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**The Effect of Lipoprotein Association on Cyclosporine  
Metabolism and Toxicity in Rats**

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**The Effect of Lipoproteins Association on Cyclosporine  
Metabolism and Toxicity in Rats**

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## **Dedication**

To My Beloved Family

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# **THE EFFECT OF LIPOPROTEINS ASSOCIATION ON CYCLOSPORINE METABOLISM AND TOXICITY IN RATS**

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Based on the hypothesis that the therapeutic and toxic effects of cyclosporine (CSA) are dependent on the *in vivo* disposition of lipoprotein-associated drug, we examined the metabolic effect and toxicity of lipoprotein-associated CSA. We also explored the regulatory effect of low density lipoprotein receptors (LDL-R) on CSA metabolism.

A 10 mg/kg/d IV CSA dose for 14 days reduced CYP3A protein and activities in all lipoprotein-associated CSA groups, with the exception of the very low density lipoprotein (VLDL)-CSA group. This reduction was attributed to a decrease in CYP3A1/2 mRNA levels. VLDL-CSA showed no changes in CYP3A levels as compared with CSA alone, suggesting a possible delay of CSA exposure

to the liver. Furthermore, VLDL-CSA did not alter lipoprotein receptors mRNA, while LDL-CSA caused a significant suppression of LDL-R. However, scavenger receptor class B type I (SR-BI) was not altered by CSA treatment. The suppression of CYP3A and CYP2C11 is more obvious when low dose (0.1 mg/kg/d) was given, suggesting an enhanced uptake of CSA into liver by LDL-R.

The specific role of LDL-R was examined with LDL-R modified rat model by either 17 $\alpha$ -ethynodiol (EE) or 2% cholesterol diet. A 28 days of 15 mg/kg/d CSA SC treatment in LDL-R suppressed rats (mimic LDL-R saturated rats by chronic CSA dose) showed a decreased suppression of CYP3A, which caused an increase in availability of CSA to other tissues such as kidney and which led to an induction of renal LDL-R. In contrast, LDL-R induced rats showed the opposite results. This study also showed that the regulation of LDL-R is sexually dimorphic and tissue-dependent. In addition, CSA caused the induction of LDL-R due to, in part, the presence of growth hormone (GH) and an altered GH pattern of secretion.

Taken together, these results demonstrated lipoproteins caused varying hepatic metabolic responses that were partially attributed to hepatic LDL-R on CSA metabolism. In addition, induction of renal LDL-R resulted in CSA-induced renal toxicity. CSA caused the saturation of LDL-R in the liver partially due to GH, which leads to the stimulation of an LDL-R-independent pathway to increase plasma lipid levels. The present study offers a better understanding of changes occurring in lipoprotein metabolism and immunosuppressant effects in transplant patients.

## Table of Contents

List of Tables .....	xii
List of Figures.....	xiii
List of Figures.....	xiii
<b>CHAPTER ONE</b>	<b>1</b>
Introduction and Literature Review.....	1
1.1 Cyclosporine .....	2
1.1.1 History .....	2
1.1.2 Chemical Structure .....	5
1.1.3 Pharmacology .....	6
1.1.4 Pharmacokinetics.....	10
1.1.5 Toxicity.....	18
1.2 Lipoproteins.....	19
1.2.1 Lipid Biochemistry.....	19
1.2.2 Low Density Lipoprotein .....	22
1.2.3 Low Density Lipoprotein Receptors .....	23
1.2.4 CSA and LDL Receptor .....	25
1.3 Cytochrome P450 .....	26
1.4 Objectives .....	29
<b>CHAPTER TWO</b>	<b>32</b>
METHODS .....	32
2.1 Liver and Kidney Microsome Preparation.....	32
2.2 Small Intestine Microsome Preparation .....	33
2.3 Liver and Kidney Membrane Preparation.....	34
2.4 Lowry Protein Assay.....	35
2.5 Gel Electrophoresis and Immunoblotting.....	36

2.6	<i>In Vitro</i> Steroid Hydroxylation Assays .....	37
2.7	Chromatography .....	38
2.8	RNA Isolation.....	41
2.9	Reverse Transcription Polymerase Chain Reaction (RT-PCR) .....	44
2.10	ALT (SGPT) and AST (SGOT) Analysis .....	54
2.11	Serum and Urine Creatinine .....	55
2.12	Cholesterol Levels .....	56
2.13	Statistical Analysis .....	57
<b>CHAPTER THREE</b>		<b>60</b>
Effect of Low Dose of HDL-Associated CSA on Hepatic Metabolism.....		60
3.1	Introduction .....	60
3.2	Experimental.....	63
3.2.1	Chemicals .....	63
3.2.2	Animals .....	63
3.2.3	Plasma and HDL-associated Drug Preparation.....	65
3.2.4	Statistical Analysis .....	66
3.3	Results .....	67
3.3.1	General Parameters.....	67
3.3.2	Serum and Urine Creatinine .....	67
3.3.3	Hepatic CYP3A2 and CYP2C11 Protein Expression .....	68
3.3.4	The Catalytic Activity of Hepatic CYP3A and CYP2C11 .....	71
3.4	Discussion.....	72
<b>CHAPTER FOUR</b>		<b>82</b>
The Effect of High Dose of Lipoprotein-Association on CSA Metabolism and Toxicity in rats.....		82
4.1	Introduction .....	82
4.2.	Experimental.....	86
4.2.1	Chemicals .....	86

4.2.2 Animals .....	87
4.2.3 Isolation of Human Plasma by Gradient Ultracentrifugation.....	88
4.2.4 Concentration of Lipoproteins.....	90
4.2.5 Extraction of CSA from the Lipoproteins .....	91
4.2.6 Chromatography for the CSA Concentration.....	92
4.2.7 Statistical Analysis .....	93
4.3 Results .....	94
4.3.1 General Observations .....	94
4.3.2 Serum Data .....	99
4.3.3 Renal Function.....	101
4.3.4 Effects of Lipoprotein associated CSA on Hepatic CYP .....	102
4.3.5 Effect of CSA on Receptors .....	106
4.4 Discussion.....	114
<b>CHAPTER FIVE</b>	<b>128</b>
Interaction between CSA and the Low Density Lipoprotein Receptors .....	128
5.1 Introduction .....	128
5.2 Methods .....	132
5.2.1 Chemicals .....	132
5.2.2 Animals .....	133
5.2.3 CSA Blood Level .....	137
5.3 Results .....	138
5.3.1 General Observations .....	138
5.3.2 Lipid Profile.....	141
5.3.3 Renal Function.....	142
5.3.4 Effect on Receptors .....	146
5.3.5 Effect on CYP Isoforms .....	147
5.3.6 <i>In Vitro</i> Metabolic Activity .....	162
5.3.7 CSA blood Concentration.....	164

5.3.8 Effect of GH on Receptors .....	171
5.4 Discussion.....	174
<b>CHAPTER SIX</b>	<b>185</b>
Summary and Conclusions .....	185
APPENDIX A .....	189
APPENDIX B.....	190
APPENDIX C .....	191
APPENDIX D .....	192
ABBREVIATION.....	193
REFERENCES.....	196
VITA .....	228

## List of Tables

Table 1.1	CSA Dosage Forms .....	3
Table 1.2	Phase I Metabolites (Nomenclature) .....	16
Table 2.1	Intraday and Interday Variability of <i>In Vitro</i> Testosterone Hydroxylation in HPLC Assay.....	40
Table 2.2	Liver Cytochrome P450 Enzyme Activities.....	43
Table 2.3	Oligonucleotide PCR Primers .....	47
Table 2.4	RT-PCR Conditions .....	48
Table 3.1	Renal Function Parameters .....	69
Table 4.1	Intraday and Interday Variability of CSA Concentration Measurement with HPLC .....	95
Table 4.2	Component in Lipoprotein Fractions (VLDL, LDL, HDL) and Plasma .....	97
Table 4.3	Summary of Study Groups .....	98
Table 4.4	Biochemical Parameters .....	100
Table 4.5	Renal Function Parameters .....	103
Table 5.1	Summary of Study I Groups .....	135
Table 5.2	Summary of Study II Groups .....	136
Table 5.3	Renal Function Parameters .....	139
Table 5.4	Liver Function Parameters .....	140
Table 5.5	Serum Lipid and Lipoprotein Levels.....	143

## **List of Figures**

Figure 1.1	Structure of CSA .....	7
Figure 1.2	Mechanism of CSA .....	8
Figure 2.1	Representative Chromatograph of Testosterone Hydroxylation	42
Figure 2.2	SR-BI DNA PCR Amplification Efficiency with Liver mRNA	49
Figure 2.3	LDL-R DNA PCR Amplification Efficiency with Liver mRNA.....	50
Figure 2.4	GAPDH DNA PCR Amplification Efficiency with Liver mRNA.....	51
Figure 2.5	LDL-R/SR-BI PCR Amplification Efficiency with Kidney mRNA.....	52
Figure 2.6	CYP2E1/GAPDH PCR Amplification Efficiency with Kidney mRNA.....	53
Figure 3.1	Comparison of Immunblot Analysis of Hepatic CYP3A and CYP 2C11 .....	73
Figure 3.2	Hepatic CYP3A and CYP2C11 Protein Expressions .....	74
Figure 3.3	The Catalytic Activity of Hepatic CYP3A.....	75
Figure 3.4	The Catalytic Activity of Hepatic CYP2C11 .....	76
Figure 4.1	Chromatograph for CSA Concentration.....	96
Figure 4.2	Effect of Lipoprotein-associated CSA on Hepatic CYP3A Protein Levels .....	107
Figure 4.3	Effect of Lipoprotein-associated CSA on Hepatic CYP2C11	

	Protein Levels .....	108
Figure 4.4	<i>In vitro</i> Testosterone Hydroxylase Activity in Hepatic Microsome .....	109
Figure 4.5	Effect of Lipoprotein-associated CSA on Hepatic CYP3A mRNA Levels .....	110
Figure 4.6	Effect of Lipoprotein-associated CSA on Hepatic CYP4A Protein Levels .....	111
Figure 4.7	Effect of Lipoprotein-associated CSA on Hepatic CYP2E1 Protein Levels .....	112
Figure 4.8	Comparison of Effect of LDL and LDL-associated CSA on Hepatic LDL-R mRNA Levels .....	115
Figure 4.9	Comparison of Effect of LDL and LDL-associated CSA on Hepatic LDL-R mRNA Levels .....	116
Figure 4.10	Comparison of Effect of LDL and LDL-associated CSA on Hepatic CYP3A mRNA Levels .....	117
Figure 4.11	Effect of Lipoprotein-associated CSA on Hepatic SR-BI Protein Levels .....	118
Figure 4.12	Comparison of Effect of LDL and LDL-associated CSA on Hepatic SR-BI mRNA Levels .....	119
Figure 5.1	Serum Lipid and Lipoprotein Content in Rats .....	144
Figure 5.2	Effect of EE and Cholesterol Diet on Hepatic LDL-R mRNA	150

Figure 5.3	Effect of CSA on Hepatic LDL-R mRNA in LDL-R Induced Rats .....	151
Figure 5.4	Effect of CSA on Hepatic LDL-R mRNA in LDL-R Suppressed Rats .....	152
Figure 5.5	Effect of CSA on Hepatic SR-BI mRNA in LDL-R Modified Rats .....	153
Figure 5.6	Effect of EE and Cholesterol Diet on Renal LDL-R mRNA ...	154
Figure 5.7	Effect of CSA on Renal LDL-R .....	155
Figure 5.8	Effect of CSA on Hepatic CYP3A1/2 Proteins in Hepatic LDL-R Modified Rats .....	156
Figure 5.9	Effect of CSA on Hepatic CYP3A1/2 mRNA in LDL-R Induced or Suppressed Rats .....	157
Figure 5.10	Effect of CSA on Hepatic CYP2C11 Expression in LDL-R Induced or Suppressed Rats.....	158
Figure 5.11	Effect of CSA on Hepatic CYP4A Protein Expression.....	159
Figure 5.12	Effect of CSA on Intestinal CYP3A Proteins in LDL-R Modified Rat.....	160
Figure 5.13	Effect of CSA on Renal CYP2E1 and CYP4A Proteins in LDL-R Modified Rats .....	161
Figure 5.14	Effect of CSA on Hepatic <i>In Vitro</i> Testosterone 6 $\beta$ -, 2 $\beta$ -hydroxylase Activity in LDL-R Induced or Suppressed Rats..	165

Figure 5.15	Effect of CSA on Hepatic <i>In Vitro</i> Testosterone 2 $\alpha$ -, 16 $\alpha$ -hydroxylase Activity in LDL-R Induced or Suppressed Rats ..	166
Figure 5.16	Effect of CSA on Hepatic <i>In Vitro</i> Testosterone 16 $\beta$ hydroxylase Activity in LDL-R Modified Rats .....	167
Figure 5.17	Effect of CSA on Hepatic <i>In Vitro</i> Testosterone 7 $\alpha$ -hydroxylase Activity in LDL-R Modified Rats .....	168
Figure 5.18	Effect of CSA on Intestinal <i>In Vitro</i> Testosterone 6 $\beta$ -, 2 $\beta$ -hydroxylase Activity in LDL-R Modified Rats.....	169
Figure 5.19	Comparison of Steady-State Whole Blood CSA Concentration .....	170
Figure 5.20	Effect of GH on Hepatic LDL-receptor mRNA Expression....	172
Figure 5.21	Effect of GH on Hepatic SR-BI mRNA Expression.....	173

# **CHAPTER ONE**

## **Introduction and Literature Review**

More than 30,000 patients (18,000 autologous, and 12,000 allogeneic) are estimated to have received transplantations worldwide and it is increasing 15-20% each year (DiPiro et al., 1999). Thus, the demand for a better immunosuppressant therapy is continuously growing. During the 1960s and 1970s, organ transplantation was restricted primarily to kidney allografts with immunosuppressant treatment limited to a small number of drugs such as azathioprine, methotrexate, cyclophosphamide, or steroids. Therefore, the discovery of cyclosporine (CSA) was a major turning point in increasing the survival rate of transplant recipients and extending the range of possible transplant procedures. Although other drugs such as FK506 (tacrolimus) and rapamycin (sirolimus) have been introduced, CSA is still considered to be the primary drug used to prevent rejection.

A tremendous number of patients with organ dysfunction also suffer adverse effects derived from current drug treatment such as CSA, which often hampers the therapy due to toxicity. The exact mechanism of those side effects is still under investigation. In the present research, CSA was used as a model drug to

examine 1) the metabolic response and toxic effect of a lipoprotein-associated CSA and 2) how the metabolic response of CSA is altered in a LDL-R (low density lipoprotein receptor) modified rat model.

## **1.1 CYCLOSPORINE**

### **1.1.1 History**

The history of CSA began in 1970. Eighty milligrams of a mixture of CSA was isolated from the fungus, *Tolypocladium inflatum Gams*, which was found in soil in Norway (Borel and Kis, 1991). This strain of fungus synthesizes a large number of metabolites and CSA is one of these metabolites. In 1972, Borel's group discovered an immunosuppressive effect from the metabolite mixture, which was different from the general cytostatic activity. In 1974, after further purification, Borel in Sandoz Pharmaceutics Inc. unveiled additional biological effects of CSA. This included selectivity for lymphocytes (especially for T helper cells), antibody- and cell- mediated immunity, and inhibition of the induction phase of lymphoid cell proliferation which affects early mitogenic

Table 1.1 CSA Dosage Forms

Sandimmune®	IV	Sol.	Cremophor®, 32.9% alcohol, 50 mg/mL, 5 mL
	Oral	Cap.	Sorbitol, 12.7% alcohol 50, 10 mg
		Sol.	Olive oil, 12.5% alcohol, 100 mg/mL, 50 mL
Neoral®	Oral	Cap.	Cremophor, 9.5% dhydrated alcohol
		Sol.	Corn & Olive oil, Cremophor, 9.5% dehydrated alcohol, 100 mg/mL, 50 mL (microemulsion)

Cremophor: Polyoxyated caster oil

triggering. In addition, Borel reported no lymphocytotoxicity. As compared with classic agents such as azathioprine and steroids, CSA showed a significant immunosuppressive effect in animals. However, in human studies, the lack of absorption of the first dosage form (oral) was a problem. A single oral dose did not reach pharmacologically active levels in the blood however this problem was solved by Wagner with olive oil as a vehicle (Borel and Kis, 1991). The first clinical trial was performed in 1978 in kidney and bone marrow transplantation recipients, and the first intravenous CSA (Sandimmune®) was approved in 1983. By 1995, a newly formulated microemulsion exhibiting better bioavailability (BA) received approval for human use (Table 1.1). Despite improvement of BA, a high incidence of kidney dysfunction (Klintmalm et al., 1981; Bennett and Norman, 1986), neurotoxicity (Atkinson et al., 1984; Thompson et al., 1984; De Groen et al., 1987) hepatotoxicity (Wang et al., 1997), hypertension and a high mortality rate (Bennett and Norman, 1986; Schachter, 1988) still remained as a problem in treatment with CSA. Those side effects even caused the loss of the kidney graft or the loss of life.

### **1.1.2 Chemical Structure**

Mapping of the chemical structure was successfully completed in 1975 using single-crystal X-ray studies and chemical degradation (Petcher et al., 1976; Borel and Kis, 1991) (Figure 1.1). The molecule was modified to create approximately 750 semi-synthetic or synthetic analogues and tested *in vitro* and *in vivo*. However, none of them had a greater immunosuppressive effect than CSA degradation (Petcher et al., 1976; Borel and Kis, 1991). CSA is a neutral cyclic polypeptide with 11 active amino acids that play an interactive role with lymphocytes resulting from formation of the lipophilic site (Wenger, 1990).

Among the series of CSAs (A, B, C, D, G), CSA was the most effective and immunosuppressive molecule (Ruegger et al., 1976; Borel and Kis, 1991). Among the 11 amino acids, biological activity is associated with amino acid 1, 2, 3, 9, 10 and 11 (Wenger, 1990). CSA is a highly lipophilic molecule weighing 1203 daltons. To date, 30 metabolites (Maurer and Lemaire, 1986; Wenger, 1990; Christians and Sewing, 1993; Christians and Sewing, 1995) have been identified with the majority of them resulting from hydrolysis and demethylation. The detailed of metabolism will be discussed in Section 1.1.4.

### **1.1.3 Pharmacology**

#### ***Mechanism***

There are more than 2000 articles examining the cellular and molecular aspect of CSA. So far, it is known that CSA's immunosuppressive activity is, at least in part, attributed to its ability in blocking T-cell proliferation in the G<sub>0</sub> phase of the cell cycle in mRNA transcription of cytokinase (Kunz and Hall, 1993; High, 1994; Sehgal, 1998). Immunophilins are categorized into two main classes. The major immunophilins are cyclophilin (17 kDa) and FKBP (FK506-binding protein, 12 kDa). These are ubiquitous throughout the body in micromolar concentrations and present in about 0.1 % of total cytosolic protein (Soldin, 1998). The minor immunophilins that exist in nanomolar concentrations in the body are those with peptidylproline cis-trans isomerase, proline rotamase (PPIase) activity (12, 25, and 50-56 kDa) that are found in the cytosol of T-cells, splenic cells, and thymic cells (Soldin, 1998). CSA binds weakly to both 25 and 52 kDa proteins (Soldin, 1998).

Cyclophilin and FKBP are the primary targets for inhibition of the immune response. These proteins are ubiquitous and abundant in both prokaryotes and eukaryotes and are readily inhibited by immunosuppressants (Harding, 1991). CSA differs from other immunosuppressants, such as FK506 and sirolimus, which bind to FKBP in that it binds to cyclophilin. Cyclophilin contains PPIase activity

Figure 1.1 Structure of CSA

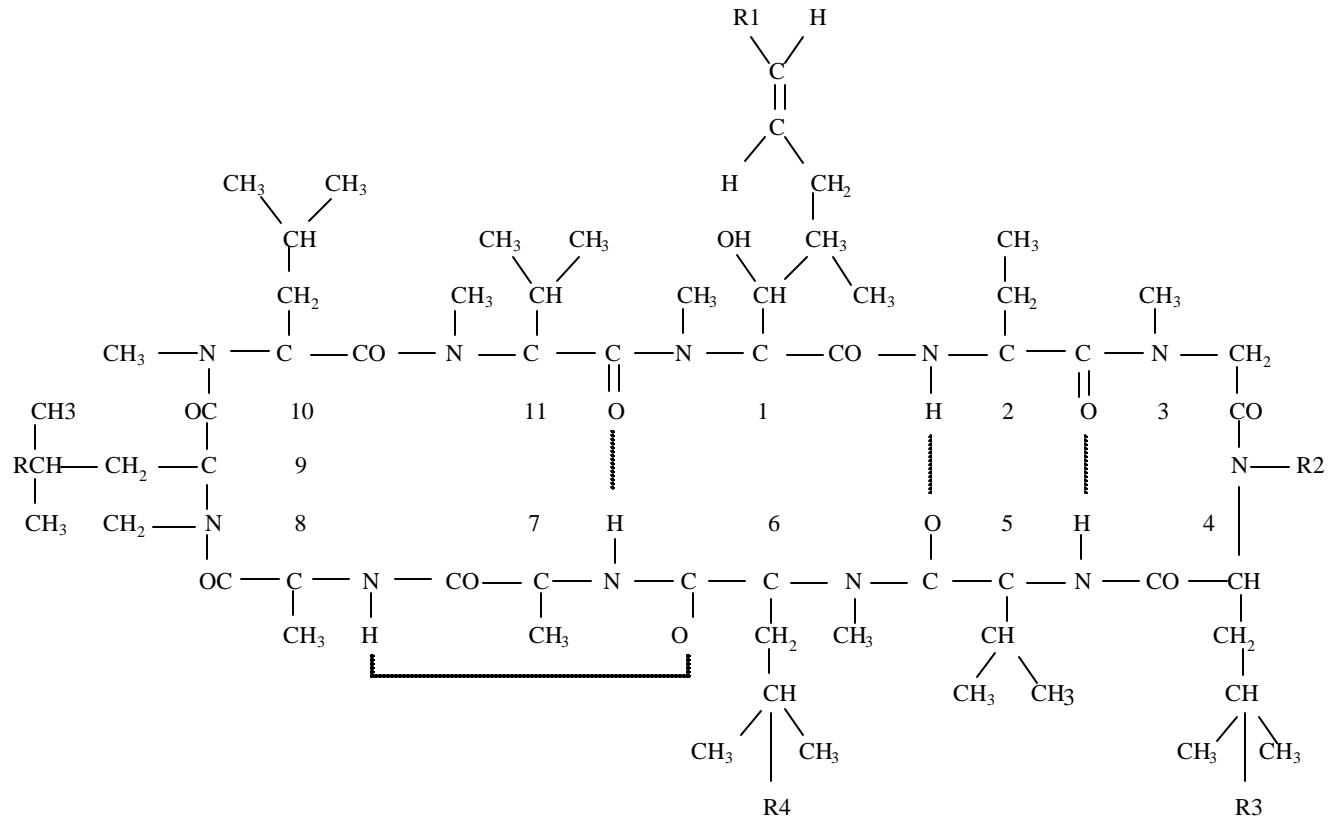
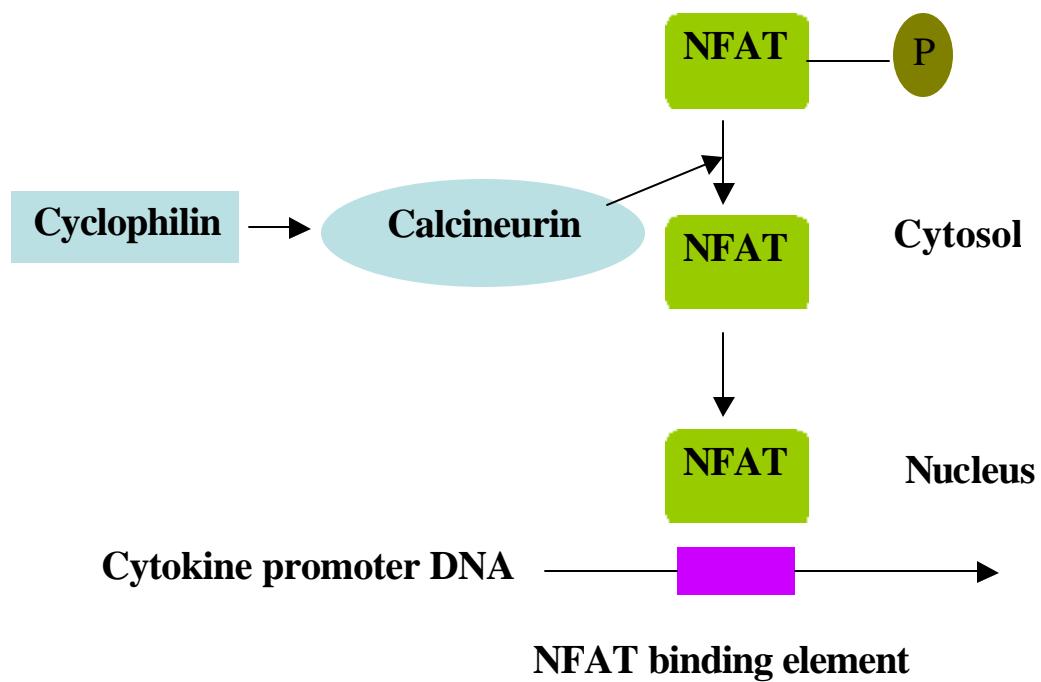


Figure 1.2 Mechanism of CSA



NFAT: Nucleus factor of activated T-cell

*in vivo* as well as *in vitro*, which plays a role in the folding of proteins and avoiding aggregation and is competitively inhibited by the CSA. The function of cyclophilins and FKPs *in vivo* is unknown in the absence of the drugs. The CSA-cyclophilin complex binds to calcineurin, which is a  $\text{Ca}^{2+}$ /calmodulin-dependent serine/threonine phosphatase (a type 2B phosphatase) (Dawson, 1996). After binding, the drug selectively inhibits the calcineurin activity in T-cells in the interleukin-2 (IL-2) signal transduction pathway (Kunz and Hall, 1993; Dawson, 1996). IL-2 gene (cytokine gene) transcription is regulated by the transcription factor, nuclear factor of activated T cells (NF-AT). Calcineurin inhibition mediates immunosuppression through regulation of IL-2 gene transcription (Soldin, 1998). Therefore, inhibition of calcineurin by CSA leads to a lack of phosphorylation of transcription factors which then prevents transcription of the IL-2 gene, which is a crucial step for activation of T-cell lymphocytes. T-cell activation increases intracellular calcium levels, which result in activation of calcineurin (Kunz and Hall, 1993; Dawson, 1996). CSA blocks the  $\text{Ca}^{2+}$ -dependent signaling pathway, but has no effect on the level of  $\text{Ca}^{2+}$  ionophore-enhanced expression of c-fos mRNA (Kunz and Hall, 1993). Although studies examining the regulation of CSA action have been conducted for almost 30 years, regulatory action of CSA still remains unclear.

In summary, the mechanism of CSA action is through the inhibition of lymphokine expression (IL2,  $\alpha$ -interferon etc.) at the level of gene transcription.

It enters the cell passively and binds to cyclophillin causing inactivation of calcium dependent activation of the cell. It blocks the signal to lymphocytes to produce IL-1, -2, -3, -4 and interferon-gamma. In addition, Wera recently demonstrated that human prolactin gene transcription was inhibited by CSA (IC<sub>50</sub> = 190 nM) (Wera et al., 1995). Since prolactin is mitogenic for T-lymphocytes, which express both prolactin and its receptor, this data could help to elucidate a possible immunosuppressive mechanism of CSA

#### **1.1.4 Pharmacokinetics**

##### ***Absorption:***

Absorption is slow and the bioavailability of CSA is highly variable in humans (20-50%) as well as in rodents (10-20%) (Wood et al., 1983). In addition, absorption by the upper small intestine is another variable to consider in oral administration since most of the absorption of CSA is observed in the upper portion of the small intestine where the majority of CYP enzymes reside. Due to the highly lipophilic nature of CSA, absorbtion is altered by the release of bile acids into the proximal jejunum (Salomon, 1991). The peak time of drug concentration in blood also varies from 1 to 8 hours depending on a patient's lipid profile, disease state, or food intake (Ptachcinski et al., 1985). In addition, the

content of P-glycoproteins (P-gp) in the lower portions of the small intestine appears to be critical for oral CSA absorption since P-gp is responsible for pumping out xenobiotics from the enterocyte to the intestinal lumen (Fricker et al., 1996).

**Distribution:**

Highly lipid-soluble CSA is expected to penetrate most biological membranes and to distribute throughout the body (Awni and Sawchuk, 1985; Lopez-Miranda et al., 1991). Due to this property, the distribution of CSA from the blood to tissues is rapid with higher concentrations of CSA found in the liver, spleen, pancreas and fat. In addition, CSA is extensively associated with lipoproteins in plasma (Awni and Sawchuk, 1985; Lopez-Miranda et al., 1991; Wasan et al., 1997). The proportion of CSA distribution in whole blood is as follows; 58% in erythrocytes, 9% in leukocytes, and 33% in plasma proteins (Lemaire and Tillement, 1982; Christians and Sewing, 1993). Of the distribution of drug in plasma, 21% is bound to lipoprotein and the remaining is bound to other proteins (8%) such as albumin,  $\alpha_1$ -acid glycoprotein or remains free (4%). Another *in vitro* study showed that distribution and binding of CSA between plasma and erythrocytes was established within 20 minutes, within the range of 25-500 ng of [<sup>3</sup>H]CSA to mL of human blood. The distribution ratio of erythrocytes to plasma was essentially constant (Christians and Sewing, 1993).

Luke et al. reported that the half-life of CSA in low density lipoprotein (LDL) and very low density lipoprotein (VLDL) was significantly lower than the half-life in high density lipoprotein (HDL) in dyslipidemic marrow transplant patients (Brunner et al., 1990a). Moreover, chronic CSA therapy caused a 75% decrease in HDL levels ( $37 \pm 8$  mg/dL to  $9 \pm 4$  mg/dL) and a 67.7% increase in LDL levels ( $65 \pm 13$  mg/dL to  $109 \pm 31$  mg/dL), while VLDL levels did not change significantly ( $17 \pm 13$  mg/dL to  $20 \pm 10$  mg/dL). They also found that a higher ratio of CSA in the HDL fraction was seen after the first dose, while a higher ratio of CSA was found in the LDL fraction following 31 days of dosing. In another set of experiments, the ratio of CSA in each lipoprotein fraction was measured in patients with renal failure (Brunner et al., 1990a). These results lead to two possible mechanisms. The first is that CSA in HDL is predisposing the patient to renal failure by preferentially delivering of the drug to sites of toxicity. The second is that CSA in LDL has been already delivered from LDL to kidney. Therefore, an increase of CSA in HDL is the consequence by preferential drug removal from LDL. This second mechanism is supported by the presence of LDL receptors, which are located in lipid-containing tissues such as liver, kidney, endocrine glands and adipose tissues (De Groen, 1988). It is highly lipophilic molecule thus preferentially bind to lipoproteins in vivo. The lipoproteins associated CSA, especially LDL-associated CSA will be transported in a similar manner as endogenous lipids.

***Metabolism:***

CSA is extensively metabolized by hepatic enzymes. More than 30 metabolites have been identified from human blood, body tissues, and urine. (Maurer and Lemaire, 1986; Wenger, 1990; Christians and Sewing, 1993; Christians and Sewing, 1995) The major pathway of metabolism is hydroxylation which produces the AM1 and AM9 metabolites, and N-demethylation which produces the AM4N metabolite. In addition, the intramolecular formation of a tetrahydrofuran derivative is also formed (Table 1.2).

Phase I metabolism: The cytochrome P450 (CYP) 3A subfamily is one of the most important enzyme metabolizing xenobiotics. It is most abundant isoform in both liver and small intestine and is account for more than 80% of CSA metabolism (Watkins et al., 1987). CYP3A4 is the major CYP3A isozyme in humans and CYP3A5 also shares some catalytic activities. For instance, CYP3A4 catalyzes CSA into two hydroxylated metabolites (AM9 and AM1) and one demethylated metabolite (AM4N), whereas CYP3A5 does not metabolize CSA to AM1 or AM4N, but does produce the AM9 metabolite. CYP will be discussed in detail in section 1.3.

Phase II metabolism: The hydroxyl group at  $\beta$ -carbon of amino acid 1 is the only functional group of unchanged CSA that is available for conjugation. Phase II metabolism of CSA consists of sulfate-conjugation and a glucuronidation. The sulfate conjugated metabolites were found in the bile and

plasma and glucuronidated metabolites were found in the bile of patients who were treated with CSA (Christians and Sewing, 1993).

Besides liver tissue, drug metabolism activity has been shown in kidney, lung and small intestine (Anders, 1980; Cunningham et al., 1982; Yoo et al., 1992; Christians and Sewing, 1995; Christians et al., 1996). However, no significant metabolism of CSA has been reported in lung and kidney (Anders, 1980; Yoo et al., 1992). Seventy percent of the CYP content in the small intestine consists of the isozyme CYP3A (Michalets, 1998), which is comparable to 20% of CYP3A in liver (Watkins et al., 1985; Christians and Sewing, 1993). The concentration of functional CYP3A in gut and liver is a major factor in determining CSA bioavailability and blood levels. Inhibition of CSA metabolism results in a rise in CSA blood levels, which causes an increase in adverse effect in general (Brunner et al., 1996). It has been known that therapeutic levels of CSA in whole blood are 100-400 ng/mL (Wilms et al., 1988; Yee et al., 1988; Yau et al., 1991).

Drug interactions: Some of CSA's metabolites also bind to cyclophilin (AM1, AM9, AM19, AM69, AM14N), suggesting that metabolites may contribute to immunosuppressive effects as well as toxicity, but to a much lesser degree than the parent compound (Fahr et al., 1990; Harding, 1991) Calcium antagonists such as verapamil, and diltiazem, which are predominantly metabolized by hepatic CYP3A4, have been reported to increase CSA blood levels. Verapamil caused a 45% increase of CSA area under the curve (AUC) and

maximum concentration (Cmax) as well as an increase in AM1 concentration (Christians and Sewing, 1993; Christians and Sewing, 1995). Diltiazem also caused a 5-fold increase in AM1 concentration, while increases in AM91c and AM19 were less pronounced (Christians and Sewing, 1993; Christians and Sewing, 1995). In addition, administration of diltiazem caused an increase in the half-life of the parent drug from 17 hours to 19.7 hours and that of AM1 from 8 hours to 45 hours. In addition, another immunosupresant, FK506, is a potent inhibitor of CSA metabolism while CSA did not inhibit FK 506 metabolism (Schreiber and Crabtree, 1992; High, 1994; Andoh et al., 1996a; Andoh et al., 1996b).

Induction of CSA metabolism caused a decrease in CSA blood levels, which carried the risk of organ rejection (Lampen et al., 1995; Bennett, 1997; Bertz and Granneman, 1997). Phenytoin (antiepileptic agent) significantly reduced the Cmax and AUC of AM1 and AM9 metabolites. Unfortunately, there are no proper animal models to accurately study the mechanism of the toxicity. Although CSA metabolites appeared to be less toxic than the parent drug in rat models (Donatsch et al., 1990), several studies showed a correlation between CSA metabolites in blood and nephro- and neurotoxicity (Kohlhaw et al., 1989; Christians and Sewing, 1995). Bleck demonstrated that there were higher blood and urine concentrations of AM19 and AM1A in kidney graft patients (Lemaire and Tillement, 1982).

Table 1.2 Phase I Metabolites (Nomenclature): (Christians and Sewing, 1993).

New Nomenclature	Old Nomenclature	R	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
<b><u>Phase I metabolites</u></b>						
AM1	M 17	H	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H
AM 9	M 1	OH	CH <sub>3</sub>	CH <sub>3</sub>	H	H
AM1c	M 18	H	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H *
AM 4N	M 21	OH	CH <sub>3</sub>	H	H	H
AM19	M 8	OH	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H
AM1c9	M 26	OH	CH <sub>2</sub> OH	CH <sub>3</sub>	H	H
AM1c4N9	-					
AM1A	M 203-218	H	COOH	CH <sub>3</sub>	H	H
AM1A4N	-					
AM1Ac	-					
Am1AL	-					
AM11d	-					
AM69	M 16	OH	CH <sub>3</sub>	CH <sub>3</sub>	H	OH
AM4N9	M 13	**				
AM14N	M 25	H	CH <sub>2</sub> OH	H	H	H *
AM14N9	-					
AM14N69	M 9	OH	CH <sub>3</sub>	H	H	OH
AM99N	-					
Dihydro-CSA M17	-					
<b><u>Phase II metabolites</u></b>						
AM1c-GLC						
Sulfates conjugated						

\* CH-O-CH-CH<sub>2</sub> of AA1, \*\*hydroxylated and N-demethylated derivative of CSA

Another study showed that liver grafted patients with nephrotoxicity showed an elevated blood concentration of AM1c9 and AM1A compared to the patients without nephrotoxicity (Christians et al., 1991). This suggests that accumulation of AM19 and AM1c9 may lead to kidney dysfunction (Christians et al., 1991). In addition, this study demonstrated that AM19 was correlated with bilirubin concentration and gamma glutamyl transferase activity in serum and that AM1A was correlated with the bilirubin concentration in serum during cholestasis (Christians et al., 1991).

***Elimination:***

Most of the unchanged CSA and its metabolites undergo biliary elimination (Yee et al., 1984). In the rat, more than 50% of radioactivity was observed in the bile following intravenous radiolabeled-CSA administration. Therefore, changes in serum bilirubin correlate with changes in CSA elimination. Enterohepatic recirculation of CSA and its metabolites also contribute to the excretion through the feces and less than 1% of an initial dose is excreted unchanged in the urine. In addition, volume of distribution (Vd) or clearance (Cl) in patients are additional variables that can change the half-life of CSA (Yee et al., 1984). For instance, hepatic dysfunction is known to delay the elimination of some drugs that are extensively metabolized such as CSA (Williams and Mamelok, 1980). An elimination half-life observed by Ptachcinski et al range from 6.2 to 23.9 hours (Ptachcinski et al., 1986). The GFR (glomerular filtration

rate) values ranges from 1.8 mL/min/kg for humans to 8.7 mL/min/kg for mice. These differences in GFR between species are mainly attributed to their relative number of glomeruli per kilogram body weight. In the rat, the ratio of kidney weight to body weight is much greater than that found in humans. The ratio is 1:125 in rat, as compared with 1:240 in humans (De Groen, 1988). Accentuating these differences is the fact that the rat GFR per gram of kidney weight is approximately twice that of humans. Thus, despite the fact that the pharmacokinetic data are similar, rats need to receive much larger doses of CSA than those given to humans in order to achieve the same effect on renal function.

### **1.1.5 Toxicity**

The accumulation of both CSA and its metabolites are involved in nephrotoxicity, hepatotoxicity, hypertension, and neurotoxicity (Bennett and Norman, 1986). Of those toxicities, the most important obstacle for the therapy is renal dysfunction. There are two types of nephrotoxicity associated with CSA therapy. One is an acute phase that is due to the direct effect of the drug. This is characterized by an increased renal vascular resistance and a decrease in GFR (Gerkens et al., 1984; Arlotto et al., 1991; Ducharme et al., 1995). The other is a chronic phase, which appears as tubulointerstitial toxicity resulting from prolonged exposure and high-doses of CSA. This toxicity is characterized by epithelial cell degeneration, interstitial reticulin deposition, and enlarged

mitochondria (Gerkens et al., 1984; Ryffel et al., 1988). Considerable damage occurs in the S3 segment of the proximal tubule (Gerkens et al., 1984), which may also inhibit the function of CYP oxidase. The prevalence of CYP oxidase in kidney is not clear yet. In order to study chronic renal failure, a rat model utilizing a low-sodium diet was used with a higher amount of CSA than the normal amount given to humans (Gerkens et al., 1984). While a high- sodium diet reduced the degree of nephrotoxicity, a low-sodium diet exacerbates it (Verrill et al., 1987). Gerken et al. explained this result in two ways. One is that salt-depleted kidneys increase proximal tubular reabsorption, which results in an increase in toxicant to nephron cells (Gerkens et al., 1984). The other is an increase of GFR due to the tubuloglumerular feedback.

## **1.2 LIPOPROTEINS**

### **1.2.1 Lipid Biochemistry**

Most of human cholesterol is present in blood as esterified cholesterol. Cholesterol is transported in the blood stream as lipid-protein complexes referred to as lipoproteins. Lipoproteins are composed of cholesteryl ester (CE) and triglyceride cores surrounded by phospholipids, cholesterol, and apoproteins that play a role in stabilizing the lipoprotein and directing its metabolism. (Niendorf

and Beisiegel, 1989) The main classes of lipoproteins are the chylomicrons, VLDLs, LDLs, and HDLs. The lipoproteins differ in their size and compositions. LDL is the major cholesterol carrying fraction in human plasma and HDL in rodent (Lusk et al., 1979; Havel and Hamilton, 1988). Most LDL is produced as the result of the metabolism of VLDL. VLDL is a very large lipoprotein (30-90 nm in diameter) synthesized by the liver. It distributes fatty acids in the form of triacylglycerols to various extrahepatic tissues, including the adipocytes. The fatty acids used in the synthesis of the VLDL's triacylglycerols are derived either from carbohydrates, via acetyl-CoA, or from fatty acids obtained from circulation. The major proteins involved in the formation of lipoprotein are Apo B, E, C (I, II, III) (Wasan and Cassidy, 1998). The Apo C-II in the VLDL activates an enzyme located on the epithelium, called lipoprotein lipase (LPL). LPL hydrolyzes the triacylglycerols in the VLDL as well as in other lipoproteins (Vaziri et al., 2000). In addition, LPL enhances cellular uptake of lipoproteins. It has been reported that LDL deficiency caused decreases in HDL and LDL cholesterol levels while it increased VLDL cholesterol levels (Oscai et al., 1990).

Disease status and drug treatments can alter the level of lipoproteins in circulation (Brunner et al., 1989; Wasan et al., 1998). Studies have demonstrated increased cholesterol levels in bone marrow transplant patients and in patients with autoimmune diseases after chronic CSA administration. (Brunner et al., 1989; Prueksaritanont et al., 1992; Gardier et al., 1993; Kahan et al., 1995)

Modification of the lipoprotein appears to influence the metabolism of lipophilic drugs such as CSA. For instance, decreases in CSA activity in patients with hypertriglyceridemia, and increases in CSA toxicity in patients with hypocholesterolemia have been reported (Nemunaitis et al., 1986; Luke et al., 1992). In addition, Luke and his co-worker have reported that greater amounts of CSA were found in the HDL fraction than the LDL fraction after a single-intravenous dose. On the other hand, CSA was decreased in HDL-fractions whereas the level of it increased in LDL-fractions, after multiple intravenous doses (De Groen et al., 1987), suggesting an important role for the lipoprotein level with which the drug associated.

The major proteins in LDL is apoprotein B (514 Kd), which covers 60-70% of the surface of the spherical LDL particles, and is considered to be responsible for LDL receptor recognition (Koelz et al., 1982). LDL consists of a cholestryl ester that is protected from the aqueous blood by an amphiphilic layer and is composed of a phospholipid and unesterified cholesterol. This unesterified cholesterol can exchange rapidly with other structures such as cellular membranes, whereas the cholestryl ester molecules remain more firmly trapped in the liphophilic core (De Smidt et al., 1990).

### **1.2.2 Low Density Lipoprotein**

Hyperlipidemia has been one of major problems after CSA therapy, due to its ability to cause secondary diseases, such as coronary heart diseases (De Groen, 1988; Markell and Friedman, 1989). The human body needs small amounts of cholesterol to maintain nerve cells and hormones. Too much cholesterol circulating in the blood stream is known as hypercholesterolemia which increases the risk of heart disease mainly because of LDL cholesterol. The majority of LDL is catabolized by LDL-R. Abnormalities in LDL-R result in diseases (Lestavel and Fruchart, 1994; Kobayashi and Kamata, 1999). It is usually caused by 1) a genetic disorder resulting in improper cholesterol metabolism; 2) a disease that raises the cholesterol levels such as diabetes mellitus, kidney or liver disease or hypothyroidism exhibiting abnormal lipoprotein metabolism (Wade et al., 1989; Liang et al., 1998; Kobayashi and Kamata, 1999). In addition, obesity also causes the body to produce excessive amounts of cholesterol. Lipoprotein lipase (LPL) synthesis is regulated by insulin, LPL deficiencies leading to Type I hyperlipoproteinemia may occur as a secondary outcome of diabetes mellitus (Wade et al., 1989). In addition, insulin and thyroid hormones positively affect hepatic LDL-R interactions which will be discussed in the last chapter.

### **1.2.3 Low Density Lipoprotein Receptors**

The function of LDL-R was recently reconfirmed by the administration of a recombinant adenoviral vector containing the normal rabbit LDL-R cDNA into the Watanabe heritable hyperlipidemic (WHHL) rabbit (Li et al., 1995). This species is genetically defective in LDL-R and subsequently develops hypercholesterolemia. Their cholesterol levels were decreased by 70% after the administration of LDL-R cDNA-containing receptors (Li et al., 1995). The LDL receptor is a glycoprotein with an apparent MW of 130-160 kDa (Lestavel and Fruchart, 1994). Two thirds of the total LDL turnover process is mediated by LDL-R which located in the liver, adrenal gland, small intestine, spleen, and kidney. The remaining one third is a receptor-independent process (Lestavel and Fruchart, 1994). The most rapid rates of transport and 80-90 % of LDL receptor activity are seen in the liver (Lestavel and Fruchart, 1994). LDL uptake is saturable in the receptor dependent route. Spady et. al. reported that the Km of LDL uptake by the receptor is approximately 100 mg/dL in all organs and the Jm (maximal transport rate) varies from 140  $\mu$ g/h/g in the liver to <10  $\mu$ g/h/g in the kidney (Spady et al., 1986). However, LDL uptake is unsaturable in the independent pathway in the range of 25-500 mg/dL, even when the levels of LDL are 20 times higher than normal (Spady et al., 1986). Verrill et al found high triglyceride (TG) and chylomicron levels in patients with type V hyperlipidemia along with high plasma CSA concentrations. These patients did not experience

nephrotoxicity up to 1500 ng/mL of CSA level in circulation, suggesting that the presence of a large amount of lipids might bind all free CSA and reduce cellular contact (Verrill et al., 1987).

The lack of LDL receptors was initially found in patients with familial hypercholesterolemia (FH). FH is a genetic disorder that is possibly caused by one of the following mutations to the LDL receptor gene (Lestavel and Fruchart, 1994); 1) a mutation that results in a complete loss of receptor synthesis; 2) a mutation that results in the synthesis of a receptor protein that is not properly processed in the golgi apparatus and is not transported into the plasma membrane; 3) a mutation that results in an LDL receptor that is incapable of binding LDLs; 4) a mutation that results in receptors that bind LDLs but do not cluster in coated pits and are not internalized. Due to these mutations, elevated LDLs lead to xanthomas by deposition of fat under the skin and tendons. A greater complication results from cholesterol deposition within the arteries, leading to atherosclerosis. Currently drug treatments considered for hypercholesterolemia are as follows (Brody et al., 1998): 1) 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, such as mevastatin and lovastatin, which increase the cellular uptake of LDLs; 2) The hepatic VLDL secretion inhibitor, nicotinic acid, which suppresses the flux of free fat acid release from adipose tissue by inhibiting lipolysis; 3) lipoprotein lipase activators (clofibrate, fenofibrate, and gemfibrozil), which promotes rapid VLDL turnover and diverse

hepatic free fatty acids from esterification reactions; 4) cholestyramine or colestipol, which are non-absorbable resins that bind bile acids. This results in the inhibition of re-absorption of bile acid by the liver which leads to accelerate excretion to feces. Through an inhibitory feedback mechanism, a greater amount of cholesterol is converted to bile acids to maintain a steady level in the circulation. In addition, the synthesis of LDL-R increases to allow increased cholesterol uptake for bile acid synthesis.

The exact mechanism by which the LDL-R is regulated is not fully understood. It has been reported that hormonal agents may regulate plasma cholesterol levels by controlling LDL catabolism. For example, pharmacological doses of estrogen stimulate hepatic LDL-R activity and remarkably decrease plasma LDL levels due to an increase of LDL-R mRNA levels (Kovanen et al., 1979; Spady et al., 1986; Rudling et al., 1992). However, estrogen administration failed to decrease serum cholesterol levels in hypophysectomized rats, indicating an indirect effect by estrogen (Steinberg et al., 1967).

#### **1.2.4 CSA and LDL Receptor**

Organ transplant patients receiving CSA are prone to cardiovascular disease resulting from arteriosclerosis or coronary artery disease (Stepkowski et al., 1996; Stepkowski et al., 1997). Patients treated with CSA have been shown to

have increased cholesterol levels, due to primarily to high LDL levels. However, the mechanism by which CSA causes the increase of LDL cholesterol levels is not fully understood. A recent study has reported that CSA inhibits ApoB100 synthesis resulting from cotranslational effects, indicating the possible role of CSA on LDL catabolism (Kaptein et al., 1994). Al Rayyes reported a contradictory finding that CSA-treated HepG2 cells showed a 25% inhibition of LDL receptor synthesis (Al Rayyes et al., 1996). In contrast, to these two previous findings, Vaziri reported that CSA-treated rats did not show altered LDL-R levels (Vaziri et al., 2000). This disagreement suggests the possibility of an indirect effect of CSA on LDL receptor activity, such as by growth hormone. The LDL receptor has been highly expressed in the estrogen treated rats however it failed to show significant results *in vitro*.

### **1.3 CYTOCHROME P450**

Cytochrome P450 (CYP) isoenzymes are a superfamily of hemoproteins that are embedded primarily in the lipid bilayer of the endoplasmic reticulum of hepatocytes (Nelson et al., 1996). It is also found in enterocytes of small intestine and kidney, and in the lung and brain to a lesser degree. By 1996, 481 CYP genes and 22 pseudogenes were known to exist (Nelson et al., 1996). Based on the

amino acid identity of the encoded genes, it is classified into families by an Arabic number for these proteins with less than 40% sequence homology and subfamilies by a letter for these proteins with more than 55% homology (Michalets, 1998). Only subfamilies 1-3 appear to be mainly responsible for the biotransformation of xenobiotics and the CYP4 subfamily is involved in the biotransformation of fatty acids and a few xenobiotics.

The levels of certain CYP are sexually different in mature rats due in large part to the patterns of secretion of growth hormone (GH) (Waxman et al., 1991). In male rats, GH secretion pattern is pulsatile with high peak (~200-250 ng/mL) levels in the circulation every 3-4 hr followed by a period of very low circulating GH levels (<1-2 ng/mL). In female rats, GH is secreted in a continuous pattern which is characterized by consistent circulating GH levels at ~15-40 ng/mL. Female GH secretion is more frequent and continually present. In humans, only pulsatile pattern are seen, but women show a higher and more frequent GH secretion pattern (<2.5 ng/mL) than men (Waxman et al., 1991; Haevey et al., 1995; Waxman et al., 1995). Male specific CYP enzymes are CYP2A2, CYP2C11/13, CYP3A2, and CYP4A2. Female-specific enzymes are CYP1A2, CYP2C12. Female-dominant CYP enzymes are CYP 2A1, CYP2C7 and CYP2E1, whereas CYP2B1/2 and CYP3A1 exist in both sexes (Lewis, 1996). Treatment with some xenobiotics causes a partial feminization of CYP enzyme expression due to the alteration of GH patterns (Shapiro et al., 1995; Pampori and

Shapiro, 1996; Pampori and Shapiro, 1999). These enzymes include CYP3A2 and CYP2C11. However, CYP3A2 is not totally dependent on the GH secretion pattern, indicating a different regulatory mechanism from CYP2C11 (Waxman et al., 1995).

To date, CYP3A has been reported as the major CSA-metabolizing enzyme in humans as well as in rodents. In rats, CYP3A1 and CYP3A2 are major isoforms catalyzing the biotransformation of CSA. These enzymes have a 89% sequence homology however, they are independently regulated. For example, CYP3A1 is inducible in mature male and female rats, whereas CYP3A2 is not inducible in mature female rats.

The levels of CYP enzymes can be monitored by changes in specific pathways of testosterone oxidation (Brunner et al., 1996). For instance, the rate of testosterone  $6\beta$ - and  $2\beta$ -hydroxylation reflects the CYP3A1/2 activity, and testosterone  $2\alpha$ - and  $16\alpha$ -hydroxylation reflects CYP2C11 activity. In addition, the formations of  $7\alpha$ - and  $16\beta$ -hydroxytestosterone are catalyzed by CYP2A1/2 and CYP2B1/2 respectively (Table 2.1) (Arlotto et al., 1989; Arlotto and Parkinson, 1989; Shimada et al., 1995).

## **1.4 OBJECTIVES**

This study was designed to investigate the effects of lipoprotein-associated CSA by measuring pharmacological and toxicological events. An abnormal lipid profile, such as hyperlipidemia and hypolipidemia demonstrates altered pharmacokinetic parameters after CSA treatment. In addition, CSA treatment itself altered the level of plasma lipoprotein. Therefore, the level of lipoprotein may be one of the critical factors to be considered in treatment. In Chapter 3 and 4, based on the data suggesting that CSA pharmacokinetics are related to the lipoproteins to which they are bound, lipoprotein associated CSA was compared with CSA alone in terms of it's metabolic response and toxicity.

In the Chapter 5, the potential mechanism for regulation of the LDL-receptor by CSA was studied. The levels of plasma lipoproteins are altered by disease status, as well as drug treatment. Chronic CSA treatment has been shown to increase the lipid level primarily resulting from rising plasma LDL levels. LDL is mainly cleared by the LDL receptor pathway. There are several possibilities to be considered for the mechanism of increased lipid levels. For instance, ApoB decreases the recognition by the LDL receptor and causes an increase in plasma LDL levels since ApoB in LDL is recognized by the LDL receptor and internalized. This may cause sustained CSA levels in the blood and thereby increase the exposure of CSA to other tissues, such as the kidney. This event would lead to lowered exposure of the drug to the livers, which could result

in other tissue-specific toxicity such as nephrotoxicity. In addition, inhibition of bile acid production by CSA may possibly lead to increasing free cholesterol levels. It has not been clear whether or not chronic CSA treatment increases the production of LDL or reduces clearance of LDL by suppressing its receptors.

**The objectives of this research are the following, based on the hypothesis:**

**Cyclosporine metabolism is modulated by LDL receptors in the rat.**

1. Investigate the effects of lipoprotein-associated CSA on drug metabolism and toxicity.
  - a. Determine the effects of lipoprotein-associated CSA on hepatic CYP enzymes.
  - b. Determine if lipoprotein-associated CSA has nephrotoxicity.
  - c. Determine whether or not lipoprotein-associated CSA modulates hepatic LDL receptors.
2. Investigate the effects of LDL receptors on CSA metabolism.
  - a. Determine whether or not LDL receptors are altered by CSA treatment.
  - b. Determine if an alteration of LDL receptors cause a modulation of hepatic and renal CYP enzymes.

- c. Determine if an induction or suppression of LDL receptors lead to renal dysfunction.
- 3. Investigate the effects of GH on LDL receptors.
  - a. Determine if there are gender differences in LDL receptor expression.
  - b. Determine the effect of impaired GH secretion on LDL receptors.

## **CHAPTER TWO**

### **METHODS**

#### **2.1 LIVER AND KIDNEY MICROSOME PREPARATION**

One gram of frozen liver or kidney tissue in three volumes of Tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.4, containing 150 mM potassium chloride and 1mM Ethylenediaminetetraacetic acid (EDTA) at 4°C, was homogenized with a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA) to isolate the microsomal fractions as described previously (Coon et al., 1978). In brief, the homogenized liver tissue was centrifuged at 9,000 x g for 20 min at 4 °C in a GS-15R centrifuge with a F630 rotor (Beckman Instruments, Inc., Palo Alto, CA). After the recentrifugation of the supernatant at 490,000 x g for 20 min at 4 °C in a tabletop ultracentrifuge (TL-100) with a TLA 100.4 rotor (Beckman Instruments, Inc., Palo Alto, CA), the pellet was suspended and washed in sodium pyrophosphate buffer, pH 7.4, containing 1mM EDTA with a tissue grinder (Wheaton Millville, NJ). The suspension was again centrifuged at 490,000 x g for 20 min at 4 °C with the same rotor. The pellet

was resuspended in a Tris chloride buffer, pH 7.4, containing 20% glycerol with the tissue grinder, and microsomes were stored at –80 °C prior to analysis. Microsomal protein concentrations were measured according to the methods of Lowry et al. (Lowry et al., 1951) using a commercial assay kit (Bio-Rad Laboratory, Hercules, CA) and bovine serum albumin as a standard. This procedure is detailed is in Section 2.4.

## **2.2 SMALL INTESTINE MICROSOME PREPARATION**

The first two-thirds of the small intestine below the pylori was used for the intestinal microsome preparation by the scraping method as previously described (Ivanetich et al., 1976). In brief, the segment was immediately washed twice in normal saline to remove blood and followed by three washes with normal saline containing 100 µM phenylmethylsulfonylflouride (PMSF; Sigma Chemical Co., St. Louis, MO) to inhibit protease and 1mM dithioerythritol (Sigma Chemical Co., St. Louis, MO) to help isolate microsome from the villus. After the lumen was cut lengthwise, the remaining deposit (fecal matter and intestinal content) was removed from the gut by shaking in the buffer and scraping with a glass slide using appropriate pressure. Consistency of the scraping throughout all samples was maintained by performing one person with the same instrument. The

harvested intestinal epithelial cells were homogenized in 5 mL homogenizing buffer, containing 0.25M sucrose, 10 mM potassium dihydrogen phosphate buffer (KPi, pH7.4), 10 mM EDTA, 100 µM leupeptin, 100 µM PMSF and 100 µM trypsin inhibitor. Centrifugation was performed at 9000 g for 20 min in a GS-15R centrifuge with a F0630 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was then centrifuged at 490,000 x g for 20 min at 4 °C in a tabletop ultracentrifuge (TL-100) with a TLA 100.4 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was discarded and the pellet was resuspended in the homogenizing buffer and stored at -80°C until used.

### **2.3 LIVER AND KIDNEY MEMBRANE PREPARATION**

One gram of rat liver or kidney was homogenized in 20 mM Tris-HCl (pH 7.5) containing, 0.2 M sucrose, 2 mM MgCl<sub>2</sub>, 5 mM PMSF (phenylmethylsulfonyl fluoride), 5 µg/mL leupeptin, 10 µg/mL aprotinin and 3 µg/mL pepstain A with a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenized sample was centrifuged at 3000 x g for 20 minutes at 4 °C to remove debris in a GS-15R centrifuge with the F630 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was centrifuged at 45000 x g for 30 min at 4 °C in a tabletop ultracentrifuge (TL-100) with a TLA 100.4 rotor (Beckman

Instruments, Inc., Palo Alto, CA). After washing the membrane protein by recentrifugation with the same condition for 30 minutes, the pellet was stored at -80°C until used. The protein concentration was measured by the Lowry assay (Lowry et al., 1951) using a bovine serum albumin as a standard.

## **2.4 LOWRY PROTEIN ASSAY**

Lowry assay (Lowry et al., 1951) was performed to determine the protein concentration. Microsomal protein concentrations were normalized prior to analyzing cytochrome P450 protein expression and catalytic activity. Normalization of the protein concentrations was critical in maintaining accuracy in each assay. The DC protein reagent was purchased from Bio-Rad laboratories (Hercules, CA). Bovine serum albumin (Sigma, Chemical Co., St. Louis, MO) was used as the standard which ranged from 0.0 – 2.0 mg/mL. In brief, 50 µL of the serial standards and 16 to 30 times diluted samples were applied with Reagent A (alkaline copper tartrate solution) and Reagent B (Folin reagent). After the incubation at room temperature for 15 min, 200 µL of each standard and sample were transferred to a 96-well microplate and the absorbance was read at 699 nm in a SLT Rainbow spectrphotometer (SLT Rainbow, SLT Lab instruments,

Grodig, Austria) using WinSelect version 2.1 software (Tecan Research Triangle Park, NC).

## **2.5 GEL ELECTROPHRESIS AND IMMUNOBLOTTING**

Immunoblots were analyzed as previously described (Laemmli, 1970) with modifications. In brief, proteins ranging from 10–130 µg were separated using an 8% polyacrylamide separating gel (SDS-PAGE) and then transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) as previously described (Brunner et al., 1996). Bio-Rad prestained SDS-PAGE broad range molecular marker was used for the estimation of each band's molecular weight. Five percent nonfat-dry milk (NFDM) in 10 mM Tris buffer, pH 7.4 with 0.9% sodium chloride (TBS) was used to block non-specific binding on the nitrocellulose membrane. After washing the nitrocellulose membrane, the blots was incubated with a 1:500-2,000 dilution of the corresponding primary antibody (goat anti-rat 3A antibody, goat anti-rat 2C11, goat anti-rat 4A, goat anti-rat 2E1 antibody, GenTest, Woburn, MA, and rabbit-anti rat SR-BI; Novus Biologicals Inc., Littleton, CO) and then with a 1:2,000-3000 dilution of corresponding secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratory, Hercules, CA) in TBS containing 1-3 % NFDM. Following each

incubation, nitrocellulose sheets were washed four times with TBS containing 0.1% Polyoxyethylenesorbitan monolaurate (Tween 20) followed by 3 transitional washes with TBS. Immune complexes were visualized by oxidation of the luminol component of the ECL detection kit by horseradish peroxidase (Amersham, Arlington Heights, IL) on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA). The density of protein bands were measured and analyzed on either a Power Macintosh 7200/90 computer using a scanner (LaCie, Company, Rochester, NY) and the public domain NIH Image program version 1.60 (developed at the US National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>) or a Kodak documentation and image analysis system EDAS 290 (Eastman Kodak Company, New Haven, CT). The protein levels are reported as the ratio of density of the sample and the standard with arbitrary units.

## **2.6 IN VITRO STEROID HYDROXYLATION ASSAYS**

To measure the specific P450 enzyme activity, *in vitro* testosterone hydroxylation assays were performed as reported previously (Brunner et al., 1996). Each incubation mixture contained either 200 µg of liver microsomal protein or 1000 µg of intestinal microsomal protein in 500 µL of 0.2 M potassium

phosphate, pH 7.4, 250 µM testosterone in methanol, 100 µL nicotinamide adenine dinucleotide phosphate (NADP) regeneration system composed of 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>), 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 5 U glucose-6-phosphate dehydrogenase. Deionized water was added to a final mixture volume of 1000 µL. After preincubation for 3 minutes at 37 °C, glucose-6-phosphate dehydrogenase was added to initiate the reaction and the addition of 5 mL of dichloromethane stopped the reaction. The reaction was maintained at the temperature of 37 °C for 15 min for liver and 60 min for small intestine. After the addition of an internal standard, 3.6 nmol of 11-hydroxy-progesterone, the mixture was vortexed, and the organic phase was isolated and dried by a stream of air. The dried samples were dissolved in 200 µL methanol and stored it at 4 °C before high pressure liquid chromatographic (HPLC) analysis. The samples were stable at 4°C for 1 month. Each peak represents the specific CYP P450 activities as shown in Table 2.1. Activity unit was expressed in pmol/min/mg protein.

## 2.7 CHROMATOGRAPHY

High pressure liquid chromatograph (Shimazu, Columbia, MD) was used to separate and quantify testosterone and its metabolites. In brief, 20 µL of the

metabolic extract was injected by an automatic injector (SIL-10A) and resolved at 40 °C (Eppendorf Model CH-30 column heater, Eppendorf Model TC-50 controller, Brinkman Instruments, Westbury, NY) on a 150 x 4.6 mm C-18 column (Supelco, Bellefonte, PA) proceeded by a 10 mm x 4.3 mm C-18 guard column (Upchurch Scientific, Oak Harbor, WA) along with 90 % solvent A (Methanol: water: acetonitrile 39: 60: 1) and 85% solvent B (methanol: water: acetonitrile 80: 18: 2) for 20 min at an average flow rate of 1 mL/ min by a dual solvent pump (LC-10AS). All system function was maintained by a system controller (SCL-10A). The absorbance was monitored at 238 nm. Activities were expressed as the ratio of peak area of each metabolite peak to that of the internal standard ( $11\alpha$ -hydroxyprogesterone) (figure 2.1). The limit of detection and quantification of the HPLC assay is 0.05  $\mu\text{g/mL}$  and 0.15  $\mu\text{g/mL}$  for all testosterone metabolites, respectively (Bai, 2001). The intraday and interday variability of this assay is within 10% except  $7\alpha$ -OHT in medium activities sample (approx. 1.3  $\mu\text{g/mL}$ ) and  $2\alpha$ -OHT in low activities samples. The intraday and interday variability appeared to be less than 10 % for all metabolites (Table 2.1).

Table 2.1 Intraday and Interday Variability of *In Vitro* Testosterone Hydroxylation in HPLC Assay

Metabolites at 7.5 ng/ $\mu$ L		metabolite/IS Mean $\pm$ SD	%CV
6 $\beta$ -hydroxy-testosterone	Intraday	1.28 $\pm$ 0.04	3.05
	Interday	1.31 $\pm$ 0.07	5.06
7 $\alpha$ - hydroxy-testosterone	Intraday	1.46 $\pm$ 0.03	1.76
	Interday	1.46 $\pm$ 0.08	5.17
16 $\alpha$ -hydroxy-testosterone	Intraday	0.82 $\pm$ 0.02	1.89
	Interday	0.81 $\pm$ 0.03	3.88
16 $\beta$ -hydroxy-testosterone	Intraday	1.34 $\pm$ 0.08	6.07
	Interday	1.46 $\pm$ 0.14	9.48
2 $\alpha$ -hydroxy-testosterone	Intraday	1.14 $\pm$ 0.01	1.27
	Interday	1.08 $\pm$ 0.09	8.05
2 $\beta$ -hydroxy-testosterone	Intraday	1.28 $\pm$ 0.03	2.17
	Interday	1.24 $\pm$ 0.08	6.23
Androstendione (AD)	Intraday	2.64 $\pm$ 0.004	0.46
	Interday	2.51 $\pm$ 0.24	9.56

11 $\alpha$ -hydroxyprogesterone was used as an internal standard (IS)

## **2.8 RNA ISOLATION**

RNA was isolated using a single step guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). In brief, tissues weighing approximately 100 mg were isolated immediately upon sacrifice and rinsed in ice-cold saline and 0.1% DEPC (Diethylpyrocarbonate) treated water. Tissue was frozen by liquid nitrogen and stored at –80 °C until used.

To isolate RNA, the frozen tissue was placed in 1 mL of cold (4°C) RNAWIZ (Ambion, Inc., Austin, TX). The tissue was immediately homogenized with a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA) with disposable generator probes (Omni Tips, Omni International Inc., Warrenton, VA). The homogenates were incubated at room temperature for 5 min in order to dissociate the nucleoprotein from nucleic acids. Following incubation, 0.2 mL of chloroform was added and vortexed. After another 10 min incubation at room temperature, centrifugation was performed at 12,000 x g for 15 min at 4°C. The clear upper aqueous phase, containing most the RNA, was taken and mixed with 0.5 mL DEPC water, and 1mL isopropanol. After incubation at room temperature for 10 min, centrifugation was performed at 12,000 x g for 15 min at 4 °C to pellet RNA. The pellet was again washed with 1 mL ice-cold 75% ethanol. The washed samples were then centrifuged at 12,000 x g for 5 min at 4°C. After the removal of ethanol by air-dry at room temperature for 10 min, the

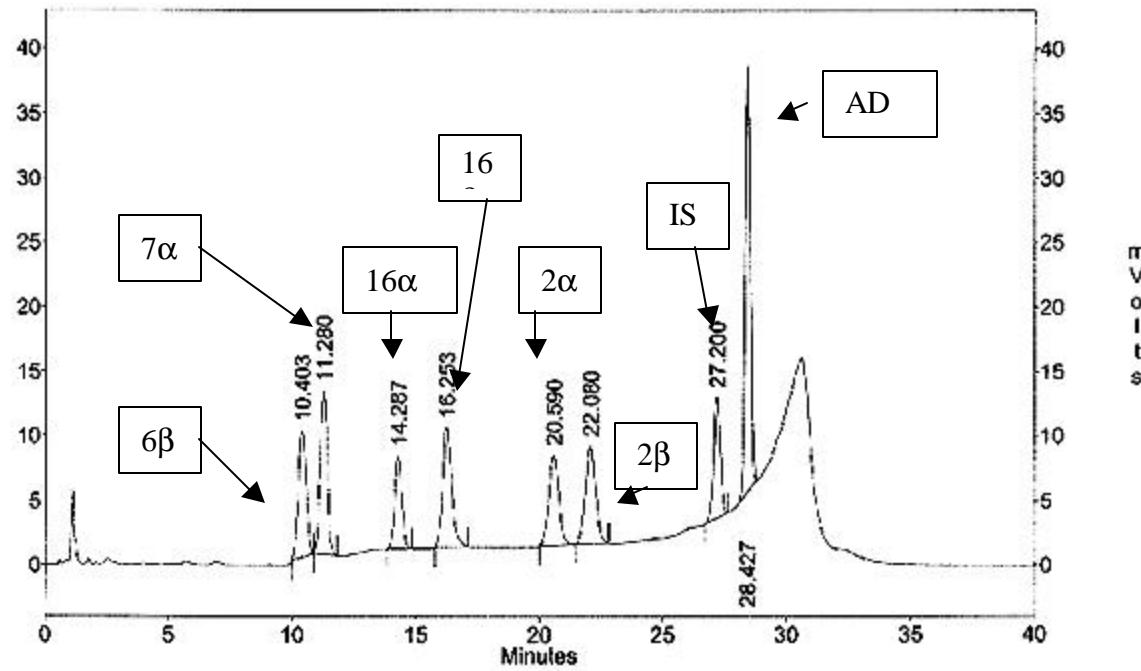


Figure 2.1 Representative Chromatograph of Testosterone Hydroxylation

Twenty  $\mu\text{L}$  of metabolic extraction dissolved in methanol were resolved at  $40^\circ\text{C}$  with a flow rate of  $1\text{mL}/\text{min}$ . Each peak was observed at  $238\text{ nm}$ . The concentration of each metabolite was  $7.5\text{ ng}/\mu\text{L}$  (See Section 2.7).

Table 2.2 Liver Cytochrome P450 Enzyme Activities.

Activity	Appro. Retention Time (min)	Enzyme in Sprague Dawley Rat
Testosterone 6 $\beta$ -hydroxylation	10	*3A1/2, 2C13, 2A2, 1A1/2,
Testosterone 7 $\alpha$ - hydroxylation	11	*2A1/2
Testosterone 16 $\alpha$ -hydroxylation	15	*2C11, 2B1/2, 2C7, 2C13
Testosterone 16 $\beta$ -hydroxylation	17	*2B1/2, 3A1
Testosterone 2 $\alpha$ -hydroxylation	21	*2C11
Testosterone 2 $\beta$ -hydroxylation	22	*3A1/2, 1A1
Internal Standard (IS)	27	N/A
Androstendione (AD)	28	*2B1/2, *2C11, 2A2, 3A1

*\*Major contributing enzyme*

RNA pellet was resuspended in 100  $\mu$ L RNA storage buffer, pH 7.5 (Ambion, Inc., Austin, TX). In order to remove residual DNA, the RNA was treated with 10  $\mu$ L of 10X Deoxyribinuclease (DNase) I buffer and 2 units of DNase at 37 °C for 25 min in a MICRO-Hybridization incubator. The DNase was deactivated by the addition of 10  $\mu$ L DNase inactivation reagent. The amount of RNA in each diluted sample (1:100 for liver and 1:20 for kidney) was quantitated using a UV-visible Spectrophotometer at 260 nm (Cary 50, Varian, Australia), where one optical density unit at 260 nm is equal to 40  $\mu$ g of RNA ( $1A_{260}$  Unit = 40  $\mu$ g/mL). If the ratio of ABS at 260 nm/ ABS 280 nm was between 1.8-2.1, the RNA was considered to be highly purified. The RNA samples were stored at –80 °C until analysis.

## **2.9 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)**

Semiquantitative RT-PCR was used to determine P450, LDL receptor mRNA expression. The first strand cDNA template was synthesized using a Retroscript kit (Ambion, Austin, TX) according to the manufacturer's protocol. In brief, the reaction mixture containing 2  $\mu$ g mRNA, 2  $\mu$ L random decamer (50

$\mu$ M), and DEPC-treated, autocleaved-deionized water to make either 12.5  $\mu$ L or 20  $\mu$ L, was heated to 80 °C for 3 or 5 min in order to denature RNA and was cooled on ice for 2 min. The reverse transcription was performed in the reverse transcription mixture containing 4  $\mu$ L dNTP (2.5 mM), 1  $\mu$ L of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM MgCl<sub>2</sub>), 1  $\mu$ L of placental RNase inhibitor (10 U/ $\mu$ L), and 1  $\mu$ L Superscript Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (100 U/ $\mu$ L) at 42°C for 1 hour and then heated at 92°C for 10 min (PTC-100 programmable thermal controller, MJ Research, Inc). The samples were chilled on ice. The cDNA samples were stored at –80 °C until use.

cDNA was synthesized from 2  $\mu$ L of each RT reaction by employing a DyNAzyme II DNA polymerase kit (Ambion, Austin, TX). The reaction mixture contains a 5  $\mu$ L 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 15 mM MgCl<sub>2</sub>), 1  $\mu$ L primers (100  $\mu$ M), 1 U thermostable DNA polymerase and 2.5  $\mu$ L dNTP mix (2.5 mM each) (Table 2.3). The PCR amplification was carried out for 18-35 cycles (based on signal intensity) after initially heating the mixture at 94 °C for 3-5 min (Figure 2.3-6). The amplification conditions (1. Denaturation, 2. Annealing, and 3. Extension) are in Table 2.4. The optimum cycles were determined by experimentally. Figure 2.1-2.5 represent the PCR yield versus the number of cycles. The number of cycles was selected from the

exponential phase of amplification rather than the plateau phase since there is a high degree of variability within product accumulation at the plateau phase.

The PCR products were stored at -80°C until used with a secure seal to prevent freeze drying. 20 µL of the PCR product mixed with 2 µL of 10x loading buffer (0.2% Ficoll 400, 0.1 M EDTA, pH 8.0, 0.1% SDS, 0.25% bromophenol blue and 0.25% xylene cyanol) was separated on a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. In addition, three negative controls which are missing either RNA, reverse transcriptase, or DNA to insure the absence of contaminants and positive control which is a purified cDNA from company were run together along with 100 bp DNA molecular marker (Introgen, Therapeutic Inc. Austin, TX). In addition, each gel has an internal standard to reduce the variability between gels. A gel was run in 1X TBE buffer (89 mM Tris base, 89 mM boric acid and 2mM EDTA, pH 8.0) containing 0.2 µg/mL ethidium bromide at 100 V for 2 hours. Each band was analyzed using Kodak electrophoresis documentation and analysis system EDAS 290 (Eastman Kodak Company, New Haven, CT). The details for each mRNA were described in Table 2.2. Each mRNA level was expressed in the ratio of target gene to GAPDH in arbitrary units.

Table 2.3 Oligonucleotide PCR Primers

RNA	5' sense primer	Tm	3' antisense primer	Tm	Size (bp)	References
CYP3A1	CAGCTCTCACACTGGAAACCTGGG	71.9	CTCATATACTGGCGTGAGGAATGG	67.3	689	(Zhang et al., 1996)
CYP3A2	TTGATCCGTTGTTCTTGTCA	61.7	GGCCAGGAAATACAAGACAA	61.4	323	(Zhang et al., 1996)
CYP2E1	CCTGGATCCAGCTTACAATAA	61.7	AACAGGTCGGCCAAAGTCAC	66.3	252	(Zhang et al., 1996)
LDL-R	ATTTGGAGGATGAGAAGCAG	62.1	CAGGGCGGGGAGGTGTGAGAA	74.7	931	(Fukushima et al., 2000)
HDL-R (SR-BI)	GTCAGCACCTGCAGGTTGG	68.1	TTTCTCCTGGCTGCGCAGTTG	71.7	417	(Liang and Vaziri, 1999)
GAPDH	CATGTTGTGATGGGTGTGAACCA	70.6	GTTGCTGTAGCCATATTG	63.3	556	-

GAPDH: Glyceraldehyde phosphate dehydrogenase

Table 2.4 RT-PCR Conditions

	RNA amount ( $\mu\text{g}/20 \mu\text{L}$ )	RT volume ( $\mu\text{L}/20$ or $12.5 \mu\text{L}^*$ )		Time for each PCR step		Cycles
			Denature	Annealing	Extention	
<u>Liver</u>						
CYP3A1	2	0.5 <sup>*</sup>	94°C, 15''	55°C, 15''	72°C, 30sec	22
CYP3A2	2	0.5 <sup>*</sup>	94°C, 15''	55°C, 15''	72°C, 30sec	22
CYP2E1	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	20
LDL-R	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	22
HDL-R (SR-BI)	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	25
GAPDH	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	18
<u>Kidney</u>						
CYP2E1	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	23
LDL-R	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	29
HDL-R (SR-BI)	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	28
GAPDH	2	2	94°C, 30''	55°C, 30''	72°C, 1 min	21

\*Total volume was  $12.5 \mu\text{L}$

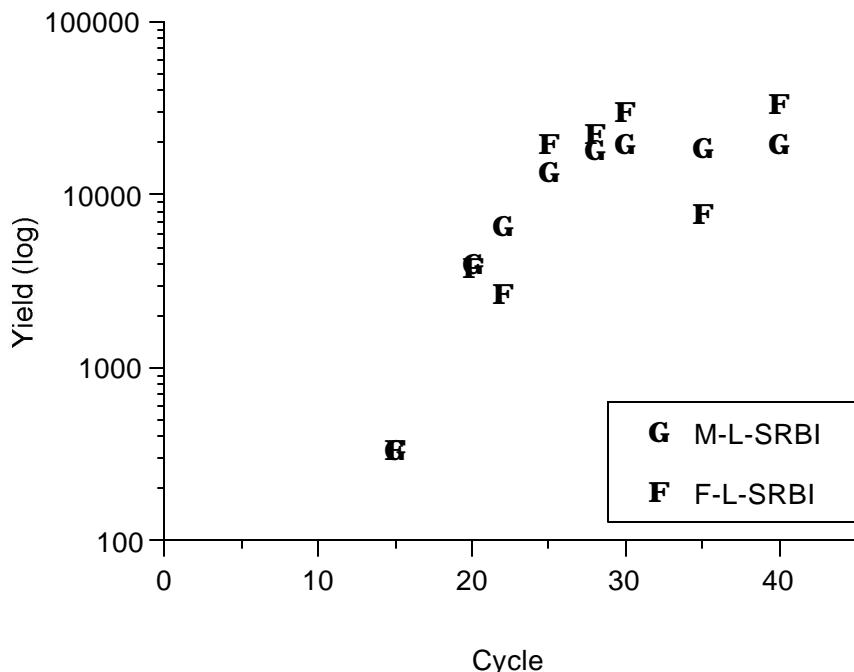


Figure 2.2 SR-BI DNA PCR Amplification Efficiency with Liver mRNA

A 20  $\mu$ L sample of the PCR product obtained from liver mRNA, for both female and male rats, was quantified on a 1.5% agarose gel containing ethidium bromide. In order to provide the optimum amplification cycle number, the synthesized cDNA was amplified 10-40 times in a single run. The minimum cycling number was selected from the exponential phase.

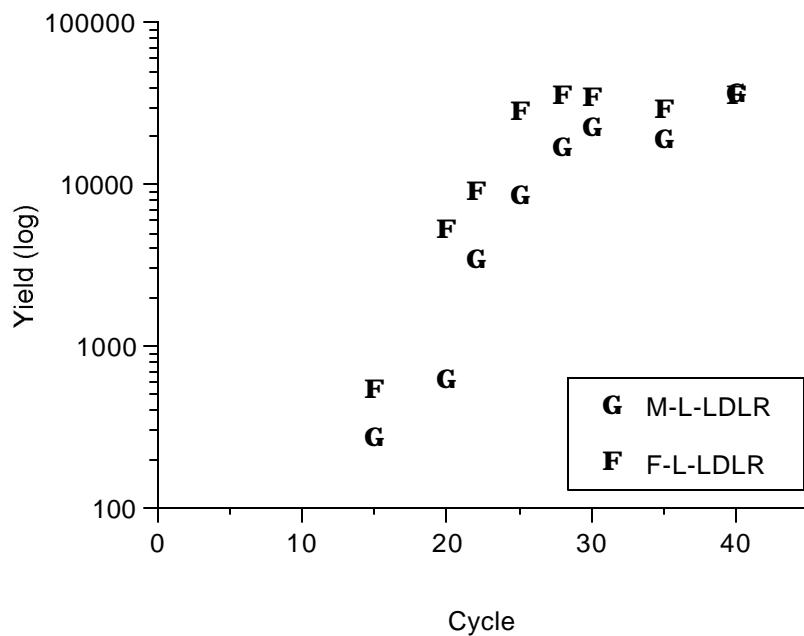


Figure 2.3 LDL-R DNA PCR Amplification Efficiency of with Liver mRNA

A 20  $\mu$ L sample of the PCR product obtained from liver mRNA, for both female and male rats, was quantified on a 1.5% agarose gel containing ethidium bromide. In order to provide the optimum amplification cycle number, the synthesized cDNA was amplified 10-40 times in a single run. The minimum cycling number was selected from the exponential phase.

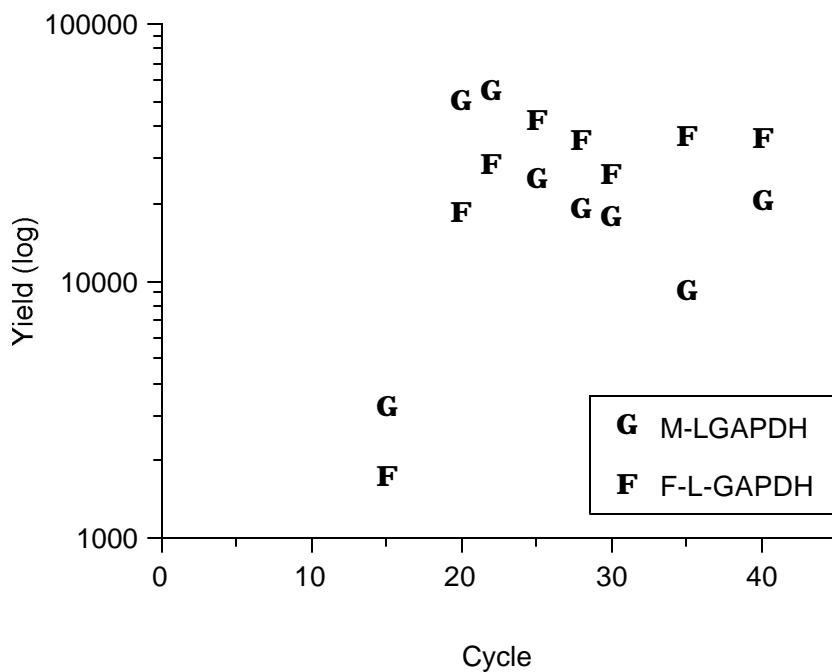


Figure 2.4 GAPDH DNA PCR Amplification Efficiency with Liver mRNA

A 20  $\mu$ L sample of the PCR product obtained from liver mRNA, for both female and male rats, was quantified on a 1.5% agarose gel containing ethidium bromide. In order to provide the optimum amplification cycle number, the synthesized cDNA was amplified 10-40 times in a single run. The minimum cycling number was selected from the exponential phase.

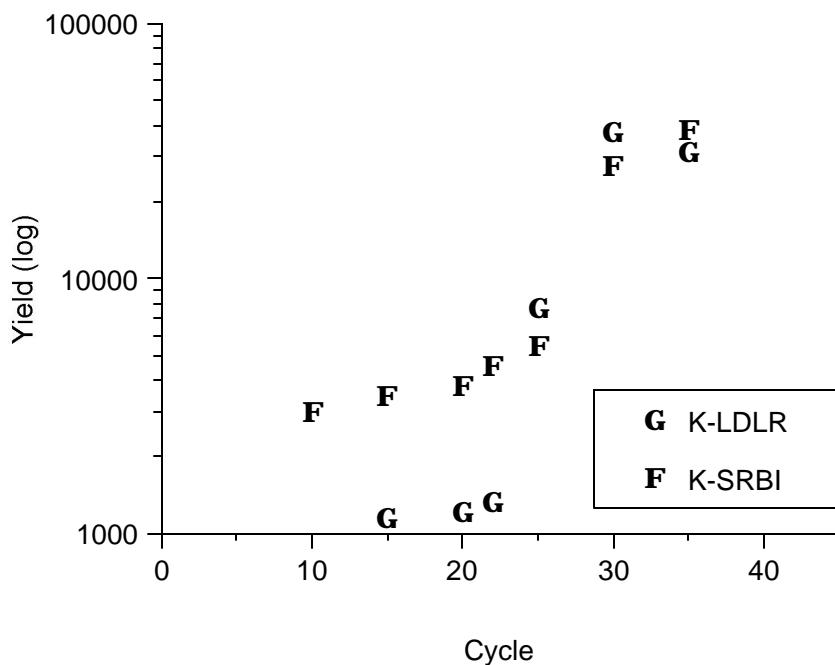


Figure 2.5 LDL-R/SR-BI PCR Amplification Efficiency with Kidney mRNA

A 20  $\mu$ L sample of the PCR product obtained from kidney mRNA was quantified on a 1.5% agarose gel containing ethidium bromide. In order to provide the optimum amplification cycle number, the synthesized cDNA was amplified 10-40 times in a single run. The minimum cycling number was selected from the exponential phase.

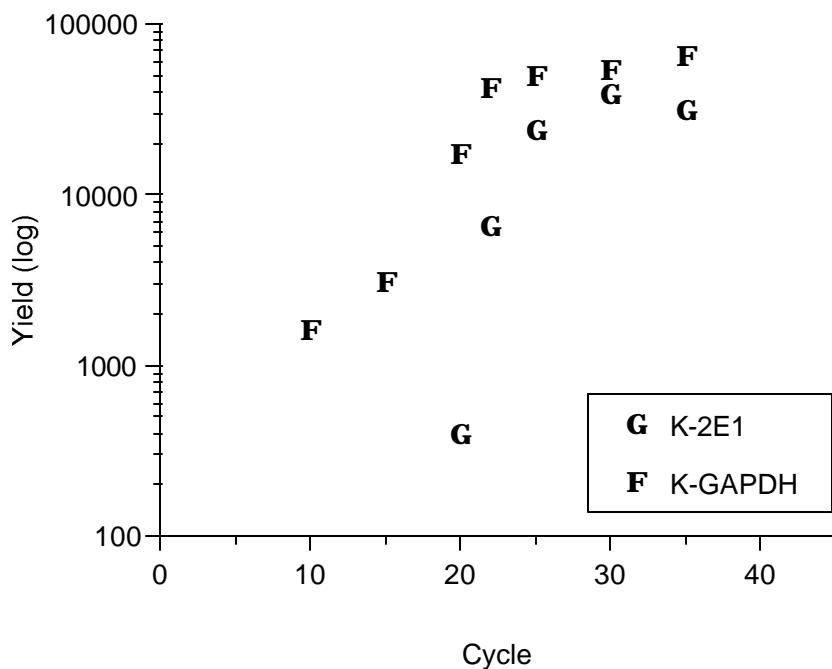


Figure 2.6 CYP2E1/GAPDH PCR Amplification Efficiency with Kidney mRNA

A 20  $\mu$ L sample of the PCR product obtained from kidney mRNA was quantified on a 1.5% agarose gel containing ethidium bromide. In order to provide the optimum amplification cycle number, the synthesized cDNA was amplified 10-40 times in a single run. The minimum cycling number was selected from the exponential phase.

## **2.10 ALT (SGPT) AND AST (SGOT) ANALYSIS**

The principle of analysis is based on an enzyme-coupled oxidation of NADH to NAD<sup>+</sup>. In the assay for ALT (alanine aminotransferase, serum glutamic pyruvate transaminase), the L-alanine is transferred to  $\alpha$ -ketoglutarate in the presence of pyridoxal-5-phosphate to produce glutamate and pyruvate. Then pyruvate is dehydrogenated to lactate by dehydrogenase (LDH/ MDH) in the presence of NADH, which is oxidized to NAD<sup>+</sup>. In the aspartate aminotransferase oxaloacetate transaminase (AST) assay, L-aspartate undergoes a similar procedure to produce oxaloacetate. Oxaloacetate is then dehydrogenated by malate dehydrogenase (MDH) to form malate. The rate of oxidation of NADH is used to calculate enzyme activity. In order to perform the assay, blood was collected from the vena cava and centrifuged at 10,000 x g for 10 minutes at 4 °C. Serum was stored at -80 °C until further analysis. The level of ALT/AST was measured using a commercial ALT/AST DT slides in a Vitros DTSC II Module (Ortho-Clinical Diagnostics, Inc., Rochester, NY).

## **2.11 SERUM AND URINE CREATININE**

Urine was collected passively by using metabolic cages. Blood was collected through the vena cava as described previously in section 3.10. Serum creatinine was measured by a colorimetric creatinine kit (Sigma Diagnosis, St. Louis, MO). In order to measure the creatinine concentration, 25 µL of serum or diluted urine (1:100) was added to a working solution which is a mixture of creatinine color reagent (0.6% picric acid) and sodium hydroxide solution (1N) in a ratio of 5 to 1. Initial absorbance and final absorbance was read at 500 nm after incubating at 30 °C for 20 seconds and again 60 seconds after the initial reading with a CH-300 (Texas International Laboratory, Austin, TX).

Creatinine levels in Chapter 5 were determined by a Vitros DTSC II Module autoanalyzer (Ortho-Clinical Daignostics, Inc., Rochester, NY) according to the manufacturer's protocol. In brief, a 10 µL sample of serum was pipetted onto a pretreated slide and incubated at 37°C for 5min. During the incubation, triarylimidazole leuco dye is oxidized to produce a colored product. The formation of the colored product, or rate of oxidation, is measured at a wavelength of 550 nm. Between the two methods described, there were no significant differences in creatinine levels.

Creatinine clearance was calculated by the following equation:

$$\frac{\text{Urine creatinine (mg/mL)} \times \text{Urine volume (mL)}}{\text{Serume Creatinine (mg/mL)} \times \text{collection time (min)}}$$

## **2.12 CHOLESTEROL LEVELS**

In order to measure lipoprotein metabolism disorders, cholesterol, HDL Cholesterol and triglyceride were measured using Vitros DT slides in a Vitros DT 60 Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY). The principle of reaction for the cholesterol is that cholesterol is dissociated from the carriers by the surfactant in the slides and cholesterol esters are hydrolyzed to free cholesterol by the cholesterol ester hydrolase. The free cholesterol is oxidized and produced  $\text{H}_2\text{O}_2$  which causes the production of a colored dye. Light reflection is observed at 555 nm after 5 min incubation at 37°C. The limit of assay using this machine was from 50 mg/dL to 325 mg/dL, since certain types of rat cholesterols was lower than this limit. Some lower cholesterol serum was mixed with a control (120 mg/dL) and obtains the level by subtraction from the blank (saline). HDL cholesterol was measured in the same way after the separation of HDL by precipitation from LDL and VLDL by using dextran sulfate and magnesium chloride. The reflection was observed at 660 nm after 5 min incubation. The limit of HDL is 1-110 mg/dL. VLDL and LDL cholesterol levels were calculated from the following equation:

$$\text{VLDL (mg/dL)} = \text{Triglyceride}/5$$

$$\text{LDL (mg/dL)} = \text{Total cholesterol} - \text{HDL} - \text{VLDL}$$

## 2.13 STATISTICAL ANALYSIS

Based on the assumption that the data obtained from the rats are normally distributed and represent a large population (parametric analysis), the statistical analysis for each of the experiments was null hypothesis-driven and based on parametric analysis (Bolton, 1997). Making a decision on the basis of probability, such as statistical significance tests, there is some chance that the decision will be wrong. The four possibilities that can occur are:

		<u>REALITY</u>	
		H0: No treatment effect	H1: Treatment effect
DECISION	H0: No treatment effect	Correct (1)	Type II (3)
	H1: Treatment effect	Type I (2)	Correct (4)

In case 1, the difference between the sample and the population is caused by only the sampling error and is found to be non-significant. The conclusion is there is no difference between the two comparison groups that would correspond to reality. In case 2, the statistical analysis shows the difference between the two groups is only due to the sampling error. This conclusion falsely leads to the assumption that there is a significant difference although in reality there is none. This type I error can be reduced by choosing more stringent levels of significance, e.g. 0.01 vs. 0.05. In case 3, the difference between the sample and the population is either too small to be detected, or is small enough to be wrongly attributed to sampling error. This results in Type II error. The conclusion falsely drawn is that

there is no difference between the two groups even though there is a difference in reality. In case 4, the difference between the sample group and the population group is statistically significant. This conclusion would correspond to reality. Therefore, the difference between a type I and II error is that a type I error is caused by sampling error which results in a significant difference where one is not present. On the other hand, due to too small of a difference, a type II error incorrectly draws no difference between the sample and the population although a difference is present.

Based on our previous studies, we have estimated that a 25% change in the measured statistic (e.g. CSA through concentrations) would be of importance and demonstrate a significant effect. This value was used in determining the minimum number of animals needed per group in order to determine if a statistical difference exists at  $\alpha=0.05$ ,  $\beta=0.20$ , and  $\Delta=0.25$  (Stolley and Strom, 1986). The formula used is:

$$n = \frac{2(Z_a + Z_b)^2 \sigma^2}{\Delta^2}$$

Where  $n$  is the sample size,  $Z_\alpha$  is the value for the two-tailed  $\alpha$ ,  $Z_\beta$  is the value for the one-tailed  $\beta$ ,  $\sigma$  is the sample standard deviation, and  $\Delta$  is the smallest difference between the study groups, to be considered important. For example, the minimum number of rats to determine a 25% difference in CSA through concentrations, assuming a mean value of  $3600 \pm 900$  ng/mL, would be

approximately five. Six rats were chosen per group to allow for possible differences in mean values from those which were estimated.

Treatment-induced effects were analyzed by one-factor analysis of variance with either Scheffe's post-hoc analysis or Bonnfaroni-Dunn post hoc analysis with. These two post hoc analyses are the most conservative of the paired comparisons procedures (Gagnon et al., 1989; Lomax, 2001). This procedure is not invalidated by differences in the number of animals per group and is not limited in the number of groups it can compare. Statistical analysis was performed using a computer and commercial statistical software (Statview; Abacus concepts, Berkeley, CA). A significant difference will be determined if the probability of chance explaining the results is reduced to less than 5% ( $P<0.05$ ).

## **CHAPTER THREE**

### **Effect of Low Dose of HDL-Associated CSA on Hepatic Metabolism**

#### **3.1 INTRODUCTION**

Although effective in enhancing the survival rate of patients after organ transplantation, the use of immunosuppressants is hampered due to considerable nephrotoxicity resulting in a decrease in glomerular filtration rate (Hows et al., 1981; Klintmalm et al., 1981; Shulman et al., 1981; Atkinson et al., 1983; Myers et al., 1988) and hepatotoxicity which is characterized by hyperbilirubinemia and elevated serum transaminases (Yee et al., 1984). Nephrotoxicity, which is dose-dependent, appears to be the most important problem to be considered when dosing immunosuppressants clinically (Myers et al., 1988).

Lipoproteins are biologically important and play a major role in diverse processes, such as immune reactions (Lopez-Miranda et al., 1993), coagulation, (Ostermann et al., 1986; Basile-Borgia and Abel, 1997) and tissue repair (Harmony and Aleson, 1981; Mbewu and Durrington, 1990). A change of cholestrylester (CE) and triglyceride (TG) in the rate of transfer (Mahley, 1988) and content seems to be a consequence of treatment with a lipophilic drug such as

CSA (Wasan et al., 1997; Wasan et al., 1998). In addition, more than 60% of CSA distributed in the plasma is bound to lipoproteins (Lemaire and Tillement, 1982). Pharmacokinetic profiles of CSA can be altered by the level of lipoproteins which is affected by disease status, drug treatments, and diet (Brunner et al., 1988; Brunner et al., 1990b; Yau et al., 1991). For instance, an increased cholesterol level after achronic administration of CSA or a lower unchanged CSA blood level has been observed in patients with hypocholesterolemia, which leads to higher drug-induced toxicities. Several studies also showed that immunosuppressive effect and drug-induced toxicities are correlated with lipoprotein levels (Brunner et al., 1990a; Yau et al., 1991; Luke et al., 1992). They also reported the time-dependent alteration of CSA amount in HDL and LDL (Brunner et al., 1990a; Luke et al., 1992).

Numerous drugs on the market are known to interact with cytochrome P450 (CYP). CSA undergoes hepatic metabolism primarily by CYP and the modulation of hepatic CYP levels resulted in the changes of CSA levels and of toxicity (Kolars et al., 1992; Brunner et al., 1996). The CYP3A gene family is reported to be the primary enzyme responsible for CSA metabolism (Brunner et al., 1998). Therefore, CSA blood levels and toxicity are markedly affected by the modulation of CYP3A levels. This presents a major problem, since drug interactions can result from chronic multiple-drug therapy. The inducers of CYP3A lower CSA levels in the blood and lessen the degree of renal toxicity

conversely, inhibitors of CYP3A increase the risk of CSA-induced toxicity (Pichard et al., 1990; Brunner et al., 1996).

In present study, based on the fact that the activity and toxicity of lipophilic drugs such as CSA are correlated to the specific classes of lipoproteins, we examined the *in vivo* disposition of HDL-associated CSA in rats by measuring the hepatic levels of microsomal CYP3A and CYP2C11, and marker for nephrotoxicity, which can allow for the prediction of drug effect and modification of biological significance. CYP3A and CYP2C11 are the male-specific isoforms in rat liver (Brunner et al., 1996). It is well established that gender-specific expression of CYP isoforms is regulated via the GH secretion pattern by drug treatment (Waxman et al., 1991; Waxman et al., 1995). Furthermore, HDL is the major carrier of cholesterol among lipoproteins in the rat, and LDL is the major carrier in humans. Therefore, the alteration of HDL levels is considered to possibly be a major factor among lipoprotein classes to influence the pharmacokinetic profile of CSA in rats.

## **3.2 EXPERIMENTAL**

### **3.2.1 Chemicals**

Intravenous CSA solution was generously donated by Norvartis Pharmaceuticals (Sandimmune®, East Hanover, NJ). CSA powder was obtained from Sandoz Research Institution (East Hanover, NJ). Furosemide was purchased from American Reagent Laboratories, Inc. (Shirley, NY). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XII),  $\beta$ -nicotinamide adenine dinucleotide (NADP; grade III), testosterone, 11 $\alpha$ -hydroxyprogesterone, xylazine, ketamine, acetopromazine and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide was purchased from National Diagnostics (Atlanta, GA). All other chemicals were purchased in the highest purity available from EM Science (Gibbstown, NJ).

### **3.2.2 Animals**

Thirty-five adult male Sprague-Dawley rats weighing 200-250 g were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) and randomly divided into five groups. Nine rats were in the saline group, seven rats were in the

vehicle group, eight rats were in the CSA group and seven rats were in the plasma-CSA group (plasma-CSA). Four rats in the HDL-associated CSA (HDL-CSA) treated group were used due to the limited amount of HDL-CSA. HDL-CSA was kindly provided by Dr. K.M. Wasan (Pharmaceutical Science, The University of British Columbia, Vancouver, Canada). Rats were pair-fed a rice diet (8.5% protein, 76.6% carbohydrate, 4.3% fat, 0.05% salt enriched with ferric orthophosphate and thiamin mononitrate; Harlan Teklad, Indianapolis, IN) for seven days followed by a single dose of furosemide 4 mg/kg ip. Rats were individually housed in wire-bottom cages in a 12-hour light/dark cycle animal facility with controlled temperature and humidity throughout this experiment. CSA, plasma-CSA, and HDL-CSA at a concentration of 0.1 mg/kg were given to rats via an indwelling jugular catheter following two days postsurgery for 14 days. In equivalent volumes with the above treatment, 0.5 mL/kg of CSA in either intravenous vehicle (Cremophor®) or saline was administered to serve as controls.

In surgery, approximately 11 cm of intramedic polyethylene tubing (PE50, 0.58 x 0.965 mm, Becton Dickinson, Franklin Lakes, NJ) attached to an 1cm/kg of a Silastic Lab Tubing (0.64 mm OD x 1.19 mm ID, Dow Corning Co, Midland, MI) was used for the catheter. An intramuscular dose of an anesthetic mixture (ketamine 100 mg/mL, acepromazine 10 mg/mL and xylazine 20 mg/mL at a

volume ratio of 1:1:1, 1 mL/kg) was used prior to surgery. Rats recovered within 1 hour after surgery. The total surgery time was approximately 15-20 min.

After the final CSA or vehicle dosing, rats were placed into rodent metabolism cages for 24 hours in order to collect urine and they were humanely sacrificed. After being sacrificed, blood was collected and liver was removed and immediately frozen in liquid nitrogen and stored at -80°C until microsomal fraction preparation. Daily weight was recorded throughout the experiment.

### **3.2.3 Plasma and HDL-associated Drug Preparation**

K<sub>2</sub>EDTA-treated plasma was obtained from normal rats. HDL fractions were prepared by sequential centrifugation (Wasan et al., 1999) and store at -80 °C. Frozen plasma or HDL was thawed at 4°C. Fifty mg/mL of CSA (from the powder form) dissolved in ethanol was prepared and stored at -80 °C until used. Plasma- or HDL-CSA (0.2 mg/mL) were prepared by the incubation of plasma or HDL with CSA at 37 °C for 2 hours in a glass bottle wrapped with foil to protect then from light. The prepared plasma- or HDL-CSA solution were stored at -20°C for long-term storage and 4°C during the dosing period in a salt solution to prevent microbial contamination. The protein and cholesterol levels in the HDL

fraction, which was used for HDL-CSA, were 2.7 mg/dL 193.42 mg/dL, respectively (provided by Dr. K.M. Wasan). Before use, a dialysis of plasma- or HDL-CSA solution (MWCO 500) was performed with 4 changes of PBS buffer, pH 7.5 at 4 °C for 24 hours and filtrated with a 0.2 µm filter.

### **3.2.4 Statistical Analysis**

Differences between treatment groups were determined by one factor analysis of variance with Scheffe's post-hoc analysis. All data were expressed as mean ± standard error. This procedure is not invalidated by differences in the number of animals per group and is not limited in the number of groups in which it may compare. A significant difference was considered if the probability of chance explaining the results is reduced to less than 5 % ( $P<0.05$ ).

### **3.3 RESULTS**

#### **3.3.1 General Parameters**

The study consisted of five groups. The intravenous CSA-treated group served as a control group to compare with two newly formulated CSA groups. The CSA vehicle (Cremophor®) and saline-treated groups were also compared to examine the possible effect of vehicle treatment. During the dosing period, rats did not show any sign of morbidity. Rats were pair-fed based on the average food intake of the HDL-CSA treated group. The mean food intake per day was  $20.1 \pm 0.8$  g for all groups and did not show any significance between groups. All CSA-treated groups showed less gain weight as compared with the vehicle-treated group, although they had been pair-feed. However, in comparison with intravenous CSA alone, none of the treatment groups showed any significant weight changes.

#### **3.3.2 Serum and Urine Creatinine**

The function of glomerular filtration was estimated by the measurement of serum and urine creatinine and calculation of creatinine clearance (Table 3.1). The high level of serum creatinine is a general representation of renal

dysfunction. The serum creatinine in the HDL-CSA treated group was 15% ( $P=0.094$ ) higher than the CSA group, but the differences were not statistically significant compared with other control groups. The plasma-CSA-treated group showed an increased serum creatinine level that reflects possible renal dysfunction. The plasma-CSA group showed a 22- 49% higher serum creatinine level than the other CSA groups and was statically significant ( $P=0.0039$ ) compared with the CSA-treated group. In addition, the creatinine clearance in the plasma-CSA treated group showed the lowest value. HDL-CSA appeared to have a higher level of serum creatinine than the CSA-treated group, although the level of serum creatinine was within the normal range in the HDL-CSA treated group. In addition, there were no significant changes in creatinine clearance.

### **3.3.3 Hepatic CYP3A2 and CYP2C11 Protein Expression**

Specific CYP3A and CYP2C11 expression were measured by immunoblot analysis. Hepatic CYP3A protein levels in the HDL-CSA treated group and in the plasma-CSA-treated group were suppressed approximately 27% ( $P=0.0311$ ) and 38% ( $P= 0.0080$ ), respectively, as compared with the CSA-treated group.

Table 3.1 Renal Function Parameters

<b>Parameter</b>	<b>Saline</b>	<b>Vehicle</b>	<b>CSA</b>	<b>Plasma-CSA</b>	<b>HDL-CSA</b>
No. of Rat	7	7	8	7	4
Initial Wt. (g)	249 ± 6	235 ± 9	251 ± 7	237 ± 2	261 ± 6
Final Wt. (g)	317 ± 6	314 ± 17	304 ± 5	289 ± 1	323 ± 10
Weight Change (%)	27.1 ± 1.4	33.2 ± 2.5	21.6 ± 2.2*	21.9 ± 1.1*	23.6 ± 1.6*
Urine Volume (mL/kg)	26.9 ± 2.5	30.8 ± 6.9	32.3 ± 7.8	31.9 ± 4.3	36.9 ± 5.4
Scr (mg/dL)	0.48 ± 0.03	0.46 ± 0.07	0.39 ± 0.03	0.58 ± 0.03**	0.45 ± 0.04
Ucr (mg/dL)	125 ± 9	107 ± 17	85 ± 15	112 ± 17	92 ± 17
Clcr (µL/min/100g)	477 ± 35	544 ± 134	476 ± 76	426 ± 81	498 ± 51

Comparison of general parameters following 14 days of intravenous administration of CSA. Urine was collected for 24 hr in a metabolic cage after the last dose. \* P < 0.05 as compared with vehicle-treated group. \*\*P < 0.05 as compared with CSA-treated group

In addition, CYP2C11 enzyme levels were reduced by 39% ( $P=0.21$ ) in the HDL-CSA treatment group and 40% ( $P=0.0044$ ) in the plasma-CSA treatment group as compared with the CSA control group (Figure 3.1 and 3.2). Even though only plasma-CSA treated rats showed a significant difference when compared to CSA alone. The suppressed both isoform levels indicated that a higher level of lipoprotein may be contributing to greater pharmacological and toxic effects of CSA in the low-sodium diet rat model. In addition, approximately 33% ( $P=0.0252$ ) and 34% ( $P=0.0099$ ) less 6 $\beta$ -hydroxytestosterone was produced in the HDL-CSA treated group and plasma treated group, respectively, as compared with the CSA-treated group indicating there was a suppression of CYP3A activity in both HDL-CSA and plasma-CSA group as well (Figure 3.3). Some studies have addressed the correlation between lipoprotein and CSA-induced toxicities (De Groen, 1988), but the mechanism of CSA-induced nephrotoxicity remains uncertain at this point. It has been previously described that the Scavenger receptor class B type I (SR-BI) which is known as the HDL receptor (HDL-R) has a greater binding rate with HDL in plasma (Acton et al., 1996). We hypothesized that the HDL receptors, since HDL is a major lipid carrier in rat, may contribute to a predisposition of CSA in liver, which results in a higher suppression of CYP3A in larger dosing regimen. Therefore, we suspect that HDL may play a role in metabolism in the rat at low concentrations of CSA.

### **3.3.4 The Catalytic Activity of Hepatic CYP3A and CYP2C11**

In order to measure the activities of CYP3A and CYP2C11, *in vitro* testosterone hydroxylation was performed. The testosterone hydroxylation at the positions of  $6\beta$  and  $2\beta$  is mainly catalyzed by CYP3A and CYP2C11 catalyzes the hydroxylation of testosterone at the  $2\alpha$  and  $16\alpha$  position (Arlotto et al., 1991; Ducharme et al., 1995). Testosterone hydroxylation data was consistent with results from CYP3A protein expression (Figure. 3.3). Low amounts of the HDL-CSA reduced CYP3A2 activities in the liver by 33% ( $P=0.0252$ ) as compared with CSA alone. The plasma-CSA treated group showed a significant reduction in  $6\beta$ -hydroxylase by 34% ( $P=0.0099$ ) activity as compared with the CSA alone treated group. Both the protein level, as well as the activity of CYP3A in the HDL- and the plasma-CSA treated group was suppressed below the 3A2 levels in the saline and vehicle groups. Considering the loading amount of CSA, in the HDL- and plasma-CSA treatment groups, was lower than a normal therapeutic dose, the effects seen on CYP3A are attributed to the specific lipoprotein bound to CSA. Although an effect was seen, these results were not found to be statistically significant in HDL-CSA-treated rats. Because CYP3A is primarily responsible for the breakdown of CSA, the suppression of this enzyme may cause an increasing circulating CSA level in blood which leads to either an increasing drug effect and/or possibly higher toxicity.

In contrast, 2 $\alpha$ - and 16 $\alpha$ -hydroxylase activities, CYP2C11 activity markers, were found to exhibit no differences between groups (Figure 3.4). Furthermore, both CYP3A2 and CYP2C11 activities showed a similar pattern after CSA treatment. However, in this study, CYP2C11 activities in CSA-treated rats remained the same as the vehicle-treated group.

### 3.4 DISCUSSION

Disease states, age, and sex have all been reported to influence the metabolism of numerous drugs, including CSA, as well as lipoprotein content and its composition (De Groen, 1988; Brunner et al., 1996; Wasan and Cassidy, 1998). In addition, changes in the lipoprotein profile can affect the distribution and toxicities of CSA (Luke et al., 1992). For instance, increases in LDL and/or VLDL levels were often seen after transplantation (Brunner et al., 1988; Markell and Friedman, 1989; Luke et al., 1992) and patients with hyperlipidemia showed higher blood CSA concentrations up to 2000  $\mu\text{g/L}$  (Verrill et al., 1987). CSA is effective for the prevention of graft-versus-host disease after transplantation, which provides a higher survival rate for recipients of transplantation (i.e. bone marrow transplantation).

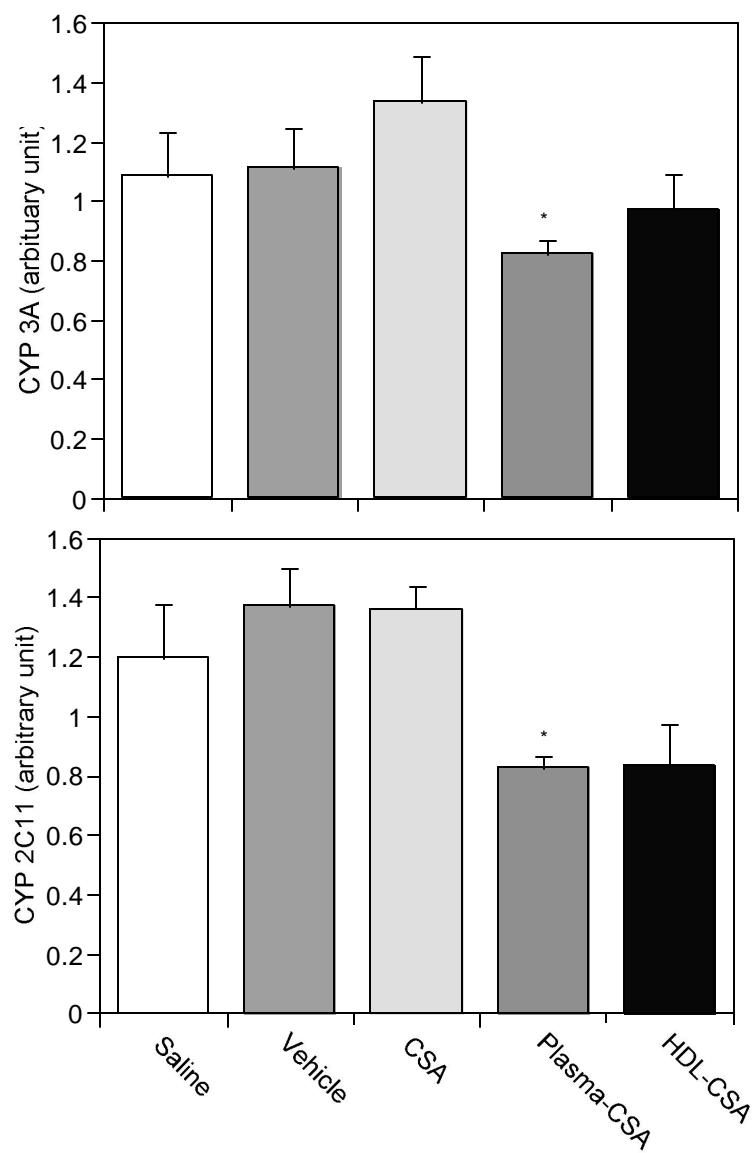


Figure 3.1 Comparison of Immunoblot Analysis of Hepatic CYP3A and CYP 2C11

Microsomes were prepared from rats following by 0.1 mg HDL-CSA and CSA and 0.5 mL saline and vehicle for 2 weeks via intravenous injection. \*P < 0.05 as compared with CSA. Units are arbitrary.

Hepatic CYP3A



Hepatic CYP2C11



↑	↑	↑	↑	↑	↑
STD	Saline	Vehicle	CSA	Plasma-CSA	HDL-CSA

Figure 3.2 Hepatic CYP3A and CYP2C11 Protein Expressions

Proteins were separated by SDS-PAGE using 1:2000 dilution of either goat-anti-3A or goat-anti-2C11 antibody in 3 % NFDM and 1:2000 dilution of rabbit anti-goat HRP. The standard (STD) represents the hepatic CYP3A induced microsomes by phenobarbital in rats.

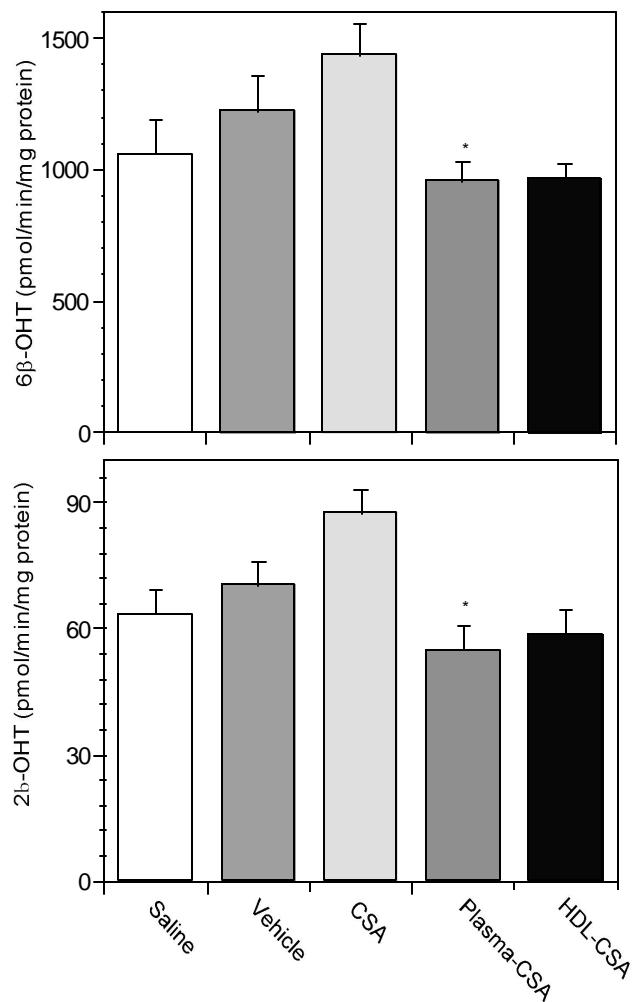


Figure 3.3 The Catalytic Activity of Hepatic CYP3A

*In vitro* testosterone hydroxylase activities were measured using 200  $\mu$ g of hepatic microsomal protein isolated from rats given an intravenous injection of CSA, Plasma-CSA and HDL-CSA for 14 days. 250  $\mu$ M testosterone was incubated for 15 min in a regenerating system and 3.6 nmol of 11  $\alpha$ -hydroxyprogesterone served as an internal standard. \*P< 0.05 as compared with CSA.

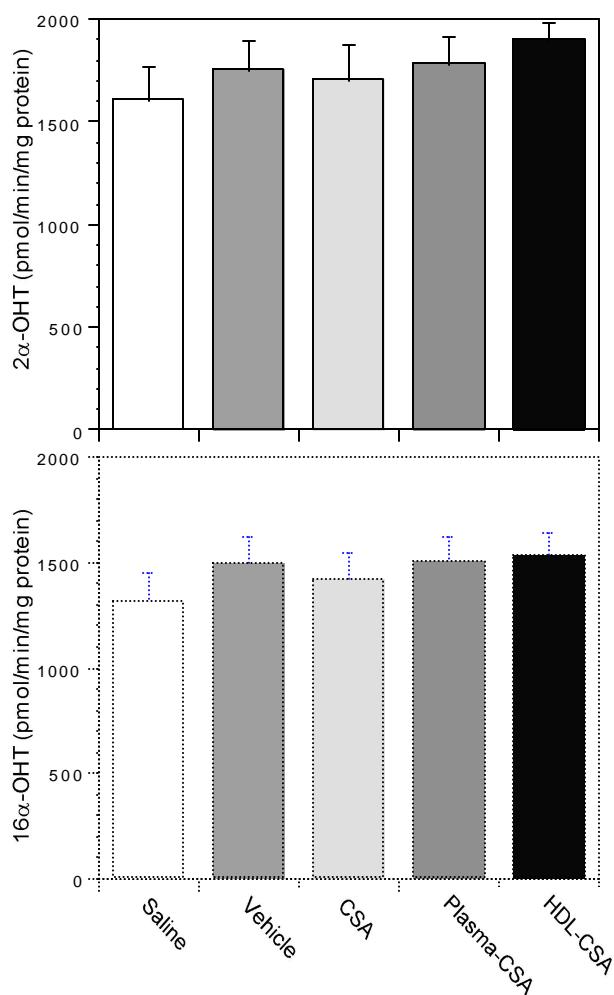


Figure 3.4 The Catalytic Activity of Hepatic CYP2C11

*In vitro* testosterone hydroxylase activities were measured using 200  $\mu$ g of hepatic microsomal protein isolated from rats given an intravenous injection of CSA alone, Plasma-CSA and HDL-CSA for 14 days. 250  $\mu$ M testosterone was incubated for 15 min in a regenerating system and 3.6 nmol of 11  $\alpha$ -hydroxyprogesterone served as an internal standard. \*P< 0.05 as compared with CSA.

Transplantation patients often showed altered lipid metabolism and this event appeared to be related with toxicity. Yau found that patients with renal failure showed an increase in the ratio of CSA in the HDL fraction compared to the LDL fraction (Yau et al., 1991). This suggested the possible mechanism that CSA in the HDL fraction may deliver the drug to the sites of toxicity and, consequently, the drug may predispose patients to renal failure. CSA metabolized by CYP3A in the rat, extensively interacts with lipoproteins in blood with 64% of CSA in plasma bound predominantly to lipoproteins. Therefore, the change of the component of the lipoproteins in patients may be the critical factor that changes the distribution and toxicity of the drug.

This study attempted to examine the effect of specific lipoprotein associated-CSA in renal toxicity and in metabolism in rats since different amounts of lipoprotein components can affect drug metabolism (Wasan et al., 1990; Wasan, 1996). In this study, we only selected the HDL fraction since it is the most abundant lipoprotein found in rats. Differences in lipoprotein catabolism exist between humans and rats. Rats lack the choesterylester transfer protein (CETP), which is responsible for both the transfer of cholesterol between lipoproteins and the activation of VLDL lipolysis. This lack of CETP results in very low levels of LDL cholesterol in rats. For this reason, HDL is considered to be the major factor involved in both drug distribution and metabolism.

Due to the limitation of the loading amount of CSA into rat HDL fractions, 0.1 mg/kg CSA and 0.5 mL/kg vehicle and saline were administered intravenously via a inserted cannular to adult male rats for 14 days.

According to the observance of renal function parameters, only plasma- CSA showed renal dysfunction which is characterized by increased serum creatinine and decreased creatinine clearance than CSA alone treated group. This may be due to either an increase in protein levels or a rapid uptake to the kidney mediated by receptors. Since the plasma contains all lipoprotein fractions, VLDL, LDL, and HDL, a rapid uptake of CSA could be the result of a single fraction, all fractions, or a combination of fractions taking up the CSA. VLDL, LDL and HDL receptors have been shown to exist in both the liver and kidney (Lestavel and Fruchart, 1994; Acton et al., 1996; Vaziri and Liang, 1997; Acton et al., 1999; Vaziri et al., 2000). Since HDL is the major lipoprotein fraction in rats, it is believed to affect either metabolism or toxicity.

Therefore, we hypothesized that SR-BI (HDL-R) contribute to the metabolism and toxicity in rats. We found significantly increased serum creatinine levels and a decreased creatinine clearance in the plasma-CSA compared to the CSA group due to the high level of plasma protein administration (6.2 mg/dL) that is known to cause glomerular injury. However HDL-CSA did not showed differences in both parameters as compared with CSA, indicating no drug-induced nephrotoxicity. In animal models, cyclosporine is usually administered at amounts higher than a standard human

dose in order to study CSA induced nephrotoxicity. Elizinga and colleagues developed a low salt diet rat model that reproduces the physiologic and pathologic renal changes seen in humans receiving chronic cyclosporine therapy (Rosen, Greenfield et al. 1990; Elzinga, Rosen et al. 1993). In rats, dietary sodium restriction enhances nephrotoxicity caused by cyclosporine, whereas a similar dose of CSA alone does not produce structural damage in non salt-depleted animals.

Figure 3.1 and Figure 3.2 showed the relative protein amounts of hepatic CYP3A and CYP2C11 between the different groups. Even though only plasma-CSA treated rats showed statistical significance, CYP3A and CYP2C11 protein expression in the HDL- also showed suppression. The suppression of CYP3A protein expression was consistent with the testosterone hydroxylation results for both groups as compared to the group treated with CSA alone (Fig. 3.3). In addition, when 0.1 mg/mL of CSA was given alone there was a slight induction of CYP3A and CYP2C11, indicating a dose dependent regulation. It has been previously reported that subcutaneous and oral administration of CSA results in a suppression of both CYP3A2 protein expression and catalytic activity in a dose-dependent manner in the range of 5mg/kg to 50 mg/kg (Brunner et al., 2000). Our study showed an induction of CYP3A, indicating there is a dose independent regulation of CYP3A with non-therapeutic CSA administration.

The content of CYP2C11 in both the HDL- and plasma- CSA groups was suppressed as compared with the CSA-treated group.  $2\alpha$ -hydroxylase activity, which is a CYP2C11 activity marker, showed no difference. This could reflect the fact that enzymes other than CYP2C11 may be involved in the decomposition of testosterone at  $2\alpha$  position in the HDL- and Plasma- CSA treated group. The other enzymes would be CYP2B1/2, since it has been reported that the production of  $16\alpha$ -hydroxytestosterone are catalyzed by CYP2B as well (Imaoka et al., 1996).

In summary, this study investigated altered hepatic drug metabolism and renal function by 0.1 mg/kg of intravenous HDL- and plasma- associated CSA administration in the rat. The higher suppression of CYP3A and CYP2C11 were seen in HDL-CSA and plasma-CSA as compared with CSA alone administration. In addition only plasma-CSA treated rats showed a higher probability of renal toxicity due to extraneous protein levels in the plasma vehicle. The results demonstrated that intravenous plasma- and HDL-CSA administration altered the metabolism of CSA in the rat differently from an CSA alone administration, even though HDL-CSA treated rats appeared to be no significance as compared with CSA treated rats. These results underscore the complex interaction of CSA with lipoprotein in hepatic CYP and explained, in part, the difficulties with dosing transplant patients. Although no renal function changes were observed, the suppression of major metabolic enzymes

can cause an increased blood level of CSA which can lead to unexpected to drug-induced toxicity. In addition, this study suggests the possibility of effective CSA therapy at doses lower than those, which are known to be non-therapeutic doses.

## **CHAPTER FOUR**

### **The Effect of High Dose of Lipoprotein-Association on CSA Metabolism and Toxicity in rats**

#### **4.1 INTRODUCTION**

As described in Chapter 1, clinically, blood lipid levels can affect the immunosuppressant activity and toxicity of CSA. It has also been reported that patients with a lower lipid profile showed a larger range of drug induced toxicity such as renal or central nervous system toxicities (De Groen, 1988). For instance, patients with type V hyperlipidemia characterized by high hepatic triglyceride and chylomicrons showed high plasma CSA concentration up to 1500 ng/mL without evidence of nephrotoxicity (Verrill et al., 1987). In the presence of a large amount of lipids, more CSA circulates in the blood in the form of a complex with lipoproteins that makes it unavailable to the cell. Rodl et al has also shown that the lipoprotein profile in the blood is one of the important factors in determining the effectiveness of CSA as an immunosuppressant (Rodl et al., 1990).

CSA is a highly lipophilic molecule, which can penetrate biological membranes and distribute throughout the body. As previously described in

section 1.1.4, CSA is bound to cells and lipoproteins within the blood. It has been previously shown that when blood is separated at 37°C a higher percent of CSA is recovered from lipoprotein fractions, whereas when blood is separated at lower temperatures CSA is more highly distributed into blood cells such as erythrocytes or leucocytes (Lemaire and Tillement, 1982; Niederberger et al., 1983). The binding of CSA to plasma components is independent of drug concentration when drug levels are significantly above the therapeutic range (Niederberger et al., 1983; Gurecki et al., 1985).

Cholesterol released from degradation of LDL may be either incorporated into membranes or esterified for storage within cells (Dietschy, 1990). This can result in an increased risk for heart-related diseases such as coronary heart disease or atherosclerosis. (Ballantyne et al., 1989; Luke et al., 1990; Ginsberg, 1996) LDL is mainly removed by hepatic LDL-R, which appears to have similar role in hamster, rats, mice, rabbits, dogs, monkey and human (Dietschy, 1990). The alteration of LDL-R can be made by diet or compounds such as ethinyl estradiol (Kovanen et al., 1979; Ma et al., 1986; Srivastava, 1996; Parini et al., 1997). However, the effect of CSA on LDL-R is not clear due to conflicting reports (Al Rayyes et al., 1996; Vaziri and Liang, 1997). Other than LDL-R, we measured the scavenger receptor class B, type I (SR-BI), which is the HDL receptor (HDL-R) (Ji et al., 1997; Acton et al., 1999) since the major cholesterol carrier in rat is HDL. The procedure, which is called “selective cholesterol uptake”, differs from that of the LDL-R. LDL-Rs

are able to uptake LDL particles by recognition of ApoB. The whole particle is taken up into the cells and undergoes a degradation process. With the SR-BI only the lipid part, not the protein part, is taken up.

As demonstrated in Chapter 3, low doses of lipoprotein associated CSA showed different metabolic response and the possibility of more toxicity. In general, 1-3 mg/kg of CSA is used in humans whereas higher amounts, 10-50 mg/kg of CSA, are used in rats due to physiological differences as described in chapter 1. In the present study, we dosed rats with a higher dose of CSA, which was bound to each of the individual lipoprotein fractions, as compared to the previous study. Our goal was to examine metabolic interactions of these CSA-lipoprotein complexes in comparison to the current commercial CSA product. Drug disposition is often related to drug-induced toxicity. Alterations in disposition, as a result of dyslipidemia, can result in either toxicity or therapeutic failure. The mechanism of this phenomenon is unknown at this time. In addition, little is known about parental dosage forms in which the drug has been associated with specific lipoproteins prior to administration to an animal or a patient.

It has been reported that cell proliferation is inhibited to a greater extent when lipoprotein-associated CSA was added to peripheral blood mononuclear cells as compared to the addition of both lipoproteins and CSA added separately (Rodl et al., 1990). This result indicates that lipoproteins alone do not have an effect on cell proliferation. Additionally, when cholesterol is taken

up by the liver it undergoes metabolism by CYP7A, indicating cholesterol metabolism is not related to CSA metabolism (Gullberg et al., 2000; Rodriguez et al., 2001). In our studies we were primarily concerned with LDL and its role in CSA metabolism, for this reason we looked at the administration of LDL alone. However as a result of the above studies and we did not investigate the effects of other lipoprotein fractions alone, but rather only lipoprotein-CSA complexes.

The objectives of this study were to examine the effect of lipoprotein-associated CSA on metabolic modulation and lipoprotein receptors. This study may explain the variation in immunological and toxic effects within and between individual patients. In our study, we used concentrated human plasma rather than rat plasma. We chose to work with human plasma rather than rat plasma because in the rat there is little LDL in rat plasma thus making it difficult to harvest, also there are loading amount limits associated with rat lipoprotein fractions.

## **4.2. EXPERIMENTAL**

### **4.2.1 Chemicals**

IV CSA vehicle, Cremophor® (Polyoxyated caster oil) was purchased from Sigma Chemical Co (St. Louis, MO). Intravenous cyclosporine (IV CSA) in Cremophor® was generously donated by Norvartis Pharmaceuticals (Sandimmune®, East Hanover, NJ). Powder CSA and cyclosporine D (CSD) were obtained from Sandoz Research Institution (East Hanover, NJ). Furosemide was purchased from American Reagent Laboratories, Inc. (Shirley, NY). Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase (type XII),  $\beta$ -nicotinamide adenine dinucleotide (NADP; grade III), testosterone, 11 $\alpha$ -hydroxyprogesterone, xylazine, ketamine, acetopromazine, Tween 20 and phenylmethylsulfonylfluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide was purchased from National Diagnostics (Atlanta, GA). All other chemicals were purchased in the highest purity available from EM Science (Gibbstown, NJ).

#### **4.2.2 Animals**

Adult male Sprague Dawley rats weighing 230-250 g (8 weeks old) from Harlan Sprague Dawley Inc. (Indianapolis, IN) were purchased. Following a 7-day acclimation period, rats were randomly divided into seven groups consisting of 5-8 rats per each group. The number of rats in each group was different since the inserted cannula occasionally clotted during the study period. A single 4 mg/kg intraperitoneal dose of furosemide was administered to initiate salt depletion. The rats were maintained on a low sodium diet. This model is well-established for chronic renal disease (Gerkens et al., 1984; Elzinga et al., 1993)]. Each rat was housed in individual wire-bottom cages in a 12-hour light/dark cycle animal facility with controlled temperature and humidity throughout the experiment. Rats were pair-fed a rice diet (8.5% protein, 76.6% carbohydrate, 4.3% fat, 0.05% sodium enriched with ferric orthophosphate and thiamin mononitrate; Harlan Teklad, Indianapolis, IN) and had tap water *ad libitum*. Daily body weight was recorded throughout the experiment.

CSA was administered intravenously via an indwelling jugular catheter at a concentration of 10 mg/kg starting 2-days after surgery. Lipoproteins-associated CSA (VLDL-, LDL-, and HDL-CSA) and plasma-associated CSA (Plasma-CSA) were given to rats. Equivalent volume with the LDL-associated CSA, 0.7-1.5 mL/kg vehicle and saline were administered to the other control groups as a daily intravenous bolus injection. Dosing volumes of other

treatment groups was in the range of 0.7-1.8 mL/kg depending on the loading CSA concentration. After the final CSA or vehicle dose, rats were placed into rodent metabolism cages for 24 hours to collect urine. Rats were sacrificed, blood collected, and liver harvested for further investigation. This study includes 3 control groups (saline, vehicle and IV CSA) to examine the possible effect of vehicle administration on CYP-mediated metabolic activity as compared with the saline group.

#### **4.2.3 Isolation of Human Plasma by Gradient Ultracentrifugation**

Human plasma was purchased from The Blood Bank of Austin (Austin, TX). Liquid plasma was stored at -80 °C after removal of chylomicrons by centrifugation at 1000 x g for 15 min. There are several techniques to separate lipoproteins, including ultracentrifugation, sequential precipitation, size exclusion chromatography, affinity chromatography, and fast protein liquid chromatography (Ferreri 1984; Wasan, Cassidy et al. 1999). The method of ultracentrifugation for lipoprotein separation is the most acceptable and widely used technique since it is relatively straightforward, requires less time, and allows larger volumes to be harvested at one time. In addition, the purity of the separated lipoproteins was confirmed in several studies either by absorbance

readings at 280nm or by SDS-PAGE (Chung et al., 1986; Sattler et al., 1994).

It was shown that there is a  $98.5 \pm 3.5\%$  recovery of lipoprotein fractions by ultracentrifugation and the coefficients of variability in VLDL, LDL, and HDL peaks are 4.8, 2.6, 2.2%, respectively. This method is advantageous because it requires a decreased spin time, resulting in decreased lipoprotein degradation, in addition to resulting in a more rapid lipoprotein separation with equal resolution. Additionally, ultracentrifugation does not require the exact density of each lipoprotein fraction to be known as sequential flotation does. This is beneficial when work is being done with various species. However rat's density levels for each fraction are not well defined.

Lipoprotein fractions were isolated from plasma and placed into tubes that contain citrate phosphate dextrose as an anticoagulant. Lipoproteins were separated using ultracentrifugation with a fixed rotor (Type 50.2 Ti) operating at  $227,220 \times g$  and maintained at  $4^{\circ}\text{C}$ . Centrifugation time varied from 9 to 24 hours depending on the batch of plasma. Spinning time was determined with 3 mL of plasma from each batch prior to a large scale operation. In brief, 140  $\mu\text{L}$  of 250 mM PMSF in isopropanol was added to 70 mL of frozen plasma to prevent possible oxidation and the density was adjusted to 1.24 g/mL using NaBr. PMSF (MW 174.2) would be removed in the process of dialysis at the final procedure. Twenty-one mL of PBS containing 5 mM  $\text{Na}_2\text{EDTA}$  was placed into the tube and 6 mL of plasma was added into the bottom of the tube

using a blunt-end needle. All samples were purged with nitrogen gas to prevent oxidation.

#### **4.2.4 Concentration of Lipoproteins**

Following centrifugation, each fraction was collected using a blunt-end needle. The layer between LDL and HDL is distinguishable as a result of the  $\beta$ -carotene present in human plasma.

Each 40 mL lipoprotein fraction was placed into a Centricon 80 (Millipore, MA) with 40 mL of PBS, it was then concentrated/desalted by centrifugation (Allegra 6R, GH 3.8 rotor) at 3,500  $\times g$  for 40 min at 4°C. This was repeated 2 additional times for a total of 3 spins. New PBS was added with each new spin. The concentrated lipoproteins were stored at 2°C in the dark under nitrogen gas. Lipoproteins in plasma were stable at -80°C, but the structure of separated lipoproteins changes slightly at this temperature. The separated fractions are only stable at 2-8°C for 3-4 months. Each fraction was characterized by 6-10% SDS gel electrophoresis and a modified Lowry-Folin protein assay (Vega and Grundy, 1996). Prior to the electrophoresis, lipoproteins were delipidated (Converse and Skinner, 1992). In brief, 1 mL of lipoprotein fraction was mixed with 2:1 (v/v) ice-cold chloroform-methanol for 10 min and mixed with 0.2 mL of 4.9 M trichloroacetic acid (TCA) and 0.2 mL

of 3.6 mM sodium deoxycholate. After 1 hr, a pellet was obtained by centrifugation at 3000 x g for 20 min. The delipidated proteins were sedimented by 3000 x g for 20 min at 4°C, the organic solvent was discarded, and the protein pellet was dried under an nitrogen stream. The protein pellets were dissolved in sampling buffer and electrophoresed on 6-10% SDS-PAGE gel as described in Chapter 2. The levels of total cholesterol and triglycerides in each fraction were measured by use of a Sigma diagnostic kit (St. Louis, MO). Each fraction (10 mL) was incubated with 120 mg CSA in 40 mL of ethanol at 37 °C for 24 hr and then dialyzed with membrane (MWCO 2,000) against 4 L PBS for 24 hours with 3 buffer changes to remove the free unassociated CSA. The procedure was maintained at 4 °C and in the dark.

#### **4.2.5 Extraction of CSA from the Lipoproteins**

Three extractions were performed by adding 200 µL of n-propanol into 50 µL of sample. The sample mixture was centrifuged for 5 minutes at 5000 x g at 4°C in a GS-15R Beckman centrifuge with an S4180 rotor (Beckman Instruments, Inc., Palo Alto, CA). After the centrifugation, the supernatant was harvested. This step was repeated two additional times and the supernatant

from each spin was combined. The supernatant was dried under a stream of air and then reconstituted in 500  $\mu$ L of isopropanol. Reconstituted samples were filtered with a 0.2  $\mu$ m pore nylon membrane (Nalgene 4 mm syringe filter, Rochester, NY), and upon further dilution, samples were analyzed by HPLC the same day. This assay showed 92.3% recovery efficiency.

#### **4.2.6 Chromatography for the CSA Concentration**

High pressure liquid chromatography (Shimazu, Columbia, MD) was used to separate and quantify CSA. In brief, 20  $\mu$ L of CSA extracts were injected by an automatic injector (SIL-10A) and were resolved at 50 °C (Eppendorf Model CH-30 column heater, Eppendorf Model TC-50 controller, Brinkman Instruments, Westbury, NY) on a 250 mm x 4.6 mm Spherisorb S5 CN column (Waters, Milford, MC) preceded by a 10 mm x 4.3 mm CN guard column (Upchurch Scientific, Oak Harbor, WA) along with solvent A composed of 5% n-propanol in n-hexane (98% pure) and solvent B composed of 20% n-propanol in n-hexane (98% pure) for 55 min at an average of 1 mL/min flow rate by dual solvent pump (LC-10AS) in binary gradient mode. Absorbance of each peak was monitored at 214 nm (SPD-10AV). A system controller (SCL-10A) manages the entire system.

cyclosporine D (CSD) 50 µg/mL was used as an internal standard. CSD and CSA peaks appeared approximately 10 min and 29 min, respectively (Figure 4.1).

The standard curve was in the range of 2.5-50 µg/mL. The interday and intraday variability is within 6% and 3%, respectively in this assay. (Table 4.1) The signal to noise ratio (S/N) is the quotient of the base peak and the standard deviation of all data points below a set threshold. The limit of detection is calculated by taking the concentration (determined by the user) of the peak of interest and dividing it by 3-5 times the S/N. The limit of quantification is the minimum detectable quantity; it is the amount of sample capable of producing a signal three times the noise level of the base line. The limit of detection and quantification for the HPLC assay used in these studies is estimated to be 2.5 µg/mL and 0.8 µg/mL, at a signal-to-noise ratio of 5:1.

Other methods used in this chapter were described the detailed in Chapter 2.

#### **4.2.7 Statistical Analysis**

Differences between treatment groups were determined by one factor analysis of variance with Scheffe's post hoc analysis. All data are expressed as

mean  $\pm$  standard error. A significant difference was considered if the probability of chance explaining the results is reduced to less than 5% ( $P < 0.05$ ).

## 4.3 RESULTS

### 4.3.1 General Observations

Each lipoprotein fraction was concentrated based on protein level. Plasma was not concentrated. Each vehicle component is shown in Table 4.2. Plasma cholesterol had higher levels of cholesterol as compared with that from normal rats. Only one rat dosed with LDL-CSA was seriously sick at the end of the study. This animal ceased to eat or drink regularly and excreted high urine volume indicating the possibility of severe toxicities, including nephrotoxicity. Data was excluded from rats that either developed a cannular dysfunction due to a clot or is sick. Each treatment group is described in Table 4.3.

Table 4.1 Recovery Efficiency and Intraday/Interday Variability of CSA Concentration Measurement with HPLC

		CSA conc. ( $\mu\text{g/mL}$ )	CSA/CSD Mean $\pm$ SD	%CV
50.0	Intraday		$1.003 \pm 0.004$	0.4
	Interday		$1.042 \pm 0.047$	4.5
25.0	Intraday		$0.520 \pm 0.006$	1.2
	Interday		$0.500 \pm 0.029$	5.8
12.5	Intraday		$0.247 \pm 0.001$	0.3
	Interday		$0.243 \pm 0.003$	1.2
5.0	Intraday		$0.084 \pm 0.003$	3.3
	Interday		$0.088 \pm 0.005$	6.0
2.5	Intraday		$0.040 \pm 0.004$	9.7
	Interday		$0.046 \pm 0.002$	5.3
Theoretical Conc. ( $\mu\text{g/mL}$ )		CSA		
		Mean $\pm$ SD ( $\mu\text{g/mL}$ )	Recovery %	
30.0		$27.7 \pm 2.9$	92.3	

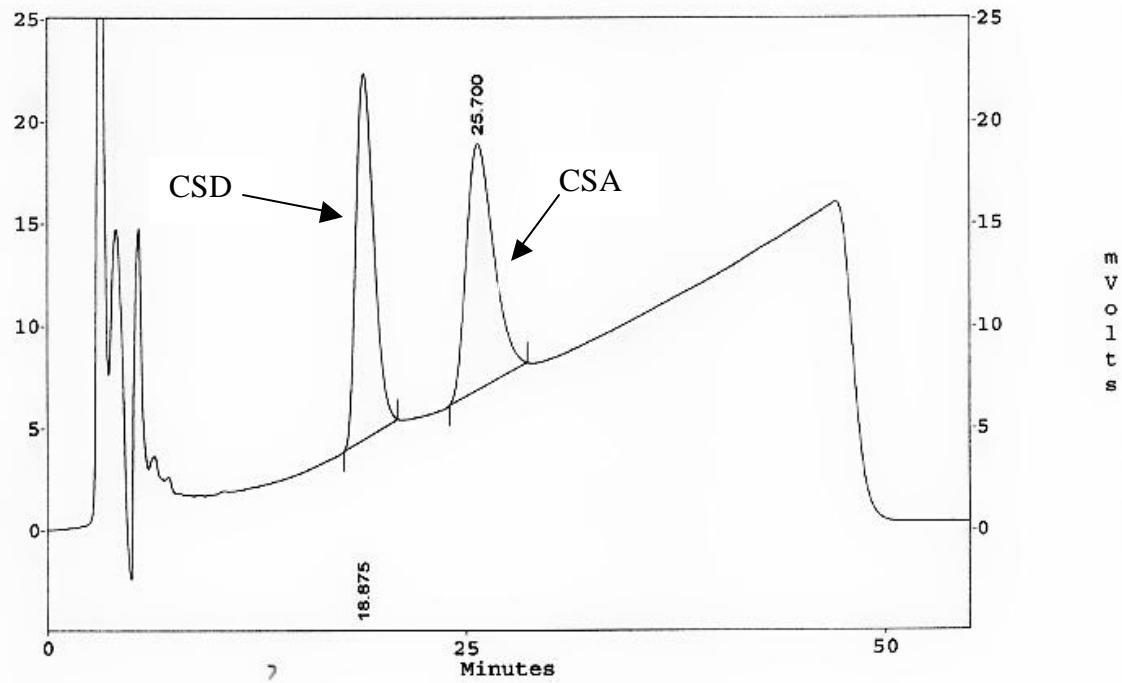


Figure 4.1 Chromatograph for CSA Concentration

Twenty  $\mu\text{L}$  of samples were resolved at 50°C on a Spherisorb S5 CN column in gradient mode. Each peak was observed at a 214 nm. The concentration of both components was 50  $\mu\text{g/mL}$ .

Table 4.2 Component in Lipoprotein Fractions (VLDL, LDL, HDL) and Plasma

Unit (mg/dL)	VLDL	LDL	HDL	Plasma	Normal rat
Protein	0.4-0.8	0.7-1.00	2.4-3.9	3.8	6 ± 0.7
TG	45-176	150-219	45-77	171	65 ± 7
Cholesterol	38-113	126-161	158-171	166	70 ± 10

Table 4.3 Summary of Study Groups

Group 1	Saline	1 mL/kg/d
Group 2	Intravenous CSA vehicle	1 mL/kg/d
Group 3	Intravenous CSA	10 mg/kg/d
Group 4	Plasma-associated CSA	10 mg/kg/d
Group 5	VLDL-associated CSA	10 mg/kg/d
Group 6	HDL-associated CSA	10 mg/kg/d
Group 7	LDL-associated CSA	10 mg/kg/d

There were no significant changes in weight, water intake or urine volume between groups, except the LDL-CSA treated rats, which appeared to drink significantly less water than the vehicle group. However, water intake and urine volume in all treatment groups were less than the IV-CSA group (Table 4.5).

#### **4.3.2 Serum Data**

The lipid profile was also observed by measuring the level of total cholesterol, triglyceride, and HDL cholesterol (Table 4.4). VLDL and LDL levels were calculated from the obtained values as described in Chapter 2. All treatment groups showed a lower cholesterol level by 19-48% as compared with that of IV-CSA. Although it was not statistically significant, rats treated with vehicle, IV-CSA, and HDL-CSA exhibited lowered LDL-cholesterol indicating enhanced clearance of LDL from plasma. Furthermore greater than 90% of the cholesterol was from HDL. The most significant decrease in HDL-cholesterol was seen in the LDL-CSA treated group where only 53% was present as compared with the levels of the CSA alone group. Serum in this group was composed of 63% HDL-cholesterol out of the total cholesterol indicating an increase in relative unesterified cholesterol level. On the other hand, LDL-CSA treated rats showed a

Table 4.4 Biochemical Parameters

<b>Parameter</b>	<b>Saline</b>	<b>Vehicle</b>	<b>IV CSA</b>	<b>Plasma-CSA</b>	<b>VLDL-CSA</b>	<b>LDL-CSA</b>	<b>LDL</b>	<b>HDL-CSA</b>
ALT (U/dL)	32.8 ±3.2	31.0 ± 8.4	31.7 ± 3.3	34.0 ±2.3	32.0 ± 2.6	32.3 ± 2.3	37.7 ± 4.8	30.6 ± 2.0
AST (U/dL)	73.4 ± 25.8	64.8 ± 8.9	56.3 ± 3.5	61.8 ± 2.4	60.2 ± 3.7	62.0 ± 6.6	74.3 ± 4.6	57.1 ± 4.1
Total Cholesterol (mg/dL)	61.6 ± 4.7	82.3 ± 5.3.	83.6 ± 6.0	62.1 ± 11.5	61.0 ±17.2	55.3 ± 14.6	67.7 ± 6.2	42.8 ± 15.5
VLDL (mg/dL)	11.9 ± 3.6	8.3 ± 2.0	8.8 ± 1.6	6.9 ± 1.0	6.7 ± 1.0	8.6 ± 2.1	8.7 ± 2.1	5.0 ± 0.5
LDL (mg/dL)	13.3± 4.9	ND	ND	7.8 ± 12.3	10.7 ± 6.4	8.6 ± 14.6	7.7 ± 14.4	NS
HDL (mg/dL)	36.4 ± 5.8	77.2 ± 4.7	75.2 ± 4.1	47.3 ± 2.8	61.7 ± 11.5	35.5 ± 8.8 <sup>b,c</sup>	51.3 ± 10.3	53.7 ± 5.3
Chol/HDL	1.7	1.0	1.1	1.3	1.0	1.6	1.3	0.8

Serum samples were obtained from rats 24 hr after the last dose. Values are expressed in mean ± SE. Number of rats is expressed in parenthesis. ND: non detectable.<sup>b</sup> P<0.05 as compared with vehicle group, <sup>c</sup> P<0.05 as compared with IV CSA group.

similar level of LDL cholesterol. There was a significant HDL-cholesterol reduction of 53% ( $P=0.0014$ ) and 54% ( $P=0.005$ ) as compared with the IV-CSA group and vehicle group, respectively. Although there were 22% and 31% lower total cholesterol and HDL cholesterol levels in LDL-CSA administered rats than LDL alone administered rats, no significant differences were found.

#### **4.3.3 Renal Function**

One-way ANOVA revealed no significant difference in serum and urine creatinine between groups. However, significant difference was noted between the control group and plasma-CSA group (Table 4.5). In addition, the VLDL-CSA group showed a decrease in creatinine clearance by 28% ( $P=0.1555$ ) as compared with the IV CSA treatment group, suggesting the vehicle may produce the least nephrotoxicity among all the treatment groups. Plasma group showed a decreased creatinine clearance of 56% ( $P<0.05$ ) as compared with the vehicle treatment group and a decreased clearance of 44% ( $P=0.024$ ) as compared with the IV CSA treated group. The second most common CSA-induced toxicity is hepatotoxicity. In order to observe if different vehicles caused different types of toxicity, hepatic function was measured by ALT and AST

levels as described in 2.10 (Table 4.4). However no significant differences were found between groups.

#### **4.3.4 Effects of Lipoprotein associated CSA on Hepatic CYP**

Hepatic CYP3A is the major CYP isoform that metabolizes CSA. The significant suppressions of CYP3A protein was found in plasma-, LDL-, and HDL-CSA treated rats as compared with the vehicle treated group (Figure 4.2). However, no statistically significant differences were seen in the enzyme expression between these groups and the IV-CSA treated rats with the exception of the VLDL-CSA group. Unexpectedly, a 49% ( $P=0.0025$ ) induction of CYP3A was found in VLDL-CSA treated rats as compared with IV-CSA treated rats, suggesting that VLDL sustains the exposure of CSA to cells. A similar pattern was seen in CYP2C11 protein expression, but no statistical significance was found (Figure 4.3).

Consistent with the protein expression, the activities of these two enzymes were suppressed (Figure 4.4) IV CSA treatment markedly reduced the hepatic microsomal drug metabolism of testosterone, which is a marker of CYP-dependent metabolism. The suppression primarily involved two gender-

Table 4.5 Renal Function Parameters

<b>Parameter</b>	<b>Saline</b>	<b>Vehicle</b>	<b>IV CSA</b>	<b>Plasma-CSA</b>	<b>VLDL-CSA</b>	<b>LDL-CSA</b>	<b>HDL-CSA</b>
No. of Rat	5	5	6	6	5	7	7
Initial Wt. (g)	264 ± 8	270 ± 7	263 ± 11	277 ± 7	268 ± 10	283 ± 6	284 ± 5
Final Wt. (g)	286 ± 12	270 ± 6	279 ± 11	293 ± 6	278 ± 11	290 ± 8	297 ± 5
Weight Change (%)	8.3 ± 4.1	0.7 ± 1.2	6.7 ± 1.2	5.8 ± 1.8	3.8 ± 1.2	2.9 ± 2.6	4.4 ± 0.9
H <sub>2</sub> O intake (mL/kg)	67.6 ± 10.3	150.1 ± 33.3	129.2 ± 23.7	78.8 ± 9.4	67.7 ± 14.5	71.1 ± 9.3 <sup>b</sup>	94.4 ± 8.5
Urine Volume (mL/kg)	34.8 ± 7.3	114.5 ± 33.3	96.4 ± 21.6	46.7 ± 6.9	36.5 ± 7.2	47.2 ± 7.2	56.8 ± 6.8
Scr (mg/dL)	0.35 ± 0.17	0.29 ± 0.05	0.35 ± 0.18	0.4 ± 0.05	0.43 ± 0.09	0.37 ± 0.18	0.41 ± 0.06
Ucr (mg/dL)	106 ± 16	41 ± 8 <sup>a</sup>	47 ± 11 <sup>a</sup>	82 ± 23	98 ± 27	82 ± 10	65 ± 9
Clcr (µL/min/100g)	653 ± 62	951 ± 132	746 ± 100	417 ± 89 <sup>b</sup>	533 ± 101	593 ± 36	669 ± 137

All values are expressed as mean ± SE, <sup>a</sup>P<0.05 as compared with saline group, <sup>b</sup> P<0.05 as compared with vehicle group.

specific CYP isoforms, CYP3A and CYP2C11. Production of  $6\beta$ -hydroxy testosterone ( $6\beta$ -OHT), a marker of CYP 3A activity, was reduced in the IV CSA, plasma-, VLDL-, LDL-, and HDL-CSA groups as compared with the vehicle or saline group. In comparison with the IV CSA group, both the HDL- and, LDL-CSA groups showed a reduced  $6\beta$ -OHT production of 42% ( $P=0.557$ ) and 18% ( $P=0.615$ ), respectively. However the VLDL-CSA group showed an increase in  $6\beta$ -OHT production by 85% ( $P=0.035$ ).

Similar results were seen with  $2\beta$ -OHT production with a decrease in the LDL-CSA group by 51% ( $P=0.083$ ), and in the HDL-CSA group by 55% ( $P=0.097$ ) accompanied by an increase in the VLDL-CSA group. The plasma group showed the same levels as the IV CSA group, suggesting no difference in metabolic interaction from IV CSA. Although the plasma contained a large amount of protein (3.8 mg/dL), these proteins, mainly albumin, do not affect CSA metabolism.

However, HDL-CSA and LDL-CSA, which contain a large amount of apolipoproteins, suppressed CYP3A activity compared with the IV CSA group, implying LDL-R involvement. It has been reported that ApoB100 containing LDL and only high cholesterol HDL have a high affinity for the LDL-R (Lopez-Miranda et al., 1993). Considering the major lipid-carrying lipoprotein in rats is HDL, this result shows that lipid levels play a role in CSA metabolism. ApoE containing VLDL has been also postulated to be internalized by the LDL-R

however the production of  $\alpha$ - and  $\beta$ -OHT in the VLDL-CSA group showed similar levels as compared with the saline or the vehicle groups. This may result from VLDL carrying CSA for a longer time in circulation and a delayed exposure time of the drug to the liver resulting in a decreased suppression of CYP3A activity. The VLDL-CSA group also showed a slightly higher production of  $2\alpha$ - and  $16\alpha$ -OHT along with the plasma-CSA treatment group as compared with the IV CSA treatment group. CYP2A1 activity was reduced by 22% ( $P=0.256$ ) and 25% ( $P=0.2334$ ) in the LDL and the VLDL group, respectively, however this was not statistically significant. The differences may be caused by PBS since PBS was used to dialyze lipoprotein-associated CSA in the final step.

Since an immunoblot with currently available polyclonal antibodies can not separate CYP3A1 and CYP3A2 due to an 89% amino acid similarity, RT-PCR was performed to distinguish which one is responsible for the regulation. It appeared that both genes were involved in the CYP3A protein expression (Figure 4.5).

Since vehicles contained different types of lipid, CYP4A protein expression, which is known to be related to lipid metabolism, was measured (Figure 4.6). We observed an increase in the level of CYP4A1/2 in all groups as compared with the saline group. Since all other groups contained lipid-containing vehicle no treatment groups did not show any difference as compared with the IV CSA group. This indicates that the induction of CYP4A is only due to the oily

nature of the vehicles themselves and it was not CSA that altered CYP4A. The content of CYP2E1 also did not show any significant differences between groups, however, a 29% ( $P=0.0048$ ) suppression in the plasma-CSA group was found as compared with IV CSA (Figure 4.7). Therefore, lipoprotein-associated CSA did not alter CYP4A or CYP2E1.

#### **4.3.5 Effect of CSA on Receptors**

The purpose of this study was to examine the correlation of specific lipoprotein effects on the metabolic response by the alteration of specific receptors such as LDL-R or SR-BI. SR-BI is a scavenger receptor that has been shown to interact with lipoproteins. It has also been termed as HDL-R, since HDL has a high interaction with SRBI (Acton et al., 1996). The contents of receptors were measured by either Western immunoblot or RT-PCR. We found that commercially available LDL-R antibody (Cortex Biochem Inc., Leandro, CA) was not specific enough to detect LDL-R. The band obtained was the same band as the one obtained from the incubation with secondary antibodies (goat anti-mouse HRP). Therefore, LDL-R effect was measured using RT-PCR. Only LDL-

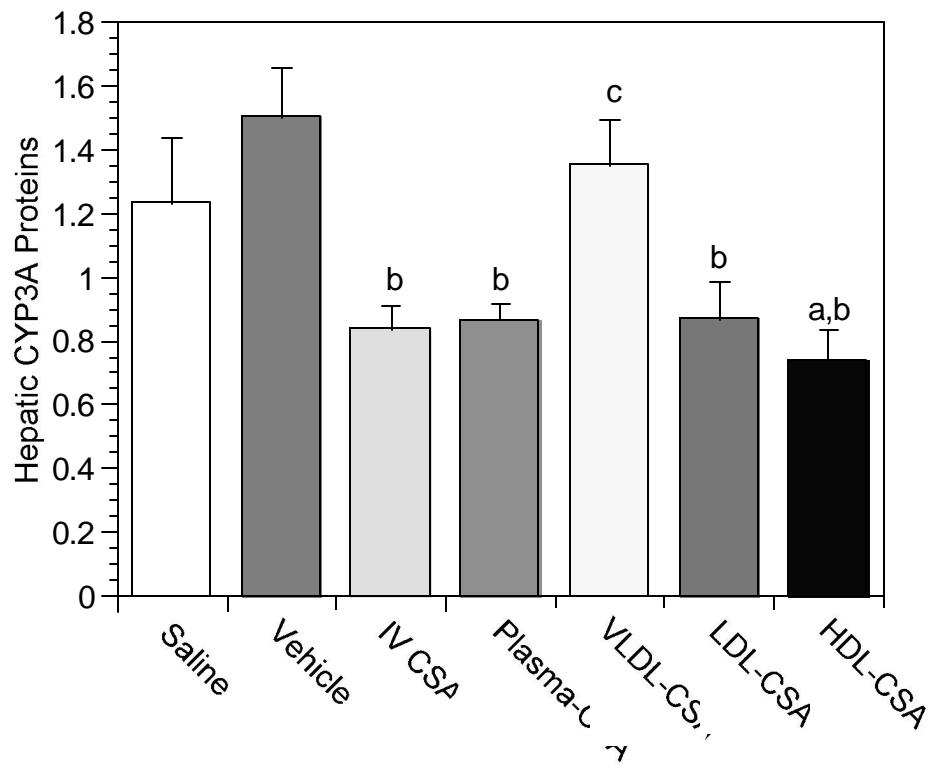


Figure 4.2 Effect of Lipoprotein-associated CSA on Hepatic CYP3A Protein Levels

Immunoblot analysis of CYP3A from hepatic microsomes isolated from rats following 10 mg/kg of lipoprotein-associated CSA for 14 days via intravenous injection. Units are arbitrary. <sup>a</sup> P<0.05 as compared with saline group, <sup>b</sup> P<0.05 as compared with vehicle group, <sup>c</sup> P<0.05 as compared with IV CSA group.

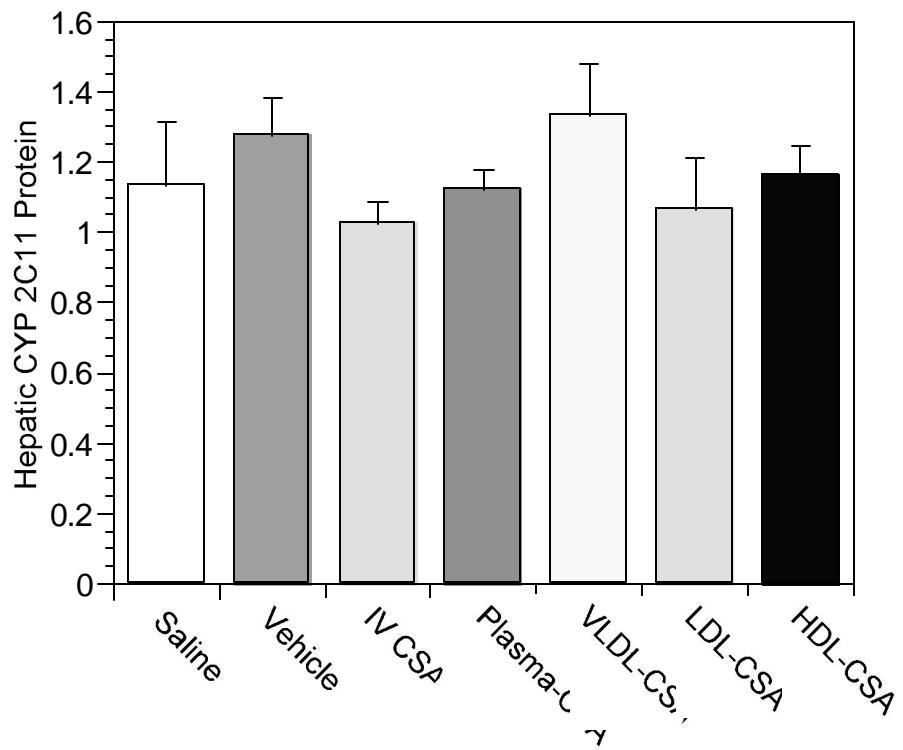


Figure 4.3 Effect of Lipoprotein-associated CSA on Hepatic CYP2C11 Protein Levels

Immunoblot analysis of CYP2C11 from hepatic microsomes isolated from rats following 10 mg/kg of lipoprotein-associated CSA for 14 days via intravenous injection. Units are arbitrary.

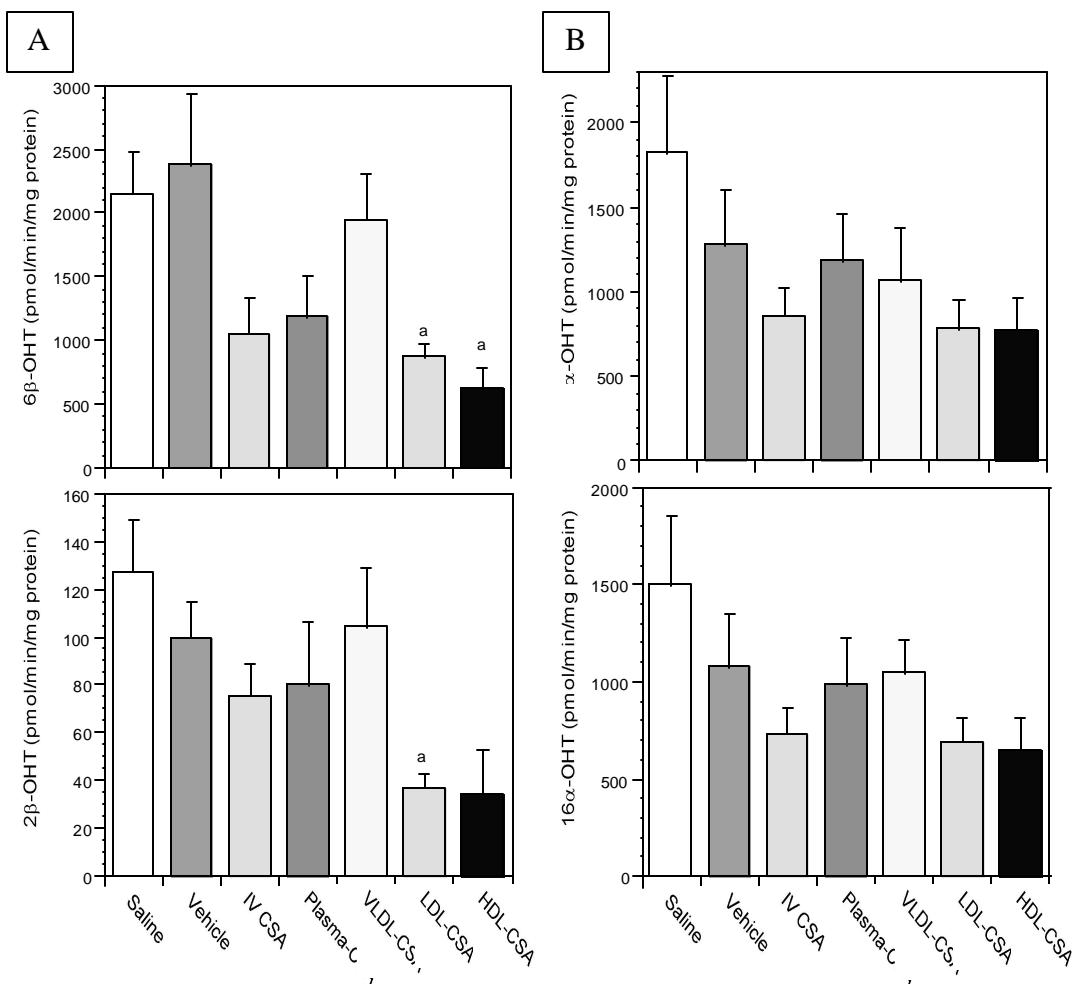


Figure 4.4 *In vitro* Testosterone Hydroxylase Activity in Hepatic Microsome

Hepatic microsomes isolated from rats given an intravenous injection of 10 mg/kg cyclosporine for 14 days. 250  $\mu$ M testosterone was incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone was used as an internal standard. Panel A and B refers CYP3A and CYP2C11 activities, respectively. <sup>a</sup>P<0.05 as compared with saline group.

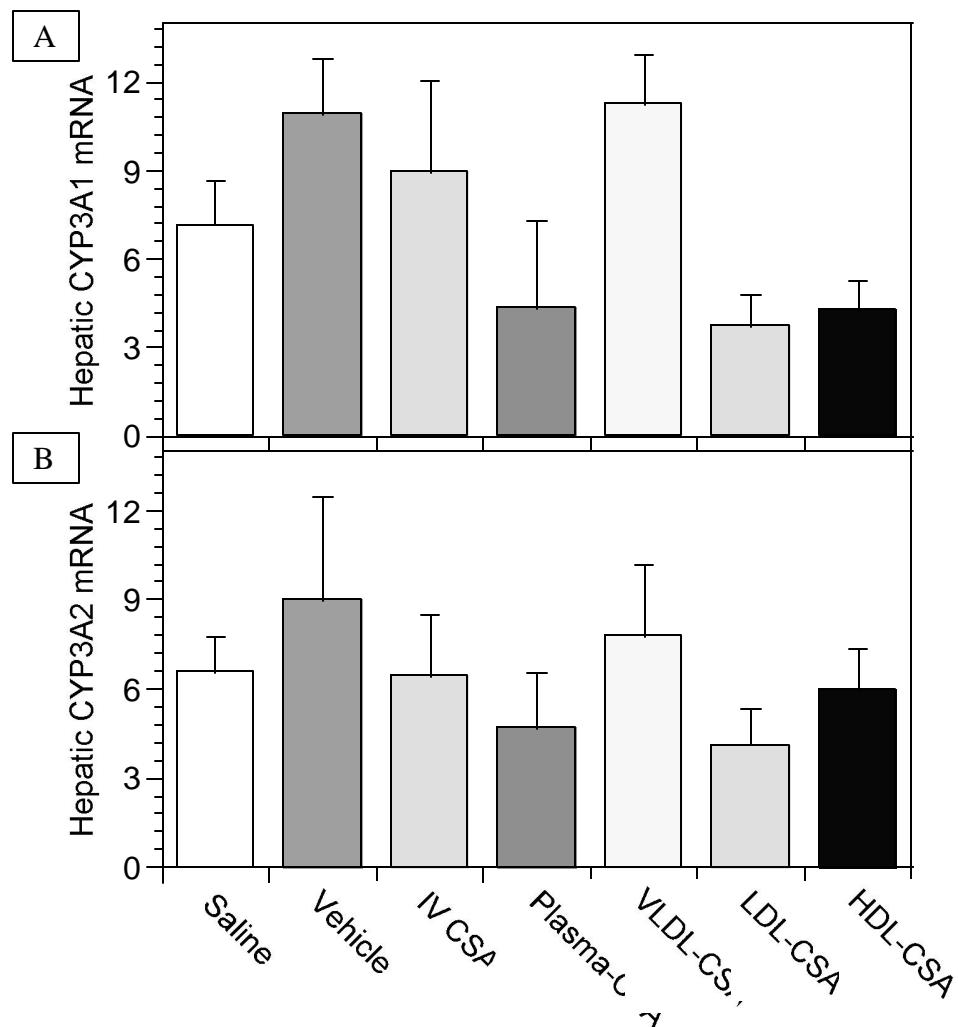


Figure 4.5 Effect of Lipoprotein-associated CSA on Hepatic CYP3A mRNA Levels

After the intravenous administration of 10 mg/kg of CSA for 14 days, 100 mg of liver was used to isolate mRNA to perform RT-PCR. Panel A and B refers to CYP3A1 mRNA and CYP3A2 mRNA, respectively. Units are arbitrary.

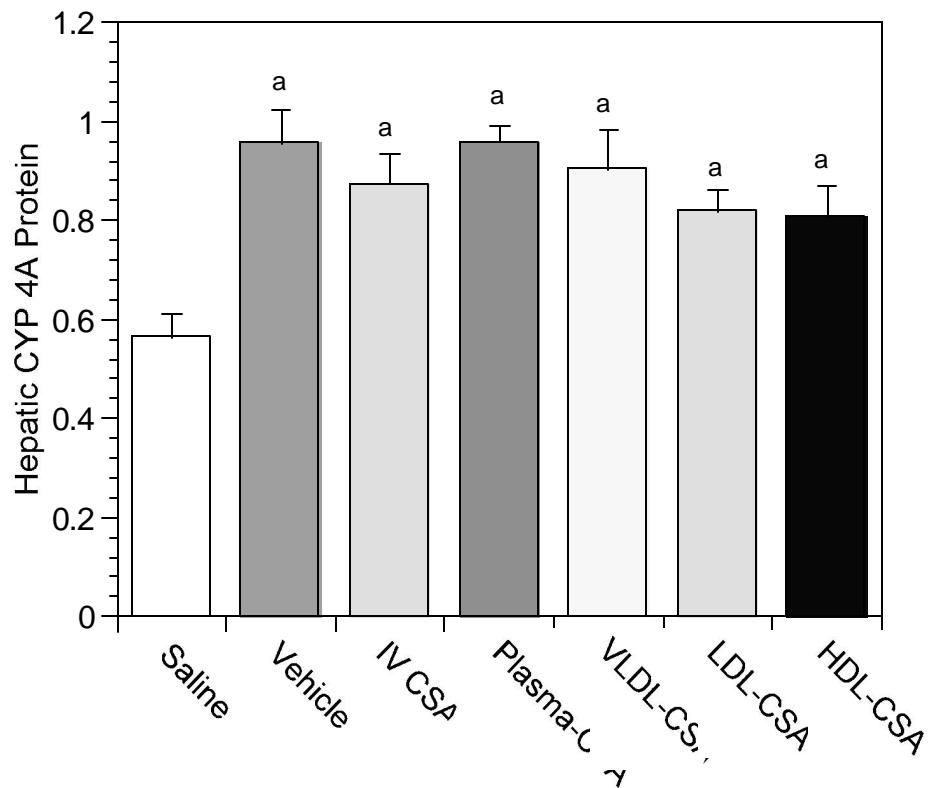


Figure 4.6 Effect of Lipoprotein-associated CSA on Hepatic CYP4A Protein Levels

Immunoblot analysis of CYP4A from hepatic microsomes isolated from rats following 10 mg/kg of lipoprotein-associated CSA for 14 days via intravenous injection. Unit is arbitrary. <sup>a</sup> P<0.05 as compared with saline group.

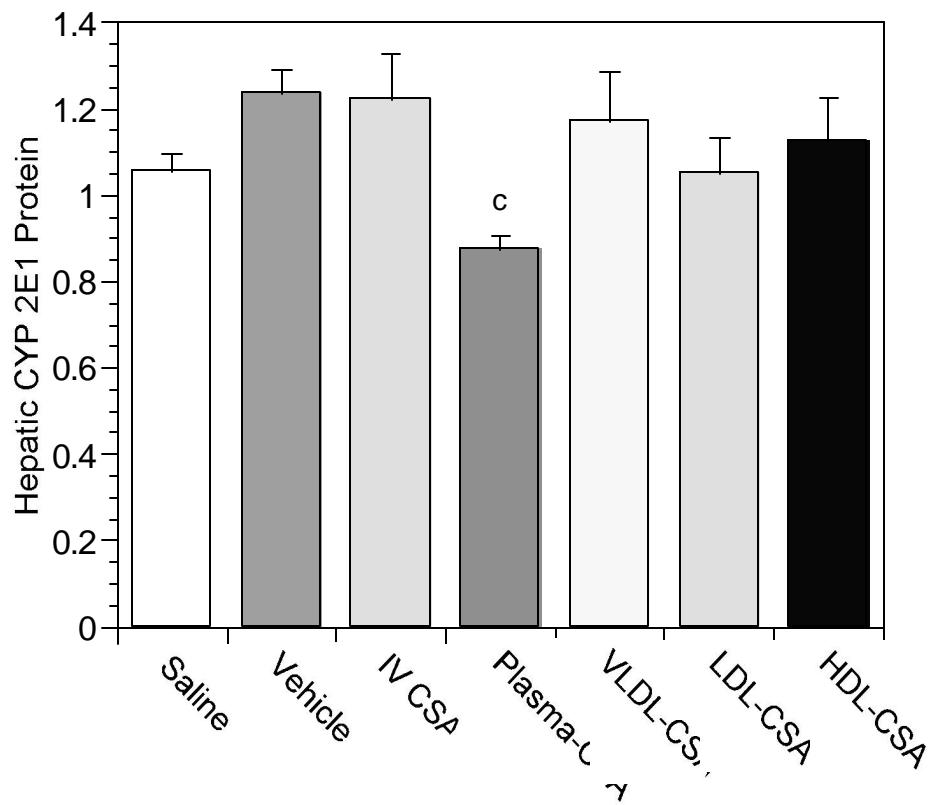


Figure 4.7 Effect of Lipoprotein-associated CSA on Hepatic CYP2E1 Protein Levels

Immunoblot analysis of CYP2E1 from hepatic microsomes isolated from rats following 10 mg/kg of lipoprotein-associated CSA for 14 days via intravenous injection. Units are arbitrary. <sup>c</sup> P<0.05 as compared with CSA alone group.

CSA treated rats showed significant suppression of LDL-R mRNA levels by 50% ( $P=0.0333$ ) (Figure 4.8). Although a 40% ( $P=0.1138$ ) suppression of LDL-R mRNA levels was seen in plasma-CSA treated rats, it was not statistically significant. In order to investigate if the difference of LDL-R mRNA in LDL-CSA treated rats was attributed to LDL itself rather than CSA, the LDL-R mRNA levels in LDL treated rats were examined (Figure 4.9). The result showed that there is no effect of LDL alone on LDL-R mRNA levels. In addition, we measured the expression of CYP3A1 and CYP3A2 mRNA when treated with LDL alone. It appears that administration of LDL alone does not have an effect on CYP3A1/2 mRNA expression (Figure 4.10). This result indicates a posttranscriptional effect of LDL-CSA on CYP3A.

Slight suppression of liver SR-BI protein expression was found in vehicle, IV CSA, plasma-, LDL-, and HDL-CSA groups but VLDL-CSA treated rats showed slight increases compared with IV-CSA treated rats (Figure 4.11A). In comparison with the saline group, CSA-containing treatment groups, except LDL-CSA, showed approximately 30% suppression (Figure 4.11B). The protein results were consistent with the mRNA expression. However, plasma-CSA treated rats showed a 78% ( $P=0.0195$ ) suppression of SR-BI mRNA levels.

CSA alone appeared to have no effect on SR-BI. Slight suppression can be attributed to the vehicle and the drug. IV-CSA and LDL-CSA also appeared to have a similar effect on liver SR-BI according to protein content, as well as

mRNA level. However, LDL alone suppressed SR-BI mRNA by 66% ( $P=0.2533$ ) more than LDL-CSA (Figure 4.12). LDL treated rats compared to VLDL-CSA treated rats showed a 45% increase in HDL cholesterol. This increase contributed to the 22% increase in total cholesterol by the LDL treated rats (Table 4.4).

#### **4.4 DISCUSSION**

This research was designed to investigate the effects of lipoprotein-associated CSA by measuring metabolic response and toxic effect. In addition, we also hoped to provide a possibility for use of a more viable intravenous vehicle for lipid-soluble drugs to reduce the toxicity. Patients who have hyperlipidemia or hypolipidemia have shown different pharmacokinetic parameters after CSA treatment (Nemunaitis et al., 1986; De Groen et al., 1987; Brunner et al., 1989; Luke et al., 1992). In addition CSA treatment alters the level of plasma lipoprotein (Yau et al., 1991; Luke et al., 1992; Wasan et al., 1997). Therefore, the level of lipoprotein may be one of the critical factors to be considered in treatment.

Currently the vehicle of the commercially-available intravenous formulation is nephrotoxic, polyoxylated caster oil (Cremophor®) which contains 32.5% (v/v)

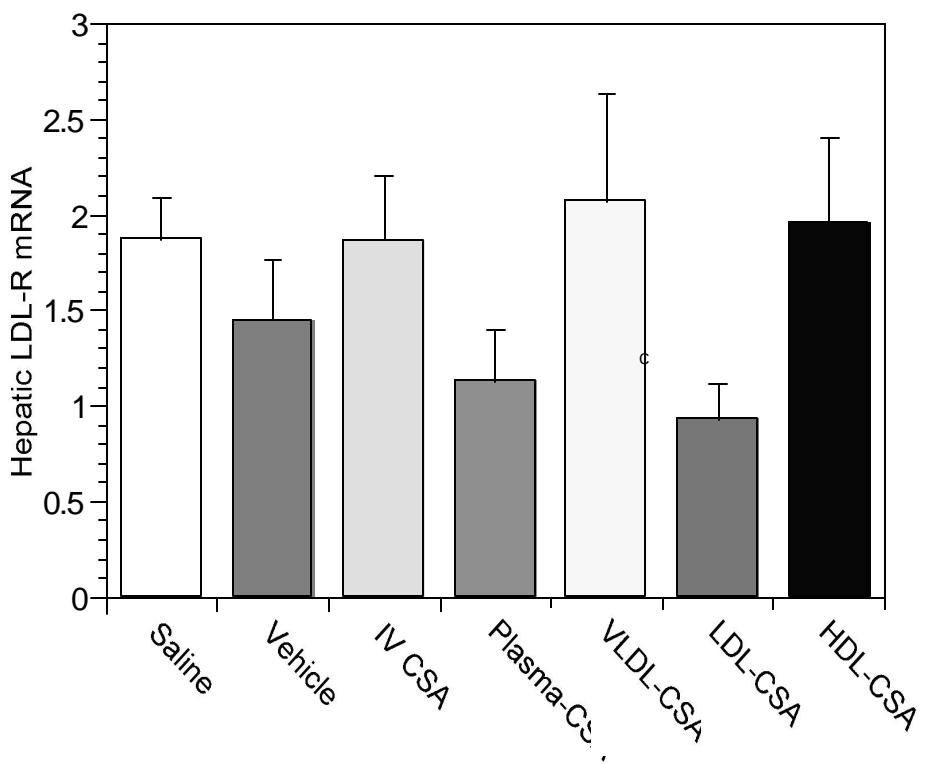


Figure 4.8 Comparison of Effect of LDL and LDL-associated CSA on Hepatic LDL-R mRNA Levels

After the intravenous administration of 10 mg/kg of CSA for 14 days, 100 mg of liver was used to isolate mRNA to perform RT-PCR. Unit is arbitrary. <sup>c</sup>P<0.05 as compared with IV-CSA.

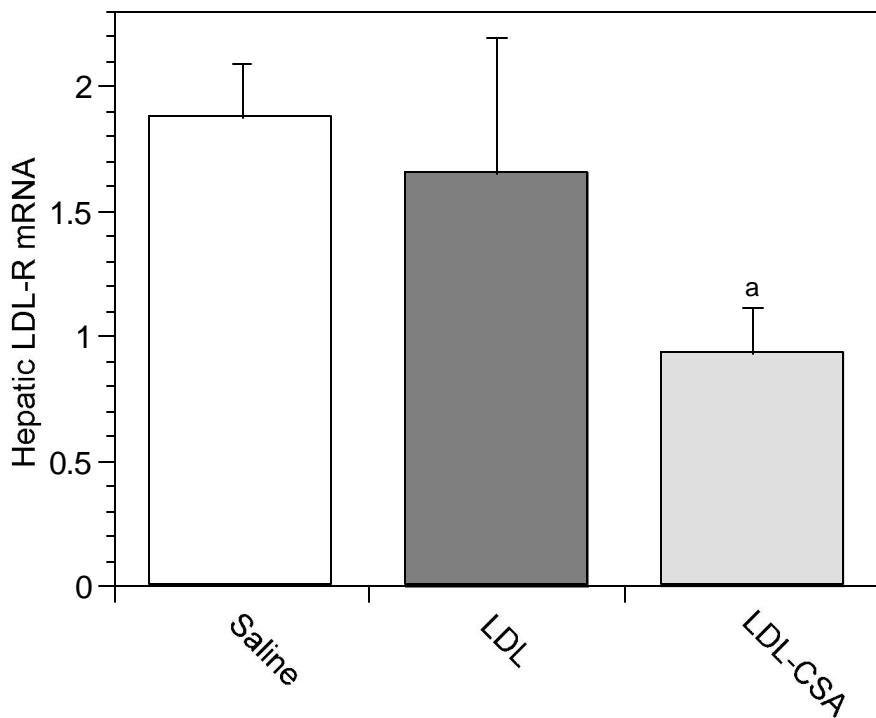


Figure 4.9 Comparison of Effect of LDL and LDL-associated CSA on Hepatic LDL-R mRNA Levels

After the intravenous administration of 1 mL/kg of saline or LDL or 10 mg/kg of LDL-CSA for 14 days, 100 mg of liver was used to isolate mRNA for RT-PCR. Units are arbitrary. <sup>a</sup>P<0.05 as compared with saline.

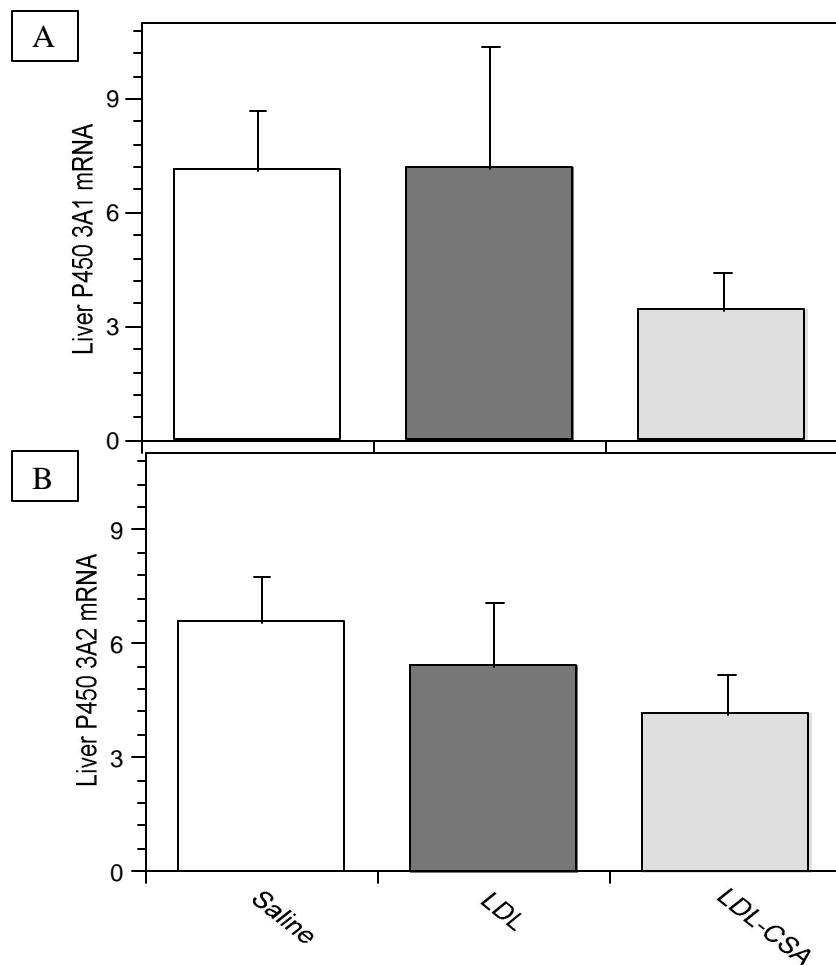


Figure 4.10 Comparison of Effect of LDL and LDL-associated CSA on Hepatic CYP3A mRNA Levels

After the intravenous administration of 1 mL/kg of saline or LDL, or 10 mg/kg of LDL-CSA for 14 days, 100 mg of liver was used to isolate mRNA for RT-PCR ( $P<0.05$ ). Panel A and B refers to CYP3A1 mRNA and CYP3A2 mRNA, respectively. Units are arbitrary.

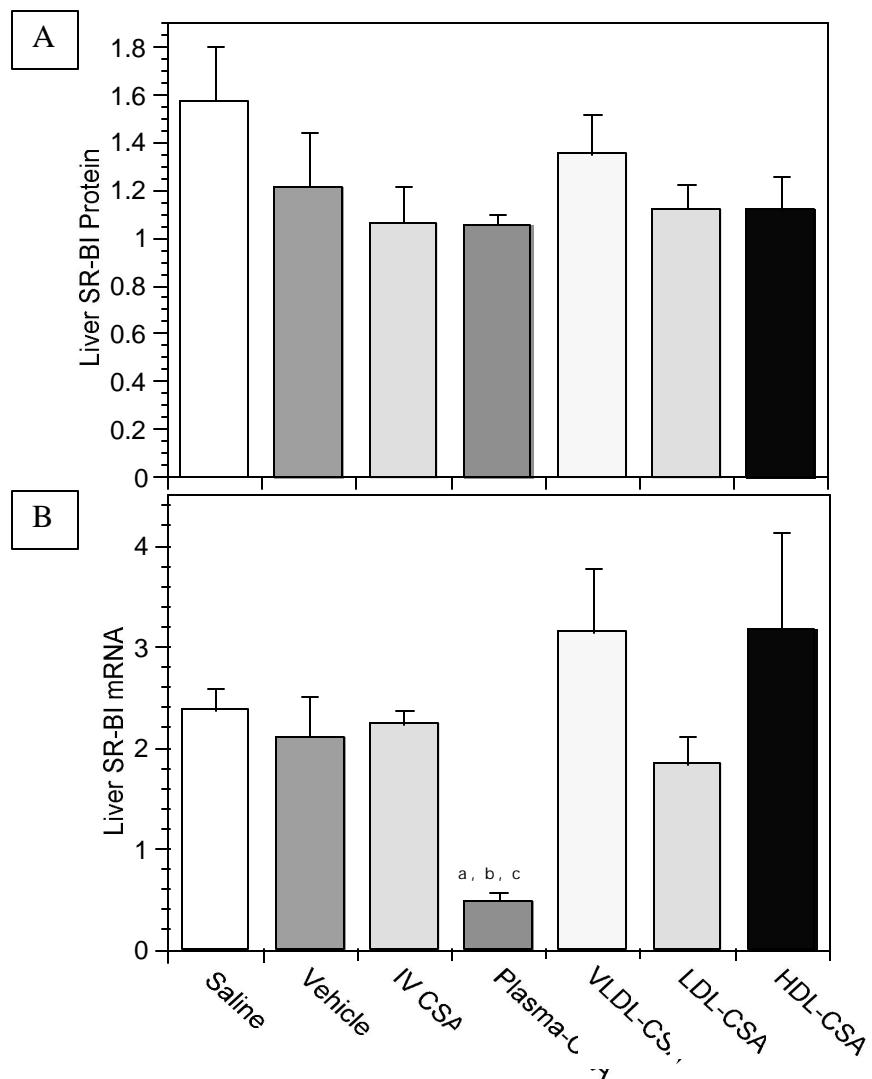


Figure 4.11 Effect of Lipoprotein-associated CSA on Hepatic SR-BI Protein Levels

Immunoblot analysis of SR-BI from hepatic microsomes from rats following 10 mg/kg of lipoprotein-associated CSA for 14 days via intravenous injection. Panel A and B refers to SR-BI proteins and SR-BI mRNA, respectively. Units are arbitrary. <sup>a</sup> P<0.05 as compared with saline group, <sup>b</sup> P<0.05 as compared with vehicle group, <sup>c</sup> P<0.05 as compared with IV CSA group.

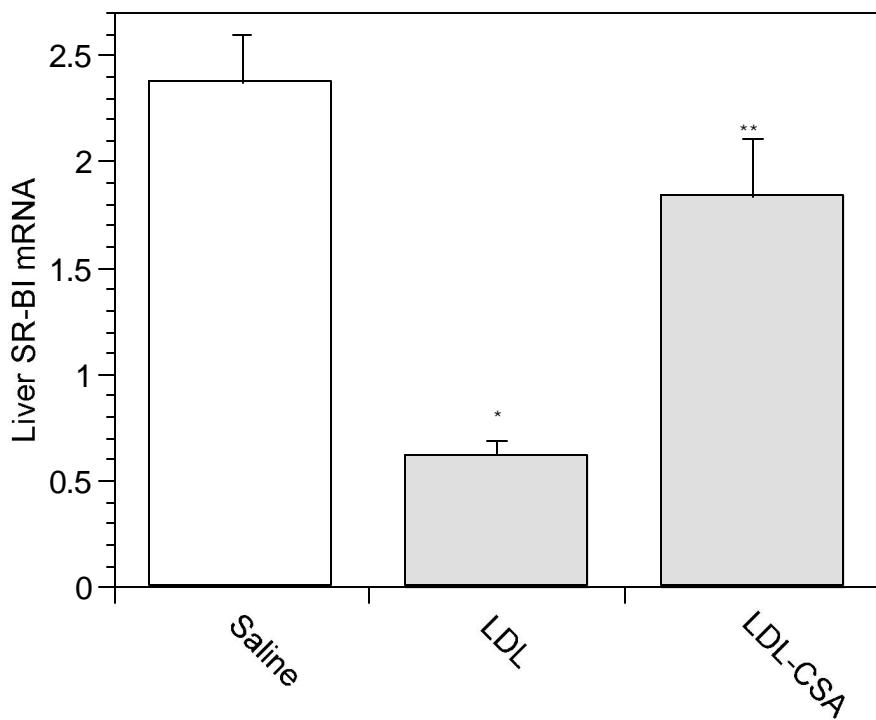


Figure 4.12 Comparison of Effect of LDL and LDL-associated CSA on Hepatic SR-BI mRNA Levels

After the intravenous administration of 1 mL/kg of saline or LDL, or 10 mg/kg of LDL-CSA for 14 days, 100 mg of liver was used to isolate mRNA for RT-PCR ( $P<0.05$ ). Units are arbitrary. \* $P<0.05$  as compared with saline. \*\* $P<0.05$  as compared with LDL.

ethanol. A number of studies reported that the use of Cremophor® resulted in hypersensitivity, extraction of plasticizer from the intravenous infusion line, as well as neurotoxicity (Dorr, 1994; Goldspiel, 1994). In addition, lipoprotein can also altered by Cremophor® resulting in a decreased density of HDL (Kongshaug et al., 1991), or a different mobility of lipoproteins (Ramaswamy et al., 1997). The use of plasma apheresis, which is patient's own LDL, for the preparation of drug associated with drug has been proposed. However, a nontherapeutic range of drug can be loaded (Vitols, 1991). Some other processes proposed involve the use of organic solvents such as heptane or diethylether (Krieger et al., 1978; Lundberg, 1987), or toxic detergents such as sodium deoxycholate (Masquelier et al., 1986) which is used to reconstitute lipoprotein particles, especially LDL. These may cause toxic events due to organic solvents or detergent residue. In addition, these procedures are time and labor intensive. Furthermore, it is frequently in low recovery of drug in the final preparation.

LDL is mainly cleared by the LDL receptors pathway. It has not been clear whether or not chronic CSA treatment increases the production of LDL or reduces clearance of LDL by receptor suppression. However, Keptein et al reported that CSA inhibited ApoB100 synthesis (Kaptein et al., 1994). In addition, he also reported that CSA inhibited bile acid production which may lead to increased free cholesterol. Since ApoB in LDL being recognized by the LDL receptor is what makes internalization possible, reduced ApoB100 causes

increasing plasma LDL levels as the same time decreased bile acid which was utilized from free cholesterol in liver results in less demand of cholesterol uptake into liver leading to an accumulation of cholesterol in circulation. It has been proposed that an increase in cholesterol may cause sustained CSA levels in the blood thereby increasing the length of time that tissues are exposed to CSA. An increased exposure to CSA by the kidney can result in renal toxicity (De Groen, 1988).

Based on the hypothesis that CSA metabolism is modulated by LDL-R, rats were intravenously administered with 10 mg/kg of plasma-, VLDL-, LDL-, and HDL-CSA for 14 days. Commercially available intravenous CSA was used as a control. We hypothesized that CSA is either incorporated into the oil core of the carrier particle or covalently bound to an apolipoprotein or lipid component. The first scheme is the ideal scheme. The surface attachment has a better chance to aggregate the lipoprotein particles due to loss of charge. In addition, covalent binding to Apo that is recognized by receptors can alter the receptor binding sites (Mahley et al., 1977).

Although the rat is not the ideal model for the study of lipoproteins, it is the most well documented animal model in regards to cytochromes P450 and CSA metabolism (Guengerich, 1993). Since our primary research interest was CSA metabolism, and not lipoprotein metabolism, we performed our studies in rats. Hepatic CYP enzymes metabolize CSA (Brunner et al., 1998). As a result of

rats having high hepatic drug-metabolizing activity (Maurer et al., 1984; Arlotto and Parkinson, 1989; Guengerich, 1993) rats were dosed with a higher than clinical dose in order to reach the appropriate activity levels of the drug. In humans and rats, the CYP3A sub-family is primarily responsible for the metabolism of CSA (Guengerich, 1993). In humans CYP3A4 is one of the major hepatic isoforms, a similar isoform CYP3A2 is found in male rats (Augustine and Zemaitis, 1986). As a result of this similarity it is anticipated that the data from our study can be extrapolated to humans.

The creatinine clearance was found to be lower in all treatment groups by 10 - 44% compared to control (IV CSA), but it was not shown to be statistically significant, with the exception of plasma-CSA treatment group (Table 4.5). The creatinine clearance in plasma-CSA treated rats was found to be significant as compared with IV CSA. This decrease may be the result of a high concentration of protein in the plasma vehicle. Rats in all the treatment groups (HDL-, LDL-, VLDL-CSA) drank less water as compared with the IV CSA and the vehicle group, which led to a lower urine volume. This is possibly caused by nephritis due to an increase in serum protein levels, characterized by proteinurea and hypoalbuminea in the serum. However we did not examine nephritis in this experiment. Although the level of creatinine in other treatment groups (VLDL, LDL and HDL) was found to be slightly lower than the control group (IV-CSA), it was not significant, suggesting no severe renal dysfunction. Therefore, we can

draw the conclusion that lipoprotein vehicles themselves may not cause any additional nephrotoxicity as compared with IV CSA.

Lipoproteins- and plasma-CSA caused a decreased cholesterol level, mainly due to decreased HDL (Table 4.4). However, LDL-CSA caused a 44% VLDL and 70% LDL cholesterol increase as compared with IV CSA treated rats, indicating a reduced LDL-R activity in LDL-CSA treated rats. Some of the rats in the HDL-CSA treated group showed very low or undetectable cholesterol levels. These results suggest two possibilities: 1) the injected HDL-CSA caused reverse cholesterol transport, 2) increased physiological utilization of LDL such as in the production of bile salt.

All CSA treatments showed a suppression of CYP3A protein expression with the exception of the VLDL-CSA group (Figure 4.2). This data was consistent with the catalytic activity data (Figure 4.4). The increased suppression of catalytic activity, seen within the HDL- and LDL-CSA treatment groups, is the result of CYP2B1/2 activity. In addition to CYP3A, CYP2B1/2 also contributes to the production of  $2\beta$ - and  $6\beta$ -hydroxytestosterone. In all CSA treatment groups hepatic CYP3A1 and CYP3A2 mRNA expressions were suppressed (Figure 4.5). However, the suppression seen in IV CSA treated rats were not as significant as that seen with the plasma-, LDL-, and HDL-CSA treated rats. This result suggests the possibility that HDL- and LDL-CSA complexes are more capable of suppressing protein expression as well as activity in long-term therapy. The

suppressive effects seen in the plasma-CSA treatment group are the result of the lipoprotein contents, primarily LDL. Although there was a suppression of CYP3A in all CSA treatment group (except VLDL-CSA), CYP2C11 protein expression and activity (production of 2 $\alpha$ - and 16 $\alpha$ -OHT) were not affected by the treatment (Figure 4.3-4). This result indicates that these two isoforms are independently regulated.

CYP4A is related to fatty acid metabolism (Gonzalez and Gelboin, 1994). CYP4A was induced in the vehicle, IV CSA-, plasma-, VLDL-, LDL-, and HDL-CSA treated rats as compared with saline-treated controls (Figure 4.6). However, when treatment groups were compared to one another there were no statistical differences between the groups. Although each treatment group contained a different lipid, this result suggests that different types of lipoproteins do not have a distinguishable effect on CYP4A.

When hepatic CYP2E1 was examined no difference within treatment groups were seen, with exception of the plasma-CSA group (Figure 4.7). We are unable to explain why there was a suppression of CYP2E1 as compared to CSA alone. The suppression may be the result of the extraneously high albumin levels present in the human plasma that was used.

One of the most interesting findings in this study was the significant suppression of LDL-Rs associated with LDL-CSA treatment (Figure 4.8). Plasma-CSA treatment also caused a suppression of LDL-Rs, although not

statistically significant, but this suppression was attributed to the LDL present in the plasma. Thus we investigated the administration of LDL alone groups in order to determine if LDL alone has an effect on LDL-R which led to alternation of CSA metabolism.

As Figure 4.8 and Figure 4.9 indicated, LDL itself did not have an effect on either hepatic CYP3A mRNA or hepatic LDL-R mRNA. However, LDL did cause the suppression of SR-BI mRNA (Figure 4.12). This result is consistent with an in vitro study conducted with mononuclear cells (Rodl et al., 1990). In comparison to LDL-CSA-treated rats, a significant suppression was seen in rats treated with LDL alone thus leading to an increase in HDL cholesterol levels. The most abundant apoprotein in HDL is ApoA with small amounts of ApoE and ApoCII/III, while LDL only contains ApoB 100 (Myant, 1990). VLDL contains distinct amounts of ApoC in addition to small amounts of ApoE. Since Apo in each lipoprotein was confirmed by SDS-PAGE, we were able to rule out that this was the result of contamination of HDL in the LDL fraction. There are two possible reasons for the suppression of SR-BI when LDL is administered alone. The first being an increase in LDL-cholesterol levels by LDL caused an increase in the reverse transport process, which leads to an accelerated uptake of HDL by SR-BI. Too much uptake results in suppression, by a feedback mechanism. Secondly, the cholesterol could have been taken up by the LDL-R, as a result of a protective mechanism, resulting in a suppression of the SR-BI.

When SR-BI protein expression was examined, an effect was only seen with the administration of plasma-CSA. Plasma-CSA caused a significant suppression of mRNA levels as compared with the saline, vehicle, and CSA alone treatment groups (Figure 4.11). Since other lipoprotein-CSA treatment groups showed no effect on SR-BI, the suppression seen is probably due to the presence of albumin. Further studies investigating the effects of albumin on SR-BI would be interesting.

VLDL-CSA treated rats did not show a suppression of the CYP3A2 protein, activity or mRNA expression suggesting a higher blood concentration by sustaining exposure of CSA to cells or by saturating receptors that take up CSA into cells. VLDL as a drug carrier could be a benefit for patients who develop toxicity and it showed the possibility of a new treatment with a reduced dose. SR-BI in VLDL-CSA treated rats showed the slight induction of protein and mRNA levels indicating the benefit of SR-BI in treatment.

In summary, specific lipoprotein-CSA complexes appear to alter metabolic responses differently from CSA alone. LDL-CSA and HDL-CSA showed a greater suppression of hepatic CYP3A, which is major enzyme responsible for CSA metabolism, while VLDL-CSA did not have an effect on hepatic CYP3A. None of the lipoprotein-CSA treatment groups showed inefficient glomerular filtration. In addition to these findings, LDL-CSA treatment resulted in a significant suppression of LDL-R mRNA levels suggesting that

LDL-Rs may be one regulatory factor responsible for altering CSA metabolism as a result of an increased uptake of CSA into hepatocytes. In addition, this study showed the different role of each fraction of lipoproteins as a drug carrier. VLDL showed the most plausible possibility as a drug carrier with less renal toxicity.

## **CHAPTER FIVE**

### **Interaction between CSA and the Low Density Lipoprotein Receptors**

#### **5.1 INTRODUCTION**

In 1973 it was shown that the use of steroid hormone agents or antimetabolites (azathioprine, imidazole derivative of 6-mercaptopurine) resulted in hyperlipidemia (Ghosh et al., 1973). It was believed that the introduction of CSA would help to reduce or replace steroid treatment, thus resulting in a decreased incidence of post-transplant hyperlipidemia. However, Cyclosporine (CSA) itself appears to cause an abnormal lipid profile that leads to deteriorated renal function (Luke et al., 1988; Berens et al., 1990).

CSA is the primary drug used following organ transplantation due to its powerful immunosuppressive effects. (Borel, 1989; Borel and Kis, 1991) Although CSA produces excellent immunosuppression, its clinical use is hampered by its toxicity and side effects (Sullivan et al., 1985; Borel, 1989; Bennett, 1990; Mason, 1990) CSA treatment can result in elevated cholesterol levels, primarily LDL levels, which can result in secondary conditions such as

atherosclerosis or hypertension (Ballantyne et al., 1989; Luke et al., 1990; Ginsberg, 1996) In addition, the inter- and intra-patient variability seen with CSA therapy makes predicting therapeutic levels difficult. (Lindholm et al., 1988; Lemaire et al., 1990; Lampen et al., 1995; Kahan et al., 1996; Lampen et al., 1996) De Groen proposed that an increase in cholesterol may cause sustained CSA levels in the blood which leads to an increase in the length of time that tissues are exposed to CSA. For example, an increased exposure to CSA by the kidney can result in renal toxicity (De Groen, 1988).

In normal subjects, LDL cholesterol constitutes about two-thirds of the total plasma cholesterol. This lipoprotein has a fairly long half-life, approximately 4-6 days (Brown et al., 1981). Approximately 50% of patients treated with CSA show an increase in cardiovascular diseases such as hypertension or atherosclerosis (Carrier et al., 1994). These diseases are primarily attributed to increased levels of LDL cholesterol. (Ginsberg, 1998) It has also been reported that lipoproteins, such as chylomicron, remnant, and LDL, produce fulminant cardiovascular related diseases when they become elevated in human plasma. The lipoprotein fraction VLDL appears to be neutral (Zilversmit, 1979). A recent study showed that CSA reduces apoprotein B secretion resulting in an increase in VLDL secretion (Kaptein et al., 1994). Therefore, elevated LDL-cholesterol levels in CSA-treated patients are not the result of increased secretion of VLDL,

but rather the result of some other mechanism, such as an interference with LDL catabolism.

Low density lipoprotein receptors (LDL-Rs) are primarily responsible for LDL uptake into the liver. (Ginsberg, 1998) The mechanism by which CSA causes increased levels of LDL cholesterol in humans is not fully understood. It has been suggested that the dispositions of lipophilic drugs, such as CSA, are regulated by the LDL-R (Ho et al., 1978; Goldstein et al., 1979; Al Rayyes et al., 1996; Vaziri et al., 2000). Al Rayyes has also demonstrated an inhibitory effect by CSA on LDL-R synthesis in Hep G2 cells (Al Rayyes et al., 1996). In contrast, Vaziri has shown that CSA-treated rats did not have alteration in LDL-R levels (Vaziri et al., 2000). This result provides the possibility that CSA effects LDL-R activity thru an indirect regulatory effect such as by growth hormone (GH).

It is well known that GH is the pituitary factor that is responsible for expression of different CYP isoforms resulting in gender differences in CSA metabolism. GH secretion patterns are characterized by a pulsatile secretion pattern in male rats and a continuous secretion pattern in female rats (Waxman et al., 1991). The pulsatile secretion pattern seen in male rats is defined by high peaks of GH secretion followed by periods of undetectable GH, whereas in female rats, there is a frequent and continuous secretion of GH. These gender specific secretion patterns result in the expression of gender specific CYP enzymes. For instance, the male pulsatile secretion of GH results in the expression

of CYP2C11 and the female continuous secretion of GH results in the expression on CYP2C12.

CYP3A is one of the most important catalyzing enzymes for more than 60% of xenobiotics including CSA metabolism (Kronbach et al., 1988; Brunner et al., 1990a; Kolars et al., 1992). In male rats, CYP3A is the major enzyme responsible for the biotransformation of CSA. It has been previously reported that administration of CSA to male rats for 28 days resulted in a significant suppression of hepatic CYP3A and CYP2C11 (Brunner et al., 1990a). Inhibition of CYP enzymes is known to be time-dependent. Two weeks of treatment with CSA resulted in CYP3A and 2C11 suppression, however 28 days of CSA treatment resulted in an even greater suppression of CYP3A and 2C11.

It has been postulated that the administration of estrogen causes the stimulation of hepatic LDL-R in addition to a decrease in plasma cholesterol levels mainly by increasing LDL clearance (Kovanen et al., 1979; Colvin, 1996; Marsh et al., 1999; Inukai et al., 2000). However the mechanism by which estrogen stimulates hepatic LDL-R is unclear. The stimulatory effect on hepatic LDL-R by estrogen could not be reproduced *in vitro* (Semenkovich and Ostlund, 1987). Similarly, in hypophysectomized rats, the administration of estrogen failed to stimulate LDL-R (Steinberg et al., 1967).

Based on the fact that the activities of CSA are related to specific lipoproteins, we hypothesized that CSA's disposition is regulated by LDL-R. In

order to examine the effect of LDL-R on the metabolic response of CSA, a LDL-R modified rat model was used. Rat were either administered 17 $\alpha$ -ethynodiol (EE) (Kovanen et al., 1979; Ma et al., 1986; Srivastava, 1996; Parini et al., 1997) or fed a high cholesterol diet (2%) (Lopez and Ness, 1997) in order to induce or suppress hepatic LDL-R, respectively. The measurement of hepatic microsomal CYP isoforms and markers of nephrotoxicity will allow for prediction of drug effect and modification of biological significance.

## 5.2 METHODS

### 5.2.1 Chemicals

Oral CSA solution (100 mg/mL) was generously provided by Norvartis Pharmaceuticals (Sandimmune®, East Hanover, NJ). This commercial solution was diluted in olive oil containing 12.5% (v/v) ethanol to a concentration of 15 mg/mL. 17 $\alpha$ -ethynodiol and propylene glycol were purchased from Sigma Chemical Inc. (St Louis, MO). 17 $\alpha$ -ethynodiol was dissolved in propylene glycol at a concentration of 5 mg/mL. Purified rat growth hormone (GH) was provided by Dr. A.F. Parlow (Pituitary Hormones and Antisera Center,

Harbor-UCLA Medical Center, Torrance, CA). GH was dissolved in saline containing 30 mM NaHCO<sub>3</sub>. Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase (type XII), β-nicotinamide adenine dinucleotide (NADP; grade III), testosterone, 11α-hydroxyprogesterone, xylazine, ketamine, acetopromazine and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide was purchased from National Diagnostics (Atlanta, GA). All other chemicals were purchased in the highest purity from EM Science (Gibbstown, NJ).

### **5.2.2 Animals**

For experiment I, 8-week-old adult male Sprague Dawley rats were purchased from the Harlan Sprague Dawley Inc. (Indianapolis, IN). Following a 7-day acclimation period, rats were randomly divided into dosing groups consisting of 6 rats per group. Each rat was housed in an individual wire-bottom cage in a 12-hour light/dark cycle animal facility with controlled temperature and humidity throughout the experiment. Rats were pair-fed with either a low-sodium diet (0.05% sodium) or 2% cholesterol diet containing 0.05% sodium (8.5% protein, 76.6% carbohydrate, 4.3% fat, 0.05% sodium; Harlan Teklad,

Indianapolis, IN) and had free access to tap water. The groups were divided as follows (Table 5.1); 1) normal LDL-R, 2) induced LDL-R and 3) suppressed LDL-R , and 4) normal rats fed a standard diet. Group 4 was studied in order to ensure that a low salt diet itself does not change metabolism.

Subcutaneous doses of 5 mg/kg of EE were administered daily for the first 4 days (5-6 p.m.), additional doses were given at 2 mg/kg every other day (5-6 p.m.) for 28 days. In order to minimize stress-related variability, an equivalent volume of saline was given to all groups in the afternoon. In the last group, rats were fed a diet containing high cholesterol from the 4th week to the 8th week to suppress LDL-R. During this period, rats had free access to food. The rats fed a regular diet also were compared to examine if low sodium diet itself affected metabolic interaction. Daily body weight was recorded throughout the experiment. Each dose of CSA or vehicle/saline was administered subcutaneously at a concentration of 15 mg/kg or 1 mL/kg, respectively, every morning (8 to 9 a.m.) for 28 days. After the final dose, rats were placed into rodent metabolism

Table 5.1 Summary of Study I Groups

Group	Rat	Treatment	
NS	Normal LDL-R (Normal Diet)	N	CSA, 15 mg/kg/d, SC
LS-Sal	Normal LDL-R	LS	Saline, 1 mL/kg/d, SC
LS-Veh	(Low Sod. Diet)	Appendix A	Vehicle, 1 mL/kg/d, SC
LS-CSA			CSA, 15 mg/kg/d, SC
E-Sal		EE	Saline, 1 mL/kg/d, SC
E-Veh	Induced LDL-R		Vehicle, 1 mL/kg/d, SC
E-CSA			CSA, 15 mg/kg/d, SC
HC-Sal		HC	Saline, 1 mL/kg/d, SC
HC-Veh	Suppressed	Appendix B	Vehicle, 1 mL/kg/d, SC
HC-CSA	LDL-R		CSA, 15 mg/kg/d, SC

(N=6/group)

Table 5.2 Summary of Study II Groups

Group	Rat	Treatment
F-NS	Female Normal	Saline 1 mL/kg/d
F-Hx-NS	Female Hypophysectomized	Saline 1 mL/kg/d
F-Hx-GH	Female Hypophysectomized	GH 20 ng/g/hr
M-NS	Male Normal	Saline 1 mL/kg/d
M-Hx-NS	Male Hypophysectomized	Saline 1 mL/kg/d
M-Hx-GH	Male Hypophysectomized	GH 20 ng/g/hr

(N=6/group)

cages for 24 hours for urine collection. At the time of sacrifice, blood was collected and liver, small intestine, and kidney were harvested for further investigation.

For experiment II, 8-week-old female and male hypophysectomized Fischer 344 rats were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). For the first 7-10 days, rats were initially fed a 5% glucose solution to aid in the recovery from the hypophysectomy surgery. At 12 weeks of age an osmotic minipump (Alzet model 2001, Palo Alto, CA) was implanted bilaterally under the skin of the hind limb. Either GH or saline was infused at the rate of 20 ng/g/hr or 1 $\mu$ L/hr for 7 days. Rats were maintained at 25°C with free access to standard rat chow and water (Table 5.2).

### **5.2.3 CSA Blood Level**

Monoclonal antibody-mediated fluorescence polarization immunoassay (FPIA) using a commercially available kit (Abbott Diagnostics Laboratories, Abbott Park, IL) was used for CSA concentrations determinations in whole blood, according to the manufacturer's instructions. Calibration curves were developed using a whole blood matrix spiked with 0, 100, 250, 500, 1000, and 1500 ng/mL

CsA. Assay quality was assured using concurrent analysis of three levels (150, 400, and 800 ng/mL) of quality control reagents (Abbott Diagnostics Laboratories, Abbott Park, IL).

## **5.3 RESULTS**

### **5.3.1 General Observations**

Rats did not show any signs of morbidity during the treatment period. However, all groups except the LS-Veh group lost weight (Table 5.3). The LDL-R induced group showed the most significant weight loss, although it consumed 13-34% more food compared with normal LDL-R group. The CSA treatment

Table 5.3 Renal Function Parameters

Parameter	N-CSA	LS-Sal	LS-Veh	LS-CSA	E-Sal	E-Veh	E-CSA	HC-Sal	HC-Veh	HC-CSA
Initial Wt. (g)		312 ± 3.2	312 ± 3.0	304 ± 4.0	277 ± 6.9	295 ± 8.5	264 ± 3.3	257 ± 2.3	262 ± 5.5	271 ± 4.5
Final Wt. (g)	257 ± 6.0 *	301 ± 5.3	314 ± 4.1	299 ± 2.5	242 ± 5.7*	251 ± 6.0*	222 ± 2.9*	296 ± 3.3	304 ± 6.7	310 ± 2.3
Weight Change (%)	-16.4 ± 2.1*	-3.5 ± 0.9	0.5 ± 0.7	-1.5 ± 1.7	-12.6 ± 1.4 *	-14.8 ± 1.5 *	-15.9 ± 1.7*	14.8 ± 0.7 *	16.2 ± 1.5 *	14.8 ± 1.2 *
Food Intake (g/kg)	56 ± 1.5 <sup>b</sup>	50 ± 0.8	48 ± 0.7	50 ± 0.4	58 ± 1.0 *	62 ± 1.1 *	68 ± 4 <sup>a, a, *</sup>	51.6 ± 0.5	50.6 ± 1.0	49 ± 0.3
H <sub>2</sub> O intake (mL/kg)	82.6 ± 9.2	95 ± 15.7	82 ± 13.7	78 ± 5.6	43 ± 6.5	53 ± 22.4	78 ± 15.9	113 ± 10.1	106 ± 15.0	86 ± 7.8
Urine Vol (mL/kg)	28.9 ± 6.6	55.1 ± 13.1	40.1 ± 13.3	34.7 ± 8.0	40.3 ± 6.9	45.3 ± 11.1	57.7 ± 13.0	48.4 ± 8.4	52.3 ± 12.2	48.4 ± 8.4
Scr (mg/dL)	0.63 ± 0.03	0.38 ± 0.05	0.32 ± 0.03	0.63 ± <sub>a, b</sub> 0.8	0.47 ± 0.04	0.45 ± 0.02	0.87 ± <sub>b</sub> 0.1 <sup>a</sup>	0.38 ± 0.02	0.40 ± 0.0	0.45 ± 0.05
Ucr (mg/dL)	113 ± 14	100 ± 17	152 ± 37	143 ± 27	108 ± 25	92 ± 20	50 ± 10	96 ± 16	77 ± 11	130 ± 14
Clcr (µL/min/ 100g)	321 ± 42	882 ± 108	868 ± 69	468 ± <sub>b</sub> 47 <sup>a</sup>	559 ± 75*	510 ± 59*	206 ± <sub>b, *</sub> 33 <sup>a</sup>	735 ± 53	587 ± 46	658 ± 55*

All values are expressed as a mean ± SE, <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup> P<0.05 as compared with corresponding Veh controls, \* P<0.05 as compared with treatment-matched controls.

Table 5.4 Liver Function Parameters

<b>Group</b>	<b>No. of Rat</b>	<b>AST (U/dL)</b>	<b>ALT (U/dL)</b>
N-CSA	6	$66.2 \pm 3.6$	$40.5 \pm 2.6$
LS-Sal	6	$60.5 \pm 3.6$	$44.5 \pm 2.3$
LS-Veh	6	$67.3 \pm 2.6$	$46.5 \pm 3.8$
LS-CSA	6	$72.0 \pm 13.2$	$43.7 \pm 0.1$
E-Sal	6	$58.0 \pm 6.3$	$45.8 \pm 4.7$
E-Veh	6	$61.5 \pm 5.1$	$57.5 \pm 5.4$
E-CSA	6	$91.8 \pm 12.1$	$75.3 \pm 14.6$
HC-Sal	6	$68.3 \pm 2.5$	$53.2 \pm 2.8$
HC-Veh	6	$81.7 \pm 5.5$	$48.5 \pm 5.1$
HC-CSA	6	$82.3 \pm 7.6$	$47.3 \pm 4.6$

All values are expressed in mean  $\pm$  SE.

group fed regular diet, showed 11 fold greater weight loss as compared with LS-CSA. ALT and AST, hepatic function indicators, did not show any statistical differences between groups (Table 5.4).

### 5.3.2 Lipid Profile

Lipid levels were measured as described in Chapter 2. N-CSA and LS-CSA treatment groups showed no lipid profile changes, thus indicating the low sodium diet did not have an effect on the lipid levels (Table 5.5).

All LDL-R suppressed rats showed significantly increased levels of total cholesterol, primarily as a result of increased LDL-cholesterol. Total cholesterol levels of the E-CSA, HC-CSA, and LS-CSA treatment groups were 75% ( $P=0.2801$ ), 63% ( $P<0.001$ ), and 35% ( $P=0.0818$ ) higher, respectively, as compared with vehicle matched controls, indicating CSA caused hyperlipidemia in all models. EE treated rats and high cholesterol fed rats were found to have significantly altered total cholesterol levels. The former showed a 63% ( $P<0.001$ ) decrease whereas the later showed a 121% ( $P<0.001$ ) increase, as compared with LS-Sal treated rats. LDL cholesterol levels were altered in a similar pattern to that of total cholesterol. Examination of triglycerides showed that E-CSA rats had levels approximately 2 times and 4 times higher than HC-CSA and LS-CSA

treated rats, respectively. VLDL levels are mirrored TG levels. This data suggests that CSA treatment in the EE-pretreated rats may cause delayed lipid catabolism.

In contrast, HDL-cholesterol levels in the LDL-R suppressed groups were decreased. Rats treated with saline, vehicle, or CSA in addition to a high cholesterol diet had significantly reduced HDL-cholesterol levels. Levels were decreased by 24% ( $P=0.0032$ ), 41% ( $P<0.001$ ), and 37% ( $P<0.001$ ), respectively, as compared with treatment-matched controls. HDL-cholesterol levels of all of the LDL-R induced groups are 3.5-7 folds lower when compared to treatment-matched controls. As shown in Table 5.5 and Figure 5.1 the majority of the cholesterol in normal LDL-R rats (LS-Sal) is carried by HDL rather than LDL. The LDL-C values in LS-Sal were so low to be detected.

### **5.3.3 Renal Function**

Creatinine is a waste product, which is produced by the muscles and excreted by the kidney (Brody et al., 1998). Serum creatinine levels are function of the amount of creatinine produced by muscles and the kidney ability to excrete waste products (i.e. kidney function). It has been previously shown that

Table 5.5 Serum Lipid and Lipoprotein Levels

<b>Group</b>	<b>Total Cholesterol (mg/dL) 1</b>	<b>VLDL-C (mg/dL)</b>	<b>LDL-C (mg/dL)</b>	<b>HDL-C (mg/mL)</b>	<b>TG (mg/dL) 2</b>
N-CSA		3.8 ± 0.4	56.2 ± 9.8	36.2 ± 2.1	20.8 ± 1.2
LS-Sal	18.5 ± 9.9	6.3 ± 0.5	ND	25.5 ± 0.8	31.5 ± 1.9
LS-Veh	68.2 ± 4.1 <sup>a</sup>	7.7 ± 0.6	27.7 ± 5.1	32.8 ± 1.4 <sup>a</sup>	38.0 ± 2.3
LS-CSA	92.3 ± 5.7 <sup>a</sup>	5.3 ± 0.4	56.3 ± 8.0 <sup>a</sup>	35.7 ± 2.2 <sup>a</sup>	26.8 ± 2.0
E-Sal	44.2 ± 13.2	8.8 ± 1.5	28.1 ± 12.2	7.2 ± 0.7*	43.0 ± 7.5
E-Veh	19.7 ± 8.6*	6.7 ± 1.3	5.6 ± 7.5 <sup>c</sup>	7.3 ± 1.1*	33.0 ± 6.6
E-CSA	34.5 ± 7.6*	20.2 ± 3.7 <sup>a,b,*</sup>	9.3 ± 5.5*	5.0 ± 1.2*	101.2 ± 17.9 <sup>a,b,*</sup>
HC-Sal	132.0 ± 6.12*	6.2 ± 0.4	106.3 ± 7.2	19.5 ± 1.6	30.2 ± 1.8
HC-Veh	125.5 ± 9.5*	5.7 ± 0.6	100.3 ± 8.8	19.5 ± 1.0*	32.3 ± 1.8
HC-CSA	204.0 ± 16.2*	10.2 ± 3.6	171.2 ± 16.6*	22.7 ± 0.8*	49.8 ± 17.7

All values are mean ± SE, <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \* P<0.05 as compared with treatment-matched controls. ND, nondetectable.

<sup>1</sup> Cholesterol mmol/L = 0.02586 x mg/dL

<sup>2</sup> Triglyceride mmol/L = 0.01129 x mg/dL

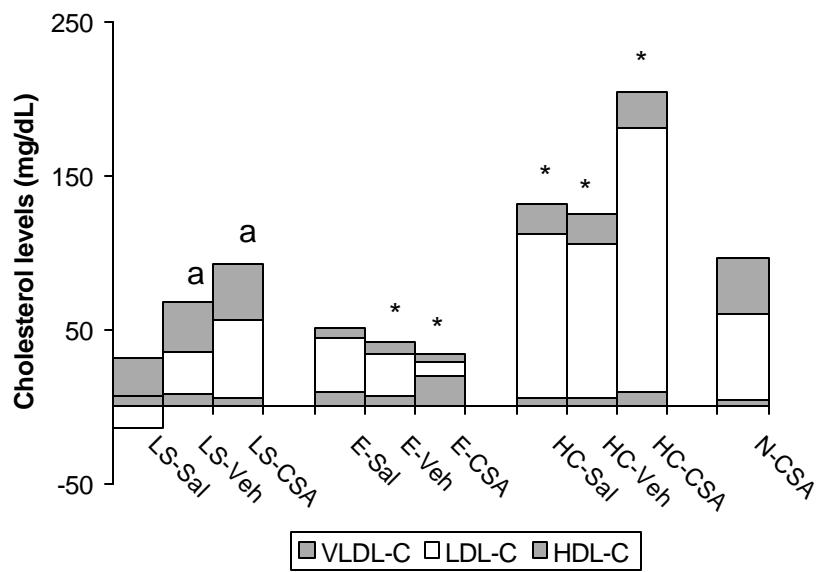


Figure 5.1 Serum Lipid and Lipoprotein Content in Rats

Serum samples were obtained from rats 24 hours after the last dose. All values are expressed as mean (N=6). <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \* P<0.05 as compared with treatment-matched controls.

serum creatinine levels are not affected by diet, although protein restriction may reduce the rate of progression of some kidney diseases and thus protect the ability of the kidneys to excrete waste products (including creatinine) (Baker et al., 1999). In general, rising creatinine levels indicate decreased kidney function. Normal serum creatinine levels in humans and rats are approximately 0.6-1.5 mg/dL and 0.4-0.6 mg/dL, respectively.

In order to examine renal toxicity, serum and urine creatinine were measured (Table 5.3). Both the LS-CSA and E-CSA treatment groups had significant nephrotoxicity as indicated by an increase in serum creatinine levels and a decrease in creatinine clearance ( $P<0.001$ ) in comparison with two control groups, LS-Saline and LS-Veh groups. As expected, the HC-CSA groups did not show any signs of renal toxicity as compared with HC-Sal or HC-Veh. The high levels of total cholesterol in this group may be holding CSA in the blood and reducing LDL receptors in each tissue, resulting in CSA having a decreased toxic effect on cells. In saline- and vehicle-treated rats in the LDL-R induced groups, serum creatinine levels were not changed. However creatinine clearance was significantly reduced by 36% ( $P=0.006$ ) and 41% ( $P=0.002$ ), respectively, as compared with those of rats in the normal low-sodium diet group. These results indicate that 17 $\alpha$ -EE induced nephrotoxicity. This study was consistent with the work of Brunner et al (Brunner et al., 1996), in which subcutaneous CSA treatment resulted in a decrease in creatinine clearance by 46% ( $P<0.001$ ) in rats

maintained on a low-sodium diet as compared with vehicle controls. In addition, this study showed that rats treated with CSA and fed a normal diet had an increased creatinine clearance by 31% ( $P=0.1$ ) as compared with the low sodium diet group (LS-CSA).

#### **5.3.4 Effect on Receptors**

EE and HC have been used to either induce or suppress hepatic IDL-R, respectively. EE pretreatment caused a 2.9-fold induction in LDL-R mRNA while HC caused a 1.5-fold suppression of LDL-R mRNA respectively (Figure 5.2). Although EE-CSA treated rats showed a 74% ( $P =1.383$ ) increase in LDL-R mRNA in comparison to LS-CSA, it appeared to be the result of EE not CSA (Figure 5.3). No effect by CSA on LDL-R was seen in the HC-CSA treatment group (Figure 5.4). A chronic olive oil vehicle administration triggered a compensatory effect to balance lipid level in circulation by increasing levels of hepatic LDL-R in HC fed rats 2-fold.

SR-BI was shown to be decreased in EE pretreated rats, However HC pretreated rats did not show this (Figure 5.5). Therefore, LDL-R and SR-BI are negatively correlated in EE treatment. HC-Veh caused an induction of SR-BI as compared with HC-Sal. EE-CSA treatment suppressed SR-BI 2.2-fold ( $P=0.0290$ )

more than LS-CSA. This suppression is more likely to be the result of EE rather than CSA itself.

Renal LDL-R mRNA and SR-BI mRNA showed different regulation pattern, indicating tissue dependent regulation (Figure 5.6). In contrast to the liver, both are positively associated. Induction of the LDL-R mRNA by EE was not seen whereas a 50% suppression in both receptors was seen with the cholesterol diet. EE-CSA showed approximately a 30% increase in LDL-R indicating an additive effect of CSA (Figure 5.7).

### 5.3.5 Effect on CYP Isoforms

Immunoblot analysis and semiquantitative RT-PCR was applied to assess CYP protein and gene expression. EE itself significantly suppressed hepatic CYP3A protein expression, mRNA expression, and CYP3A activity. CSA treatment also suppressed hepatic CYP3A protein expression in varying degrees in different models (Figure 5.8). LS-CSA, HC-CSA and E-CSA showed a suppression of CYP3A protein expression by 39% ( $P=0.0095$ ), 55% ( $P=0.0786$ ), and 34% ( $P=0.2736$ ), respectively, as compared with vehicle matched controls. EE-CSA and HC-CSA treatment had a greater suppressive effect on CYP3A protein expression, although statistical significance was not found. There was no

alteration in CYP3A protein expression in rats treated with CSA and fed a low-sodium diet as compared with the rats treated with CSA and fed a normal diet. A low-sodium diet could be responsible for the decreased CYP3A mRNA levels. In high cholesterol fed rats, CSA caused a 75% ( $P = 0.0238$ ) decrease in CYP3A2 mRNA levels, but it was not a statistically significant difference. Both CYP3A1/2 mRNA levels were higher than LS-CSA by approximately 2-fold (Figure 5.9). Apparently, either a high cholesterol level or suppressed LDL-R may result in a decreased metabolism of CSA.

Another male-specific hepatic isoform, CYP2C11, was decreased but the changes were not found to be statistically significant (Figure 5.10). Immunosuppressant therapy with CSA in each different condition did not have a significant effect on hepatic CYP2C11 protein expression. Only the EE-treated group showed a significant decrease of CYP2C11 protein. The most significant decrease was seen in the EE-CSA treatment group where CYP2C11 was decreased by 50% ( $P<0.001$ ) as compared with LS-CSA. EE, the female hormone, caused a suppression of CYP2C11 similar to that seen with CYP3A2 mRNA.

Hepatic CYP4A is known to be responsible for the hydroxylation of medium chain fatty acids. As figure 5.11 shows, EE-CSA and HC-CSA treated rats showed an increase in hepatic CYP4A levels by 178% ( $P=0.0013$ ), and 80% ( $P=0.1323$ ), respectively as compared with LS-CSA. In comparison, with in each

of the pre-treatment control groups, CSA in LDL-R suppressed rats had a decrease in CYP4A levels by 43% ( $P=0.0101$ ). CSA treated rats in LDL-R induced groups had CYP4A levels which were increased by 37% ( $P=0.0013$ ). CSA treatment in combination with both low-sodium diet and regular diet did not show any differences.

It has been accepted that hepatic CYP3A and SI CYP3A are regulated independently (Figure 5.12). Furthermore, CYP3A in the small intestine can vary up to 30 folds. We found an induction of intestinal CYP3A proteins after CSA treatment in LDL-R induced rats in comparison to the vehicle group. The vehicle caused the suppression of intestinal CYP3A. The reason for this is unknown. EE-CSA and N-CSA induced CYP3A by 2-fold ( $P<0.001$ ) and 3.4-fold ( $P<0.0001$ ), respectively. In addition to hepatic CYP isoforms, renal CYP4A and CYP2E1 were observed, however no significant changes were found (Figure 5.13).

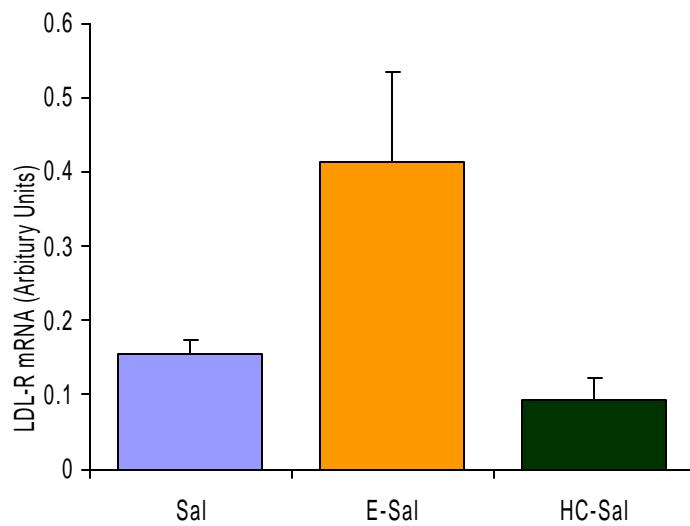


Figure 5.2 Effect of EE and Cholesterol Diet on Hepatic LDL-R mRNA

Hepatic LDL-R mRNA isolated from rats following pretreatment either EE to induce LDL-R or 2% cholesterol diet to suppress hepatic LDL-R as described in the section 5.2.2. Units are arbitrary.

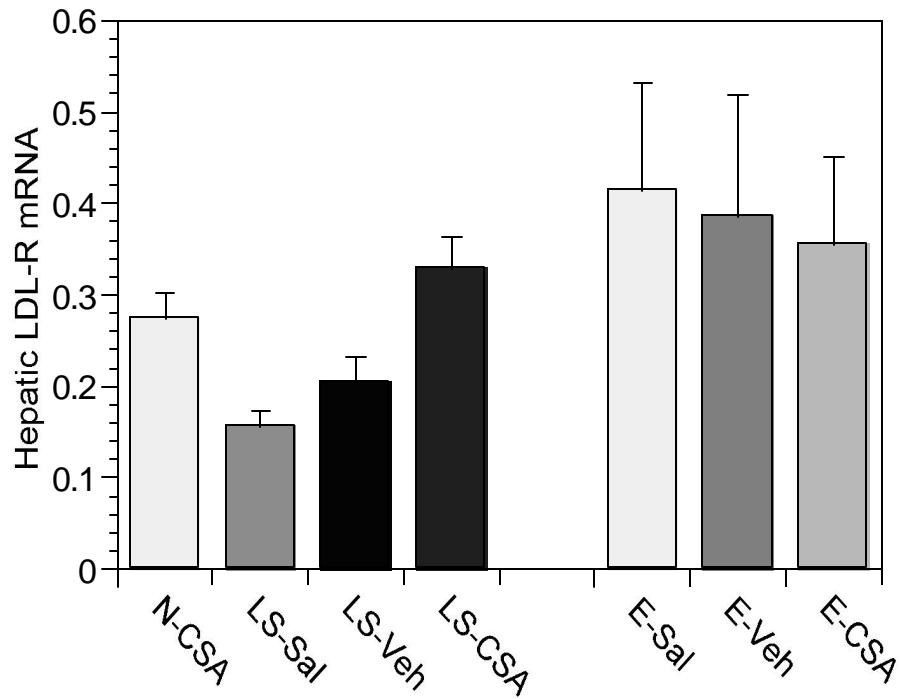


Figure 5.3 Effect of CSA on Hepatic LDL-R mRNA in LDL-R Induced Rats

Hepatic LDL-R mRNA was isolated from rats following treatment with saline, vehicle, or CSA in LDL-R normal or LDL-R induced rats as described in the section 5.2.2. CSA treated rats fed with normal diet (N-CSA) also compared. Units are arbitrary.

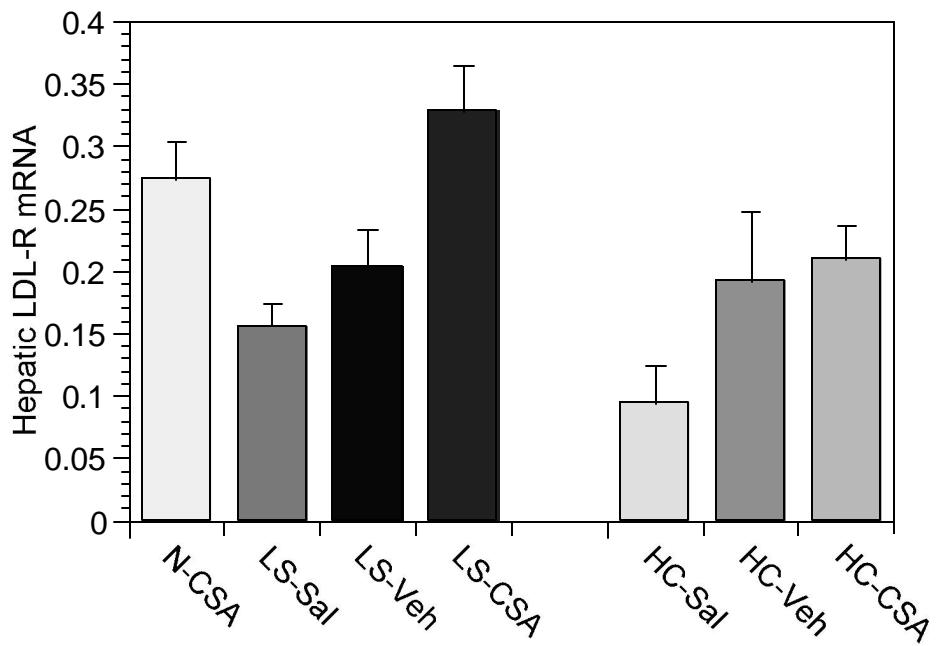


Figure 5.4 Effect of CSA on Hepatic LDL-R mRNA in LDL-R Suppressed Rats

100 mg of liver was used to isolate hepatic LDL-R mRNA for RT-PCR. Rats were administered saline, vehicle, or CSA in LDL-R normal or LDL-R suppressed rats. Units are arbitrary.

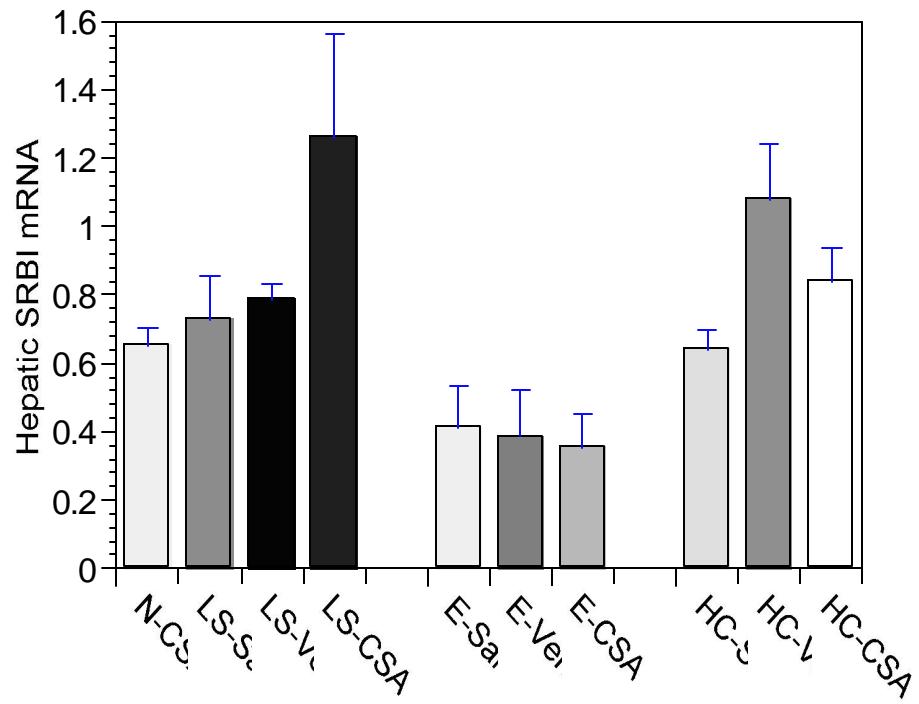


Figure 5.5 Effect of CSA on Hepatic SR-BI mRNA in LDL-R Modified Rats

100 mg of liver was used to isolate mRNA for RT-PCR. Units are arbitrary.

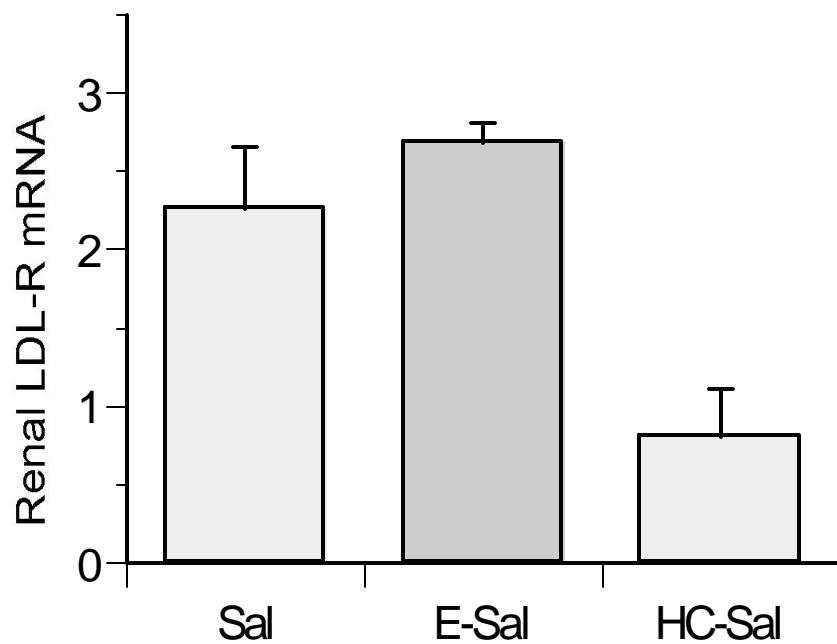


Figure 5.6 Effect of EE and Cholesterol Diet on Renal LDL-R mRNA

Hepatic LDL-R mRNA was isolated from rats following treatment with saline n LDL-R normal, induced, or suppressed rat models. Units are arbitrary.

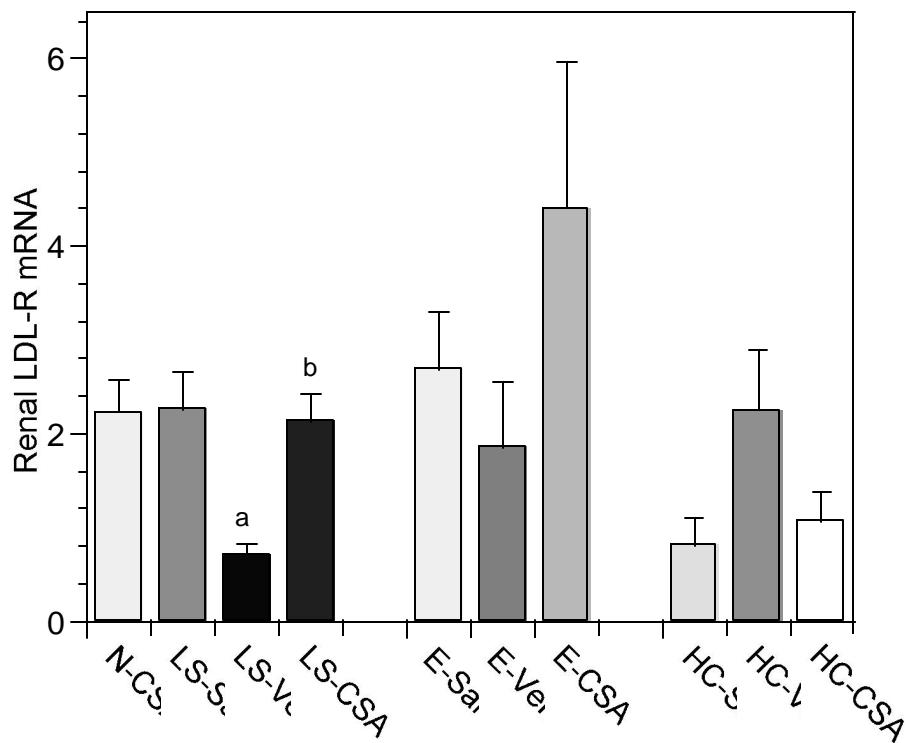


Figure 5.7 Effect of CSA on Renal LDL-R

100 mg of kidney were used to isolate mRNA to perform RT-PCR. <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup> P<0.05 as compared with corresponding Veh controls, Units are arbitrary.

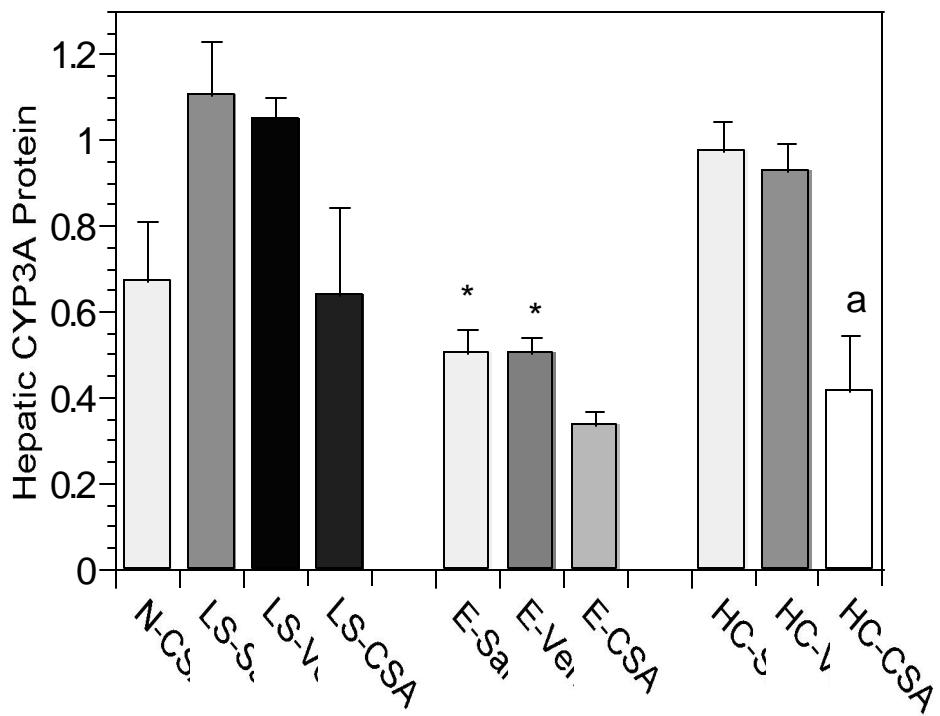


Figure 5.8 Effect of CSA on Hepatic CYP3A1/2 Proteins in Hepatic LDL-R Modified Rats

Immunoblot analysis of CYP2E1 and CYP4A from renal microsomes isolated from rats following subcutaneous injection of saline, vehicle, or CSA for 28 days in hepaticLDL-R normal , induced or suppressed rat models. Units are arbitrary.

<sup>a</sup>P<0.05 as compared with corresponding Sal controls \* P<0.05 as compared with treatment-matched controls.

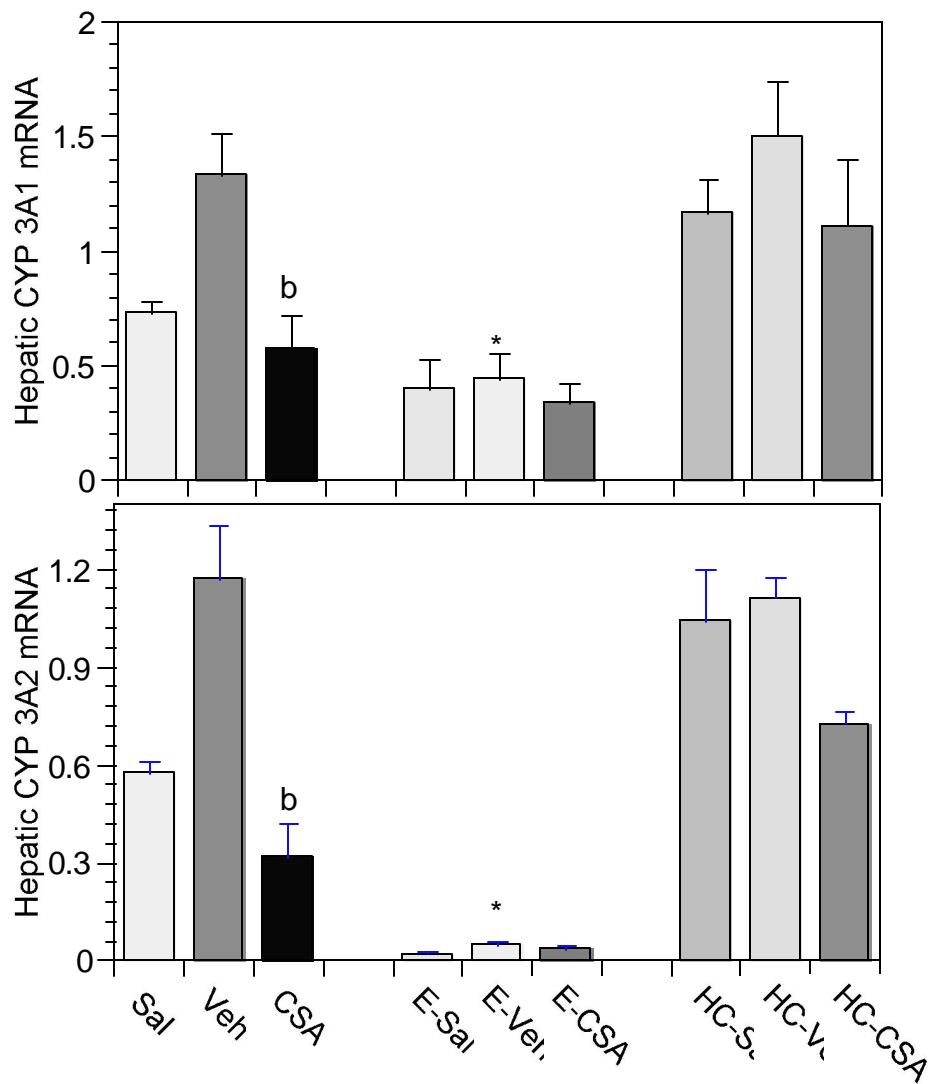


Figure 5.9 Effect of CSA on Hepatic CYP3A1/2 mRNA in LDL-R Induced or Suppressed Rats

Hepatic CYP3A mRNA was isolated from rats following treatment with saline, vehicle or CSA in LDL-R normal, induced, or suppressed rat models. Units are arbitrary.

<sup>b</sup> P<0.05 as compared with corresponding Veh controls, \* P<0.05 as compared with treatment-matched controls.

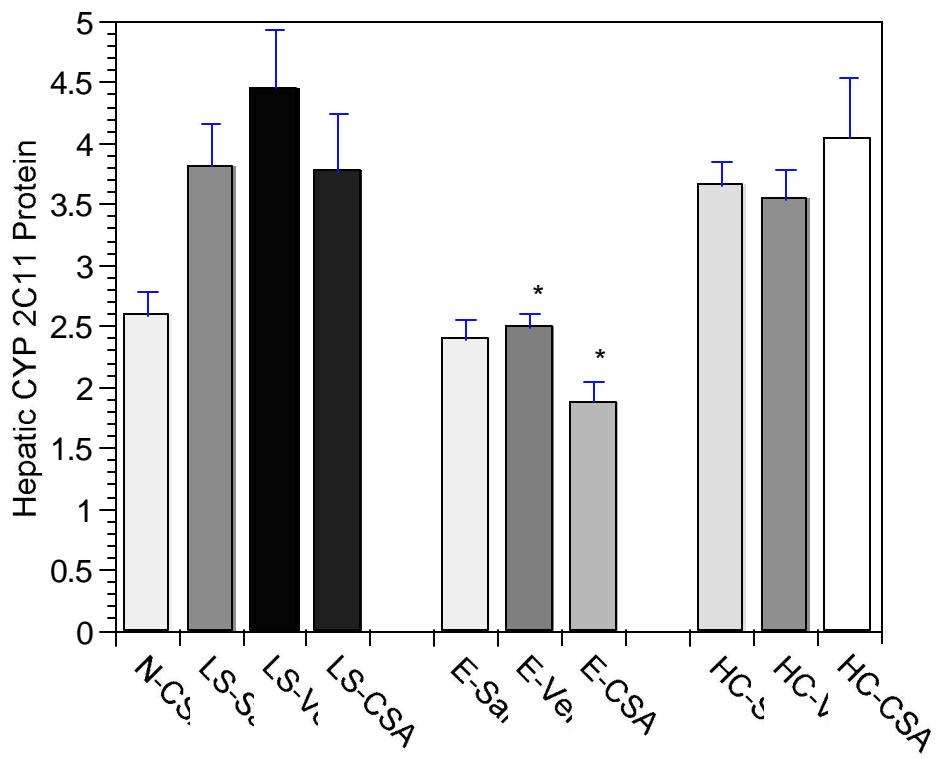


Figure 5.10 Effect of CSA on Hepatic CYP2C11 Expression in LDL-R Induced or Suppressed Rats

Immunoblot analysis with hepatic CYP2C11 microsomes isolated from rats following subcutaneous injection of saline, vehicle, or CSA for 28 days in hepatic LDL-R normal, induced or suppressed rat models. Units are arbitrary.

\* P<0.05 as compared with treatment-matched controls.

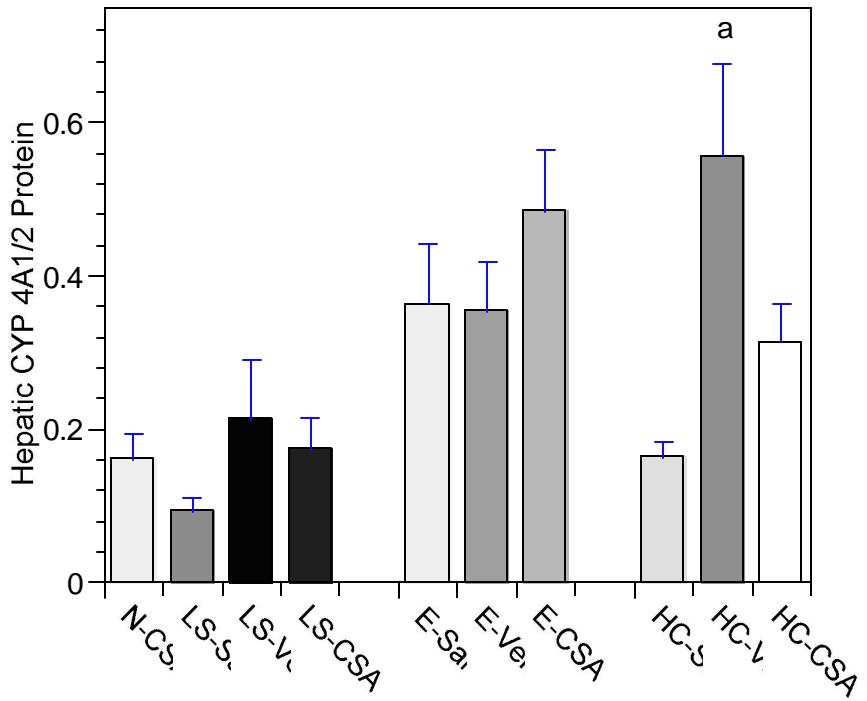


Figure 5.11 Effect of CSA on Hepatic CYP4A Protein Expression

Hepatic microsomes isolated from rats given a subcutaneous injection for 28 days.  
Units are arbitrary. <sup>a</sup>P<0.05 as compared with corresponding Sal control.

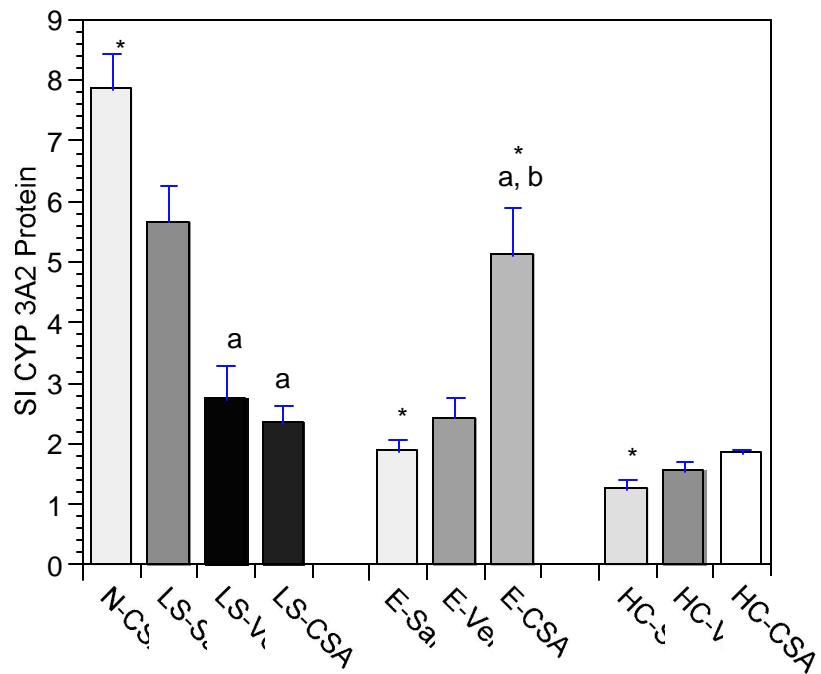


Figure 5.12 Effect of CSA on Intestinal CYP3A Proteins in LDL-R Modified Rat

Immunoblot analysis of CYP3A2 and CYP2C11 from intestinal microsome isolated from rats following by saline, vehicle and CSA treatment in LDL-R induced or suppressed and normal rat models. Units are arbitrary. <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \*P<0.05 as compared with treatment-matched controls.

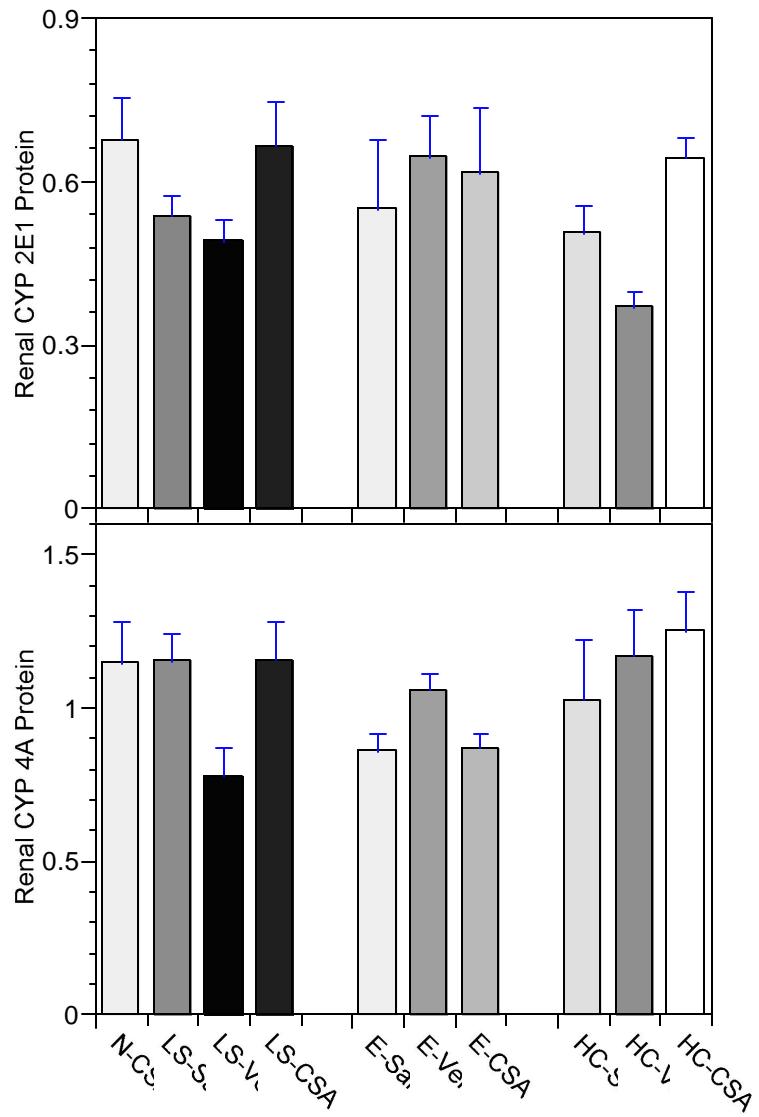


Figure 5.13 Effect of CSA on Renal CYP2E1 and CYP4A Proteins in LDL-R Modified Rats

Immunoblot analysis of CYP2E1 and CYP4A from renal microsomes isolated from rats following saline, vehicle, or CSA treatment in hepatic LDL-R normal, induced or suppressed rat models. Units are arbitrary.

### **5.3.6 *In Vitro* Metabolic Activity**

The regio- and stereospecific *in vitro* testosterone hydroxylation was performed to monitor the metabolic activities of the specific CYP isoforms. In agreement with CYP3A protein expression, all CSA treatments group had a decreased production of  $6\beta$ - and  $2\beta$ -hydroxytestosterone, markers of CYP3A activity, as compared with vehicle-matched controls (Figure 5.14). The metabolic activity results were consistent with protein and mRNA expression.

All of the LDL-R induced rats (EE-treated) had suppressed  $6\beta$ - and  $2\beta$ -testosterone hydroxylase activities. In comparison with LS-CSA, EE-CSA diminished the formation by 66% ( $P=0.5045$ ) and 50% ( $P=0.6409$ ), whereas HC-CSA increased it by 82% ( $P=0.4057$ ), and 111% ( $P=0.3040$ ) indicating either high cholesterol or LDL-R resulted in a decreased inhibition of CYP3A. the administration of LS-CSA resulted in a greater suppression of both  $6\beta$ - and  $2\beta$ -OHT demonstrating less inhibition of CYP3A in comparison to N-CSA treated rats (Figure 5.14).

$2\alpha$ - and  $16\alpha$ -OHT are effective markers of CYP2C11 catalytic activity. All EE treated rats were found to have significantly decreased formations of both testosterone metabolites (Figure 5.15)  $16\beta$ - and  $7\alpha$ -OHT formations in all LDL-R induced groups were significantly increased, indicating an induction of CYP 2B1/2 and CYP 2A1/2, respectively (Figure 5.16 and Figure 5.17). EE treatment increased the basal production level of  $16\beta$ -OHT 3 fold and  $7\alpha$ -OHT 4.5 folds

(EE-Sal vs LS-Sal). Moreover, a 3-fold increase in the production of  $16\beta$ -OHT was seen in CSA-treated rats in the LDL-R induced group. LS-CSA and EE-CSA caused a suppression of  $16\beta$ -OHT production by 51% ( $P<0.001$ ) which is in conflict with the results of Prueksaritanont (Prueksaritanont et al., 1993). There are two factors to be considered between two studies. One is low sodium diet and the other is that his study administered EE for 5 days and scarified rats whereas our study design was conducted for 28 days with a half dose given every the other day. Both are plausible possibilities. In our experiment, low sod seemed to have an effect on CYP2B activities since N-CSA showed a 2 fold increase of  $16\beta$ -OHT production ( $P=0.0241$ ) although it was not significant. The idea that a regulation of EE is time-dependent has not been reported yet (Figure 5.16)

Unexpectedly, CSA treatment caused a decrease in the production of  $7\alpha$ -OHT (Figure 5.17). Since hepatic CYP2A1 is the female-dominant isoform and CSA is known to cause demasculinization of hepatic protein expression, hepatic CYP2A1 activity was expected to be increased. The formation of androstanedione from testosterone, which is mainly catalyzed by CYP2C11 and CYP2A2, showed the same trend as  $16\alpha$ -OHT formation. Those results are in agreement with previous report by Prueksaritanont (Figure 5.9) (Prueksaritanont et al., 1993).

In intestinal CYP3A protein expressions, all CSA treatment resulted in a increase in as compared with vehicle-matched controls, however the LS-CSA group showed an induction of CYP3A2 activities ( $6\beta$ -OHT) by more than 2-fold

which is different from the protein expressions (Figure 5.18). It has been reported that testosterone (16 $\beta$  position) is the most specific substrate of the CYP2B family in rats (Figure 5.14). Due to CYP2B low turnoverrate, it is not considered to be useful for humans (Waxman et al., 1991).

### **5.3.7 CSA blood Concentration**

EE-CSA treated rats showed a statistically significant decrease in CSA blood concentration by 45% ( $P=0.020$ ), 53% ( $P<0.001$ ), and 48% ( $P=0.006$ ) as compared to HC-CSA, LS-CSA, and N-CSA treated rats, respectively (Fugure 19). HC-CSA treated rats did not show any differences as compared to LS-CSA or N-CSA treated rats.

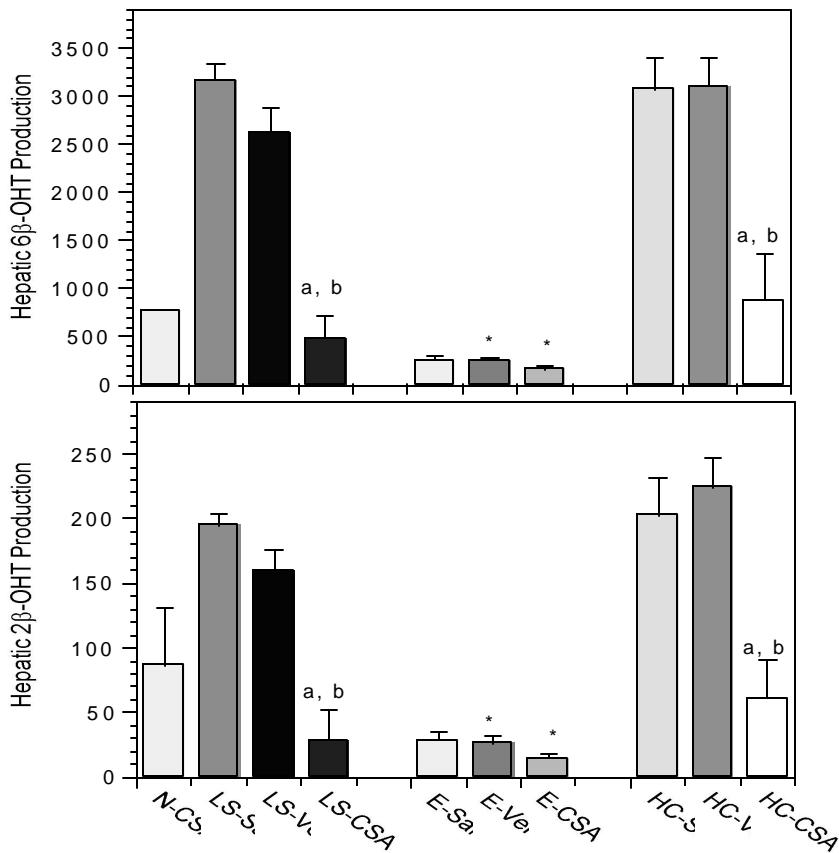


Figure 5.14 Effect of CSA on Hepatic *In Vitro* Testosterone 6 $\beta$ -, 2 $\beta$ -hydroxylase Activity in LDL-R Induced or Suppressed Rats

Hepatic 6 $\beta$ - 2 $\beta$  hydroxytestosterones, CYP3A2 activity markers, were measured with microsomes isolated from rats given a subcutaneous injection for 28 days.

250  $\mu$ M testosterone were incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone were used as an internal standard. <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \* P<0.05 as compared with treatment-matched controls. Units of catalytic activity are pmol/min/mg protein.

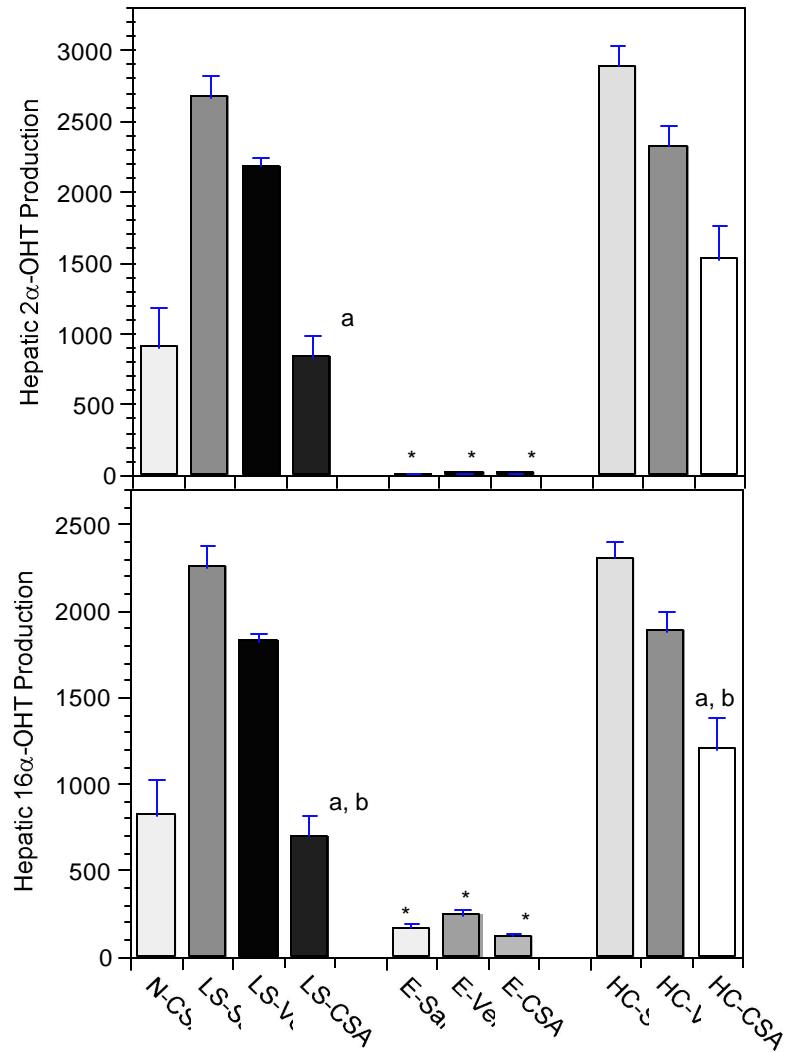


Figure 5.15 Effect of CSA on Hepatic *In Vitro* Testosterone 2 $\alpha$ -, 16 $\alpha$ -hydroxylase Activity in LDL-R Induced or Suppressed Rats

Hepatic 2 $\alpha$ -, 16 $\alpha$ -hydroxytestosterones, CYP2C11 activity markers, were measured with microsomes isolated from rats given a subcutaneous injection for 28 days. 250  $\mu$ M testosterone were incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone were used as an internal standard.

<sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \*P<0.05 as compared with treatment-matched controls. Units of catalytic activity are pmol/min/mg protein.

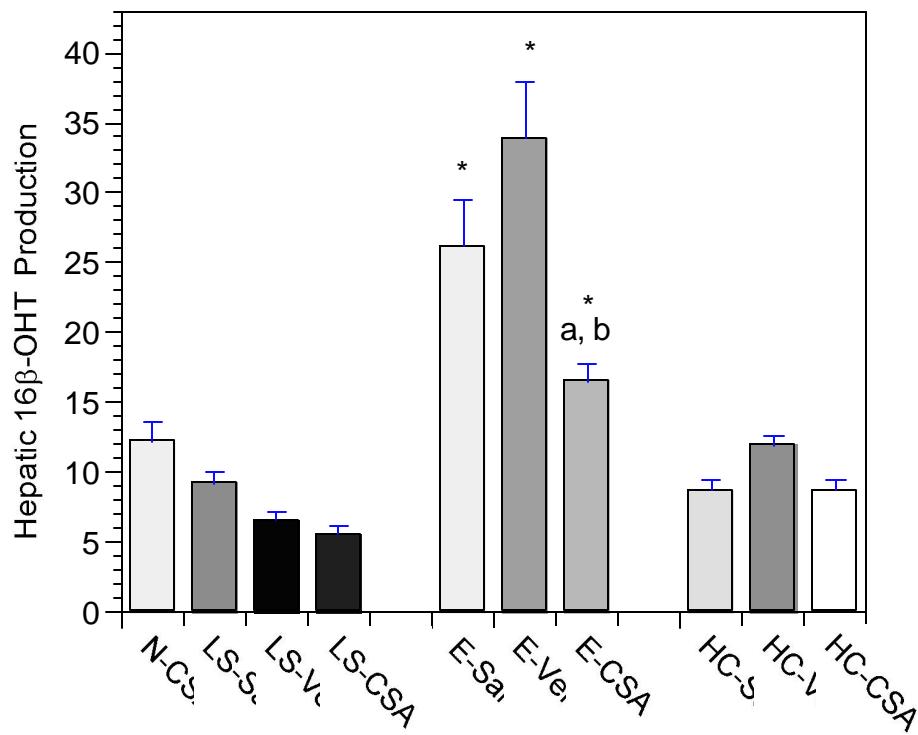


Figure 5.16 Effect of CSA on Hepatic *In Vitro* Testosterone 16 $\beta$  hydroxylase Activity in LDL-R Modified Rats

Hepatic 16 $\beta$ -hydroxytestosterones, CYP 2B1/2 marker, were measured with microsomes isolated from rats given a subcutaneous injection for 28 days. 250  $\mu$ M testosterone were incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone were used as an internal standard. <sup>a</sup>P<0.05 as compared with corresponding Sal controls, <sup>b</sup>P<0.05 as compared with corresponding Veh controls, \*P<0.05 as compared with treatment-matched controls. Units of catalytic activity are pmol/min/mg protein.

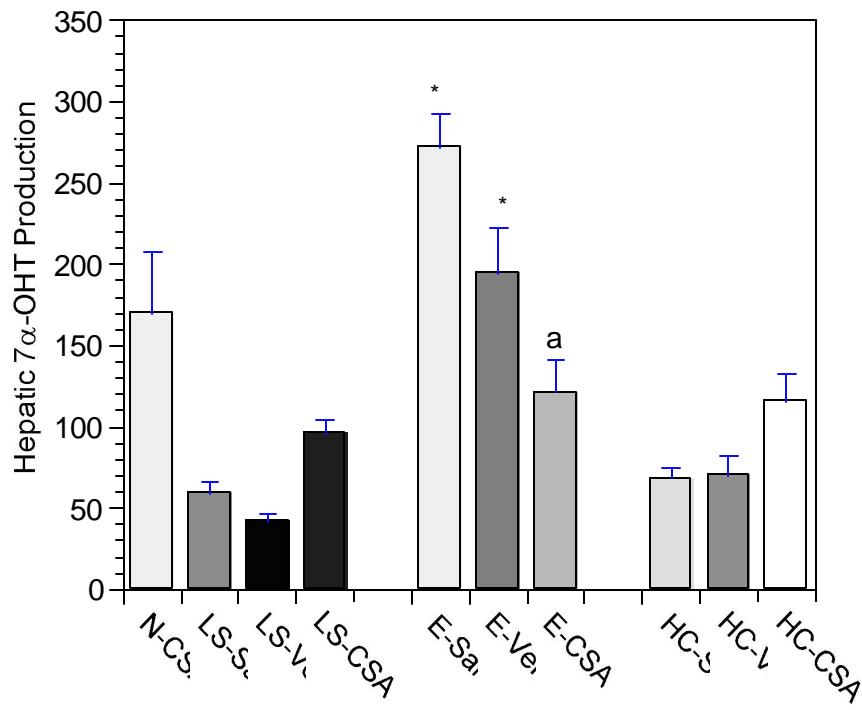


Figure 5.17 Effect of CSA on Hepatic *In Vitro* Testosterone 7 $\alpha$ -hydroxylase Activity in LDL-R Modified Rats

Hepatic 7 $\alpha$ -hydroxytestosterones, CYP 2A1/2 marker, were measured with microsomes isolated from rats given a subcutaneous injection for 28 days. 250  $\mu$ M testosterone were incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone were used as an internal standard  $^aP<0.05$  as compared with corresponding Sal controls,  $*P<0.05$  as compared with treatment-matched controls. Units of catalytic activity are pmol/min/mg protein.

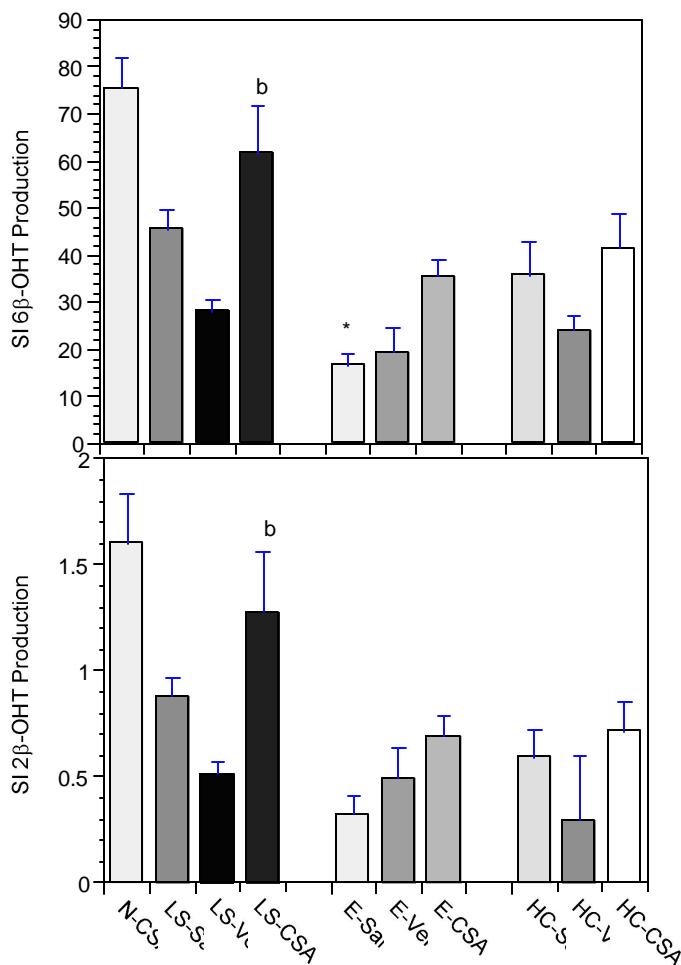


Figure 5.18 Effect of CSA on Intestinal *In Vitro* Testosterone 6 $\beta$ -, 2 $\beta$ -hydroxylase Activity in LDL-R Modified Rats

Intestinal 6 $\beta$ - 2 $\beta$  hydroxytestosterones, CYP3A2 activity markers, were measured with microsomes isolated from rats given a subcutaneous injection for 28 days. 250  $\mu$ M testosterone were incubated for 15 min in regenerating system and 3.6 nmol of 11 $\alpha$ -hydroxyprogesterone were used as an internal standard. <sup>b</sup> P<0.05 as compared with corresponding Veh controls, \*P<0.05 as compared with treatment-matched controls. Units of catalytic activity are pmol/min/mg protein.

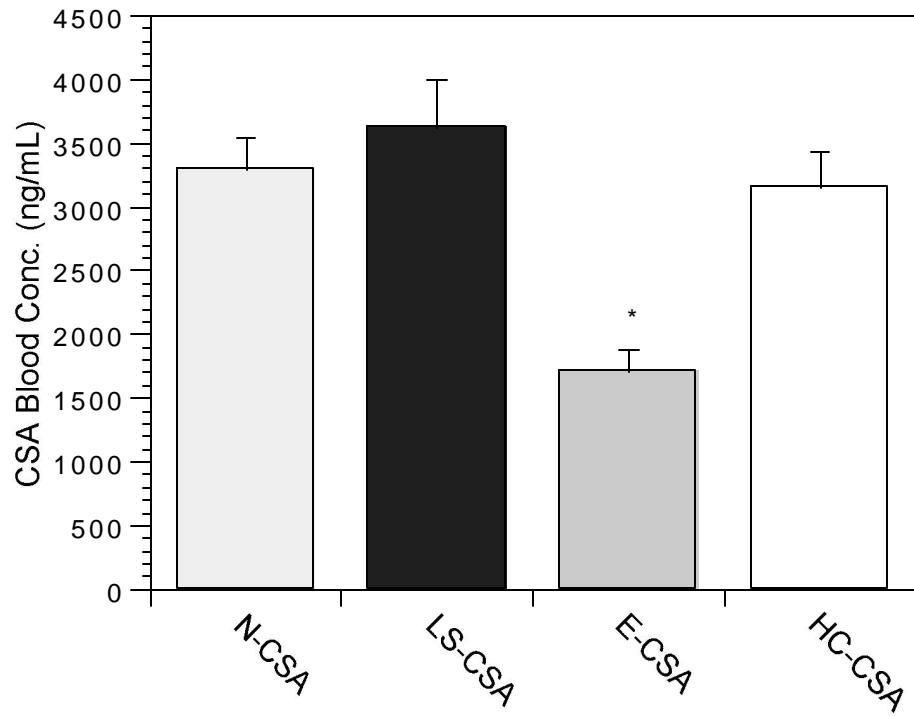


Figure 5.19 Comparison of Steady-State Whole Blood CSA Concentration

CSA concentration in whole blood was measured using *Monoclonal antibody-mediated fluorescence polarization immunoassay (FPIA)*, following 28 days of CSA subcutaneous administration. Units are ng/mL. \*P<0.001 as compared LS-CSA.

### **5.3.8 Effect of GH on Receptors**

Lipoprotein receptors are one of many integrated transport systems that continuously carry endogenous and exogenous lipid (Bilheimer et al., 1984). Following immunosuppressant treatment, changes in lipids levels are seen mainly as increasing plasma LDL-cholesterol levels (De Groen et al., 1987; De Groen, 1988; Brunner et al., 1990a). However the correlation has not been clear. In addition, in vivo and in vitro studies have shown a conflict in regulation. Our recent study showed that rats had sexually dimorphic regulation of hepatic LDL-R (Figure 5.20). Hepatic LDL-R mRNA levels in female rats were 3.7 folds higher ( $P=0.0025$ ) than that of male rats. Hypophysectomy resulted in a suppression of LDL-R mRNA levels, by 51% ( $P=0.0017$ ) in female rats, and an 80% ( $P=0.0819$ ) induction in male rats. Examination of SR-BI mRNA levels showed no differences between groups (Figure 5.21). This study indicated that LDL-R regulation is sex dependent and GH is one of the factors that affect the regulation.

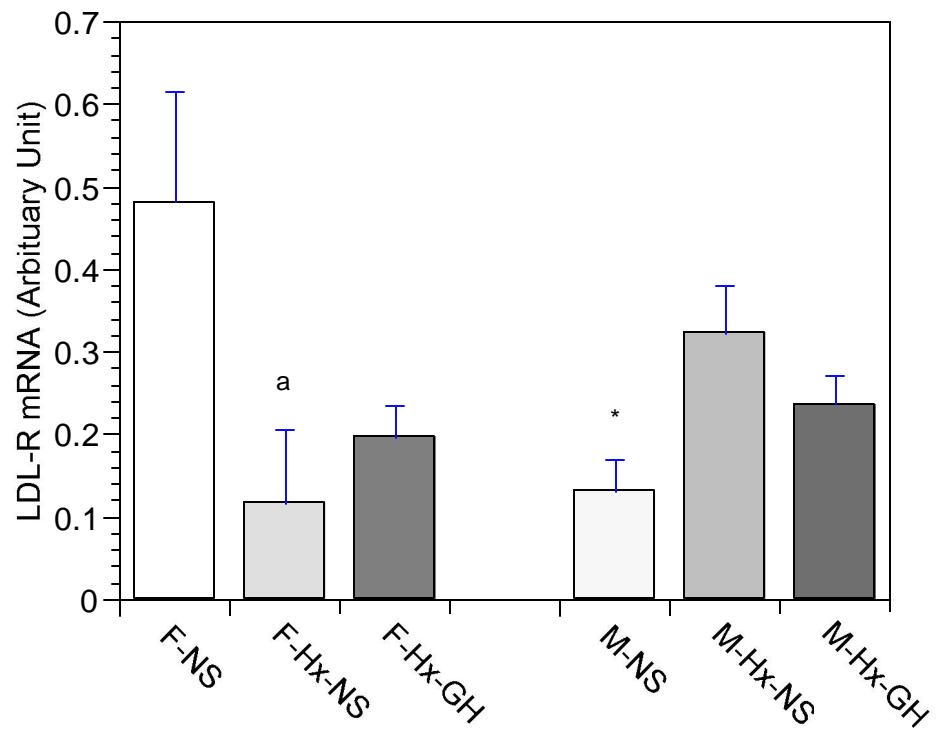


Figure 5.20 Effect of GH on Hepatic LDL-receptor mRNA Expression

100 mg of liver was used to isolate mRNA for RT-PCR. <sup>a</sup>P<0.05 as compared with respective controls, \* P<0.05 as compared with sex-matched controls. Units are arbitrary.

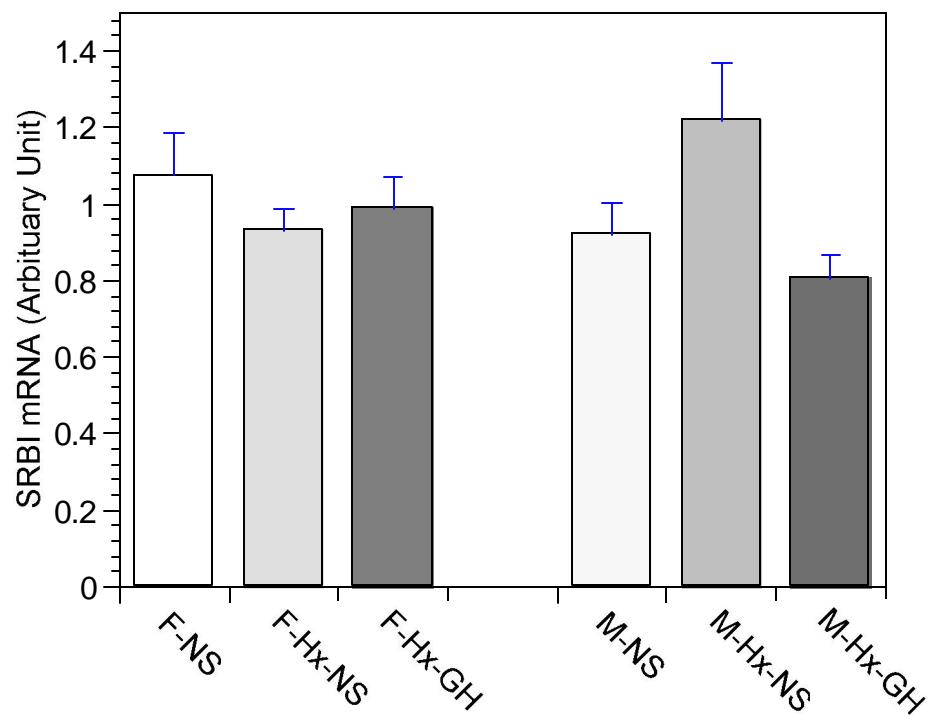


Figure 5.21 Effect of GH on Hepatic SR-BI mRNA Expression

100 mg of liver was used to isolated mRNA for RT-PCR. Units are arbitrary.

## **5.4 DISCUSSION**

In this study, we investigated 1) whether or not LDL-Rs alter the metabolic response and toxicity, 2) whether or not CSA regulates LDL catabolism, and 3) whether or not LDL-Rs are modulated by growth hormone.

LDL-R levels were modified by 17 $\alpha$ -ethynodiol (EE) administration and 2% cholesterol diet (Kovanen et al., 1979; Ma et al., 1986; Srivastava, 1996; Lopez and Ness, 1997; Parini et al., 1997). EE is the major estrogen hormone in oral contraceptives, which is metabolized by 2-hydroxylation (Guengerich, 1990). EE is a CYP suicide inactivator both in vivo and in vitro (White and Muller-Eberhard, 1977). Although drug-drug interactions were possible in the groups that were administered EE, EE was still used because it is the most effective drug to induce LDL-R in rats (Kovanen et al., 1979). Therefore, each pretreatment group has corresponding controls, which were dosed with either saline or vehicle, in order to verify that any effects seen were the result of drug and not vehicle treatment.

The regulation of LDL-R by a cholesterol diet is both species- and strain-dependent (Roach et al., 1993). Several studies have shown that the rat is remarkably resistant to hypercholesterolemia when fed a high cholesterol diet, and in some cases a cholesterol diet can upregulate LDL-receptors. (Horton et al., 1995; Pitman et al., 1998; Gatto et al., 2001) In the case of Sprague-Dawley rats,

LDL-Rs are efficiently downregulated at the transcriptional level (Roach et al., 1993). It has also been reported that increases in LDL-R activity occurs with polyunsaturated fats (i.e. linoleic acid) (Spady et al., 1995) indicating a different pathway of LDL regulation.

In comparison to the respective vehicle controls, CSA treatment resulted in increased LDL cholesterol levels in both the normal LDL-R and the induced LDL-R rats. Increased LDL cholesterol levels are often seen in patients receiving CSA therapy. We also found that in induced LDL-R rats, there was an increase in VLDL cholesterol levels, indicating that CSA caused either an increased synthesis of VLDL cholesterol or an inhibition of VLDL clearance. Kaptein also reported the same result and suggested that the inhibition of LDL catabolism caused an increase in VLDL levels (Kaptein et al., 1994).

It has been previously reported that 1% ethanol levels *in vitro* causes an increase in triglycerides, unesterified cholesterol, cholesteryl ester, ApoA and ApoE (Dashti et al., 1996). Although the CSA vehicle contains 12.5% ethanol, the amount given to rats receiving treatment is below the levels to have an effect on lipids. Therefore increased LDL cholesterol levels were likely not due to ethanol.

A chief concern during this study was salt intake. We used a low-sodium rat model in order to induce histological changes seen with chronic CSA treatment. CSA nephrotoxicity is characterized by chronic tubulointerstitial injury. (Gerkens et al., 1984; Burdmann et al., 1993) The LS-Sal treatment caused

a decrease in total cholesterol, as well as LDL-cholesterol. This result is different from a report that very low sodium intake (<50 mmol/d) is associated with increased levels of total and LDL cholesterol (Egan and Lackland, 2000). This discrepancy is likely to be the result of different lengths of treatment. The previous study investigated rats on sodium restriction for 7 days, whereas our rats were on a low sodium diet for a total of 34 days (acclimation and study period combined). CSA treatment in both the normal diet and the low sodium diet groups resulted in increased total cholesterol levels primarily as a result of increased LDL levels (Table 5.3).

Although it was not statistically significant, an HC diet resulted in a downregulation of hepatic LDL-R mRNA whereas EE pretreatment resulted in an upregulation of LDL-R mRNA (Figure 5.2). HC diet and EE pretreatment resulted in contrasting regulation of hepatic SR-BI mRNA (Figure 5.5). Hepatic LDL-R normal rats had higher HDL cholesterol levels, but pretreatment caused an increase in LDL cholesterol levels (Table 5.5). An increase in LDL cholesterol along with a slight increase in renal LDL-R was seen following CSA treatment, indicating that CSA upregulates LDL-R *in vivo* (Figure 5.4). This data contradicts previous *in vitro* data (Al Rayyes et al., 1996; Al Rayyes et al., 1997). On the other hand, CSA treatment in high-cholesterol fed rats did not change the level of LDL-R mRNA as compared with vehicle controls. In addition, no signs of nephrotoxicity and hepatotoxicity were seen (Table 5.3-4). This study suggests

that the effect of CSA on LDL-R is saturable. LDL-R induced rats treated with CSA showed signs of significant renal toxicity and hepatotoxicity due to induced renal LDL-R.

EE pre-treatment resulted in a pronounced decrease in CYP3A, and CYP2B protein expression as well as a decrease in 6 $\beta$ -, 2 $\beta$ -, and 16 $\beta$ -hydroxylase activities. A significant decrease of CYP2C11 function, as monitored by the selective probes 2 $\alpha$ - and 16 $\alpha$ -hydroxylase, was also observed (Pichard et al., 1990; Prueksaritanont et al., 1993) (Figure 5.14-17). Our study showed a disagreement with the results Prueksaritanont reported (Prueksaritanont et al., 1993). They reported a decrease in CYP2B1/2 activities (16 $\beta$ -OHT formation) after 5 days of a 5 mg/kg/day treatment of EE in rats fed a standard diet. However, all EE-pretreatment groups in our study showed an induction. Different experimental factors between the two were dosing amount, period and time rhythm. We dosed our rats with 5 mg/kg of EE for the first 4 days then reduced the dose to 2 mg/kg of EE every other day for 28 days. Prueksaritanont et al reported results following a short term treatment (5 days) of a 5 mg/kg dose of EE. In addition, our dosing time was between 5:00 p.m. to 6:00 p.m., whereas Prueksaritanont dosed rats between 9:30 a.m. to 10:30 a.m.

Our purpose of dosing time was to avoid any unexpected drug-drug interaction with CSA. CSA was dosed every 8:00 a.m. In lipid metabolism, estrogen treatment appears to show diurnal variation (Erickson et al., 1989).

However, none of these studies provide concrete evidence to support the effect on CYP2B by EE. While this *in vivo* study showed a suppression of CYP2B activity, an *in vitro* study showed the induction of CYP2B mRNA (Kocarek et al., 1994). We also found that CSA with EE pretreatment resulted in a decline in CYP2A1 activities (7 $\alpha$ -OHT formation), a female-dominant isoform, suggesting a possible inhibitory effect by a combination with a thyroid hormone (Arlotto and Parkinson, 1989). Overall, high cholesterol fed rats showed less inhibitory effect on the activities of CYP3A, CYP2B, and CYP2A2, suggesting the possibility of a higher blood concentration of CSA.

The regulatory effect of CSA in the small intestine was decreased in comparison to the liver (Figure 5.12, Figure 5.14). In the liver testosterone metabolite production was 300 times greater than in the intestine. In addition, metabolism in the small intestine is independently regulated in comparison to the liver.

CYP2E is known to be positively correlated with both plasma cholesterol and HDL cholesterol (Boucher et al., 2000). In addition to causing an increase production of LDL oxidation in a time dependent manner (Aviram et al., 1999). We examined relationship between renal toxicity and renal CYP2E and found that renal microsomes showed no difference in regulation of CYP2E1 between groups (Figure 5.13). We also investigated renal CYP4A, which contributes to lipid metabolism, no difference in regulation between groups

(Figure 5.13). These results indicate that alterations of lipid metabolism do not affect renal CYP2E1 and CYP4A expression.

A 45% increase in the LDL-R mRNA expression was seen in CSA-treated rats with a low sodium alone (Figure 5.3). This result conflicted with the result reported by Vaziri et al (Vaziri et al., 2000). The lack of changes in LDL-R expression may be due to a compensatory effect in response to chronic CSA treatment. Another possibility may be related to the dosing amount. The Vaziri's study group dosed 18 mg/kg of oral CSA, this is a much lower dose than normally used especially considering the low BA or associated with the use of the oral formulation. This lower dose may not cause toxicity or have the same immunosuppressive effects as higher doses. *In vitro* experiments have shown CSA to have an inhibitory effect on LDL catabolism, decreasing LDL receptor activity in HepG2 cells by 25% (Al Rayyes et al., 1996). These results suggest an indirect effect of CSA on LDL-Rs.

Chronic administration of oil-based vehicle triggered an increase in the levels of LDL-R in HC fed rats (Figure 5.4). In LDL-R suppressed models, no effect of CSA on the level of LDL-R mRNA was seen. However, the total level of cholesterol primarily as a result of LDL-cholesterol was increased in all treatment groups of CSA-treated rats, indicating an upregulation of LDL-R by an independent pathway. Renal LDL-R mRNA showed a different regulatory pathway as compared with hepatic LDL-R, indicating there is tissue dependent

regulation (Figure 5.6-7). These levels of renal LDL-R mRNA are associated with nephrotoxicity, whereas the levels of hepatic LDL-R are not. The suppression of renal LDL-R mRNA by HC diet was seen, however the induction of renal LDL-R mRNA by EE administration was not seen in comparison with the saline control. The levels of these receptors appear to be associated with CSA-induced nephrotoxicity measured by creatinine clearance. The higher variability of renal LDL-R mRNA, in the induced LDL-R groups, may be attributed to differences in regulation resulting from the EE pretreatment. Lipid variation is often seen in patients following CSA treatment. This study suggests that when hepatic LDL-Rs are saturated, the CSA bound to LDL is taken up into kidney leading to drug-induced renal toxicity. This uptake is mediated by the renal LDL-Rs.

In addition, Both the LS-CSA and E-CSA treatment groups had significant nephrotoxicity in comparison with respective two control groups, (Table 5) while the HC-CSA groups did not show any signs of renal toxicity as compared with HC-Sal or HC-Veh. The high levels of total cholesterol in this group may be holding CSA in the blood and reducing LDL receptors in each tissue, resulting in CSA having a decreased toxic effect on cells. In saline- and vehicle-treated rats in the LDL-R induced groups, serum creatinine levels were not changed. However creatinine clearance was significantly reduced as compared with those of rats in the normal low-sodium diet group. These results indicate that 17 $\alpha$ -EE also induced nephrotoxicity.

In order to examine an indirect effect on LDL-R, the effect of GH on LDL-R was monitored using hypophysectomized rats. This animal model used to study the specificity of GH effect in reduction of various physiological or endocrine parameters. The amount of CYP is appeared to be different between sex in rats. Male rats have more P450 in liver, excess of 10-30% and some cases the oxidation rate is up to 20 times higher (Chung, 1977; Kobliakov et al., 1991; Kobliakov et al., 1993). Human also exhibit sex differences in drug metabolism by different regulation on hepatic monooxygenases (Redmond et al., 1980; Liddle et al., 1994; Cheung et al., 1996). Our model drug, CSA is known to regulate CYP P450s in sex dependent manner by feminizing GH pattern.

Our study indicates that the regulation of LDL-R is sex dependent and GH is one of the factors that regulate LDL-R (Figure 5.20). However, GH has no effect on SR-BI (Figure 5.21). A similar effect was observed by Rudling et al. with human growth hormone treatment in rats (Rudling et al., 1992; Rudling et al., 1996). Several hormonal regulatory effects on LDL-R activity and mRNA levels have previously been reported. Estrogen has been shown to effect on a stimulatory effecton hepatic LDL-R mRNA levels in normal rats (Kovanen et al., 1979; Rudling et al., 1992) whereas it has little effect in hypophysectomized rats (Rudling et al., 1992). In addition, it was shown that endogenous estrogens did not control LDL-Rs. Treatment with anti-estrogen agent (tamoxifen or clomiphen) in intact rats or the removal of endogenous estrogen by surgery (ovariectomized

rat) did not have an effect on LDL-R levels (Parini et al., 1997; Parini et al., 2000). However, LDL-R mRNA levels were increased by estrogen in the presence of human GH in hypophysectomized rats (Kovanen et al., 1979; Rudling et al., 1992). Aging and weight loss in rats is negatively correlated LDL-R mRNA levels and increasing plasma cholesterol levels (Walker et al., 1994). Insulin (100 mU/ml) and thyroid hormones also appeared to have a positive effect on LDL-R mRNA (Steinberg et al., 1967; Wade et al., 1989; Ness and Lopez, 1995).

It has been previously shown that when animals with chronic renal failure and nephrotic syndrome are treated with CSA there is a significant down-regulation of lipoprotein lipase, this condition is associated with hypertriglyceridemia and impaired TG-rich LP clearance thus leading to the accumulation of VLDL (Vaziri et al., 2000). VLDL concentrations are known to occur in CSA treated patients as a result of lipoprotein lipase deficiency.

It has been reported that after a single dose of CSA there were increased CSA levels in HDL, while the CSA levels in LDL were increased after chronic dosing of CSA (Yau et al., 1991; Luke et al., 1992). Furthermore, patients with chronic renal failure showed increased CSA levels in HDL after chronic therapy suggesting that lipoproteins have specific roles when bound to CSA.

A significant finding of this study was that CSA treatment resulted in the induction of hepatic LDL-Rs initially, but chronic treatment did not have an effect on hepatic LDL-Rs as a result of saturation. As a result of hepatic LDL-R

saturation, probably there is an increase in circulating CSA levels, which could result in an induction of renal LDL-Rs. Therefore, an induction of LDL-Rs will result in a greater uptake of CSA by the liver. CSA will therefore be more likely to get metabolized and circulating CSA levels will be decreased, primarily in LDL fractions.

With chronic CSA treatment LDL-Rs become saturated, therefore the excess CSA bound to LDL, will not be taken up by the liver. This results in a decreased metabolism of CSA by a decreased suppression of CYP3A. The CSA that is not metabolized most likely accumulates in circulation and can result in drug-induced toxicity to other tissues. CSA treatment in hepatic LDL-R induced rats (E-CSA) caused a decrease in CSA blood concentration by 53% and 48% as compared with LS-CSA and N-CSA treatment, respectively (Figure 12). This is due to the induction of hepatic LDL-R.

It was hypothesized that the high levels of total cholesterol in the LDL-R suppressed group may be sequestering CSA in the blood and causing a reduction of LDL receptors in each tissue, resulting in CSA having a decreased toxic effect on cells. However, CSA treatment in LDL-R suppressed rats showed only a slight decrease in CSA blood concentration, although these rats showed a significant increase in lipid level. Considering that the level of LDL-R mRNA in the HC-CSA group was lower than the mRNA level in the LS-CSA group, indicating that drug metabolism is dependent on hepatic LDL-R availability rather than lipid

levels in the blood. As a result of hepatic LDL-R saturation, kidney had more chance to take up CSA by LDL-R and resulted in an induction of renal LDL-Rs.

We found that 28 days of CSA administration in normal and induced LDL-R rats caused an induction of renal LDL-Rs. The higher levels of CSA in HDL fractions, seen in patients with acute renal failure are the result of an induction of renal LDL-R. Our findings from this study help to clarify the dyslipidemia which is often seen in patients following CSA treatment. In addition, this study aids in the prediction of the pharmacological and toxicological effects seen with CSA treatment.

In summary, this study indicated that CYP3A was inhibited at the posttranscriptional level in LDL suppressed rats fed a high cholesterol diet, whereas it was inhibited at the transcriptional level in LDL induced or normal rats. CSA caused an increased LDL-R mRNA levels in intact rats but both LDL-R modulated rats did not show any effects, suggesting an increased receptor-independent pathway for lipid catabolism. Nephrotoxicity appeared to be positively associated with the level of renal LDL-R mRNA. The level of LDL-R mRNA appeared to be sexually dimorphic and regulated, in part, by GH. These data may provide a better understanding of abnormal lipid episodes and changes in immunosuppressive activity in organ transplant patients after CSA therapy.

## **CHAPTER SIX**

### **Summary and Conclusions**

CSA has potent immunosuppressant effects on both humoral and cellular systems. The use of CSA has made transplant treatment much easier, however, the long term consequences of suppressing immune function are not yet clear.

In general, intravenous CSA administration is required during the initial post-transplantation period and up to several weeks afterwards to prevent disruption of the gastrointestinal tract by chemotherapeutic agent and total body irradiation. The current available intravenous formulation contains a renal toxic polyoxylated castor oil, thus limiting its use. New approaches have been demanded to improve the usefulness of the parental dosage form that can be also applied to the other lipid soluble agents.

The pharmacokinetics and toxicity of lipid soluble drugs, such as CSA, are influenced by lipoproteins due to their hydrophobic properties. Previous research has shown that the distribution and toxicity of CSA in human is closely related to the lipoprotein components, particularly LDL in human. Therefore, the levels of plasma LDL along with CSA pharmacokinetic parameters may be important in monitoring CSA therapy. To date, abnormal lipid metabolism by chronic CSA treatment is not fully understood. Majority of LDL fractions are cleared by two

pathways, either by LDL-R-dependent or LDLR-independent pathway from the body. This is especially true in the liver. Since the major pathway of clearance is the LDL dependent pathway, the availability of LDL receptors is considered to be the most important factor.

Our study was conducted based on the hypothesis that CSA metabolism is modulated by LDL-R. We examined the metabolic and toxic effect of lipoprotein-associated CSA at low and high concentrations. We also investigated the effect of LDL receptors on CSA metabolism using LDL-R modified rat models.

A 0.1 mg/kg/d dose of CSA resulted in the suppression of CYP3A2 and CYP2C11 proteins and activities in plasma- and HDL-CSA (0.1 mg/kg/d)-treated groups. These suggested a possible synergistic effect with lipoproteins in the liver as well as an improved uptake into cells, possibly mediated by lipoprotein receptors. In addition, plasma-CSA-treated rats showed a higher probability of renal toxicity, possibly due to protein levels in plasma vehicle.

When CSA was dosed at 10 mg/kg/d, CYP3A activities were reduced in all lipoprotein associated CSA groups were reduced, except the VLDL-CSA treated group. This was attributed to a decrease in CYP3A1 and CYP3A2 mRNA. VLDL-CSA treated rats did not show a suppression of the CYP3A2 protein expression, activity or mRNA expression. These suggest there was a higher blood concentration of CSA as a result of sustained exposure of CSA to the hepatic cells. The sustained exposure may be the result of the construction of a stable

formation of CSA in the circulation, thus resulting in a decreased exposure to CYP enzymes. VLDL-CSA treatment did not alter lipoprotein receptors, while LDL-CSA treatment caused a significant suppression of LDL-R mRNA levels. Therefore, VLDL as a drug carrier could be beneficial to patients who develop toxicity in addition it offers the possibility of a new treatment with a reduced dose. SR-BI levels in VLDL-CSA treated rats showed the induction of protein and mRNA expression, indicating the benefit of SR-BI in treatment.

Serum lipid levels appeared to be negatively correlated with hepatic LDL-R mRNA levels whereby increased lipid levels caused less of a suppression of male specific isoforms, CYP3A and CYP2C11 as compared with CSA therapy alone. The activity of the female-dominant isoform, CYP2A1 was induced by CSA therapy but in LDL-R induced rats CSA suppressed CYP2A1 activity, indicating different regulatory mechanisms between the two metabolic effects. For instance, weight loss in the LDL-R induced model possibly caused an increase in GH basal level. CSA caused an increase in LDL-R mRNA levels in intact rats, but in both LDL-R modulated rats mRNA levels were not affected, suggesting an upregulation of a receptor independent pathway for lipid catabolism. CSA-induced renal toxicity was positively correlated with renal LDL-R mRNA levels. This study also showed that the regulation of LDL-R is sexually dimorphic and tissue-dependent. In addition, CSA caused the induction of LDL-R

due to, in part, the presence of growth hormone (GH) and an altered GH pattern of secretion.

Our studies show the difference in metabolic interactions with different lipoproteins and suggest the possibility of a new era for intravenous vehicles and the role of lipoproteins in CSA metabolism. Lipoproteins caused various hepatic metabolic changes that were partially attributed to hepatic LDL-R levels. The induction of renal LDL-R resulted in CSA-induced renal toxicity. CSA caused a saturation of LDL-R in the liver. This was partially due to GH, which leads to increases lipid levels. The present studies offer a better understanding of changes occurring in lipoprotein metabolism and immunosuppressant therapy in transplant patients.

## APPENDIX A

### Adjusted sodium (0.05%) Diet

Ingredient	g/kg
Casein, High Protein	200.0
DL-Methionine	3.0
Sucrose	507.69
Corn Starch	150.0
Corn Oil	50.0
Cellulose	50.0
Vitamin mix, Takelad (40060)	10.0
Ethoxyquin (antioxidant)	0.01
Calcium Phosphate, diabasic, CaHPO <sub>4</sub>	17.5
Potassium Citrate, monohydrate	7.7
Potassium Sulfate, K <sub>2</sub> SO <sub>4</sub>	1.82
Sodium Chloride, NaCl	1.02
Magnesium Oxide, MgO	0.84
Ferric Citrate	0.21
Manganous Carbonate	0.123
Zinc Carbonate	0.056
Chromium Potassium Sulfate , CrK(SO <sub>4</sub> ) <sub>2</sub> . 12 H <sub>2</sub> O	0.0193
Cupric Carbonate	0.011
Potassium Iodate, KIO	0.0004
Sodium Selenite, Na <sub>2</sub> SeO <sub>3</sub> 5H <sub>2</sub> O	0.0004

Ingredient from the Harlan Teklad, Indianapolis, IN)

## APPENDIX B

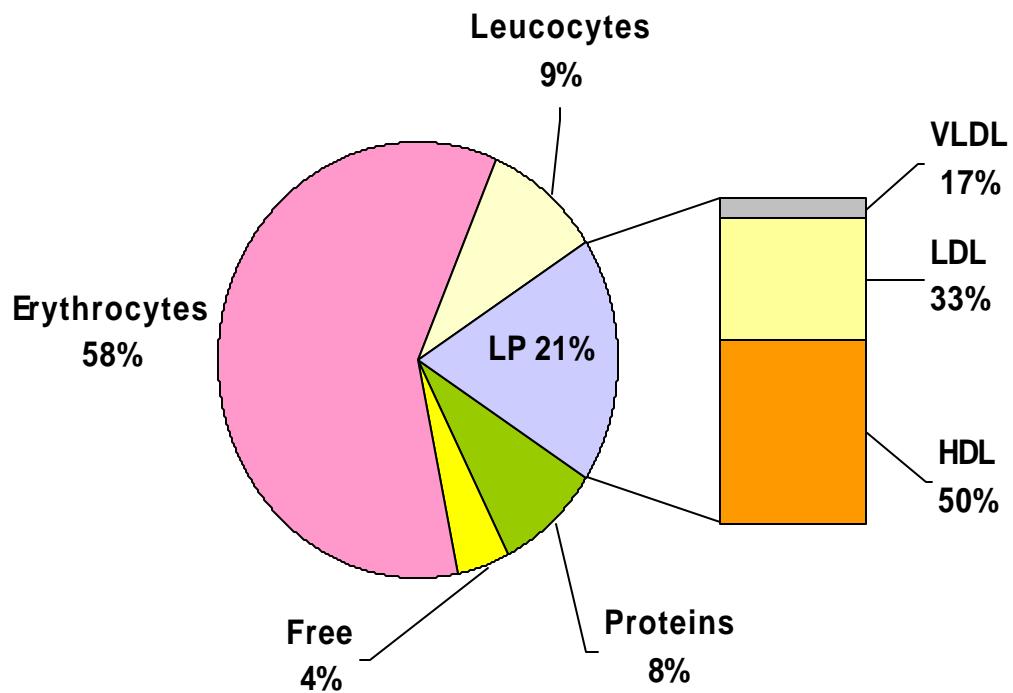
### Adjusted sodium (0.05%) Diet containing 2% Cholesterol

Ingredient	g/kg
Casein, High Protein	200.0
DL-Methionine	3.0
Sucrose	507.69
Corn Starch	150.0
Corn Oil	50.0
Cellulose	30.0
Vitamin mix, Takelad (40060)	10.0
Ethoxyquin (antioxidant)	0.01
Cholesterol	20.0
Calcium Phosphate, diabasic, CaHPO <sub>4</sub>	17.5
Potassium Citrate, monohydrate	7.7
Potassium Sulfate, K <sub>2</sub> SO <sub>4</sub>	1.82
Sodium Chloride, NaCl	1.02
Magnesium Oxide, MgO	0.84
Ferric Citrate	0.21
Manganous Carbonate	0.123
Zinc Carbonate	0.056
Chromium Potassium Sulfate , CrK(SO <sub>4</sub> ) <sub>2</sub> . 12 H <sub>2</sub> O	0.0193
Cupric Carbonate	0.011
Potassium Iodate, KIO	0.0004
Sodium Selenite, Na <sub>2</sub> SeO <sub>3</sub> 5H <sub>2</sub> O	0.0004

Both diets contain 17.7 % protein, 5% fat, 65% carbohydrate, and 0.05% Sodium.  
 Both diets provide the same calories. Ingredient from the Harlan Teklad,  
 Indianapolis, IN).

## APPENDIX C

Distribution of CSA in Plasma



In the range of 500 ng/ $\mu$ L of CSA in human plasma (In vitro).

(Lemaire and Tillement, 1982; Christians and Sewing, 1993)

## APPENDIX D

### Lipoprotein Composition

Lipoproteins	HDL		LDL		VLDL		Chylomicron	
Density	1.063-1.21 gm/mL		1.006-1.063 gm/mL		0.95–1.006 gm/ mL		< 0.95 gm / mL	
Apoprotein (MW)	A I AII CIII D E (CI, II)	27K 17K 8.8K ~20K 33K	B100	550K	B100 CII E (CI)	550K 10K 33K	B48 C I	275K 7K
Phospholipid Unesterified- cholesterol Cholesteryl- ester Triglyceride Protein	24% 2%  20%	22% 8%  37%	19% 7%  13%	7% 2%  5%				
Apoproteins	50:50 = Protein: Lipid 70% ApoAI 2% ApoCI 1% ApoCII 2% ApoCIII 1-2% ApoE	25:75 = Protein:Lipid (0.7-1 mg/mL)  Apo B100 degrading to ApoB74, 26	10:90 = Protein:Lipid 50% ApoCIII 10% ApoCI 10% ApoCII 10-20% ApoE	Rat: Apo B48: 210k				

(Harmony and Aleson, 1981; Converse and Skinner, 1992)

## **ABBREVIATION**

ALT	Alanine aminotransferase
ApoB	Apolipoprotein
AST	Aminotransferase oxaloacetate transaminase
BA	Bioavailability
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CE	Cholesterylester
CSA	Cyclosporin A
CSD	Cyclosporin D
CYP	Cytochrome P450
DNase	Deoxyribinuclease
DTT	Deoxyrebonuclease
EDTA	ethylenediaminetetraacetic acid
EE	17 $\alpha$ -ethynylestradiol
FH	Familiar hypercholesterolemia
FKBP	FK-506 binding portin
GAPDH	Glycerylaldehyde phosphate dehydrogenase
GH	Growth hormone
HC	High cholesterol diet
HDL	High density lipoproteins
HDL-R	High density lipoprotein receptors

HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	high-performance liquid chromatography
Hx	Hypophysectomized
IL-2	Interleukin-2
IS	Internal standard
IV	Intravenous
kDa	Kilodalton
LDL	Low density lipoproteins
LDL-R	Low density lipoprotein receptors
LPL	Lipoprotein lipase
LS	Low-sodium diet
mRNA	Messenger ribonucleic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NF-AT	Nucleous factor of activated T-cell
OHT	Hydroxytestosterone
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulfonyl fluoride
PPIase	Peptidylproline cis-trans isomerase, proline rotamase
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SR-BI	Scavenger receptor class B type I
TBS	Tris buffer with 0.9% sodium chloride
TG	Triglyceride

Tris	Tris[hydroxymethyl]aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate
VLDL	Very low density lipoproteins
WHHL	Watanabe heritable hyperlipidemic

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## VITA

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**Recent Journal Publications:**

1. Bai S, **Kim T**, Brunner LJ. *Regulation of P-glycoprotein by growth hormone in rats*. Journal of Pharmaceutical Science. (In review).
2. Bai S, **Kim T**, Stepkowski SM, Kahan BD, Brunner LJ. *Metabolic interaction of CSA and sirolimus through cytochrome P450 and P-glycoprotein*. Drug Metabolism and Disposition. (In review).
3. **Kim T**, Wasan K, Brunner LJ. *Effect of lipoprotein associated cyclosporine on hepatic metabolism*. The Journal of Pharmaceutical Research. (In preparation).
4. **Kim T**, Brunner LJ. *Regulation of lipoprotein receptors on drug metabolism*. (In preparation)
5. **Kim T**, Liu S, Callahen, S, Brunner LJ. *CSA metabolism in LDL-receptors altered rat model*. (In preparation)
6. **Kim T**, Bai S, Brunner LJ. *Regulation of lipoprotein receptors by growth hormone* (In preparation)
7. **Kim T**, Brunner LJ, *The regulation of LDL-R by estrogen in time manner*. (In preparation)

### **Recent Conference Presentations:**

1. Bai S, **Kim T**, Brunner LJ. *Regulation of P-glycoprotein by growth hormone in rats.* Seventeenth Annual American Association of Pharmaceutical Scientists Meeting, Denver, CO; 2001.
2. Bai S, **Kim T**, Brunner LJ. *Effect of CSA and sirolimus on hepatic and intestinal cytochrome P450 3A2 and P-glycoprotein.* Seventeenth Annual American Association of Pharmaceutical Scientists Meeting, Denver, CO; 2001.
3. Bai S, **Kim T**, Napoli KL, Stepkowski SM, Kahan BD and Brunner LJ. *The Metabolic Interaction of CSA and Sirolimus via Oral administration in the Rat.* Sixteenth Annual American Association of Pharmaceutical Scientists Meeting, Indiana, IN; 2000.
4. Bai S, Liu J, **Kim T**, Brunner LJ. *The in vivo induction of hepatic P-glycoprotein by CSA in the rat.* Sixteenth Annual American Association of Pharmaceutical Scientists Meeting, Indiana, IN; 2000.

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