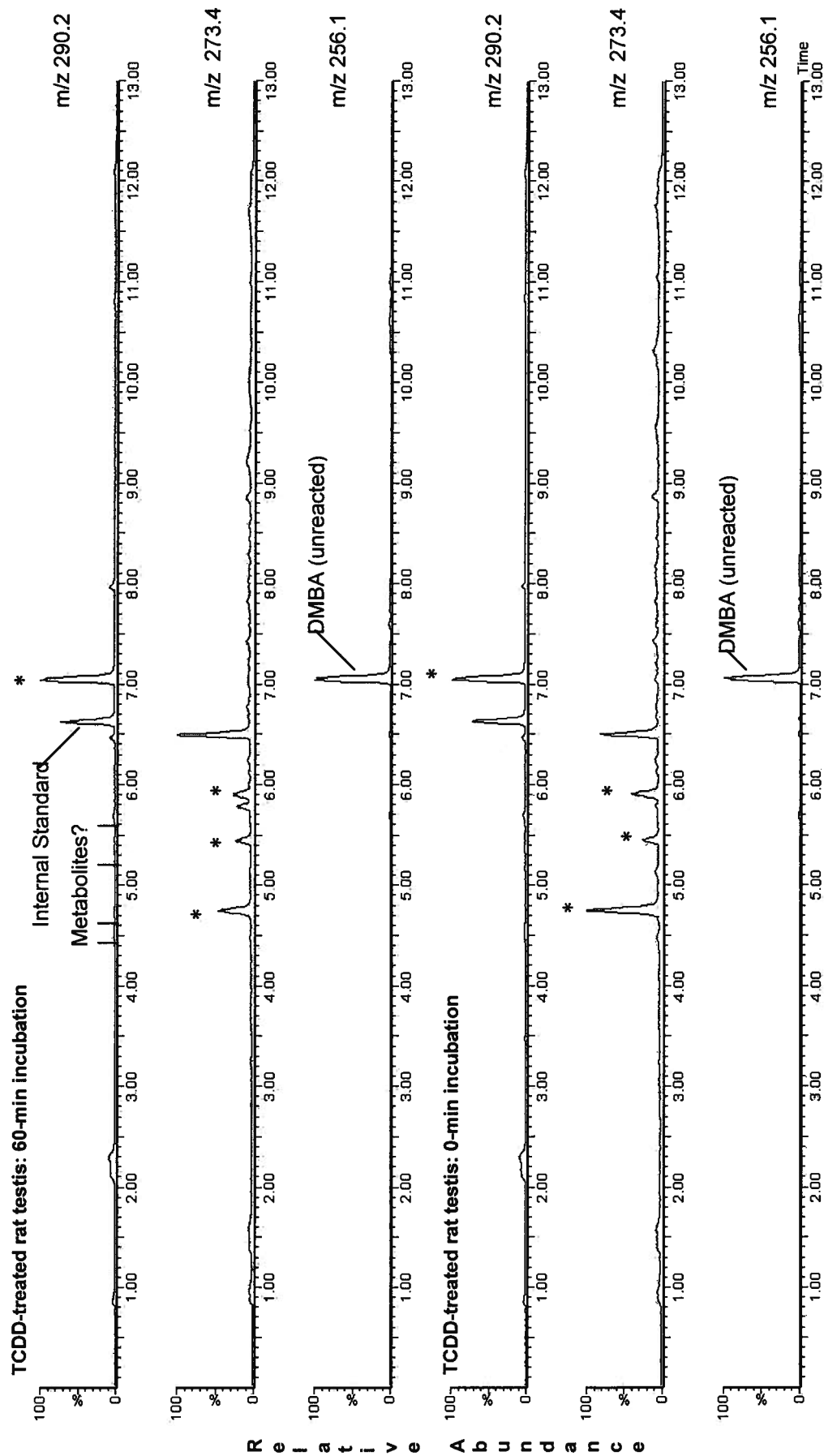


Figure 3.43B: Representative LC/MS chromatogram showing metabolites from a standard mixture after incubation of DMBA with microsomes from TCDD-treated rat testis for 0- and 60-min. The internal standard was epi-testosterone. *, peaks that were present in both 0-min incubation and 60-min incubation mixtures.

PART 2: RAT CYP2S1 EXPRESSION

Like CYP1B1, CYP2S1 is a CYP enzyme that is thought to be expressed in extrahepatic tissues, is presumed to be regulated by the AhR and its catalytic function is unknown.

3.17. CYP2S1 mRNA Expression in Rat Tissues

To investigate sex- and tissue-specific expression of CYP2S1 in rats, CYP2S1 mRNA levels were determined, relative to β -actin, in various tissues prepared from corn oil-treated male and female rats. Following RT-PCR, CYP2S1 mRNA was detected, using ethidium bromide-stained agarose gels, in liver and all extrahepatic tissues examined, except for the adrenal gland (Figure 3.44). For comparative purposes, CYP1A1 mRNA expression was measured in the same tissues. CYP1A1 mRNA was detected in all tissues examined, except kidney and adrenal gland (Figure 3.44).

Determination of relative CYP2S1 levels using densitometric analysis of ethidium bromide-stained bands showed that relative CYP2S1 mRNA levels in lung were significantly greater than in liver, kidney, stomach, duodenum, jejunum, ileum, spleen, and skin of female rats (Figure 3.45). In male rats, however, relative CYP2S1 mRNA levels in lung were not significantly greater than in kidney, stomach, duodenum, jejunum, and ileum but were greater than in liver, spleen and skin (Figure 3.45). For any individual tissue examined in this study, CYP2S1 mRNA levels were not significantly different between male and female rats (Figure 3.45).

The results of RT-PCR analysis demonstrate that CYP2S1 mRNA was expressed in liver and extrahepatic tissues of male and female rats. Relative CYP2S1 mRNA levels were higher in lung than in liver. There was no sex-dependent difference in constitutive CYP2S1 mRNA expression in adult rats.

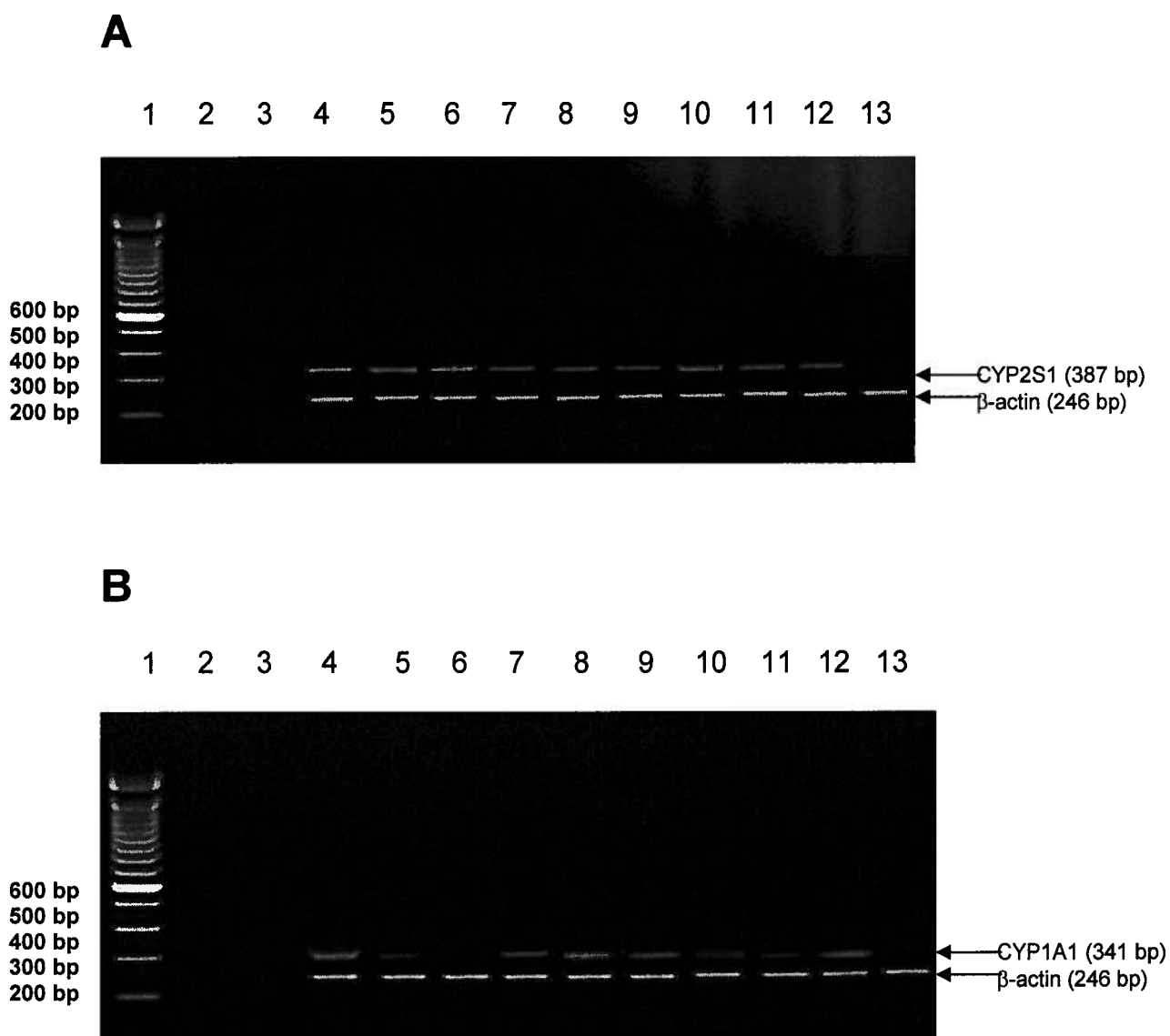


Figure 3.44: Representative ethidium bromide-stained agarose gel showing hepatic and extrahepatic CYP2S1 mRNA expression in male rats (photograph A) and CYP1A1 mRNA expression in female rats (photograph B). Total RNA was isolated and the block cyclor RT-PCR was performed for co-amplification of CYP2S1 or CYP1A1 cDNA with β -actin cDNA as the internal control as described under “Materials and Methods”. Agarose gels were stained with ethidium bromide. Lane 1: DNA ladder; lane 2- negative control (no cDNA); lane 3-negative control (no primer); lane 4-lung; lane 5-liver; lane 6-kidney; lane 7-stomach; lane 8- duodenum; lane 9-jejunum; lane 10-ileum; lane 11-spleen; lane 12-skin; lane 13-adrenal.

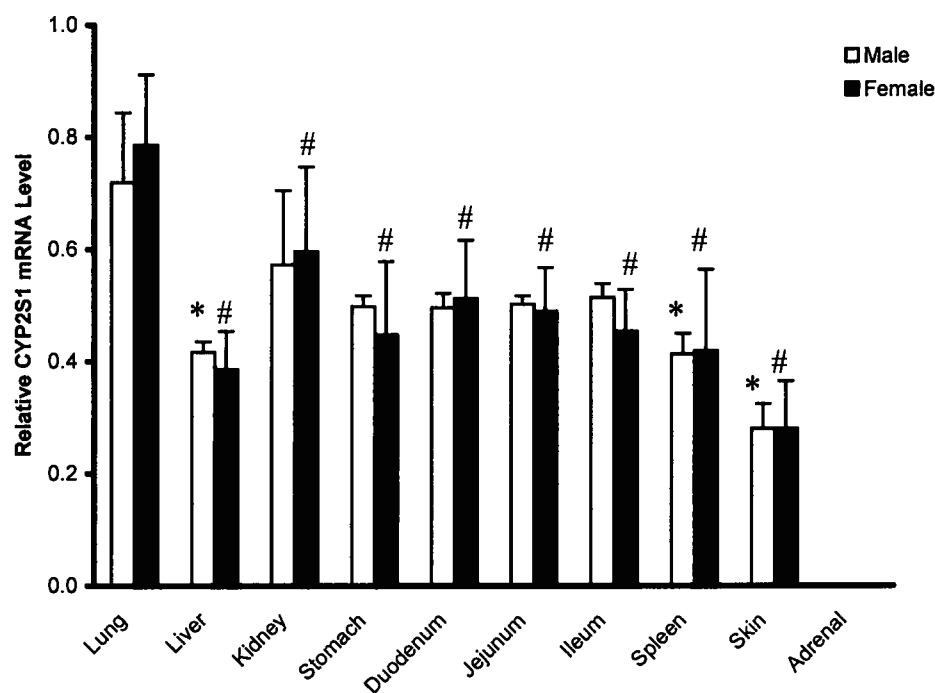


Figure 3.45: Comparative CYP2S1 mRNA expression in various tissues from adult male and female SD rats (n=4). CYP2S1 mRNA levels were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR and data are expressed as the ratio of the intensity of the CYP2S1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of four male or female rats. Data were analyzed using Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from CYP2S1 mRNA levels in lung from male rats with a *P* value <0.05 . #, Significantly different from CYP2S1 mRNA levels in lung from female rats with a *P* value <0.05 .

3.18. Effect of Treatment with AhR agonists on CYP2S1 mRNA Expression

Due to the presence of XRE sequences in the CYP2S1 promoter region, similar to XRE sequences found in AhR agonist-inducible CYP1 enzymes, CYP2S1 mRNA expression is thought to be regulated by the AhR-mediated pathway (Smith et al., 2003). AhR agonists such as 3-MC and TCDD are known to induce CYP1 enzymes in rats (Jones and Riddick, 1996; Walker et al., 1999) and were used to determine if CYP2S1 mRNA levels are inducible after AhR activation. Treatment of adult female SD rats with 3-MC (25 mg/kg/day for three consecutive days) increased relative CYP2S1 mRNA levels by approximately 3-fold in lung and to a lesser degree in stomach, jejunum and ileum. CYP2S1 mRNA levels were not increased in other tissues (Figure 3.46A). For comparison, CYP1A1 mRNA levels were measured in the same tissues. CYP1A1 mRNA levels were increased 34-fold in liver, 6-fold in lung, 2-fold in stomach, and 2-fold in duodenum, relative to the level measured in vehicle-treated rats (Figure 3.46B).

Since 3-MC modestly increased CYP2S1 mRNA levels in rats, TCDD, the most potent AhR agonist, was used to further investigate if CYP2S1 mRNA expression is regulated by the AhR-mediated pathway. Adult male rats were treated with various dosages of TCDD to determine if the increase in CYP2S1 mRNA levels occurs in a dose-dependent manner. CYP2S1 mRNA levels were increased in lung following treatment with TCDD at dosages of 5, 10, 50 and 100 µg/kg, whereas CYP2S1 mRNA levels in liver and kidney were increased at dosages of 10, 50 and 100 µg/kg. At the highest dosage tested, TCDD increased relative CYP2S1 mRNA levels by 7-fold in lung, 3.6-fold in liver and 2.7-fold in kidney, in comparison to the vehicle-treated group (Figure 3.47A). In comparison, CYP1A1 mRNA levels were increased by 12-fold in lung and 60-fold in liver, relative to the vehicle-treated group, at the highest dosage of TCDD tested (Figure 3.47B).

The results of treatment of rats with 3-MC and TCDD suggest that expression of CYP2S1 mRNA in lung is regulated by the AhR pathway. As expected, TCDD was a more potent inducer of CYP2S1 mRNA expression than 3-MC. CYP2S1 mRNA expression in liver was less responsive to treatment with AhR agonists than CYP1A1 mRNA expression.

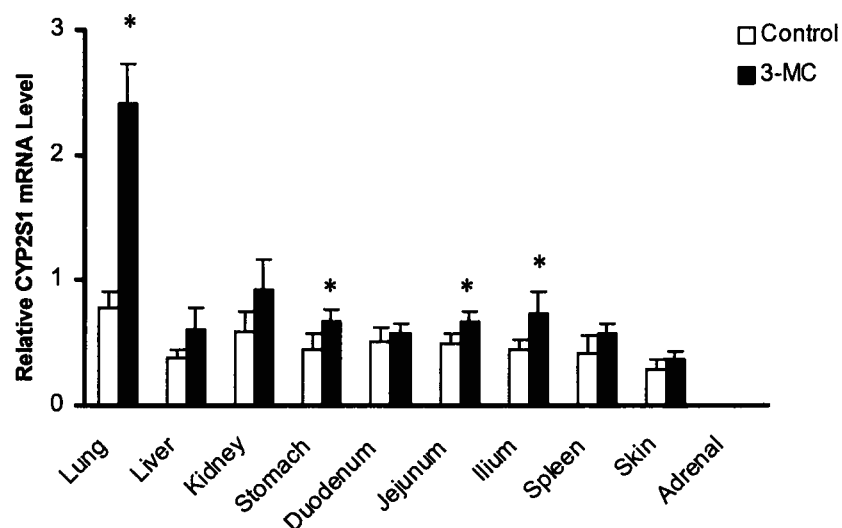
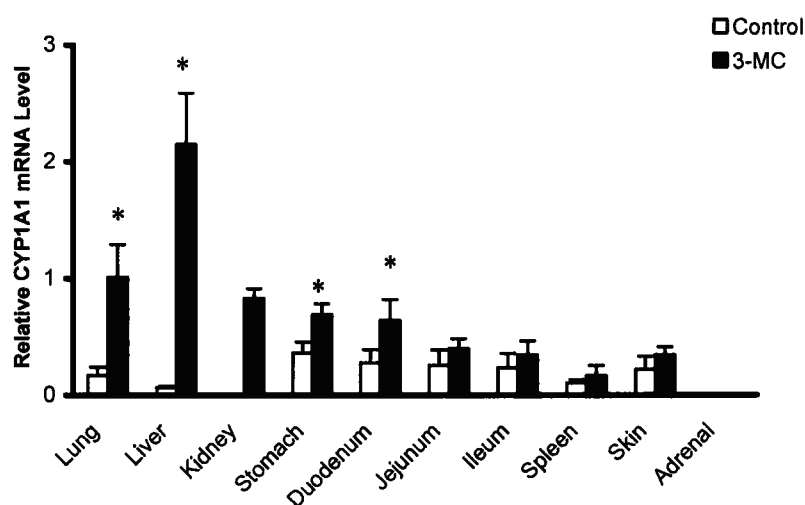
A**B**

Figure 3.46: Effect of treatment with 3-MC on CYP2S1 mRNA (plot A) and CYP1A1 mRNA (plot B) expression. Adult female SD rats (n=4) were treated with 3-MC (25 mg/kg/day) for three consecutive days. Relative CYP2S1 mRNA and CYP1A1 mRNA levels were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR and data are expressed as the ratio of the intensity of the CYP2S1 mRNA or CYP1A1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of four rats per treatment group. Data were analyzed using unpaired student's t-test (parametric). *, Significantly different from the corresponding control (corn oil-treated) group with a P value <0.05 .

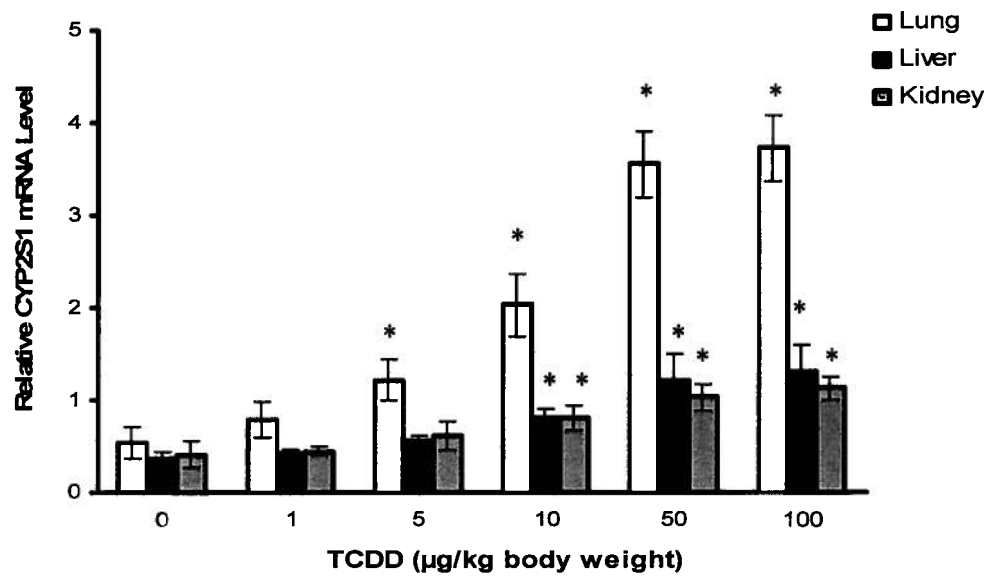
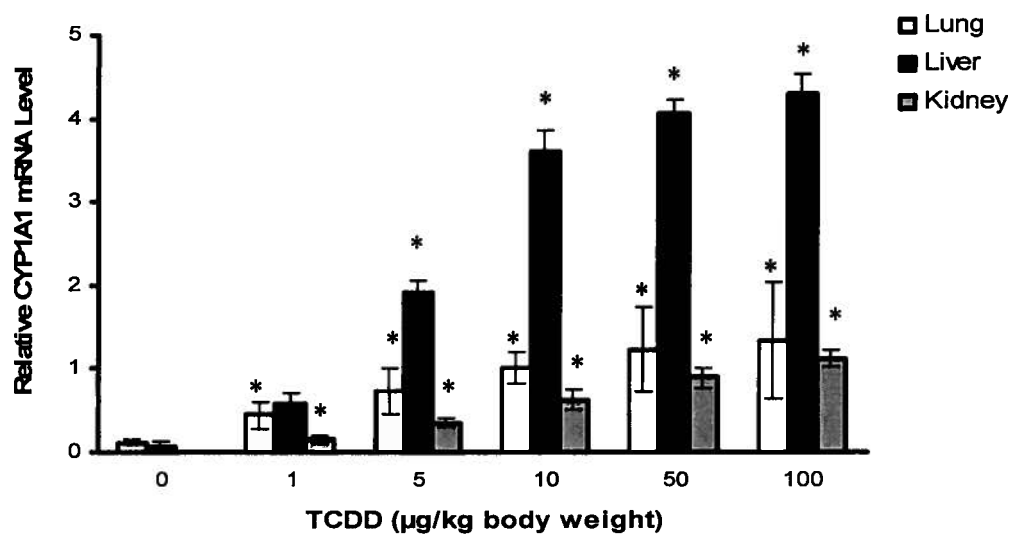
A**B**

Figure 3.47: Effect of treatment with varying dosages of TCDD on CYP2S1 mRNA (plot A) and CYP1A1 mRNA (plot B) expression. Adult male SD rats (n=3) were treated with TCDD (0, 1, 5, 10, 50, 100 µg/kg with a single intraperitoneal injection). Relative CYP2S1 mRNA and CYP1A1 mRNA levels were determined by densitometric analysis of ethidium bromide-stained agarose gels after RT-PCR and data are expressed as the ratio of the intensity of the CYP2S1 or CYP1A1 mRNA band to that of the β -actin mRNA band (reference gene). Values shown are the mean \pm SD of three rats per treatment group. CYP2S1 mRNA data from TCDD-treated lung and kidney and CYP1A1 mRNA data from TCDD-treated lung and liver were analyzed using one-way analysis of variance (parametric), followed by Student Newman Keul's multiple comparison test. CYP2S1 mRNA data from TCDD-treated liver and CYP1A1 mRNA data from TCDD-treated kidney were analyzed using Kruskal-Wallis one-way analysis of variance test (nonparametric), followed by Student Newman Keul's multiple comparison test. *, Significantly different from the corresponding control (dioxane:corn oil-treated) group with a *P* value <0.05.

3.19. Characterization of Anti-CYP2S1 Peptide Serum

Antibody against rat CYP2S1 was not commercially available. Thus, anti-rat CYP2S1 serum was generated using an anti-peptide approach, as described in Materials and Methods section. The reactivity of rabbit sera prepared against two CYP2S1 peptides (C-terminus amino acid sequence or an internal amino acid sequence) was assessed by immunoblot analysis and noncompetitive ELISA. Both anti-rat CYP2S1 sera preparations showed a strong reaction, as determined by noncompetitive ELISA, with their corresponding free synthetic peptides (either C-terminus or internal sequence). Both anti-rat CYP2S1 sera preparations either did not react, or reacted weakly, with a panel of recombinant and purified CYP enzymes and other proteins (Table 3.1 and Table 3.2). When evaluated by immunoblot analysis, the anti-rat CYP2S1 serum preparation that was raised against the internal amino acid sequence of rat CYP2S1 (i.e. CLKMAQEKQDPGTEF) did not react with protein bands in the predicted CYP2S1 region on blots containing rat and mouse microsomal preparations (Figure 3.48). On the basis of the poor reactivity of this serum preparation on immunoblots, it was not used in further experiments.

The immunoreactivity profile of the anti-rat CYP2S1 serum preparation that was raised against the C-terminus peptide (CDFQLRVWPTGDQSR), in the ELISA assay, is summarized in Table 3.1. The immunoreactivity of this serum preparation was further characterized by immunoblot analysis using rat and mouse tissue microsome samples. Based on the predicted molecular weight of CYP2S1 (using prestained protein standards), we tentatively identified a band of approximately 50 kD, which reacted with anti-rat CYP2S1 serum prepared to the C-terminus sequence, as CYP2S1 in rat lung microsomal samples (Figure 3.49A). This band was not detected when the same blots were probed with preimmune serum (Figure 3.49C). Anti-rat CYP2S1 serum (C-terminus sequence) was also able to detect CYP2S1 protein in mouse lung microsomes. The reactivity of CYP2S1 antibody (C-terminus sequence) with mouse CYP2S1 protein is not surprising because thirteen of the fourteen amino acid residues in the C-terminus of

rat and mouse are identical (Table 3.3). We used the anti-rat CYP2S1 serum (C-terminus sequence) for all subsequent experiments.

To further evaluate the specificity of anti-rat CYP2S1 serum (C-terminus sequence), immunoblots with purified or recombinant rat CYP1A1, CYP1A2, CYP1B1, CYP2A1, CYP2A2, CYP2B1, CYP2C11, CYP2C12, CYP2D1, CYP2E1, CYP3A1 and epoxide hydrolase proteins were probed with this antiserum. The antiserum did not react with any of the purified or recombinant rat CYP standards or epoxide hydrolase (Figure 3.50). This finding is consistent with the fact that the C-terminus sequence we selected for the antiserum preparation was distinct from the C-terminus of other rat CYP enzymes, as published in National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein>).

As there was no purified or recombinant CYP2S1 protein standard available for immunoblot studies, the specificity of the CYP2S1 protein band (detected by C-terminus antibody) was investigated using the antiserum preabsorption technique. Immunoblots were probed with anti-rat CYP2S1 serum (C-terminus sequence) that was preincubated with unconjugated peptide (1.12 $\mu\text{g}/\mu\text{l}$ antiserum) or peptide-KLH conjugate (1.12, 2.25, or 5.62 $\mu\text{g}/\mu\text{l}$ antiserum) for 1 h at room temperature. Results were compared to immunoblots that were probed with anti-rat CYP2S1 serum that was not preincubated with unconjugated peptide or peptide-KLH conjugate. The 50 kD protein band on immunoblots containing rat and mouse microsomal samples that we had tentatively identified as CYP2S1 was not detected when the serum was preabsorbed with unconjugated peptide or peptide-KLH conjugate (Figure 3.49B). Furthermore, the reaction of preabsorbed anti-rat CYP2S1 serum with unconjugated synthetic peptide was attenuated, relative to anti-rat CYP2S1 serum that was not preincubated, in the ELISA assay (Figure 3.51).

The results obtained from ELISA and immunoblot analysis suggest that anti-rat CYP2S1 serum prepared against the C-terminus peptide of rat CYP2S1 was immunoreactive with

CYP2S1 protein, whereas anti-rat CYP2S1 serum prepared against an internal sequence of rat CYP2S1 was poorly reactive in immunoblot analysis. On the basis of this reactivity, a CYP2S1 protein was identified in rat and mouse lung with a molecular weight of approximately 50 kD.

Table 3.1: Reactivity of anti-rat CYP2S1 serum (generated against the C-terminus of rat CYP2S1) determined by noncompetitive enzyme-linked immunosorbent assay (ELISA)^a.

Proteins Tested	Absorbance at 490 nm		
	Preimmune Serum ^b	Anti-rat CYP2S1 Serum ^b	Anti-rat CYP2S1 Serum ^c
Synthetic peptide ^d	0.07	0.35	0.13
Synthetic peptide ^e	0.07	1.40	0.69
CYP1A1	0.06	0.12	0.08
CYP2B1	0.06	0.12	0.06
CYP2B2	0.05	0.08	0.06
CYP2C11	0.05	0.08	0.07
CYP2C13	0.06	0.09	0.06
CYP3A1	0.05	0.08	0.07
Epoxide hydrolase	0.05	0.09	0.06
Keyhole limpet hemocyanin	0.07	1.69	1.05
Carbonate buffer	0.05	0.11	0.07

^a Wells of 96-well microtest plates were coated with purified or recombinant rat CYP enzymes, epoxide hydrolase and keyhole limpet hemocyanin diluted to 0.2 µg/ml. Free synthetic peptide corresponding to C-terminus of rat CYP2S1 (i.e. CDFQLRVWPTGDQSR; 486-499 amino acids) was used at concentrations of 0.2 µg/ml or 1 µg/ml. The ELISA was carried out as described in “Materials and Methods”

^b 1:100 dilution

^c 1:250 dilution

^d synthetic unconjugated peptide concentration; 0.2 µg/ml

^e synthetic unconjugated peptide concentration; 1 µg/ml

Table 3.2: Reactivity of anti-rat CYP2S1 serum (generated against an internal sequence of rat CYP2S1) determined by noncompetitive enzyme-linked immunosorbent assay (ELISA)^a.

Proteins Tested	Absorbance at 490 nm	
	Preimmune Serum ^b	Anti-rat CYP2S1 Serum ^b
Synthetic peptide ^c	0.07	1.18
CYP1A1	0.05	0.19
CYP2B1	0.05	0.12
CYP2B2	0.06	0.08
CYP2C11	0.06	0.10
CYP2C13	0.06	0.12
CYP3A1	0.05	0.08
Epoxide hydrolase	0.06	0.08
Keyhole limpet hemocyanin	0.06	1.13
Carbonate buffer	0.04	0.46

^a Wells of 96-well microtest plates were coated with purified or recombinant rat CYP enzymes, epoxide hydrolase and keyhole limpet hemocyanin diluted to 0.2 µg/ml. Free synthetic peptide corresponding to an internal sequence of rat CYP2S1 (i.e. CLKMAQEKQDPGTEF; amino acids 272-286) was used at a concentration of 0.2 µg/ml. The ELISA was carried out as described in “Materials and Methods”

^b 1:100 dilution

^c synthetic unconjugated peptide concentration; 0.2 µg/ml

Table 3.3: Aligned amino acid sequence of the synthetic peptide with C-terminus amino acid sequences of CYP2S1 from different species^a.

C-Terminus Synthetic Peptide:	C-DFQLRVWPTGDQSR
Mouse (NP_083051)	478- AISGLFNIPP <u>DFQLRVWPTGDQSR</u> -501
Rat (NP_001100965)	476-AVRGLFNIPP <u>DFQLQVWPTGDQSR</u> -499
Human (NP_085125)	481-LFNIPPAFQLQ <u>VRPTDLHSTTQTR</u> - 504
Chimpanzee (XP_001147950)	262-LFNIPPAFQLQ <u>VRPTDLHSTTQTR</u> - 285
Rhesus monkey (XP_001094218)	367-LFNIPPAFQLQ <u>VRPTDLHSTTQTT</u> - 390
Dog (XP_541605)	471-LSLQPAVSGLFN <u>IPPAFQLRV</u> RP- 494

^a The synthetic amino acid sequence was subjected to online protein-protein BLAST search. Numbers in parentheses are the protein accession numbers obtained from the protein BLAST search at the National Center for Biotechnology Information (NCBI). Amino acids identical to the synthetic peptide are underlined and in bold type.

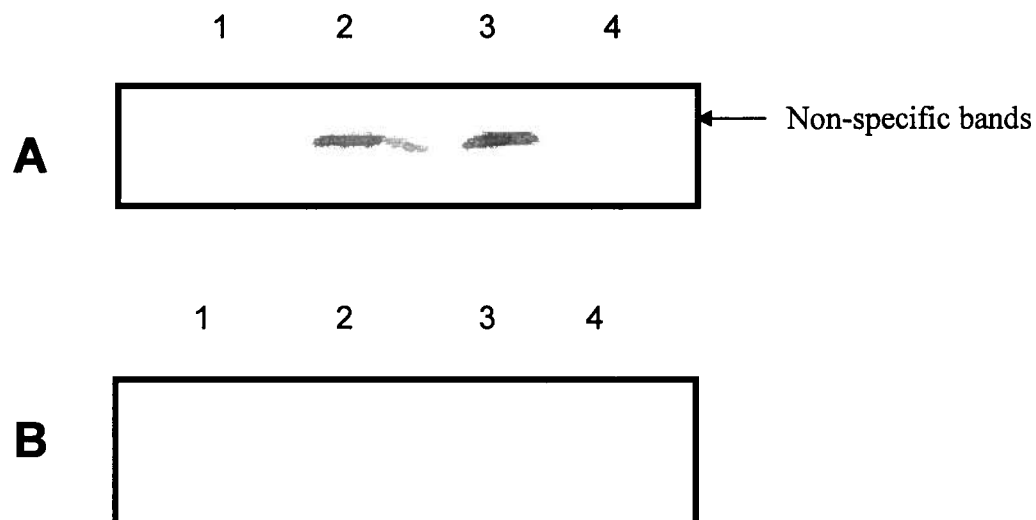


Figure 3.48: Immunoreactivity of serum, which was prepared to an internal sequence of rat CYP2S1 (i.e. CLKMAQEKQDPGTEF, amino acids 272-286), with rat and mouse lung microsomal samples. Immunoblots were developed using (A) anti-rat CYP2S1 serum (1:200 dilution) or (B) preimmune serum from rabbits. The bands shown in the blots represent non-specific protein bands. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using anti-rat CYP2S1 serum (1:200 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. Lane 1 contains untreated male mouse lung (60 μ g), lane 2 contains vehicle-treated male rat lung (60 μ g), lane 3 contains TCDD (50 μ g/kg) treated male rat lung (60 μ g), lane 4 contains untreated female rat lung (60 μ g).

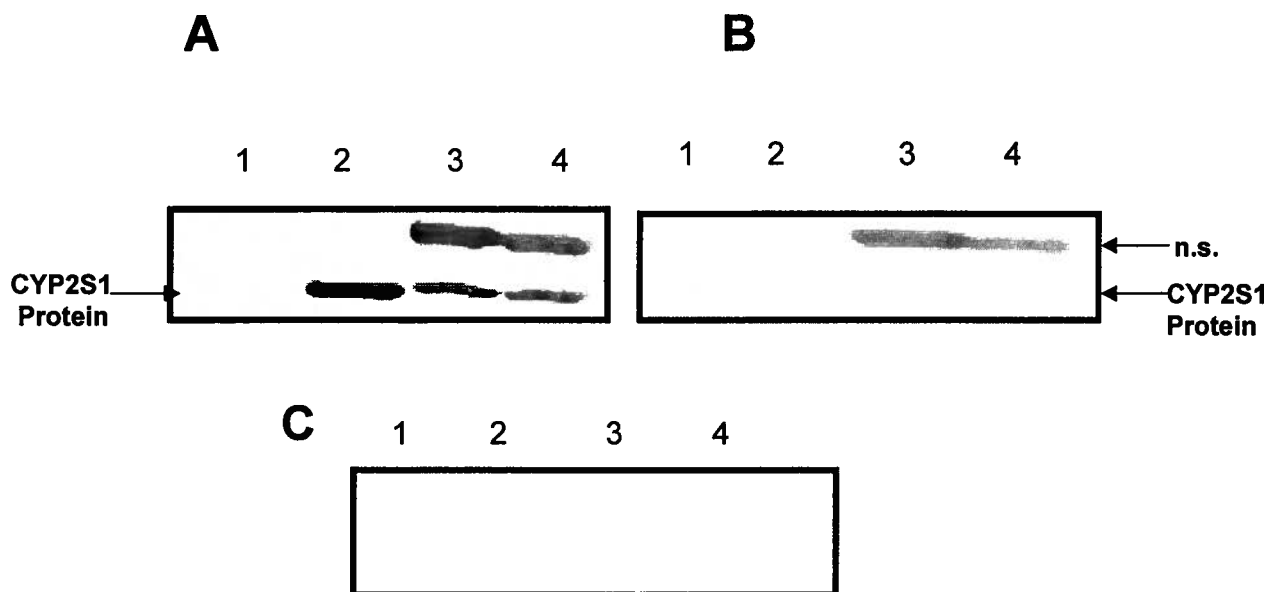


Figure 3.49: Identification of CYP2S1 protein in rat and mouse lung microsomal samples using anti-rat CYP2S1 serum, which was prepared to the C-terminal peptide sequence of CYP2S1 (i.e. CDFQLRVWPTGDQSR). Immunoblots were developed using (A) anti-rat CYP2S1 serum (1:200 dilution) or (B) anti-rat CYP2S1 serum (1:200 dilution) preincubated together at room temperature for 1 h with the unconjugated peptide, using 1.12 µg peptide/µl antiserum (C) preimmune serum from rabbits. The lower band represents CYP2S1, whereas the upper band represents a non-specific (n.s.) protein band. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using anti-rat CYP2S1 serum (1:200 dilution) or preabsorbed antiserum (1:200 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. Lane 1 contains recombinant rat CYP3A1 protein std. (0.5 pmol), lane 2 contains untreated male mouse lung (20 µg), lane 3 contains vehicle-treated male rat lung (60 µg), lane 4 contains TCDD (50 µg/kg)-treated male rat lung (40 µg).

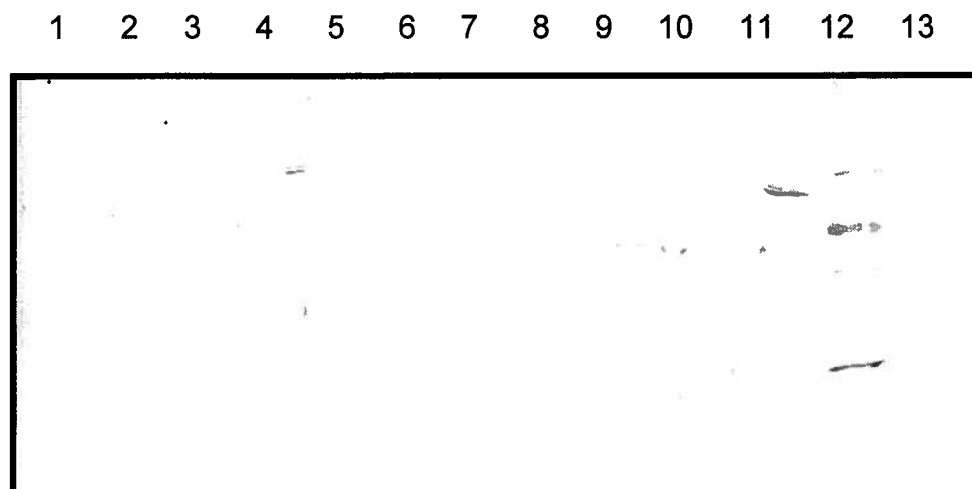


Figure 3.50: Characterization of cross-reactivity of anti-rat CYP2S1 serum, which was prepared to the C-terminal peptide sequence of CYP2S1 (i.e. CDFQLRVWPTGDQSR), using immunoblot analysis. The bands shown in the blot represent non-specific protein bands. Recombinant and purified proteins and microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using anti-rat CYP2S1 serum (1:200 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. Lane 1 contains purified rat CYP1A1 (0.5 pmol), lane 2 contains purified rat CYP1A2 (1 pmol), lane 3 contains recombinant rat CYP1B1 (0.4 pmol), lane 4 contains purified rat CYP2A1 (0.5 pmol), lane 5 contains purified rat CYP2A2 (0.4 pmol), lane 6 contains recombinant rat CYP2B1 (1 pmol), lane 7 contains recombinant rat CYP2C6 (0.4 pmol), lane 8 contains recombinant rat CYP2C11 (0.4 pmol), lane 9 contains recombinant rat CYP2C12 (0.4 pmol), lane 10 contains recombinant rat CYP2D1 (0.5 pmol), lane 11 contains recombinant rat CYP2E1 (1 pmol), lane 12 contains recombinant rat CYP3A1 (0.5 pmol), lane 13 contains purified epoxide hydrolase (0.5 μ g).

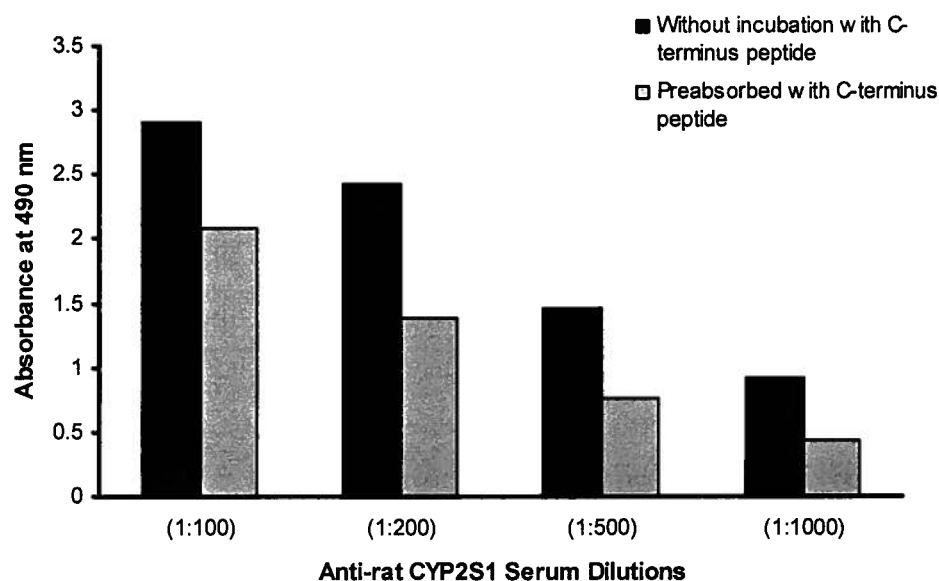


Figure 3.51: Immunoreactivity of anti-rat CYP2S1 serum, which was prepared to the C-terminal peptide sequence of CYP2S1, after preabsorption with unconjugated C-terminus peptide (i.e. CDFQLRVWPTGDQSR) determined by noncompetitive enzyme-linked immunosorbent assay (ELISA). Wells of 96-well microtest plates were coated with synthetic unconjugated peptide corresponding to C-terminus of rat CYP2S1 at a concentration of 1 $\mu\text{g/ml}$. In a second microtest plate, anti-rat CYP2S1 serum, at various dilutions, was incubated overnight with unconjugated peptide (1.12 $\mu\text{g}/\mu\text{l}$ antiserum) and subsequently the preabsorbed serum was used as primary antibody in ELISA (grey bars). Anti-rat CYP2S1 serum that was not preincubated with C-terminus peptide was also used in ELISA (black bars). Immunoreactivity of anti-rat CYP2S1 serum, with or without preabsorption, was determined by ELISA as described in “Materials and Methods”. Immunoreactivity of anti-rat CYP2S1 serum (C-terminus) is expressed as absorbance at 490 nm measured on a microplate reader.

3.20. CYP2S1 Protein Expression in Rat Tissues and Effect of AhR Agonists

Recombinant or purified CYP2S1 protein was not commercially available for use as a calibration standard for quantification of immunoblots. Hence, CYP2S1 protein levels in microsomes were measured on a relative basis. Relative CYP2S1 protein levels were calculated as a ratio of the contour quantity (i.e. intensity) of the CYP2S1 band in one sample to that of the CYP2S1 band in a reference sample. A defined constant amount of lung microsomal protein (50 μg) from vehicle-treated male rat was loaded onto gels as the reference sample and the contour quantity of the CYP2S1 band in this sample was used for the relative quantification of CYP2S1 protein in other microsomal samples.

To measure CYP2S1 expression at the protein level, microsomal fractions prepared from adult rat lung, liver, kidney and stomach were tested by immunoblot analysis when the same amount of microsomal protein (50 μg) from each tissue was applied to each lane. An immunoreactive band corresponding to rat CYP2S1 protein was observed in lung and stomach microsomal samples prepared from untreated adult male rats and corn-oil-treated adult female rats (Figure 3.52A), whereas no band was detected in microsomes prepared from liver, small intestine, spleen, thymus, brain, skin, adrenal, testis, uterus or ovary. A faint CYP2S1 protein band was detected in kidney microsomes from male rats, but it was not quantifiable by densitometric analysis. Densitometric analysis of the staining intensity of the immunoreactive CYP2S1 band in lung, stomach and kidney samples indicated that there was no difference in relative CYP2S1 protein levels between male and female rats when the same amount of microsomal protein (50 μg) from each tissue was applied to each lane (Figure 3.52B). Relative CYP2S1 protein levels were approximately 10 times greater in lung and stomach than in kidney.

Relative CYP2S1 protein levels were increased 2-fold in lung microsomes after treatment with TCDD at dosage of 50 $\mu\text{g}/\text{kg}$ but relative CYP2S1 protein levels were not increased in the 100 $\mu\text{g}/\text{kg}$ treatment group (Figure 3.53). Likewise, relative CYP2S1 protein levels were similar

in kidney microsome samples prepared from rats treated with vehicle or TCDD at all dosages. Similarly, treatment with BaP or 3-MC failed to increase relative CYP2S1 protein levels in lung and liver microsomes (Figure 3.54 and Figure 3.55).

To determine the effectiveness of the TCDD treatment, CYP1A1, CYP1A2 and CYP1B1 protein levels were measured in the same lung, liver and kidney microsomes by immunoblot analysis. Densitometric quantification of immunoblots showed a dose-dependent increase of hepatic CYP1A1, CYP1A2 and CYP1B1 protein levels with maximal levels of CYP1A1 and CYP1A2 observed after treatment with TCDD at a dosage of 100 µg/kg (Figure 3.56 and Figure 3.57). CYP1A1 protein, but not CYP1A2 protein, was detected in lung and kidney microsomes after treatment with TCDD (5 µg/kg) (Figure 3.56). CYP1B1 protein was detected in lung but not kidney microsomes after TCDD treatment (Figure 3.57).

The results of immunoblot analysis using anti-rat CYP2S1 serum (C-terminus) suggest that CYP2S1 protein was expressed in rat lung, stomach and kidney. Relative CYP2S1 protein levels were not induced in rats after treatment with 3-MC, BaP or TCDD, which indicate that CYP2S1 protein expression may not be regulated by the AhR pathway.

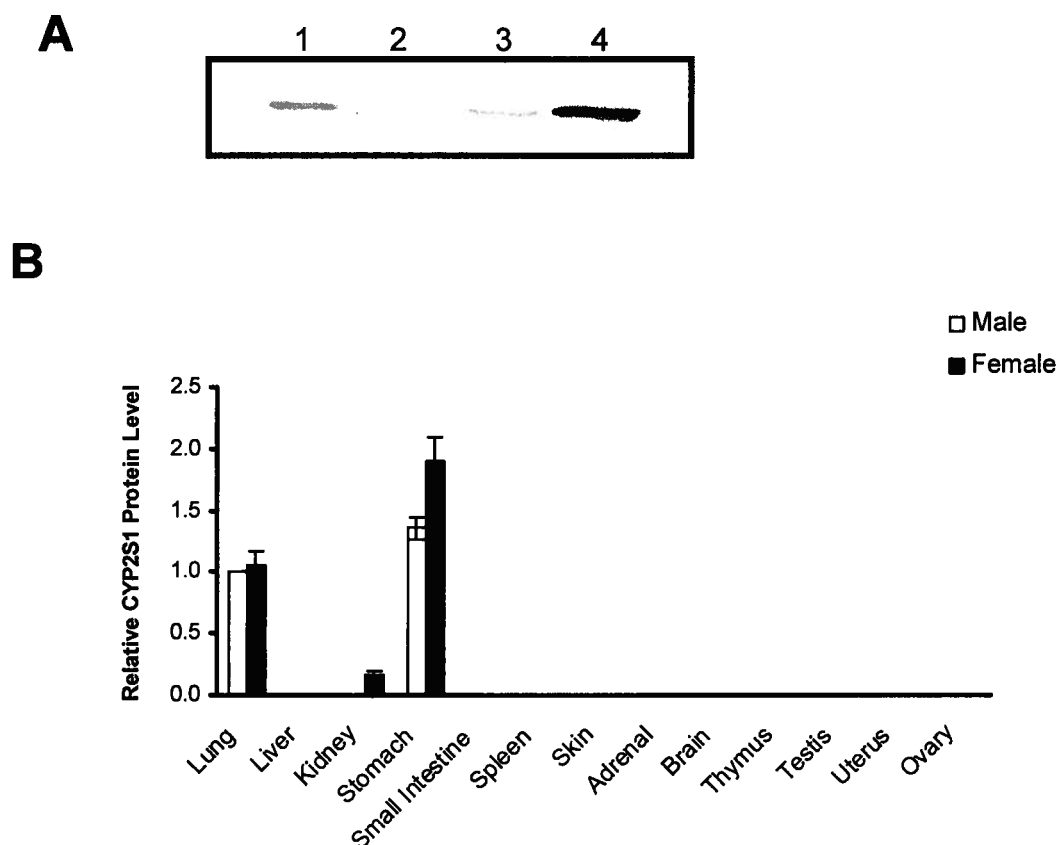


Figure 3.52: CYP2S1 protein expression in various tissues from adult male and female rats. (A) A representative immunoblot of microsomal samples from female SD rats probed with anti-rat CYP2S1 serum. (B) Relative CYP2S1 protein expression in lung, kidney and stomach microsomes from male and female rats. Microsomes were prepared from the pooled samples of adult male SD rats (three rats in each group) treated with a single intraperitoneal injection of vehicle (1 part dioxane: 6 parts corn oil) or untreated adult female SD rats. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP2S1 serum (1:200 dilution) prepared to the C-terminus sequence of CYP2S1 and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, 50 μ g total protein was loaded for each microsome sample per lane; Lane 1 contains lung, lane 2 contains liver, lane 3 contains kidney, and lane 4 contains stomach. For panel B, the intensity of CYP2S1 protein bands was measured by densitometric analysis of immunoblots. Relative CYP2S1 protein levels in microsomes are expressed as a ratio of the intensity of the CYP2S1 band to that of the reference band (i.e. CYP2S1 band obtained from 50 μ g lung microsomal protein prepared from vehicle-treated male rats). Results are expressed as mean \pm SD of three different immunoblots.

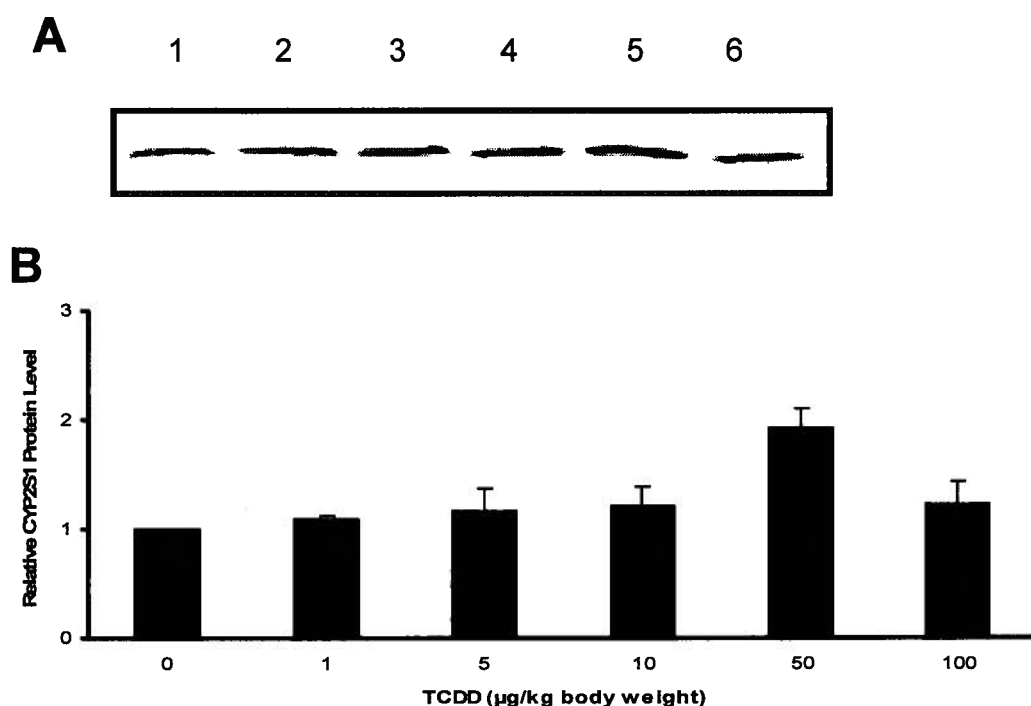


Figure 3.53: Effect of treatment with varying dosages of TCDD on pulmonary CYP2S1 protein expression in adult male SD rats. (A) A representative immunoblot of microsomal samples from TCDD-treated rat lung. (B) Relative CYP2S1 protein levels in rat lung after treatment with TCDD. Microsomes were prepared from pooled lung, liver and kidney of male rats (three rats in each group) treated with a single intraperitoneal injection of vehicle (1 part dioxane: 6 parts corn oil) or 1, 5, 10, 50, 100 µg TCDD/kg. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP2S1 serum (1:200 dilution) raised against the C-terminus sequence of CYP2S1 and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, 50 µg total protein was loaded for each microsomal sample per lane; lane 1 contains lung (from vehicle-treated rats), lane 2 contains lung (from rats treated with 1 µg TCDD/kg), lane 3 contains lung (from rats treated with 5 µg TCDD/kg), lane 4 contains lung (from rats treated with 10 µg TCDD/kg), lane 5 contains lung (from rats treated with 50 µg TCDD/kg), and lane 6 contains lung (from rats treated with 100 µg TCDD/kg). For panel B, the intensity of CYP2S1 protein bands was measured by densitometric analysis of immunoblots. Relative CYP2S1 protein levels in microsomes are expressed as a ratio of the intensity of the CYP2S1 band to that of the reference band (i.e. CYP2S1 band obtained from 50 µg lung microsomal protein prepared from vehicle-treated male rats). Results are expressed as mean \pm SD of three different immunoblots.

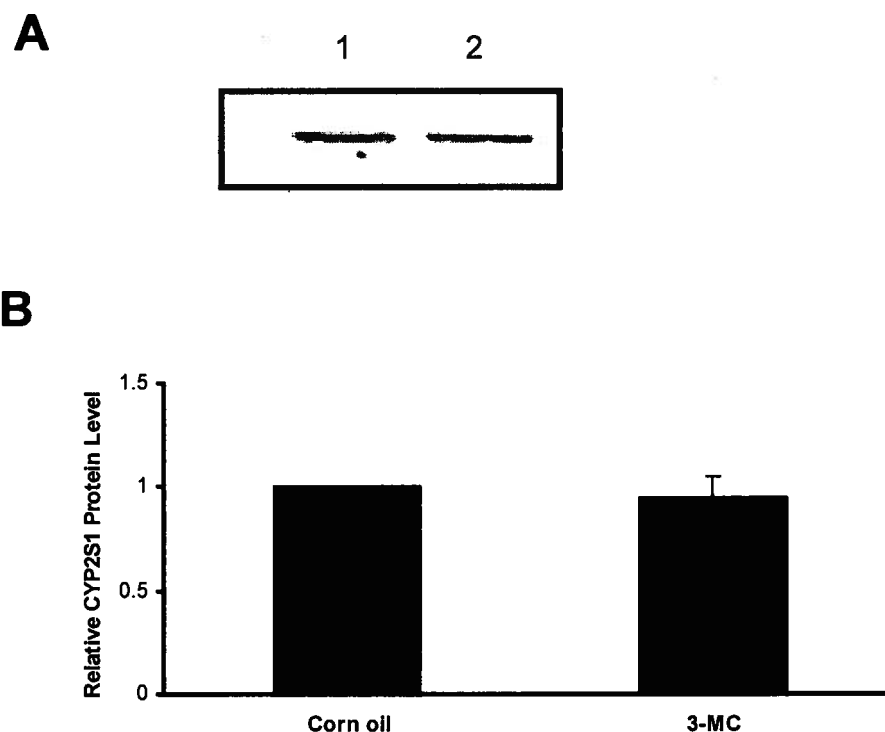


Figure 3.54: Effect of treatment with 3-MC on CYP2S1 protein expression in adult male SD rats. (A) A representative immunoblot of microsomal samples from 3-MC-treated rat lung. (B) Relative CYP2S1 protein levels in rat lung after treatment with 3-MC. Microsomes were prepared from pooled lungs of male rats (n=5) treated with 3-MC (25 mg/kg/day) or corn oil for three consecutive days. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP2S1 serum (1:200 dilution) raised against the C-terminus sequence of CYP2S1 and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, 50 μ g total protein was loaded for each microsomal sample per lane; lane 1 contains lung (from vehicle-treated rats) and lane 2 contains lung (from rats treated with 25 mg 3-MC/kg). For panel B, the intensity of CYP2S1 protein bands was measured by densitometric analysis of immunoblots. Relative CYP2S1 protein levels in microsomes are expressed as a ratio of the intensity of the CYP2S1 band to that of the reference band (i.e. CYP2S1 band obtained from 50 μ g lung microsomal protein prepared from vehicle-treated male rats). Results are expressed as mean \pm SD of two different immunoblots.

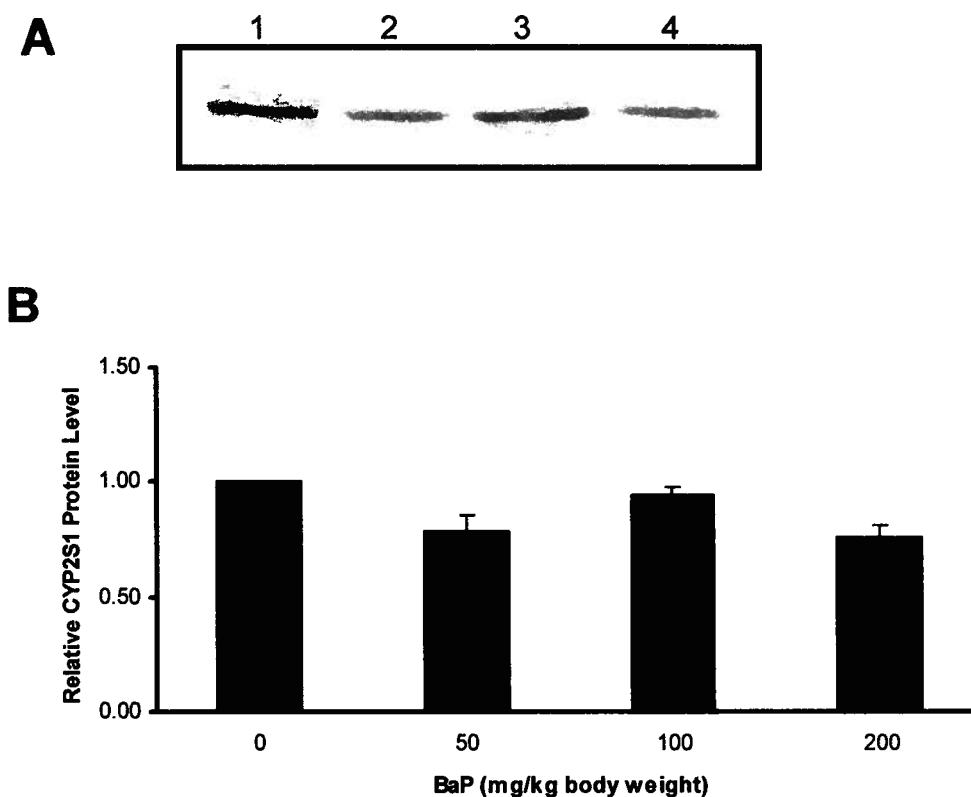


Figure 3.55: Effect of treatment with BaP on CYP2S1 protein expression in male SD rats. (A) A representative immunoblot of microsomal samples from BaP-treated rat lung. (B) Relative CYP2S1 protein levels in rat lung after treatment with BaP. Microsomes were prepared from pooled lungs of male rats (n=3) treated by oral gavage with BaP (50, 100 and 200 mg/kg/day) or corn oil for three consecutive days. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP2S1 serum (1:200 dilution) raised against the C-terminus sequence of CYP2S1 and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, 50 μ g total protein was loaded for each microsomal sample per lane; lane 1 contains lung (from vehicle-treated rats), lane 2 contains lung (from rats treated with 50 mg BaP/kg), lane 3 contains lung (from rats treated with 100 mg BaP/kg), and lane 4 contains lung (from rats treated with 200 mg BaP/kg). For panel B, the intensity of CYP2S1 protein bands was measured by densitometric analysis of immunoblots. Relative CYP2S1 protein levels in microsomes are expressed as a ratio of the intensity of the CYP2S1 band to that of the reference band (i.e. CYP2S1 band obtained from 50 μ g lung microsomal protein prepared from vehicle-treated male rats). Results are expressed as mean \pm SD of two different immunoblots.

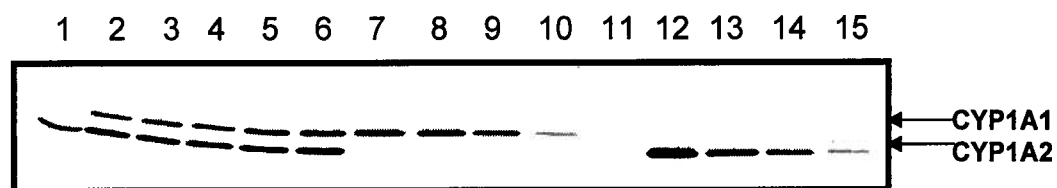
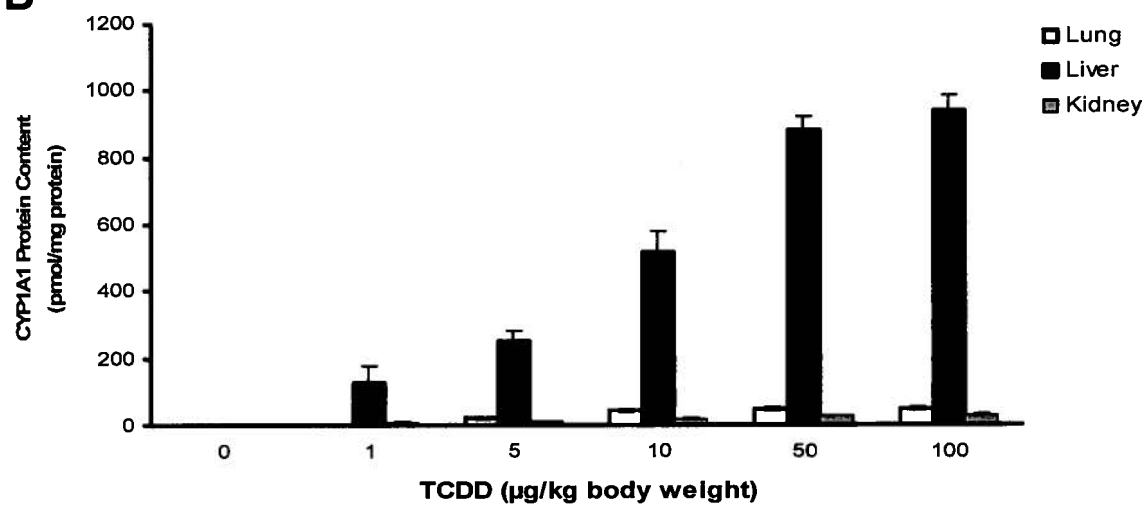
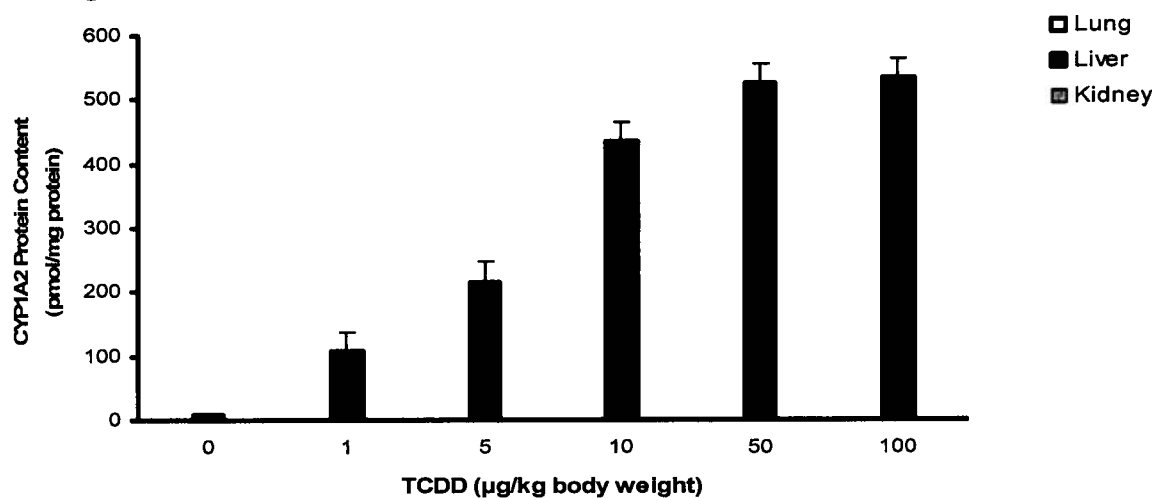
A**B****C**

Figure 3.56: Effect of treatment with varying dosages of TCDD on CYP1A1 and CYP1A2 protein expression in lung, liver and kidney microsomes in adult male SD rats. (A) A representative immunoblot of hepatic microsomal samples prepared from TCDD-treated rats. The upper band represents CYP1A1 protein and the lower band represents CYP1A2 protein. (B) CYP1A1 protein concentrations in TCDD-treated rat lung, liver and kidney. (C) CYP1A2 protein concentrations in TCDD-treated rat lung, liver and kidney. Microsomes were prepared from pooled lung, liver and kidney of male rats (n=3) treated with a single intraperitoneal injection of vehicle (1 part dioxane: 6 parts corn oil) or 1, 5, 10, 50, 100 μg TCDD/kg. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP1A serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, lane 1 contains 20 μg liver microsomes prepared from rats treated with vehicle, lane 2 contains 2 μg liver microsomes prepared from rats treated with 1 μg TCDD/kg, lane 3 contains 1 μg liver microsomes prepared from rats treated with 5 μg TCDD/kg, lane 4 contains 0.5 μg liver microsomes prepared from rats treated with 10 μg TCDD/kg, lane 5 contains 0.5 μg liver microsomes prepared from rats treated with 50 μg TCDD/kg, lane 6 contains 0.5 μg liver microsomes prepared from rats treated with 100 μg TCDD/kg. Lanes 7, 8, 9, 10 and 11 contain purified rat CYP1A1 protein at concentrations of 0.4, 0.3, 0.2, 0.1 and 0.05 pmol/lane, respectively. Lanes 12, 13, 14 and 15 contain recombinant rat CYP1A2 protein at concentrations of 0.4, 0.2, 0.1 and 0.05 pmol/lane, respectively. For panel B, CYP1A1 and CYP1A2 protein concentrations were quantified by densitometric analysis of immunoblots using calibration curves prepared by loading various amounts of recombinant or purified rat CYP1A1 and CYP1A2 protein standards, respectively. Results are expressed as mean \pm SD of three different immunoblots. Protein levels are expressed as pmol CYP1A protein/mg microsomal protein.

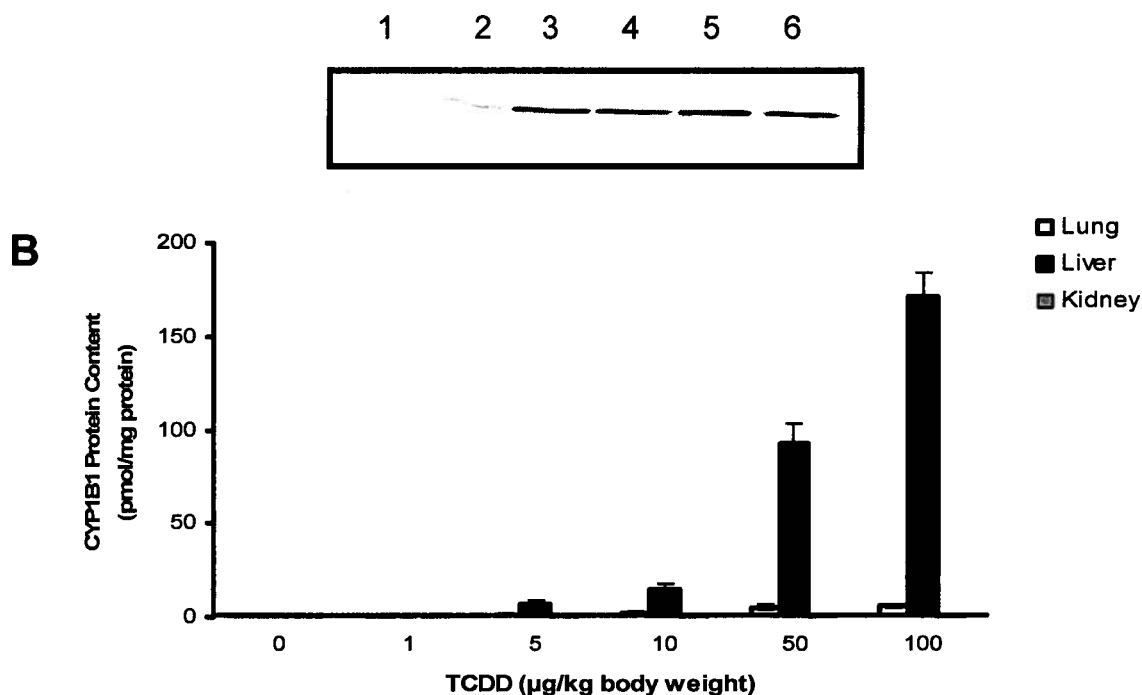


Figure 3.57: Effect of treatment with varying dosages of TCDD on CYP1B1 protein expression in lung, liver and kidney microsomes in adult male SD rats. (A) A representative immunoblot of hepatic microsomal samples prepared from TCDD-treated rats. (B) CYP1B1 protein concentrations in TCDD-treated rat lung, liver and kidney. Microsomes were prepared from pooled lung, liver and kidney of male rats (n=3) treated with a single intraperitoneal injection of vehicle (1 part dioxane: 6 parts corn oil) or 1, 5, 10, 50, 100 µg TCDD/kg. Microsomal protein samples were subjected to SDS-PAGE (one individual microsomal sample per lane) and immunoblots were developed using rabbit anti-rat CYP1B1 serum (1:500 dilution) and secondary antibody (1:3000 dilution) as described in “Materials and Methods”. For panel A, lane 1 contains 40 µg liver microsomes prepared from rats treated with vehicle, lane 2 contains 40 µg liver microsomes prepared from rats treated with 1 µg TCDD/kg, lane 3 contains 40 µg liver microsomes prepared from rats treated with 5 µg TCDD/kg, lane 4 contains 20 µg liver microsomes prepared from rats treated with 10 µg TCDD/kg, lane 5 contains 5 µg liver microsomes prepared from rats treated with 50 µg TCDD/kg, lane 6 contains 2.5 µg liver microsomes prepared from rats treated with 100 µg TCDD/kg. For panel B, CYP1B1 protein concentration was quantified by densitometric analysis of immunoblots using a calibration curve prepared by loading various amounts of recombinant rat CYP1B1 protein standard. Results are expressed as mean \pm SD of three different immunoblots. Protein levels are expressed as pmol CYP1B1 protein/mg microsomal protein.

4. DISCUSSION

4.1. CYP1B1

The regulation of testicular CYP1B1 expression was investigated in the present study. The effect of pituitary and sex hormones on testicular CYP1B1 expression in vitro and the role of LH signaling pathways in the hormonal regulation of testicular CYP1B1 expression were assessed. The effect of TCDD, the most potent AhR agonist, on testicular CYP1B1 expression was also determined. Two cell lines, specifically mouse Leydig tumor (MA-10) cells and rat Leydig tumor (R2C) cells, were used for in vitro experiments. Adult SD rats were used for in vivo experiments.

4.1.1. Effect of LH and Other Pituitary Hormones on Testicular CYP1B1 Expression

Of the pituitary hormones tested, LH (0.2 IU/ml) showed the greatest inductive effect (3.9-fold) on CYP1B1 mRNA expression in MA-10 cells. Treatment with FSH and prolactin increased CYP1B1 mRNA levels to a lesser extent (>1.5-fold). Combined treatments with LH plus FSH, LH plus prolactin, or LH plus FSH plus prolactin exerted an effect similar to treatment with LH alone.

To characterize the inductive effect of LH on CYP1B1 expression in vitro, the effect of varying concentrations of LH on CYP1B1 mRNA levels was determined. CYP1B1 mRNA levels were increased by LH in MA-10 cells in a concentration- and time-dependent manner. Similarly, in rat Leydig R2C cells, CYP1B1 protein levels were increased after treatment with LH. The increase in CYP1B1 mRNA levels in MA-10 cells was accompanied by stimulation of PKA activity. However, PKA activity was not increased after treatment of R2C cells with LH. The basal PKA activity in R2C cells was 2-fold higher than in MA-10 cells. It is possible that in R2C cells LH is unable to increase PKA activity further as PKA enzyme is already activated at a higher magnitude in untreated R2C cells than in MA-10 cells.

LH exerts its regulatory effect by binding to the LH receptor and stimulating formation of cAMP (a second messenger), leading to activation of several kinase and lipase pathways (Figure 1.10). CYP11B1 induction was not due to increased LH receptor expression because the results showed that LH downregulated LH receptor mRNA expression in a dose- and time-dependent fashion. Downregulation of LH receptor expression in MA-10 cells does not imply that LH-mediated pathways are attenuated after treatment with LH because receptor-mediated signaling pathways depend primarily on activation of receptor function (e.g. formation of second messenger) rather than induction of receptor expression. It is likely that, in spite of the decrease in LH receptor mRNA expression, binding of LH to the LH receptor was capable of stimulating formation of cAMP (a second messenger) and activating kinase pathways.

The results obtained in the present study are consistent with an earlier observation from our laboratory that testicular CYP11B1 protein expression was regulated by LH, FSH and prolactin in rats (Leung et al., 2009). Testicular CYP11B1 protein expression was suppressed after hypophysectomy of adult rats and LH, FSH and prolactin induced CYP11B1 protein expression in hypophysectomized rat testis (Leung et al., 2009). LH elicited the greatest increase in testicular CYP11B1 protein expression (among all single pituitary hormone treatments) in hypophysectomized rats. In contrast to *in vivo* data, the effect of LH on CYP11B1 mRNA expression was not potentiated by the addition of FSH or prolactin. Likewise, the effect of FSH on CYP11B1 mRNA expression in MA-10 cells is much less than that of LH. In testis, the effects of FSH are observed mainly in Sertoli cells but not in Leydig cells (Sriraman et al., 2005). It is unknown whether CYP11B1 enzyme is expressed exclusively in Leydig cells or it is also expressed in Sertoli cells. If CYP11B1 is expressed in Sertoli cells, this can explain the effect of FSH on testicular CYP11B1 expression *in vivo*.

Information on the effect of pituitary hormones on CYP11 expression is limited, but there are reports about the effect of pituitary hormones on steroidogenic CYP enzymes and CYP-

mediated activities in testis. Testicular CYP1 activity (i.e. BaP hydroxylase) was decreased following hypophysectomy of adult rats and BaP hydroxylase activity was increased in hypophysectomized rats by LH, but not FSH (Lee et al., 1980). In contrast to CYP1 enzymes, substantial work has been carried out in the regulation of testicular steroidogenic CYP enzymes including CYP11A1, CYP17A1, and CYP19A1 by pituitary hormones. CYP17A1 and CYP11A1 protein expression was suppressed in hypophysectomized rat testis and their expression was restored after administration of exogenous LH and prolactin, but not FSH (Dombrowicz et al., 1996). The effect of LH on CYP11A1 and CYP17A1 protein expression was more pronounced than that of prolactin (Dombrowicz et al., 1996). CYP17A1 activity (i.e. 17-hydroxylation) was attenuated in hypophysectomized rat testis and the activity was restored after administration of exogenous LH. Administration of FSH failed to increase the 17-hydroxylase activity in hypophysectomized rats (Takeyama et al., 1986). Expression of CYP17A1 mRNA was upregulated 2- to 3-fold by LH in MA-10 cells (Payne, 1990). Similarly, LH increased CYP19A1 and CYP11A1 mRNA expression by 2- to 3-fold in mouse and rat Leydig cells (Genissel et al., 2001; Mellon and Vaisse, 1989).

In summary, the results obtained from the present study showed that CYP1B1 expression is positively regulated by LH in mouse and rat Leydig cells; possibly via PKA-mediated pathways.

4.1.2. Involvement of Protein Kinase Pathways in Testicular CYP1B1 Expression

In this study, the role of PKA, PKC and PKG pathways in constitutive and LH-elicited CYP1B1 expression was explored using protein kinase activators and protein kinase inhibitors. Of the signaling pathways modulated by LH, cAMP-activated PKA pathways are involved mainly in the regulation of CYP11A1, CYP17A1 and CYP19A1 enzymes in Leydig cells (Payne and Hales, 2004; Stocco et al., 2005). It has been observed that the LH-mediated increase in

cAMP formation leads to stimulation of protein kinase activity, specifically PKA activity, in MA-10 cells and rat primary Leydig cells (Anakwe and Moger, 1986; Cooke et al., 1976; Dufau, 1998; Paz et al., 2002). In the present study, increased PKA activity after exposure of Leydig cells to LH suggests that the PKA pathway may be an important player in the regulation of testicular CYP11B1 expression. Hence, I hypothesized that LH exerted its inductive effect on testicular CYP11B1 expression by stimulating cAMP formation and PKA activity. 8-Br-cAMP, a synthetic cAMP analog and PKA activator, induced CYP11B1 mRNA expression in MA-10 cells in a concentration- and time-dependent manner. Similarly, CYP11B1 protein levels were increased after treatment of R2C cells with 8-Br-cAMP. PKA activity was stimulated in both MA-10 and R2C cells by treatment with 8-Br-cAMP. The results obtained from my study are in agreement with the report by Zheng et al. (2003), where they reported a 50% increase in CYP11B1 mRNA and protein expression in MA-10 cells after treatment with 8-Br-cAMP. There is no report of the effect of cAMP analogs on CYP11B1 expression in R2C cells.

Similar to LH treatment, 8-Br-cAMP downregulated LH receptor mRNA expression in a dose- and time-dependent manner. These results suggest that induction of CYP11B1 expression after LH or 8-Br-cAMP treatment is not attained through an increase in LH receptor expression. It had been shown that LH receptor mRNA expression was decreased in MA-10 cells after treatment with LH and 8-Br-cAMP (Nelson and Ascoli, 1992; Tsuchiya et al., 2003a). Nelson et al. (1992) and Tsuchiya et al. (2003a) proposed that LH-mediated stimulation of cAMP formation and PKA activity leads to overproduction of steroid hormones, which subsequently decrease the transcription of the LH receptor gene (potentially through feedback inhibition) and increase the rate of degradation of LH receptor mRNA. The results obtained from the present study indicate that LH- or 8-Br-cAMP-mediated induction of CYP11B1 expression was not due to altered LH receptor expression but was likely a result of stimulation of PKA activity.

To further explore the involvement of PKA pathways in the regulation of testicular CYP1B1 expression, the effect of PKA inhibitors on basal or LH- or cAMP-mediated CYP1B1 expression was investigated. In MA-10 cells, H89 suppressed basal CYP1B1 mRNA expression and PKA activity in a concentration- and time-dependent manner. In addition, H89 was also able to block the 8-Br cAMP- and LH-elicited increases in CYP1B1 mRNA expression and PKA activity in a competitive manner as both parameters were restored to approximately basal levels with increasing concentrations of LH or 8-Br-cAMP. The results suggest that PKA and possibly other kinase pathways (e.g. PKC, PKG) were involved in the regulation of CYP1B1 expression in Leydig cells.

Rp-CPT-cAMP suppressed basal CYP1B1 mRNA levels and PKA activity in MA-10 cells in a concentration- and time-dependent manner. The suppression of basal PKA activity in MA-10 cells by Rp-CPT-cAMP was more pronounced than suppression by H89. Similarly, in R2C cells, CYP1B1 protein levels and PKA activity were decreased after treatment with Rp-CPT-cAMP. Rp-CPT-cAMP also attenuated the LH-elicited increase in CYP1B1 mRNA and protein expression in MA-10 and R2C cells, respectively. Rp-CPT-cAMP-mediated blockade of CYP1B1 induction by LH was accompanied by concomitant attenuation of PKA activity in Leydig cells. The effect of H89 and Rp-CPT-cAMP on CYP1B1 expression and PKA activity indicates that the PKA-mediated pathway is involved in regulating CYP1B1 expression in Leydig cells.

The involvement of PKC and PKG pathways in the regulation of CYP1B1 expression was investigated using Go 6983 (a specific PKC inhibitor) and 8-Br-cGMP (a PKG activator). Treatment with Go 6983 or 8-Br-cGMP did not affect constitutive CYP1B1 mRNA and protein expression in MA-10 and R2C cells. Moreover, the LH-elicited increase in CYP1B1 mRNA expression was not blocked by co-treatment of MA-10 cells with LH plus Go 6983. The results

suggest that CYP1B1 expression is not regulated by PKC and PKG pathways in MA-10 and R2C Leydig cells.

CYP11A1 and CYP19A1 proteins are constitutively expressed in Leydig cells (Payne, 1990), whereas levels of CYP17A1 mRNA are low or undetectable in MA-10 cells (Payne, 1990). CYP11A1, CYP17A1 and CYP19A1 mRNA levels were induced 2- to 3-fold in mouse and rat Leydig cells by cAMP analogs (Mellon and Vaisse, 1989; Payne, 1990). It has been established that in MA-10 cells, LH regulates CYP11A1 and CYP17A1 mRNA expression by activating the cAMP-dependent PKA pathway (Payne and Hales, 2004; Payne and Youngblood, 1995). H89 blocked constitutive CYP17A1 mRNA expression in MA-10 cells (Laurich et al., 2002). Likewise, LH induced CYP19A1 mRNA expression in rat Leydig cells through the cAMP-activated PKA pathway (Genissel et al., 2001).

In summary, CYP1B1 mRNA and protein are induced in Leydig cells by LH and by a PKA activator, 8-Br-cAMP. A non-selective PKA inhibitor, H89, blocked the LH-mediated expression of CYP1B1 mRNA. Similarly, Rp-CPT-cAMP, a specific PKA inhibitor, blocked the LH-mediated induction of CYP1B1 mRNA and protein levels in MA-10 and R2C cells. Collectively, the results indicate that PKA activation is important in the LH-mediated induction of CYP1B1 expression. On the basis of my experiments using cAMP analogs and studies of cAMP-dependent regulation of CYP1B1 in the adrenal gland (Brake and Jefcoate, 1995; Zheng et al., 2003), I conclude that LH activates PKA pathways in Leydig cells by stimulating the formation of cAMP.

4.1.3. Possible Mechanism of Testicular CYP1B1 Regulation by LH

Results from the present study indicate that induction of CYP1B1 by LH treatment (in vitro) occurs via the cAMP-activated PKA pathway. Zheng et al. (2003) reported that the effects of cAMP analogs on CYP1B1 expression in MA-10 and adrenal cells is exerted through two

cAMP-responsive elements located in the far upstream enhancer sequence of the CYP1B1 promoter region. In LH-mediated PKA pathways, CREB and SF-1 are two main transcription factors that are phosphorylated, leading to an increase in CYP mRNA expression in Leydig cells (Payne and Hales, 2004; Payne and Youngblood, 1995). The increase in cAMP formation after LH treatment leads to activation of PKA activity and phosphorylation of downstream transcription factors such as CREB and SF-1 (Cooke et al., 1976; Stocco et al., 2005).

Transcriptional regulation of CYP17A1 by LH and cAMP is dependent on phosphorylation of SF-1, but not CREB-1, in Leydig cells (Payne and Hales, 2004). CREB and SF-1 are the primary transcription factors involved in the constitutive and cAMP-mediated expression of CYP19A1 in rat R2C cells (Carlone and Richards, 1997a). Similarly, Zheng et al. (2003, 2005) showed that cAMP-mediated CYP1B1 expression in MA-10 cells is regulated through phosphorylation of CREB and SF-1 by PKA. In mouse adrenal cells (Y-1), the interaction of CREB and SF-1 transcription factors present in the 3'-end of the gene is crucial for the activity of the far upstream enhancer sequence of CYP1B1 (Zheng and Jefcoate, 2005). Mutation of PKA phosphorylation sites of CREB, but not SF-1, attenuated the far upstream enhancer sequence activity of CYP1B1 in mouse adrenal cells (Zheng and Jefcoate, 2005). In contrast, Tsuchiya et al. (2006) reported that SF-1 may not be important in transcription of the human CYP1B1 gene.

Although LH receptor mRNA expression was downregulated after treatment with LH or 8-Br-cAMP, PKA activity was stimulated with increasing concentrations of LH or 8-Br-cAMP. This indicates that LH-mediated induction of CYP1B1 mRNA in MA-10 cells is a post-receptor event and intracellular pathways are involved in the process. The possible mechanism of testicular CYP1B1 regulation by LH is summarized in Figure 4.1. LH molecules bind to luteinizing hormone receptor, a G-protein coupled receptor, leading to stimulation of adenylyl cyclase enzyme, which catalyzes the conversion of adenosine triphosphate to cAMP (second

messenger). Subsequently, cAMP activates PKA, which phosphorylates transcription factors such as CREB and SF-1, which bind to response elements present in the CYP1B1 gene, leading to increase in CYP1B1 mRNA transcription and protein expression.

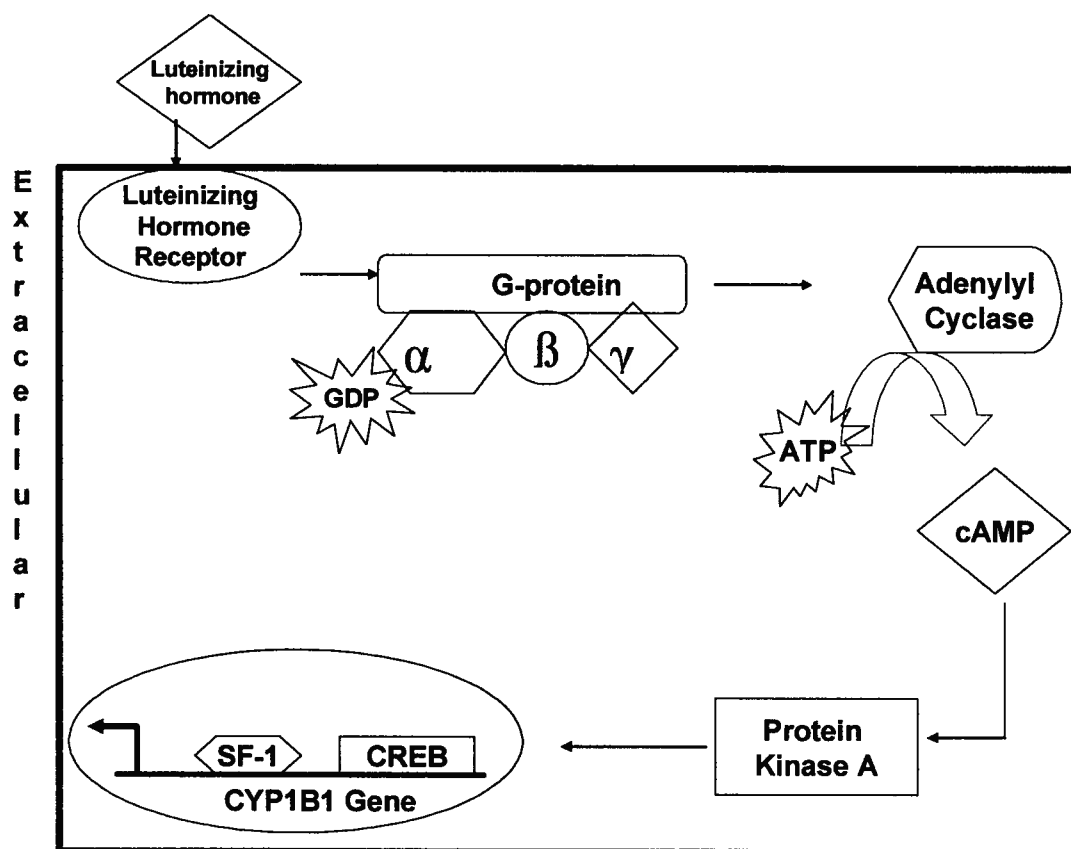


Figure 4.1: Proposed mechanism for CYP1B1 regulation by luteinizing hormone in testis. Luteinizing hormone binds to luteinizing hormone receptor, a G-protein coupled receptor, leading to stimulation of adenylyl cyclase enzyme, which catalyzes the conversion of ATP to cAMP (second messenger). Subsequently, cAMP activates PKA which phosphorylates transcription factors such as CREB and SF-1, which bind to response elements present in the CYP1B1 gene, leading to increase in CYP1B1 mRNA transcription. Abbreviations: ATP- adenosine triphosphate; GDP- guanosine diphosphate; cAMP- 3'-5'-cyclic adenosine monophosphate; SF-1- steroidogenic factor-1; CREB- cAMP responsive element binding protein.

4.1.4. Effect of Estradiol Benzoate on Testicular CYP1B1 Expression

CYP1B1 mRNA expression was suppressed after treatment of MA-10 cells with estradiol benzoate in a concentration- and time-dependent manner. Suppression of testicular CYP1B1 expression by estradiol benzoate was also observed in adult intact rats (Leung et al., 2009). Surprisingly, estradiol benzoate did not affect CYP1B1 protein expression in R2C cells. The reason for the lack of agreement between the effects of estradiol benzoate on CYP1B1 mRNA in MA-10 and CYP1B1 protein in R2C cells is unknown. It is possible that basal levels of positive regulatory pathways (e.g. PKA-mediated pathway) of CYP1B1 are high in R2C cells and the negative effect of estradiol on these pathways is not sufficient to suppress CYP1B1 protein expression in rat Leydig cells.

The involvement of estrogen receptors in estradiol-elicited suppression of CYP1B1 was investigated using ICI 182,780, a competitive antagonist of estrogen receptor. ICI 182,780 either alone or in combination with estradiol benzoate did not change CYP1B1 mRNA levels in MA-10 cells or CYP1B1 protein levels in R2C cells. This suggests that suppression of CYP1B1 mRNA expression by estradiol is independent of estrogen receptor function.

In general, estrogens have a negative effect on the expression of steroidogenic CYP enzymes in testis such as CYP17A1 and CYP11A1 (Akingbemi et al., 2003; Nozu et al., 1981c). For example, the total CYP content of rat testis was decreased by estradiol (20 µg/rat) (Nozu et al., 1981c). Moreover, in rat Leydig cells, CYP17A1 activity was inhibited by estradiol (Nozu et al., 1981c). CYP17A1 mRNA expression was downregulated by estradiol acting through the estrogen receptors in fetal and adult testis (Akingbemi et al., 2003; Delbes et al., 2005). Similarly, CYP17A1 mRNA expression was increased in estrogen receptor- α gene knockout mice (Akingbemi et al., 2003; Delbes et al., 2005). The inductive effect of ICI 182,780 on CYP17A1 expression has also been observed in mice (Akingbemi et al., 2003).

It was reported that estradiol benzoate completely suppressed LH receptor expression in rat pituitary (Fukuzawa et al., 2004). In the present study, LH receptor mRNA expression was studied to determine if downregulation of LH receptor expression was responsible for the suppressive effect of estradiol benzoate. LH receptor mRNA expression did not change after treatment of MA-10 cells with estradiol benzoate. This indicates that suppression of CYP11B1 mRNA expression by estradiol is independent of LH receptor mRNA expression.

A review of the literature showed that estradiol benzoate decreased serum LH levels in rodents. Neutralization of endogenous circulating estradiol using an estradiol specific antibody in adult male rats led to an increase in circulating LH levels (Nishihara and Takahashi, 1983). Similarly, estrogen receptor- α gene knockout mice have a 3-fold higher level of serum LH. It was suggested that estradiol suppresses constitutive CYP17A1 expression by attenuating LH secretion and LH-modulated pathways (Akingbemi et al., 2003). cAMP analog-stimulated steroidogenesis was blocked by estrogens in rat Leydig cells (Sairam and Berman, 1979). Tamoxifen (an agonist/antagonist of estrogen) blocked the LH-elicited increase in cAMP formation in Leydig cells (Lin et al., 1981). In porcine Leydig cells, estradiol and diethylstilbestrol (a synthetic nonsteroidal estrogen) decreased CYP17A1 activity through inhibition of cAMP formation (Li, 1991).

Estradiol can potentially modulate LH-mediated regulation of CYP11B1 expression in Leydig cells by interfering with PKA pathways. To determine if estradiol-mediated suppression of CYP11B1 expression occurred through interference with LH-mediated CYP11B1 regulation, mouse Leydig MA-10 cells were co-treated with estradiol benzoate plus LH. In the presence of estradiol, the effect of LH on CYP11B1 mRNA and PKA activity did not change in MA-10 cells. Results of my study suggest that the suppressive effect of estradiol on CYP11B1 expression in MA-10 cells is not an outcome of attenuation of LH-mediated signaling pathways.

4.1.5. Possible Mechanism of Estradiol-mediated Suppression of Testicular CYP1B1 Expression

Rat testicular CYP1B1 protein levels were suppressed by estradiol benzoate *in vivo* in a previous study in our laboratory. The inhibitory effect of estradiol on the hypothalamus-pituitary axis, leading to a decrease in serum LH levels (Akingbemi et al., 2003; Wersinger et al., 1999), may explain the negative regulation of testicular CYP1B1 by estradiol in adult rats (Leung et al., 2009). The mechanism of suppression of CYP1B1 mRNA by estradiol benzoate in Leydig cells (*in vitro*) is not yet understood. Initially it was hypothesized that similar to CYP17A1 (Li, 1991), inhibition of LH-mediated PKA pathway may be responsible for the estradiol-elicited downregulation of CYP1B1 in Leydig cells. However, results from the present study indicate otherwise. Our results suggest that estradiol does not block the local effect of LH on CYP1B1 expression in Leydig cells. It should be noted that estradiol is also capable of modulating testicular CYP expression (e.g. CYP11A1 and CYP17A1) without interfering with LH secretion at the hypothalamus-pituitary level and can exert a direct action on testis (Sakaue et al., 2002). It was observed that estradiol decreased CYP17A1 protein expression and activity in hypophysectomized rats, indicating that estradiol can suppress CYP expression without altering LH secretion at the distal level. Moreover, co-treatment of hypophysectomized rats with estradiol plus LH did not block the suppressive effect of estradiol on CYP17A1 (Brinkmann et al., 1980; Kremers et al., 1977). Thus, it is possible that estradiol-elicited suppression of CYP1B1 expression in Leydig cells is mediated through LH-independent pathways.

Results from the present study suggest that estrogen receptors have no role in the suppression of CYP1B1 expression in Leydig cells. Sex steroids formed during steroidogenesis play a vital role in the homeostasis of CYP expression in Leydig cells (Nozu et al., 1981b; Nozu et al., 1981c; Payne, 1990). For example, CYP17A1 expression and activity are decreased by intracellular estrogen formed from the elevated testosterone during acute stimulation of

steroidogenesis by LH in Leydig cells (Nozu et al., 1981c). Thus, estrogen and testosterone have been implicated in the desensitization of steroidogenesis in Leydig cells (Nozu et al., 1981c; Payne, 1990). The decrease in testosterone formation, following blockade of CYP17A1 activity by estrogen, leads to attenuation of overall endocrine functioning and proliferation of Leydig cells (Abney, 1999), which may be a possible reason for CYP1B1 mRNA suppression after exposure to estradiol benzoate. Moreover, estrogen has a direct suppressive effect on the proliferation of Leydig cells, primarily inhibiting the cellular events at the S-phase of the cell cycle (Abney, 1999). Testosterone binds to the active sites of CYP17A1 protein and forms a pseudosubstrate, which after multiple reactions leads to degradation of CYP17A1 enzyme (Hales et al., 1987). It is possible that the stability of CYP1B1 mRNA is decreased following exposure to estradiol benzoate in Leydig cells in the present study.

DNA methylation is another mechanism by which CYP gene expression is controlled. DNA methylation is defined as covalent binding of a methyl group to DNA bases, mainly cytosine, catalyzed by DNA methyltransferases (Bestor, 2000). CpG dinucleotides (cytosine and guanine nucleotides bonded by phosphodiesterases), also known as CpG islands, are the primary sites of DNA methylation located in and around the promoter and/or enhancer regions in genes (Bestor, 2000; Gardiner-Garden and Frommer, 1987; Jones and Takai, 2001; Larsen et al., 1992; Rountree et al., 2001). CYP genes from the CYP1 family such as CYP1A1, CYP1A2, and CYP1B1 contain one or more CpG islands in their nucleotide sequence and are regulated by the DNA methylation mechanism (Ingelman-Sundberg et al., 2007; Okino et al., 2006; Tokizane et al., 2005). In general, the methylation status of CYP genes (e.g. CYP1A1, CYP1A2) is inversely correlated with CYP protein expression levels in tissues (Ingelman-Sundberg et al., 2007). DNA methylation is hormonally regulated in testis. For example, LH caused hypomethylation of seminiferous tubule DNA in Leydig cells (Reddy and Reddy, 1990) and exposure to diethylstilbestrol, a synthetic estrogen, led to increased methylation of ribosomal DNA in mouse

uterine samples (Alworth et al., 2002). Estradiol-mediated suppression of CYP1B1 mRNA expression may occur as a result of hypermethylation of the CYP1B1 gene following treatment of Leydig cells with estradiol benzoate. Proposed mechanisms of testicular CYP1B1 regulation by estradiol are summarized in Figure 4.2.

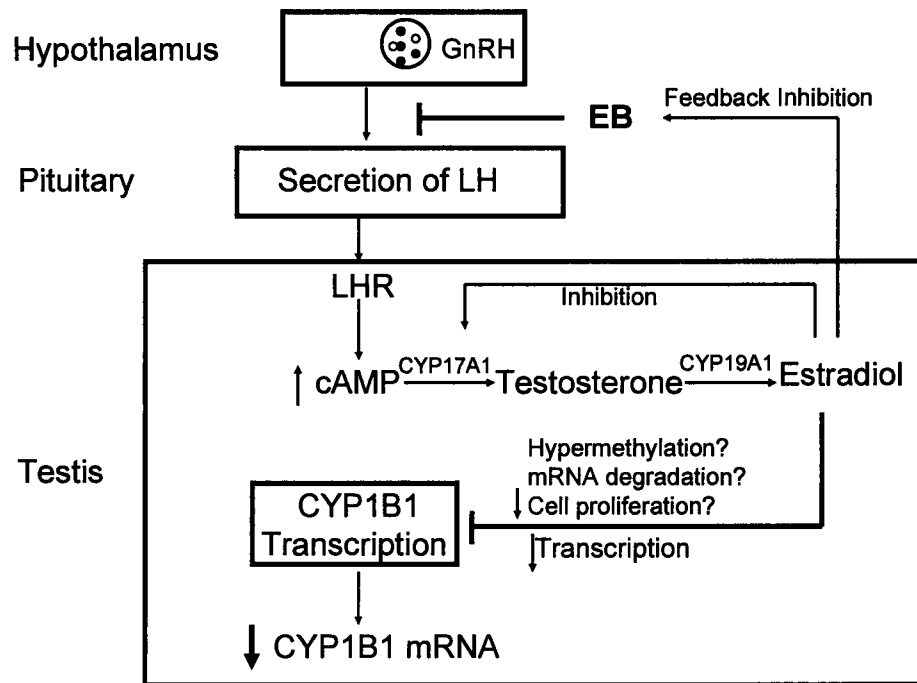


Figure 4.2: Proposed mechanism for estradiol-mediated regulation of testicular CYP1B1 mRNA expression. GnRH, a decapeptide formed in the hypothalamus, controls the pulsatile release of LH from anterior pituitary. Subsequently, LH stimulates the formation of cAMP and thus controls the CYP17A1-mediated formation of testosterone, which is converted to estradiol by CYP19A1. In addition to feedback inhibition of LH secretion, estradiol suppresses CYP1B1 mRNA expression in Leydig cells, possibly, by multiple mechanisms including hypermethylation of CYP1B1 gene and mRNA degradation. Abbreviations: GnRH- gonadotropin releasing hormone; LH- luteinizing hormone; LHR- luteinizing hormone receptor; cAMP- 3'-5'-cyclic adenosine monophosphate.

4.1.6. Effect of TCDD on Testicular CYP1B1 Expression

The effect of TCDD, the most potent AhR agonist, on CYP1B1 mRNA and protein expression was determined. It appears that in contrast to liver, TCDD was not able to exert a dose-dependent inductive effect on testicular CYP1B1 protein expression in rats at the dosages examined in this study. The results obtained are consistent with a previous study in our laboratory, in which testicular CYP1B1 protein levels remained unchanged after treatment of adult rats with BaP at dosages of 1-200 mg/kg/day for 3 consecutive days (M. Kawai, unpublished data). We thought it is possible that the lack of induction of testicular CYP1B1 by AhR agonists in testis *in vivo* could be due to the inability of AhR ligands to cross the blood-testis-barrier. Subsequently, the effect of TCDD on CYP1B1 mRNA and protein expression in Leydig cells (*in vitro*) was determined. Treatment of MA-10 and R2C cells with varying concentrations of TCDD did not change CYP1B1 mRNA and protein expression, respectively.

The effect of TCDD on testicular CYP1B1 protein expression *in vivo* observed in my study is in agreement with a previous report. Mandal et al. (2001) showed that CYP1B1 mRNA levels did not change after treatment of rats with TCDD at dosage of 50 µg/kg. However, the effect of TCDD on CYP1B1 mRNA expression in MA-10 cells observed in the present study does not agree with two other studies. Zheng et al. (2003) reported that CYP1B1 mRNA is induced approximately 3-fold by TCDD in MA-10 cells. Similarly, Mandal et al. (2001) showed that TCDD induced CYP1B1 mRNA expression in MA-10 cells.

In summary, testicular CYP1B1 mRNA and protein expression is not inducible by TCDD in rats and Leydig cells from mouse and rat.

4.1.7. Possible Mechanism of Lack of Induction of Testicular CYP1B1 Expression by TCDD

CYP1B1 protein expression is induced by AhR agonists such as TCDD and BaP in mouse and rat liver and lung (Chang et al., 2006; Galvan et al., 2005; Harrigan et al., 2006; Walker et al., 1999). However, testicular CYP1B1 mRNA levels are the same in AhR-wild type and AhR-knockout mice (Shimada et al., 2003), suggesting that basal CYP1B1 expression is not regulated by AhR in testis. Moreover, testicular CYP1B1 expression is not induced by AhR agonists in AhR-wild type or in AhR-knockout mice (Shimada et al., 2002; Shimada et al., 2003). A possible explanation of the lack of inducibility of CYP1B1 expression by AhR agonists is related to AhR repressor expression and function in testis. AhR repressor mRNA is expressed at very high levels in human and rodent testis (Bernshausen et al., 2005; Yamamoto et al., 2004). In contrast, AhR repressor mRNA levels are low in liver and lung (Bernshausen et al., 2005; Yamamoto et al., 2004). The role of AhR repressor in the expression and induction of CYP1B1 is not conclusive. It has been proposed that AhR repressor competes with AhR in the formation of AhR-ARNT dimer and subsequently, suppresses the AhR-mediated activity (Bernshausen et al., 2005; Tsuchiya et al., 2003b). Although no correlation has been found between induction of CYP1 enzymes with AhR repressor levels, it is possible that AhR repressor blocks the stimulation of CYP1B1 transcription by AhR agonists in testis. The presence of a tissue-specific promoter in the CYP1B1 gene and tissue-specific transcriptional coactivator recruitment after treatment with AhR agonists are other possible reasons for the differential regulation of CYP1B1 expression by AhR agonists in liver, lung, kidney and testis.

DNA methylation status of CYP genes can control the tissue-specific regulation of CYP enzymes (e.g. CYP1A1, CYP1B1). It has been reported that the extent of CYP gene methylation (e.g. CYP1A1 and CYP1B1) is tissue-specific (Okino et al., 2006; Tokizane et al., 2005). Expression of CYP1A1 mRNA was suppressed by TCDD in human prostate cancer cells (e.g.

LNCaP) as a result of the hypermethylation of CYP1A1 gene (Okino et al., 2006). In the present study, the lack of increase in CYP1B1 transcription after treatment with AhR agonists may be due to hypermethylation of the CYP1B1 gene in Leydig cells. It has been suggested that higher abundance of methylated cytosine bases in the promoter region of the target gene (e.g. CYP1B1) potentially block the binding of the transcription factor (AhR-ARNT dimer for CYP1B1) to the DNA binding sites of the gene (Rountree et al., 2001; Tate and Bird, 1993). A proposed mechanism of testicular CYP1B1 regulation by AhR agonists is summarized in Figure 4.3.

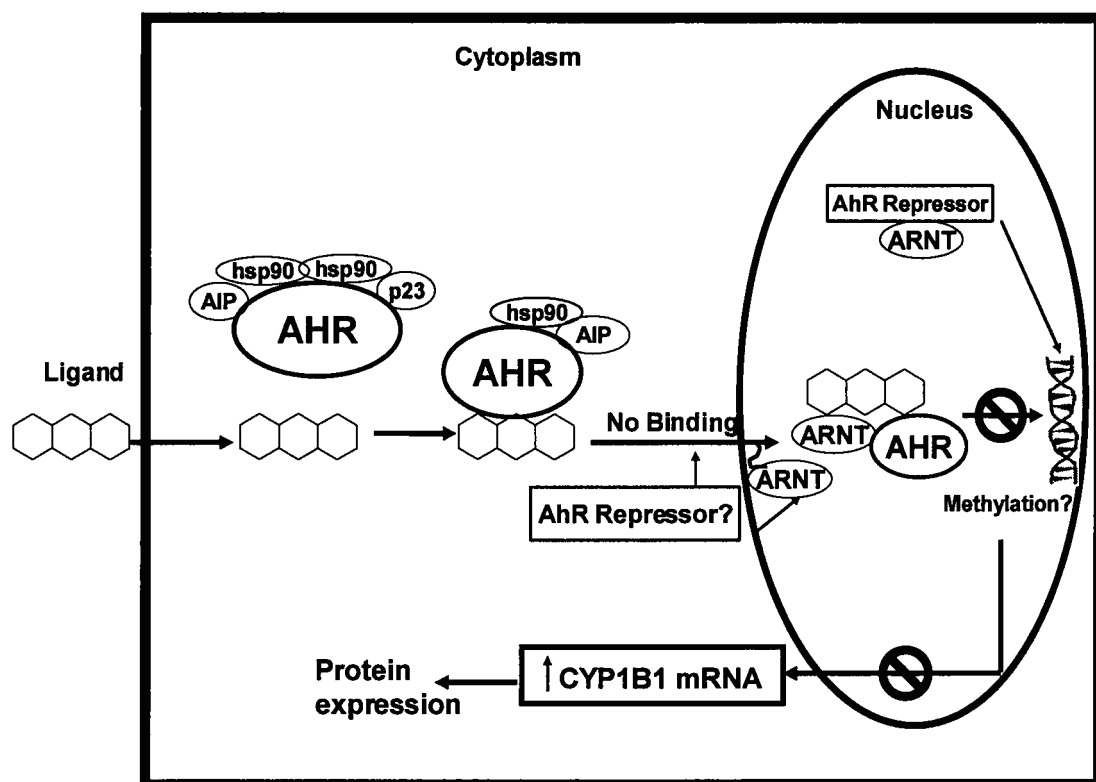


Figure 4.3: Proposed mechanism for lack of induction of testicular CYP1B1 by AhR agonists. In Leydig cells, the AhR repressor potentially competes with the AhR for binding with ARNT. Subsequently, formation of the AhR-ARNT dimer may be blocked, leading to lack of activation of CYP1B1 transcription. In addition, hypermethylation of the CYP1B1 gene following treatment with AhR agonists may also inhibit stimulation of CYP1B1 transcription. Abbreviations: AhR- aryl hydrocarbon receptor; ARNT- aryl hydrocarbon receptor nuclear translocator; AIP, aryl hydrocarbon-interacting protein; hsp90, heat shock protein 90.

4.2. CYP2S1

The tissue distribution and sex-dependent expression of CYP2S1 mRNA and protein in rats and the regulation of CYP2S1 expression by AhR agonists were investigated in this study.

4.2.1. Identification of Rat CYP2S1 mRNA and Protein

Primers for the CYP2S1 gene were selected and a block cyler RT-PCR assay was developed to detect CYP2S1 mRNA in rat tissues such as lung. The rat CYP2S1 nucleotide sequence is 88% and 82% similar to the mouse CYP2S1 and human CYP2S1 nucleotide sequences, respectively. Rat CYP2S1 mRNA is 2632 bp nucleotide sequence long (NCBI accession number NM_001107495.1) and is located on chromosome 1q (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Other CYP enzymes located on rat chromosome 1q include CYP2A1, CYP2A2, CYP2A3, CYP2B1, CYP2B2, CYP2B12, CYP2B15, CYP2F4, CYP2G1 and CYP2T1 (Hu et al., 2008).

Anti-rat CYP2S1 serum raised against the C-terminus peptide sequence showed reactivity with the synthetic peptide in the ELISA assay and with a protein on immunoblots containing microsomes prepared from rat tissues. We identified this protein as CYP2S1 with a molecular weight of approximately 50 kD. Due to a highly conserved CYP2S1 C-terminus sequence in rat and mouse, the antiserum also detected CYP2S1 protein in mouse lung. However, relative to rat and mouse CYP2S1, the C-terminus sequence is less conserved in other species such as human, rhesus monkey, and chimpanzee (Table 3.3). The rat CYP2S1 protein shows 93% and 76% amino acid sequence identity with mouse and human CYP2S1 amino acid sequences, respectively.

Rat CYP2S1 is located on chromosome 1q, which also contains CYP2A, CYP2B, CYP2F, and CYP2G enzymes (Hu et al., 2008). CYP2S1 protein exhibits 50%, 48%, 47% and 46% amino acid sequence identity with rat CYP2G1, CYP2A3, CYP2B12 and CYP2B15,

respectively. The amino acid sequence identity of rat CYP2S1 with other CYP2 enzymes present on rat chromosome 1q and their tissue distribution and substrates are summarized in Table 4.1.

Table 4.1: Amino acid sequence identity of rat CYP2S1 with other CYP2 enzymes present on rat chromosome 1q and their protein tissue distribution and substrates (compiled from Baldwin et al., 2005; Hoffman et al., 2001; Imaoka et al., 2005; Keeney et al., 1998; Robottom-Ferreira et al., 2003; Shervington, 1999; Stoltz et al., 1998; Zhuo et al., 2004).

CYP2	% Sequence Identity with CYP2S1	Tissue distribution	Substrates
CYP2S1	100	lung, stomach, kidney	ND
CYP2A1	45	liver, small intestine spleen, testis	testosterone
CYP2A2	43	ND	ND
CYP2A3	48	lung, esophagus, nasal cavity, breast, olfactory mucosa	coumarin
CYP2B1	48	liver, lung, kidney, small intestine, brain	nicotine, testosterone
CYP2B2	49	liver, kidney	
CYP2B12	47	keratinocyte, sebocytes	arachidonic Acid
CYP2B15	46	keratinocyte, sebocytes	arachidonic Acid
CYP2F4	46	lung, nasal cavity, olfactory mucosa	naphthalene
CYP2G1	50	olfactory mucosa	testosterone, progesterone
CYP2T1	43	ND	ND

ND- Not determined

4.2.2. Tissue Distribution of CYP2S1 mRNA and Protein

CYP2S1 mRNA was present in rat lung, liver, kidney and gastrointestinal tract tissues. The CYP2S1 mRNA levels were higher in lung than other tissues including liver. The tissue distribution of rat CYP2S1 mRNA expression follows the same pattern as that reported for human CYP2S1 mRNA, which is expressed in lung, liver, kidney, small intestine, stomach, and spleen (Rylander et al., 2001). RT-PCR analysis indicated that there was no sex-dependent difference in CYP2S1 mRNA expression between male and female rats. Lack of a sex difference in CYP2S1 mRNA expression suggests that sex hormones are not involved in the regulation of CYP2S1 mRNA expression. Similarly, there is no report of sex-dependent regulation of rat CYP2B12, CYP2B15, CYP2F4, CYP2G1 and CYP2T1 expression, which are also located on rat chromosome 1q. However, CYP enzymes from CYP2A and CYP2C subfamilies have been found to be regulated by estrogen and testosterone in rats (Waxman et al., 1985; Waxman et al., 1989a).

Expression of CYP2S1 protein is predominantly extrahepatic. CYP2S1 protein was identified in rat stomach, lung and kidney. Stomach had a relatively higher level of CYP2S1 protein expression than lung or kidney. Moreover, CYP2S1 protein was not detected in rat liver, small intestine, testis, ovary, spleen, thymus, brain, adrenal gland and skin. Thus, rat CYP2S1 protein expression follows a different pattern than mRNA expression.

Human CYP2S1 gene contains two CpG islands, which are potential sites for DNA methylation (Ingelman-Sundberg et al., 2007). Tissue-specific expression of CYP2S1 mRNA may be due to a differential methylation level of the CYP2S1 gene in rat tissues. Tissue-specific expression of certain CYP1 and CYP2 family enzymes such as CYP1A2, CYP1B1, and CYP2W1 is reported to be influenced by DNA methylation (Ingelman-Sundberg et al., 2007). Differences in methylation of CYP1A2 promoter sequences have been implicated in the tissue specific expression of CYP1A2 protein. The hepatic CYP1A2 promoter undergoes

hypomethylation, leading to higher constitutive expression in liver, whereas hypermethylation of the CYP1A2 promoter was observed in tissues having little or no expression of CYP1A2 protein such as lung and kidney (Jin et al., 2004). Hypomethylation of promoter or enhancer regions of the CYP1B1 gene is responsible for the higher expression of human CYP1B1 protein in prostate cancer (Tokizane et al., 2005). Gomez et al. (2007) reported that hypomethylation of the CYP2W1 gene is responsible for colon-specific expression of the gene.

Several possible mechanisms could be proposed to explain the lack of agreement between the CYP2S1 mRNA and CYP2S1 protein expression patterns. Certain CYP protein structures are altered after translation of mRNA codons, collectively known as post-translational modifications, which can regulate CYP protein expression in rodents and humans. Among several mechanisms of post-translational modifications, addition of functional groups (e.g. glycosylation, phosphorylation), altered protein structure (e.g. proteolytic cleavage) and protein stabilization due to proteasomal degradation have been implicated in the regulation of CYP protein expression (Aguiar et al., 2005). Post-translational modification during CYP2S1 protein synthesis may result in differences between CYP2S1 mRNA and protein expression. Protein expression and activity of CYP enzymes located on rat chromosome 1q such as CYP2B1 and CYP2B2 and other CYP2 enzymes such as CYP2C6, CYP2C11 and CYP2C12 are altered by phosphorylation (Aguiar et al., 2005). However, it should be understood that in spite of efforts from researchers for several decades, a definitive role for post-translational modifications such as phosphorylation in CYP expression has not been shown. Currently available reports on regulation of certain CYP expression by post-translational modifications are inconclusive and need further work.

Degradation of the CYP2S1 gene products to different extents may be a possible factor contributing towards discordant CYP2S1 mRNA and protein expression in rats (Karlgren et al., 2006). Nine allelic variants of the CYP2S1 gene in the proximity of the coding region have been

identified in a Finnish population (Saarikoski et al., 2004), whereas in a Korean population, the CYP2S1 gene has twelve variants located in the promoter and coding region (Jang et al., 2007). It has been proposed that polymorphic variants may decrease the stability of the CYP2S1 mRNA transcript, leading to altered protein expression and enzymatic activity (Saarikoski et al., 2005a). Human CYP2A6, a CYP2S1-related gene in the 19q chromosomal cluster, and CYP2A5 (mouse homolog) are regulated by similar post-transcriptional mechanisms (Gilmore et al., 2001; Tilloy-Ellul et al., 1999).

4.2.3. Regulation of CYP2S1 Expression by AhR Agonists

In the present study, the effect of AhR agonists on CYP2S1 enzyme expression was determined and was compared with classical AhR-regulated CYP enzymes such as CYP1A1, CYP1A2 and CYP1B1. CYP2S1 mRNA expression was increased in lung, stomach and jejunum after treatment of rats with 3-MC (25 mg/kg/day for three consecutive days) in rats. We investigated the dose-response effect of TCDD treatment on rat CYP2S1 mRNA expression in liver, lung and kidney. CYP2S1 mRNA levels were increased after a single intraperitoneal injection of TCDD (1-100 µg/kg) in a dose-dependent and tissue-specific manner. CYP2S1 mRNA expression showed a 7- and 3.6-fold increase in lung and liver, respectively. In comparison to CYP2S1, CYP1A1 mRNA was more responsive to 3-MC and TCDD treatment in the same samples. Nonlinear regression analysis of mRNA data from lung tissues obtained from TCDD-treated rats yielded ED₅₀ values of 16.3 and 6.6 µg/kg for TCDD-mediated increase of CYP2S1 and CYP1A1, respectively. Similarly, the ED₅₀ value for CYP1A1 mRNA induction in liver tissues obtained from TCDD-treated rats was much lower than for CYP2S1 mRNA. It was estimated that inducibility of CYP2S1 mRNA by 3-MC and TCDD was much lower than that of CYP1A1 mRNA, which suggests that CYP2S1 mRNA is weakly regulated by the AhR. Relative CYP2S1 protein levels were slightly increased (~2-fold) after treatment with TCDD at dosage of

50 µg/kg. However, relative CYP2S1 protein levels did not change in the 100 µg/kg treatment group. In contrast, CYP1A1, CYP1A2 and CYP1B1 proteins were highly induced in a dose-dependent manner.

Results from my study are in agreement with previous reports, which indicate that CYP2S1 mRNA expression is regulated by AhR through its regulatory components such as ARNT and XRE (Rivera et al., 2002; Rivera et al., 2007). Rivera et al. (2002) reported a 2-fold increase of CYP2S1 mRNA after treatment of human lung cell line A549 with TCDD (100 nM). In a mouse hepatoma cell line (Hepa-1), CYP2S1 mRNA was increased 10-fold by TCDD (10 nM) (Rivera et al., 2002). A modest increase in CYP2S1 protein expression was also observed in TCDD-treated (10 nM) mouse Hepa-1 cells (Rivera et al., 2007). No induction of CYP2S1 mRNA was observed in AhR-deficient, ARNT-deficient and XRE-deficient mouse hepatoma cells (Rivera et al., 2002; Rivera et al., 2007). A TCDD-mediated increase of CYP2S1 mRNA was observed in mouse lung and liver tissues (Rivera et al., 2002). However, there are no reports of induction of CYP2S1 protein in human cell lines or in mice after treatment with AhR agonists.

Certain CYP1 and CYP2 enzymes (e.g. CYP1A1, CYP1A2, CYP2B6 and CYP2C9) are downregulated by hypoxic (low oxygen level) conditions (Fradette and Du Souich, 2004). Hypoxia inducible factor-1 (HIF-1) is the main transcription factor responsible for altering the transcription of target genes in hypoxia (Gradin et al., 1996). It has been shown that AhR and HIF-1 α bind to ARNT, a common dimerization partner, leading to cross-talk between AhR and hypoxia signaling pathways (Lee et al., 2006). TCDD-mediated induction of CYP1A1 mRNA expression was repressed by hypoxia in hepatic cells (e.g. HepG2) (Chan et al., 1999). In contrast, Rivera et al. (2007) reported that hypoxia (1% O₂) induces CYP2S1 protein expression in Hepa-1 cells. The induction of CYP2S1 protein was additive after treatment with TCDD and 1% O₂. The authors concluded that the HIF-1 α /ARNT and AhR/ARNT dimers are responsible

for hypoxic and TCDD-mediated CYP2S1 induction, respectively, through several overlapping XRE and hypoxia response elements in the mouse CYP2S1 promoter region (Rivera et al., 2007).

Previously it was thought that AhR-mediated CYP induction is restricted to CYP1 family enzymes. However, it has been established that CYP2A subfamily enzymes are also induced by AhR agonists in rat, mouse and hamster. For example, 3-MC treatment increased pulmonary CYP2A3 (up to 5-fold) (Kimura et al., 1989; Nagata et al., 1987) and hepatic CYP2A1 (up to 2-fold) in rats (Thomas et al., 1981). Mouse CYP2A5 (a homologue of human CYP2A6) was induced 3-fold by TCDD (Arpiainen et al., 2005; Gokhale et al., 1997) and hamster CYP2A8 was induced in liver by 3-MC (Kurose et al., 1999).

Although CYP2S1 mRNA was induced by 3-MC and TCDD, lack of induction of CYP2S1 protein expression after treatment of rats with AhR agonists was intriguing to us. CYP enzymes (e.g. CYP2C18) are known to be regulated by post-transcriptional regulation (Ingelman-Sundberg, 2008). MicroRNA (miRNA), small non-coding RNAs involved in the negative regulation of genes implicated in development and apoptosis (Ambros, 2004; Wienholds and Plasterk, 2005) may be involved in the post-transcriptional regulation of CYP2S1 in rat lung. It has been observed that miRNA and CYP protein levels are inversely related (Tsuchiya et al., 2006). miRNAs form a complex with RNA-induced silencing complex and bind to the 3'-untranslated region (3'-UTR) of the gene. 3'-UTR regions have been identified in several human CYP genes including CYP1B1, CYP2C18, CYP2S1, and CYP2W1 (Ingelman-Sundberg et al., 2007). For example, human CYP1B1 protein expression is suppressed by miRNAs through an unknown mechanism. This may explain the lack of association of CYP1B1 mRNA and protein expression pattern in human tissues (Ingelman-Sundberg et al., 2007; Tsuchiya et al., 2006). Human CYP2C18 protein expression is post-transcriptionally regulated by miRNAs and consequently, higher levels CYP2C18 mRNA are not translated to CYP2C18

protein (Ingelman-Sundberg, 2008). Thus, CYP2C18 protein follows a different pattern of expression than its mRNA transcript. The human CYP2S1 mRNA transcript has a long 3'-UTR (1051 bp) region. Reports suggest that the longer 3'-UTR region increases the probability of having putative sites for miRNA binding (Ingelman-Sundberg et al., 2007). Rat CYP2S1 mRNA may be post-transcriptionally regulated by miRNAs. It is possible that AhR agonists increase miRNA levels or initiate the binding of miRNAs to the 3'-UTR region of the rat CYP2S1 mRNA leading to decreased translation of mRNA into CYP2S1 protein. The effect of TCDD and other AhR agonists on miRNAs in rat lung has not been determined. In a recent study, Moffat et al. (2007) reported that only 5 miRNAs out of 464 miRNAs examined were altered after treatment with TCDD in rat liver. Further studies are needed to understand the role of specific miRNAs in the AhR-mediated regulation of CYP2S1 protein in rats. A proposed mechanism of rat CYP2S1 regulation by AhR agonists is summarized in Figure 4.4.

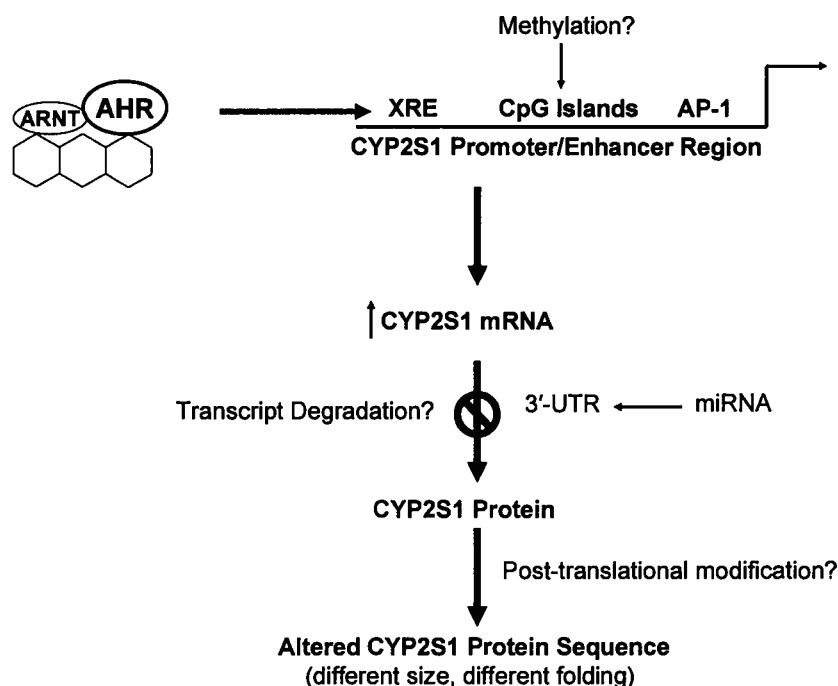


Figure 4.4: Proposed mechanism for lack of induction of CYP2S1 protein by AhR agonists in rats. CYP2S1 mRNA transcript contains a long 3'-UTR region, which may play a key role in the post-transcriptional suppression by miRNA. In addition, degradation of CYP2S1 mRNA or post-translational modifications may regulate CYP2S1 protein expression after treatment of rats with AhR agonists. Abbreviations: 3'-UTR- 3'-untranslated region; miRNA- microRNA; AhR- aryl hydrocarbon receptor; ARNT- aryl hydrocarbon receptor nuclear translocator; XRE- xenobiotic response element; CpG islands- cytosine and guanine nucleotides bonded by phosphodiester; AP-1- activator protein 1 (a transcription factor).

4.3. Limitations of the Present Study

Some of the objectives of my project were not achieved due to limitations in the study.

Anti-mouse CYP1B1 antibody, anti-rat CYP2S1 antibody and recombinant rat CYP2S1 protein were not available commercially and thus posed significant challenges.

- In the present study, mouse CYP1B1 protein levels were not measured in MA-10 Leydig cells because a suitable anti-mouse CYP1B1 antibody was not available commercially. Thus, it was not possible to determine if the effect of LH, estradiol and TCDD occurred at the protein level in MA-10 cells. It is possible that CYP1B1 protein is expressed at a very low or undetectable level in MA-10 cells. Also, the anti- mouse CYP1B1 antibody developed and provided by Alpha Diagnostics (San Antonio, TX, USA) may be a very weak antibody with a low titer, contributing towards its inability to detect CYP1B1 protein. Recombinant or purified mouse CYP1B1 protein could be used to generate an inhibitory anti-mouse CYP1B1 antibody with a higher titer and immunoreactivity. However, the effect of LH, estradiol and TCDD on CYP1B1 protein expression was determined in rat R2C Leydig cells but no corresponding mRNA data in this cell system.
- I attempted to determine CYP1B1 catalytic activity by measuring the formation of DMBA-3,4-diol and DMBA-5,6-diol metabolites after incubation of DMBA with testicular microsome samples. CYP1B1 protein activity could not be measured in testicular microsomes. The failure of the assay could be due to lack of optimization of the assay methodology in extracting the metabolites from the reaction mixture. It should be noted that even if the DMBA assay was optimized it might have been difficult to measure CYP1B1 activity due to low levels of CYP1B1 protein in MA-10 cells. Moreover, the DMBA metabolism assay is not a highly specific marker assay for CYP1B1 as other CYP enzymes such as CYP1A1 and CYP1A2 also catalyze DMBA oxidation reactions. Thus, it would have been difficult to measure the effect of LH, estradiol and TCDD on CYP1B1 activity in MA-10 cells. The LC/MS assay explored in

this project could be developed and validated by determining extraction efficiency, accuracy, sensitivity, precision and reproducibility of the assay. Use of an inhibitory antibody would be useful for determining the contribution of CYP1B1 to the formation of DMBA-3,4-diol and DMBA-5,6-diol metabolites from DMBA.

- In this study, relative CYP2S1 and CYP1B1 mRNA levels were determined using the semi-quantitative block cyler PCR technique. Thus, the amount of induction or suppression in mRNA levels observed after treatment with LH, estradiol benzoate and AhR agonists should be interpreted with caution due to the inherent non-quantitative nature of the technique.
- Rat CYP2S1 protein levels were not quantified because recombinant rat CYP2S1 or purified CYP2S1 protein were not commercially available for use as calibration standards in the immunoblots. The CYP2S1 values reported in the CYP2S1 protein studies are relative CYP2S1 protein levels and it would be useful to know the absolute levels of CYP2S1, which could be compared with the levels of other CYP enzymes in rat tissues. Development of recombinant rat CYP2S1 protein would allow us to determine the absolute levels of CYP2S1 protein in rat tissues.

4.4. Conclusions

The following conclusions could be drawn from the studies described in this thesis:

CYP1B1

- a) Of the pituitary hormones tested, LH had the greatest inductive effect on CYP1B1 mRNA expression in MA-10 Leydig cells. LH induced CYP1B1 mRNA and protein in a concentration-dependent fashion in MA-10 and R2C cells, respectively. PKA activity was stimulated in Leydig cells after exposure to LH. Basal PKA activity in R2C cells was approximately 2-fold higher than that of MA-10 cells. We show for the first time that CYP1B1 protein is expressed at readily detectable levels in rat Leydig R2C cells.
- b) LH downregulated LH receptor mRNA expression in MA-10 Leydig cells. The effect of LH on CYP1B1 mRNA expression was independent of LH receptor mRNA levels. Post-receptor signaling cascades may be involved in the regulation of CYP1B1 transcription in Leydig cells.
- c) Studies using a PKA activator (8-Br-cAMP) and PKA inhibitors (H89 and Rp-CPT-cAMP) suggest that PKA was involved in the LH-elicited induction of CYP1B1 mRNA and protein in MA-10 and R2C Leydig cells, respectively. Thus, testicular CYP1B1 regulation by LH was mediated through a cAMP-dependent PKA pathway.
- d) Kinase pathways involving PKC and PKG had little or no role in the constitutive or LH-mediated regulation of CYP1B1 expression in Leydig cells.
- e) CYP1B1 mRNA was suppressed by estradiol benzoate in MA-10 Leydig cells. However, estradiol benzoate did not decrease CYP1B1 protein levels in R2C Leydig cells.
- f) Estradiol-mediated suppression of CYP1B1 mRNA expression was not dependent on LH receptor mRNA expression or blockade of estrogen receptors by a competitive antagonist

(ICI 182,780). The decrease in CYP1B1 mRNA expression was not due to estradiol-mediated suppression of LH-mediated pathways in MA-10 Leydig cells.

- g) Regulation of CYP1B1 protein expression by AhR was tissue-specific in rats. TCDD, the most potent AhR agonist, did not induce CYP1B1 mRNA and protein expression in SD rats and MA-10 and R2C Leydig cells. In contrast, CYP1B1 protein was induced by TCDD in rat liver, lung and kidney.

CYP2S1

- a) CYP2S1 mRNA is expressed predominantly in extrahepatic tissues including lung, kidney, small intestine, stomach, spleen, and skin in a tissue-specific manner. CYP2S1 mRNA levels were higher in lung than in liver. There was no sex-dependent difference in constitutive CYP2S1 mRNA expression in adult rats.
- b) Two antibodies to rat CYP2S1 were generated using the anti-peptide approach. Peptides corresponding to the C-terminal sequence (486-499 amino acids) and an internal sequence (272-286 amino acids) of CYP2S1 protein were used for this purpose. Anti-rat CYP2S1 serum prepared against the C-terminus peptide of rat CYP2S1 was more immunoreactive to CYP2S1 protein and was used for the protein studies.
- c) CYP2S1 protein with a molecular weight of approximately 50 kD was identified in rat lung, stomach and kidney using anti-rat CYP2S1 serum (C-terminus). Relative CYP2S1 protein levels were higher in stomach than in lung and thus follows a different pattern than CYP2S1 mRNA. CYP2S1 protein was also identified in mouse lung by anti-rat CYP2S1 serum.
- d) CYP2S1 mRNA was induced by 3-MC and TCDD in rat lung, liver, kidney and small intestine. However, relative CYP2S1 protein levels were not increased in lung, liver and kidney after treatment of rats with 3-MC, BaP and TCDD, which indicates that CYP2S1

protein expression may not be regulated by AhR. CYP1A1 mRNA and protein expression were more responsive than that of CYP2S1 after exposure to AhR agonists in the same tissues.

- e) CYP1A1 and CYP1A2 protein expression were highly inducible by TCDD in rat liver, lung and kidney.

The results obtained from this work provide additional knowledge about regulation of CYP1B1 and CYP2S1, two important but poorly understood CYP enzymes. In summary, the results show that LH regulates CYP1B1 expression via cAMP-mediated PKA pathway in Leydig tumor cells. Estradiol suppresses CYP1B1 expression through an unknown mechanism in Leydig cells. The AhR may have little or no role in the regulation of testicular CYP1B1 expression in rodents.

We show for the first time that CYP2S1 mRNA and protein are expressed in the rat and the expression is mainly extrahepatic and not sex-dependent. It appears that CYP2S1 mRNA, but not protein, is responsive to treatment with AhR agonists in rats.

4.5. Global Summary

Due to the success of the human and mouse genome projects, a great deal of progress has been made in recent years in the identification of novel extrahepatic CYP enzymes. In the present study, CYP1B1 and CYP2S1, which are predominantly expressed in extrahepatic tissues and are postulated to be regulated by AhR, were studied.

CYP1B1 is expressed mainly in extrahepatic tissues and is weakly regulated by the AhR-mediated pathway. The effect of AhR agonists on CYP1B1 expression in liver, lung and kidney is much less than on CYP1A enzymes. Constitutive CYP1B1 expression in testis is very high in comparison with hepatic and other extrahepatic tissues. However, results from this study suggest that the AhR-mediated pathway is not a major factor in the regulation of testicular CYP1B1 expression. AhR repressor, tissue specific promoter(s), or DNA methylation status of the CYP1B1 gene may play an important role in the lack of induction of CYP1B1 expression by AhR agonists in rat testis.

Pituitary hormones, mainly LH, are involved in the regulation of CYP1B1 expression in rat testis and rodent Leydig cells in vitro through the PKA pathway. LH increases CYP1B1 expression via stimulation of cAMP formation and subsequently PKA activation. Increased PKA activity initiates the phosphorylation of CYP1B1 transcription factors such as CREB and SF-1 (Zheng and Jefcoate, 2005), leading to increased CYP1B1 mRNA and protein levels. Thus, therapeutic agents (e.g. flutamide, raloxifen, naloxone) that interfere with LH levels in the body (Akingbemi et al., 2003; Andrews et al., 2001; O'Connor et al., 2002) can modulate CYP1B1 levels. Another important aspect of CYP1B1 expression is its downregulation by estradiol in mouse Leydig cells. At the present time, the mechanism of this suppressive effect by estradiol is unknown. Transcriptional suppression of CYP1B1 mRNA due to higher levels of DNA methylation of the CYP1B1 gene or degradation of CYP1B1 mRNA transcript after estradiol treatment may be involved in the local effect of estradiol on CYP1B1 mRNA expression in

Leydig cells. It has been proposed that exogenous estradiol downregulates CYP1B1 protein expression *in vivo* by blocking the hypothalamus-pituitary axis, leading to decreased LH secretion from the anterior pituitary (Leung et al., 2009).

Similar to CYP1B1, expression of CYP2S1 is tissue-specific and is expressed mainly in extrahepatic tissues in rats. CYP2S1 mRNA and protein levels are highest in rat lung and stomach, respectively. The absence of a sex difference in constitutive CYP2S1 mRNA expression suggests that CYP2S1 is not regulated by sex hormones. Previously, it was believed that only CYP enzymes from the CYP1 family are regulated by the AhR. However, recent reports confirm that CYP2 family enzymes are also regulated by AhR agonists, albeit, more weakly than CYP1 family enzymes (Arpiainen et al., 2005). AhR agonists such as BaP, 3-MC and TCDD induce CYP2S1 mRNA, but not protein, expression in rat lung, liver and kidney. Post-translational modifications and miRNA-mediated post-transcriptional regulations may be involved in the lack of induction of CYP2S1 protein expression by AhR agonists.

The biological role of CYP1B1 is not yet fully understood. It has been suggested that CYP1B1 may be involved in the chemical-induced carcinogenesis in certain tissues such as skin, bone marrow and ovary in mice (Buters et al., 2002; Buters et al., 2003). CYP1B1 has also been implicated in the development of congenital glaucoma, an eye disorder, in humans (Alward, 2003; Vasiliou and Gonzalez, 2008). The function of CYP1B1 in testis is unknown. It is possible that testicular CYP1B1 is involved in the bioactivation of toxicants.

The biological role of CYP2S1 is not yet been established. Although there is no evidence, researchers have proposed several functions for CYP2S1. It has been hypothesized that similar to other AhR-regulated enzymes, CYP2S1 may be involved in the bioactivation of PAHs leading to chemical carcinogenesis (Saarikoski et al., 2005a). Due to their expression in fetal tissues and their potential role in the metabolism of endogenous substrates such as retinoids, it has been

proposed that CYP1B1 and CYP2S1 may play important roles in embryonic development (Saarikoski et al., 2005a).

4.6. Future Directions

There are several avenues of CYP1B1 and CYP2S1 research that could be proposed for future studies. Factors responsible for lack of induction of testicular CYP1B1 expression by AhR agonists can be determined. AhR repressor could be one of the potential negative factors involved in AhR-mediated regulation of testicular CYP1B1 expression. Determination of testicular CYP1B1 mRNA and protein levels in AhR repressor-knockout mice treated with TCDD will confirm any definitive role of AhR repressor in lack of induction of testicular CYP1B1 expression by AhR agonists. Promoter or enhancer sequences responsible for the expression of the CYP1B1 gene in liver and testis can be identified using computational tools. Site-directed mutation of the nucleotide sequences of CYP1B1 promoter regions could be prepared and plasmids can be used to transfect a cell line deficient in CYP1B1 gene. Subsequently, CYP1B1 mRNA and protein levels could be measured after treatment with TCDD and LH, which may explain the possible involvement of different promoter sequences in tissue-specific expression and regulation of CYP1B1 mRNA and protein.

Both CYP1B1 and CYP2S1 genes contain multiple CpG islands. The methylation status of these genes could be determined using the methylation specific PCR reaction and bisulfite sequencing technique (Gomez et al., 2007; Tokizane et al., 2005). Information on the methylation level of CYP1B1 and CYP2S1 genes in vehicle-, TCDD- and estradiol benzoate-treated samples may enable us to understand unexplained phenomena in the present study, such as lack of induction of testicular CYP1B1 mRNA and protein by AhR agonists, estradiol benzoate-mediated suppression of CYP1B1 mRNA and tissue-specific expression of CYP2S1 mRNA. 5-Aza-2-deoxycytidine, an inhibitor of DNA methyltransferase which is responsible for the methylation of DNA bases (Okino et al., 2006), could be used to determine if blockade of DNA methylation leads to higher transcription of CYP1B1 and CYP2S1 genes.

Regulation of CYP2S1 protein expression by AhR-mediated pathway is not clearly understood at present. CYP2S1 mRNA and protein levels could be measured in untreated and TCDD-treated AhR-knockout mice to determine if AhR is involved in the regulation of CYP2S1 expression.

miRNAs have been implicated in the post-transcriptional suppression of certain CYP proteins (e.g. CYP2W1) (Ingelman-Sundberg et al., 2007). The binding sites for miRNAs have been identified in human CYP1B1 and CYP2S1 mRNA (Ingelman-Sundberg et al., 2007). It will be interesting to examine if miRNAs are involved in the regulation of CYP1B1 protein expression in testis and in Leydig cell lines and in the regulation of CYP2S1 protein expression rat lung, liver and kidney. Potential miRNAs associated with CYP1B1 and CYP2S1 could be identified using computational methods and also by using miRNA overexpressed cell lines and miRNA-knockout mice models. Transfection of cells expressing high levels of CYP1B1 and CYP2S1 proteins with wild-type and mutated miRNA plasmids, followed by measurement of CYP1B1 and CYP2S1 protein levels, could be carried out to determine the importance of specific miRNA(s) in the regulation of CYP1B1 or CYP2S1 protein expression. The effect of LH, estradiol and TCDD on the expression and activity of miRNAs could also be determined.

Although it has been speculated that CYP1B1 may be responsible for bioactivation of toxicants in testis, a definitive biological role of testicular CYP1B1 needs to be determined. Tissue-specific knockout of the CYP1B1 gene could reveal the physiological role(s) of CYP1B1.

The physiological significance of CYP2S1 and its importance in drug biotransformation is unknown at present. An inhibitory antibody would be useful for screening the contribution of CYP2S1 to microsomal metabolism. Recombinant rat CYP2S1 protein can be prepared using baculovirus expression system and subsequently, metabolism assays for PAHs, sex hormones, and other potential substrates of CYP2 enzymes could be carried out to identify rat CYP2S1 substrates. Recombinant rat CYP2S1 can also be used for absolute quantification of CYP2S1

protein levels (pmol/mg microsomal protein) in microsomal samples from rat tissues using immunoblot analysis. In addition, development of a model that over-expresses CYP2S1 would be useful for elucidating its metabolic and physiological roles. It will be interesting to investigate embryogenesis and organ development in a CYP2S1 knock-out mouse model to determine the developmental role of CYP2S1, if any.

Expression, regulation and functionality of other newly identified orphan CYP enzymes (e.g. CYP4X1, CYP20A1) could also be investigated.

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