AN ABSTRACT OF THE DISSERTATION OF

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Title: Chlordecone (CD), A Mixed Steroid X Receptor (SXR) and Estrogen Receptor alpha (ERα) Agonist, Altered Hepatic Cholesterol (CH) Homeostasis and Lipoprotein Metabolism

Abstract approved:

Lawrence R. Curtis

Overtly non-toxic doses of organochlorine (OC) insecticides perturb lipid homeostasis in mice and rats. To increase understanding of how chlordecone (CD), an OC insecticide, modulates cholesterol (CH) homeostasis, we determined the effect of CD pretreatment on the tissue and hepatic subcellular distribution of exogenous [^{14}C]CH. Corn oil alone or CD (5 or 15 mg/kg) was administered to C57BL/6 mice by intraperitoneal (ip) injection. Three days later, a lipid bolus dose (5 ml corn oil/kg) containing [^{14}C]CH (10 mg/kg) was administered to corn oil or CD pretreated mice by ip injection. CD pretreatment reduced [^{14}C]CH equivalents in the plasma non-high density lipoprotein (HDL) fraction but increased total CH concentration in the same

fraction 4 hr after lipid bolus dose. Along with reduced disposition of [^{14}C]CH to liver, CD pretreatment decreased [^{14}C]CH equivalents in hepatic microsomal and cytosolic fractions 4 and 16 hr after ip bolus lipid, respectively and decreased relative distribution of [^{14}C]CH to the lipoprotein-rich fraction. However CD treatment did not change total hepatic CH concentration 16 hr after lipid bolus dose. In contrast CD pretreatment stimulated biliary excretion of [^{14}C]CH and increased total CH concentration in gallbladder 4 hr and 16 hr after ip lipid bolus, respectively. Reduced disposition of [^{14}C]CH to liver and stimulated biliary CH excretion were not associated with altered hepatic membrane scavenger receptor class B type I (SR-BI) and ATP-binding cassette transporter G8 (ABCG8) protein contents. We then determined the effect of CD on activation of nuclear receptors, especially those involved in lipid homeostasis to characterize the specificity of CD effect. CD (10 μM) strongly suppressed LXRβ activation and increased activation for SXR (human PXR homolog). The same concentration of CD increased activation of human ERα specifically and suppressed E2-mediated ERβ activation effectively. Increased hepatic CYP3A11 protein content by western blotting supported PXR activation. Finally, we determined the effect of CD on lipoprotein metabolism. CD increased apolipoprotein A-I (apoA-I) protein content in hepatic lipoprotein-rich and microsomal fractions. A trend for increased apolipoprotein B-100 (apoB-100) protein content in hepatic microsomal fraction in CD treated animals was observed. CD modulated HDL particle size and shape by increasing more non-spherical and heterogeneous particles. At 14 days after CD treatment (15 mg/kg) apoA-I and apoB-100 but not CYP3A11 protein in hepatic microsomes was similar to controls. This work indicated altered CH

homeostasis was a mode of OC insecticide action of relevance after a single low dose. These findings at least partially explain altered CH tissue distribution in CD-pretreated mice.

Chlordecone (CD), A Mixed Steroid X Receptor (SXR) and Estrogen Receptor alpha (ERα) Agonist, Altered Hepatic Cholesterol (CH) Homeostasis and Lipoprotein Metabolism

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Junga Lee, Author

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CONTRIBUTION OF AUTHORS

Richard C. Scheri assisted with sample collection and preparation in Chapter 2 and 3. Dr. David J. Mangelsdorf and Dr. Yuan Zhang assisted with the reporter gene assay in Chapter 3.

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Chapter 1

Introduction

Humans are exposed to complex mixtures of xenobiotics including drugs, pesticides and other environmental contaminants. In the body, xenobiotics, dietary nutrients, and endogenous substances may interact to modulate regulatory pathways for intermediary metabolism. Inhibition or activation of nutrient storage, transport or metabolism pathways may yield beneficial or adverse effects.

Organochlorines (OC) are a major group of persistent organic pollutants that bioaccumulate in wildlife and humans. These include chlorinated dioxins and furans, polychlorinated biphenyls (PCBs), and the OC insecticides. Even though most uses of OC insecticides were banned in the United States and Europe, they continue to occur in tissues of humans and wildlife throughout the world (Bocquene and Franco 2005; Luellen *et al.* 2006). Trophic transfer though food webs is the principle route of environmental exposure to these agents. Since they are highly lipophilic and generally very resistant to degradation, concerns about their potential health effects continue. They are neurotoxic through their interactions with ion channels (Narahashi *et al.* 1998). Some OC insecticides, chlordecone (CD) for example, are carcinogenic in rodent bioassays (Reuber 1978). Others, such as dieldrin, are tumor promoters in rodent models (Kolaja *et al.* 1998).

Cholesterol (CH) is essential for diverse cellular functions. CH is important constituent of cell membranes to maintain membrane fluidity and permeability and transcriptional regulation, necessary for synthesis of bile salts essential for digesting dietary fats and a substrate for steroid hormone production (Chen and Raymond 2006; Simons and Ikonen 2000; Tabas 2002). However, accumulation of excess CH causes several diseases especially heart attacks and stroke by promoting atherosclerosis and might contribute to the early onset of Alzheimer's disease and renal dysfunction (Abrass 2004; Kees-Folts and Diamond 1993; Krieger 1999). Liver is primary regulatory organ for CH homeostasis which is strictly maintained by many well-known mechanisms. These include plasma lipoprotein-mediated extracellular CH transport, the sterol regulatory element binding protein (SREBP) system involved in CH synthesis (Brown and Goldstein 1997), nuclear receptor-mediated CH catabolism to bile acids (Peet et al. 1998), and plasma membrane transporter-mediated excretion into bile (Yu *et al.* 2002).

Prior reports indicate that an overtly non-toxic dose of CD, an OC insecticide, alters tissue distribution of exogenous CH in mice and rats (Carpenter and Curtis 1991; Gilroy *et al.* 1994). Subsequent experiments and other work suggests that CD also modulates other aspects of lipid homeostasis (Carpenter *et al.* 1996; Chetty *et al.* 1993). Alterations in lipid homeostasis occur in rodents at doses that are not overtly neurotoxic or carcinogenic. However molecular mechanisms underlying these observations are unclear.

To increase understanding whereby overtly nontoxic doses of CD perturb lipid homeostasis we adopted the C57BL/6 mouse as a model. The characteristics of CH

homeostasis in the mouse are quantitatively and qualitatively different from those in human (Dietschy and Turley 2002). Nevertheless, phenotypic changes in mice due to deleted or overexpressed specific functions important in sterol balance are often similar to those of human with mutation or pharmacological modification of the same proteins. Moreover, the ability to manipulate its genome makes the mouse be a valuable model to study atherosclerosis (Fazio and Linton 2001; Paigen *et al.* 1994).

First, we determined the effect of CD pretreatment on the tissue distribution of exogenous [^{14}C]CH. Since liver is primary regulatory organ for CH homeostasis, hepatic subcellular distribution and hepatic compartmental redistribution of exogenous [^{14}C]CH were further assessed in control and CD pretreated mice. Blood plasma, hepatic and biliary total CH concentrations were also examined to extend investigation to the total hepatic CH pool. To determine whether reduced disposition of [^{14}C]CH to liver and stimulated biliary excretion of [^{14}C]CH were associated with alteration of SR-BI or ABCG8, hepatic membrane SR-BI and ABCG8 protein contents were assessed. This exogenous [^{14}C]CH distribution study is presented in chapter 2.

Nuclear receptors function as ligand activated transcription factor that regulate the expression of target genes. Members of nuclear receptor superfamily include liver X receptors (LXRα,β), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARα,δ,γ) and xenobiotic receptors (SXR/PXR) (Table 1-1)(Chawla *et al.* 2001). These nuclear receptors promote the storage, transport, and catabolism of sterols and fatty acids by regulating target genes. Cellular and whole-body lipid and CH level are maintained through a complex homeostatic network through coordinated transcriptional mechanisms (Edwards *et al.* 2002; Ory 2004). Therefore we assessed

CD interactions with nuclear receptors especially involved in lipid homeostasis in vitro and in vivo to characterize the specificity of CD effect. Based on nuclear receptors specificity, finally, we focused on the effect of CD on lipoprotein metabolism. CD interactions with nuclear receptors and lipoprotein metabolism are presented in chapter 3.

Table 1-1. LXR, FXR, SXR/PXR, and PPAR (liver X receptors, farnesoid X receptor, steroid/pregnane X receptor and peroxisome proliferator activated receptors) ligands and their target genes.

Nuclear receptors	Ligands	Target genes	
		CYP enzyme	ABC transporter
LXRα,β	Oxysterols	CYP7A1	ABCA1, ABCG1, ABCG4 ABCG5, ABCG8
FXR	Bile acids	CYP7A1 CYP8B1	ABCB11
SXR/PXR	Xenobiotics Steroids, Bile acids	CYP3A CYP2C	ABCB1, ABCC2
PPARα,δ,γ	Fatty acids, Fibrates Eicosanoids, Thiazolidinediones	CYP4A1 CYP4A3 CYP4B1	ABCD2, ABCD3, ABCB4

Chapter 2

Chlordecone altered hepatic disposition of [^{14}C]cholesterol and plasma cholesterol distribution but not SR-BI or ABCG8 proteins in livers of C57BL/6 Mice

Junga Lee, Richard C. Scheri, and Lawrence R. Curtis

ABSTRACT

Organochlorine (OC) insecticides continue to occur in tissues of humans and wildlife throughout the world although they were banned in the United States a few decades ago. Low doses of OC insecticide chlordecone (CD) alter hepatic disposition of lipophilic xenobiotics and perturb lipid homeostasis in rainbow trout, mice and rats. We examined the effect of CD pretreatment on the tissue and hepatic subcellular distribution of exogenous [^{14}C]cholesterol (CH) equivalents 4 and 16 hr after intraperitoneal (ip) injection of 5 ml corn oil/kg that contained 10 mg CH/kg. Blood, liver and gallbladder total CH concentration were also measured in the same mice. Finally, scavenger receptor class B type I (SR-BI) and ATP-binding cassette transporter G8 (ABCG8) proteins were quantified by western blotting in hepatic membranes from control and CD treated mice. Our results demonstrated that CD pretreatment altered tissue distribution of exogenously administered [^{14}C]CH by decreasing hepatic and renal accumulation and increasing biliary excretion. CD pretreatment altered hepatic subcellular distribution resulting in decreased hepatic cytosolic and microsomal [^{14}C]CH equivalents and lipoprotein-rich fraction-to-homogenate ratio. CD pretreatment increased the ratio of [^{14}C]CH equivalents in high density lipoprotein (HDL) to that in plasma and reduced [^{14}C]CH equivalents in the non-HDL fraction 4 hr after a bolus lipid dose. CD pretreatment increased plasma non-HDL total CH 4 hr after a bolus lipid dose. Liver membrane contents of SR-BI or ABCG8 proteins were unchanged by CD treatment. Overall these data suggest that single overtly nontoxic doses of CD alter CH homeostasis and lipoprotein metabolism.

INTRODUCTION

Organochlorines (OC) are a major group of persistent organic pollutants including chlorinated dioxins and furans, polychlorinated biphenyls (PCBs), and the OC insecticides. Despite bans on most uses of OCs in the United States and Europe, they continue to occur in tissues of humans and wildlife throughout the world (Bocquene and Franco 2005; Luellen *et al.* 2006). Because they are highly lipophilic and generally very resistant to degradation, trophic transfer though food webs is the principle route of environmental exposure to these agents.

The OC insecticides dieldrin and chlordecone (CD) alter hepatic disposition of lipophilic xenobiotics and perturb lipid homeostasis in rainbow trout, mice and rats (Carpenter and Curtis 1989, 1991; Carpenter *et al.* 1996; Donohoe *et al.* 1998; Gilroy *et al.* 1994). These effects of OC insecticides occur at doses far lower than those of traditional concern to toxicologists. Single, overtly nontoxic doses of CD and dieldrin decrease plasma triglycerides and total cholesterol (CH) in rats 21 and 60 days after administration, respectively (Ishikawa *et al.* 1978). A single dose of 5 mg CD/kg to C57BL/6 mice also significantly alters tissue distribution of exogenous [^{14}C]CD or [^{14}C]CH, resulting in a decrease in hepatic disposition and an increased distribution to other tissues (Carpenter and Curtis 1991).

CH is important as a constituent of cell membranes and as a precursor of steroid hormones and bile acids (Chen and Raymond 2006; Tabas 2002). However accumulation of excess CH contributes to several diseases especially heart attacks and stroke by promoting atherosclerosis (Krieger 1999). CH metabolism is regulated by many of well-known mechanisms. These include the plasma lipoprotein-mediated

extracellular CH transport, the sterol regulatory element binding protein (SREBP) system involved CH synthesis (Brown and Goldstein 1997), nuclear receptor-mediated CH catabolism to bile acids (Peet et al. 1998), and plasma membrane transporter-mediated excretion into bile (Yu *et al.* 2002).

Transport kinetic studies indicate that the rate of uptake of high density lipoprotein (HDL)-bound [^{14}C]CD or [^{14}C]CH is significantly lower in perfused livers from Sprague-Dawley rats pretreated with a single dose of 15 mg/kg CD (Gilroy *et al.* 1994). Gilory et al. (1994) report a significantly increased in the rate of efflux of HDL-bound [^{14}C]CD to perfusate and cumulative biliary excretion of HDL-bound [^{14}C]CH in these same preparations.

Plasma HDL transfers CH from peripheral tissue to the liver for biliary excretion by the reverse CH transport pathway (Fielding and Fielding 1995). Therefore HDL is an important source of CH for secretion into the bile in rodents (Fielding and Fielding 1995; Lee and Parks 2005). Plasma low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) deliver CH to peripheral tissues such as adipose. In contrast to other OC insecticides, such as dieldrin and DDT in which tissue distribution is proportional to tissue lipid contents (Lindstrom *et al.* 1974), CD distributes preferentially to the liver in both humans (Cohn *et al.* 1978) and rodents (Egle *et al.* 1978). Dieldrin and DDT are primarily associated with LDL and VLDL (Mick *et al.* 1971). CD binds preferentially with albumin and HDL in vitro and in vivo studies of human, rat, and pig plasma (Soine *et al.* 1984a; Soine *et al.* 1982) and this may account for its predominant distribution to the liver. These previous works indicate that a change in hepatic uptake of HDL-complexed CH is important in

alteration of lipid homeostasis by CD. Low doses of CD pretreatment may modulate membrane proteins which transport CH thereby disturbing the distribution of the exogenous CH to liver and increasing delivery of CH into bile.

Scavenger receptor class B type I (SR-BI), best known as a physiological HDL receptor, mediates selective uptake of both cholesteryl esters and other lipids from HDL (Hobbs and Rader 1999; Krieger 1999). SR-BI deficiency reduced the rate of the clearance of HDL-CH from plasma (Out *et al.* 2004). ATP-binding cassette (ABC) transporter G5 (ABCG5) and G8 (ABCG8) are members of the G family of ABC transporters. Co-expression of G5 and G8 which are obligate heterodimers promotes transport of CH from hepatocytes to bile and probably from enterocytes into the intestinal lumen (Yu *et al.* 2005; Yu *et al.* 2002).

The present studies examined the effect of CD pretreatment on the tissue distribution of exogenous [^{14}C]CH 4 and 16 hr after intraperitoneal (ip) injection of 5 ml corn oil/kg that contained 10 mg CH/kg. Hepatic subcellular distribution of [^{14}C]CH was also determined. Blood plasma, hepatic and biliary total CH were examined in control and CD pretreated mice. This extended investigation to the total hepatic CH pool. To determine whether the reduced disposition of [^{14}C]CH to liver were induced by decreased SR-BI protein, SR-BI was analyzed by immunoblotting with hepatic membranes from CD treated mice (15 mg/kg) or controls. Immunoblot analysis for ABCG8 was conducted with hepatic membrane from CD treated mice (15 mg/kg) or controls to determine whether the stimulation of biliary CH efflux by CD was coupled to the increased CH efflux pump (ABCG5/ABCG8).

Our results demonstrated that CD pretreatment altered plasma, tissue and hepatic subcellular distribution of exogenously administered [^{14}C]CH. CD pretreatment also altered the amount of total CH in blood plasma and gallbladder. The reduced hepatic [^{14}C]CH equivalents and increased biliary [^{14}C]CH equivalents were not associated with altered hepatic membrane SR-BI and ABCG8 contents.

MATERIALS AND METHODS

Chemicals

CD (99 % purity) was purchased from Chem Service (West Chester, PA) and purity was confirmed by GC-EI/MS. [4-^{14}C]CH (55 mCi/mmol) was obtained from American Radiolabeled Chemicals. Inc. (St. Louis, MO). Solvable tissue solubilizer and Hionic-fluor scintillation cocktail were purchased from PerkinElmer (Boston, MA). All other chemicals and general reagents were purchased from Sigma (St. Louis, MO).

Treatment of mice

Male C57BL/6 mice, 6 to 7-weeks-old, were obtained from Simenson Laboratory (Gilroy, CA). The mice were randomly divided into three groups; control, 5.0 mg or 15 mg CD/kg body weight treatment. The animals were housed in a temperature controlled room (22 ± 1^{o}C) with a daily cycle of 12 hr of light and 12 hr darkness and fed ad libitum with AIN93 diet (Dyets, Inc., Bethlehem, PA) with a free access to water. Treatments were initiated after seven days acclimatization.

[^{14}C]Cholesterol tissue distribution experiments

CD was dissolved in corn oil. Mice received CD (5 or 15 mg/kg body weight) or corn oil alone by intraperitoneal injection (ip, 5 ml/kg body weight). After 3 days, a bolus lipid dose of corn oil (5 ml/kg) containing [^{14}C]CH was administered (10 mg/kg body weight, ip). [^{14}C]CH injected mice were housed individually and allowed free access to water. After 4 or 16 hr animals were killed by CO_2 anesthesia and exsanguination. Blood samples and tissues (liver, kidneys, gallbladder, adrenal gland and adipose tissue) were removed and prepared as required for each analysis. The procedures for

animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).

Quantification of radioactivity in tissues, blood and hepatic subcellular fractions

Blood was collected by cardiac puncture in heparinized syringes. Plasma was isolated by centrifugation at 3,000g for 25 min at 4^{o}C. HDL fraction was separated after apolipoprotein-B containing lipoproteins were precipitated with dextran sulfate and magnesium (Rachem, San Diego, CA). Kidneys, fat, and adrenal glands were homogenized in homogenization buffer (0.01 M potassium phosphate, pH 7.5; 0.15 M KCl; 1.0 mM EDTA; 0.1 mM BHT (butylated hydroxytoluene); 0.1 mM PMSF (phenylmethylsulfonyl fluoride)) with a dounce homogenizer. Liver was homogenized in homogenization buffer with a polytron PT 3000 (Brinkmann Instruments, Westbury, NY). Liver homogenate was centrifuged at 1200 g for 30 min. The pellet was cell debris. The supernatant was centrifuged at 9,000 g for 20 min to prepare mitochondria. The supernatant was again centrifuged at 100,000 g for 90 min at 4^{o}C with Ti 70 rotor. The remaining supernatant and pellet were cytosolic and microsomal fractions, respectively. Subsamples of each tissue homogenate and each of hepatic subcellular fraction (cell debris, mitochondria, cytosol and microsomes) were solubilized with Solvable (PerkinElmer, Boston, MA) at 50 to 60^{o}C for 1.5 – 2 hr and decolorized by adding 30% hydrogen peroxide. Plasma and HDL fraction were counted without further treatment. Lipids from gallbladder were extracted with hexane after incubation in KOH/ethanol at 70^{o}C for 30 min. Ten ml of Hionic-Fluor (PerkinElmer, Boston, MA) liquid scintillation cocktail was added. After decay of chemiluminescnce liquid scintillation counting was conducted using a Beckman LS7500 liquid scintillation

counter. Protein concentration was determined by BCA assay (Pierce Biotechnology, Inc. Rockford, IL).

Plasma lipid analyses

Total plasma CH concentrations were measured enzymatically by using 2 ul of plasma and 200 ul of CH reagent from Sigma Diagnostic (St. Louis, MO). HDL-CH concentration was measured after apolipoprotein-B containing particles precipitation with dextran sulfate and magnesium (Rachem, San Diego, CA). Non-HDL-CH concentrations were taken as the difference between the two.

Western blot analysis

Liver and intestine crude membrane fractions were prepared as described (Voshol *et al.* 2001) from control or 15 mg CD/kg treated animals. Proteins were separated on Tris/glycine gels (Bio-Rad, Richmond, CA) under reducing conditions. Following gel electrophoresis at 100 V for 2 hr, proteins were electrophoretically transferred onto PVDF membranes utilizing a Mini trans-blot transfer cell (Bio-Rad, Richmond, CA). Membranes were then blotted with rabbit anti-SR-BI (Abcam, Cambridge, MA) and probed with horseradish peroxidase conjugated goat anti-rabbit IgG. Membranes were also blotted with mice anti-ABCG8 (a generous gift from Professor Helen H. Hobbs, Univ. of Texas Southwestern Medical Center) and then probed with horseradish peroxidase conjugated rat anti-mouse IgG as the secondary antibody (Bio-Rad, Richmond, CA). The proteins were detected after development with enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ). Quantification of the intensity of the protein bands was performed with NIH-image software.

Hepatic and biliary lipid analyses

For quantification of hepatic and biliary CH, tissues were incubated in KOH/ethanol for 30 min at 70^{o}C. After addition of hexane and centrifugation for 3 min, the lipid layer (upper layer) of each sample was collected and dried under nitrogen. Dried lipids were dissolved in 2-propanol containing 10 % Trinton-X-100 as assay samples. Total and unesterified CH concentration was determined using a Cholesterol/Cholesteryl Ester Quantitation Kit^{R} (BioVision, Mt. View, CA). Hepatic esterified CH content was taken as the difference between the total CH and unesterified CH concentration.

Statistical Methods

Data were expressed as means ± SE. Comparisons among groups were submitted to a one-way analysis of variance (ANOVA). For comparison between two groups, Student's t-test with equal-variance was applied. In all analyses, a 95% confidential level was used as the criterion for significance. For outlier identification, Grubbs' Test (assumes normality) was applied.

RESULTS

The mean body weights of the control, 5, and 15 mg/kg CD pretreated mice at 4 or 16 hr after 10 mg [^{14}C]CH/kg in 5 ml corn oil/kg (ip bolus lipid) were similar as were the liver weights (Table 2-1). No significant differences in gallbladder weights between control and CD pretreated mice were observed (Table 2-1). However, 16 hr following ip bolus lipid dose, gallbladder weights were significantly smaller (~9 to 10 mg) than those at 4 hr following ip bolus lipid dose (~15 to 17 mg) (Table 2-1).

Plasma [^{14}C]CH equivalents were approximately 3-fold higher at 16 hr than at 4 hr after the ip bolus lipid dose ($p < 0.05$, Fig. 2-1A, 2-1B), although plasma total CH concentration at 16 hr was not different from that at 4 hr after the ip bolus lipid (Fig. 2-1C, 2-1D). Plasma [^{14}C]CH equivalents from CD (5 or 15 mg CD/kg) pretreated mice significantly decreased 4 hr following the bolus lipid dose (31 %, $p < 0.05$)(Fig. 2-1A). CD pretreatment (5 or 15 mg CD/kg) significantly decreased [^{14}C]CH equivalents in the non-HDL fraction compared with controls (45%, $p < 0.05$)(Fig. 2-1A). On the contrary, CD pretreatment increased total CH in non-HDL fraction 4 hr following ip bolus lipid (about 1.8-fold, $p < 0.05$)(Fig. 2-1C). At 16 hr after the ip bolus lipid, neither [^{14}C]CH equivalents nor total CH concentrations in plasma were altered by CD pretreatment (Fig. 2-1B, 2-1D). No significant differences in the [^{14}C]CH equivalents and total CH in the either HDL or non-HDL fractions were observed 16 hr following ip bolus lipid (Fig. 2-1B, 2-1D). Although the [^{14}C]CH equivalents in the HDL fraction were unchanged, the ratio of [^{14}C]CH equivalents in HDL fraction to that in plasma was mildly but statistically significantly increased by CD pretreatment 4 hr after ip bolus lipid in a dose-dependent manner (Fig. 2-2A).

Consistent with previous results, CD decreased [^{14}C]CH equivalents distribution to the liver at 16 hr after the bolus lipid dose (43 %, $p < 0.05$)(Fig. 2-3A). Altered [^{14}C]CH disposition in the liver of CD pretreated mice was further characterized in hepatic subcellular fractions (homogenate, lipoprotein-rich fraction, cytosol, microsomes, mitochondria and cell debris/nuclei). At 4 hr after the bolus lipid dose, [^{14}C]CH equivalents significantly decreased about 50 % in the microsomal fraction from CD (15 mg/kg) pretreated mice compared to control animals (10.1 ± 1.3 vs 5.9 ± 0.4 pmol/mg protein, $p < 0.05$). At 16 hr after ip bolus lipid, [^{14}C]CH equivalents significantly decreased about 30 % in hepatic cytosolic fraction from CD (15 mg/kg) pretreated mice compared to control animals (5.7 ± 0.4 vs 4.0 ± 0.3 pmol/mg protein, $p < 0.05$). To assess the hepatic compartmental re-distribution of [^{14}C]CH, [^{14}C]CH equivalents from each hepatic fraction were normalized with [^{14}C]CH equivalents in homogenate. The ratios of [^{14}C]CH lipoprotein-rich fraction-to-homogenate were lower at 16 hr than at 4 hr after the ip bolus lipid in both control and CD pretreated mice. Interestingly, 15 mg CD/kg pretreatment significantly reduced [^{14}C]CH equivalents lipoprotein-rich fraction-to-homogenate ratio 16 hr after ip bolus lipid, compared with control (about 50%, $p < 0.05$)(Fig. 2-3B). A trend for decreased [^{14}C]CH in lipoprotein-rich and microsomal fraction-to-homogenate ratios at 4 and cytosolic fraction-to-homogenate ratios at 16 hr after the ip bolus lipid in CD pretreated animals was observed (Fig. 2-3B).

SR-BI was analyzed by immunoblotting with hepatic membranes from CD treated mice (15 mg/kg) or controls. Liver membrane contents of SR-BI protein in CD-treated mice were not different from control animals (Fig. 2-2B).

CD pretreatment increased [^{14}C]CH equivalents of gallbladder following 4 and 16 hr after the ip bolus lipid dose, in a dose-dependent manner (Fig. 2-4A). A statistically significant increase was observed 4 hr after the tracer of 15 mg CD/kg pretreated animals ($p < 0.05$). A trend for increased [^{14}C]CH equivalents in gallbladders at 16 hr after the ip bolus lipid in 15 mg CD/kg pretreated animals was observed ($p < 0.056$, Fig. 2-4A). CD pretreatment increased total CH concentration in gallbladder 16 hr after lipid bolus compared with control (Fig. 2-4B). Immunoblot analysis for ABCG8 was conducted with hepatic membrane from CD treated mice (15 mg/kg) or controls. Liver membrane content of ABCG8 protein was unchanged by CD treatment (Fig. 2-4C). Statistically significant reductions of [^{14}C]CH distribution to the kidney (48 %, $p < 0.05$) was observed in 15 mg CD/kg pretreated animals (data not shown). No significant change was observed in other tissues (data not shown).

DISCUSSION

The present results demonstrate that pretreatment with a single, overtly nontoxic dose of CD pretreatment alters plasma (Fig. 2-1, 2-2), tissue and hepatic subcellular distribution of exogenously administered [^{14}C]CH (Fig. 2-3, 2-4). There were a number of limitations in interpretation of plasma [^{14}C]CH equivalents for explanation of CD-altered disposition of the exogenous lipid bolus. Total plasma [^{14}C]CH equivalents included material: (1) newly absorbed from the peritoneal cavity associated with chylomicrons; (2) subjected to intraplasmic exchange between lipoprotein classes; and (3) secreted from the liver to form nascent HDL or as VLDL. Nonetheless comparisons of data for plasma [^{14}C]CH equivalents and plasma total CH in fractions treated with dextran sulfate and magnesium demonstrated CD-dependent differences (Fig. 2-1A). CD pretreatment decreased plasma non-HDL [^{14}C]CH and increased plasma total non-HDL-CH 4 after the ip lipid bolus dose (Fig. 2-1A, 2-1C). There were at least two mutually nonexclusive explanations. (1) CD delayed [^{14}C]CH incorporation into non-HDL plasma lipoproteins, perhaps into hepatic VLDL synthesized for secretion. Exogenous CH occurred in a hepatic pool distinct from that synthesized in the liver (Oram and Vaughan 2006). Consistent with this, in control mice 4 hr after the ip bolus lipid dose about 50 % of plasma [^{14}C]CH equivalents were in the non-HDL fraction, while about 17 % of plasma total CH appeared in this fraction. (2) CD perhaps increased clearance of non-HDL-[^{14}C]CH. Stimulated plasma clearance of non-HDL-[^{14}C]CH seemed unlikely since total non-HDL-CH was elevated in CD pretreated mice 4 hr after the bolus lipid (Fig. 2-1C). Plasma non-HDL-[^{14}C]CH equivalents in CD pretreated mice were lower than controls 4 but not

16 hr after the bolus lipid dose (Fig. 2-3A, 2-3B). This indicated that CD altered a CH exchange pathway or pool that delayed [^{14}C]CH incorporation into non-HDL lipoproteins. At 16 hr after ip lipid bolus dose [^{14}C]CH appeared integrated into the total plasma CH pool (Fig. 2-1B). In fact, it appeared preferentially retained or secreted in the non-HDL pool since 32 to 35% of [^{14}C]CH equivalents were in that fraction compared 18 to 20% of total CH at 16 hr.

Hepatic [^{14}C]CH equivalents were not different between control and CD pretreated mice at 4 hr after ip lipid bolus dose. CD exhibited specific binding affinity with albumin and HDL and was preferentially accumulated in the liver (Soine *et al.* 1982). Gilroy et al. (1994) suggested a competitive interaction between CH and CD by the observation of decreased uptake of HDL-bound [^{14}C]CH in the CD treated perfused rat liver. CD was an agonist for the steroid X receptor (SXR) in a reporter gene assay and this was supported by CYP3A protein induction in mouse liver by CD (Lee et al., unpublished data). PXR (SXR analog in mice) agonists but not a selective constitutive androstane receptor (CAR) agonist increased expression of the apoA-I gene in mice (Bachmann *et al.* 2004), the principal component of HDL. Sporstol et al. reported the down regulation of SR-BI by a PXR agonist in vitro (Sporstol *et al.* 2005). The HDL receptor SR-BI plays critical roles in the uptake of plasma CH by the liver. Therefore, hepatic SR-BI protein content was determined, this assessed whether hepatic uptake of HDL-CH was important in alteration of plasma CH by CD. SR-BI content in hepatic plasma membrane was not changed by CD treatment (Fig. 2-2B). Even though SR-BI was a physiological HDL receptor, it exhibited binding affinity for HDL, LDL, and VLDL (Acton *et al.* 1996; Calvo *et al.* 1998). Over expression of SR-

BI in the liver increased the clearance of LDL and VLDL (Wang *et al.* 1998). More non-spherical and heterogeneous HDL particles were observed in plasma from CD treated animals compared with controls (Lee et al., unpublished data). Therefore it was possible that CD binding to HDL particles perturbed the SR-BI mediated selective cholesteryl ester uptake from HDL while clearance of LDL and VLDL was not affected. However it seemed unlikely since total non-HDL-CH was elevated in CD pretreated mice 4 hr after the bolus lipid (Fig. 2-1C). Lower lipoprotein-rich fraction-to-liver homogenate [^{14}C]CH equivalents ratios in 15 mg CD/kg pretreated mice was consistent with suppression of incorporation of exogenous CH into lipoprotein complexes (Fig. 2-3B). Markedly reduced [^{14}C]CH lipoprotein-rich fraction-to-homogenate ratio from 4 to 16 hr after the bolus lipid dose in both control and CD pretreated mice suggested the secretory phase peaked before 16 hr (Fig. 2-3B). Elevation in plasma non-HDL-total CH at 4 hr compared to 16 hr after the lipid dose (Fig. 2-3C, 2-3D) in CD pretreated mice supported this interpretation.

Gallbladder bile of mice pretreated with 15 mg CD/kg contained 3-fold more [^{14}C]CH equivalents than controls 4 hr after the ip lipid bolus. A trend for this persisted after 16 hr but was not statistically significant. Since gallbladder weight was lower at 16 than 4 hr after the lipid bolus (Table 2-1) leakage or ejection of bile probably occurred more extensively before the later than the earlier sampling time. This likely reduced accuracy of gallbladder bile [^{14}C]CH equivalents for estimation of biliary secretion at 16 compared to 4 hr after the ip lipid bolus. Biliary CH secretion is one of the major pathways to eliminate excess CH from body. Heterodimers of ABCG5 and ABCG8 regulate and the whole-body retention of plant sterols and

promote hepatobiliary secretion of CH (Kosters *et al.* 2006; Yu *et al.* 2002). CH feeding upregulates expression of ABCG5/ABCG8 coordinately through activation of LXR (liver X receptor) although the binding sites of LXR to these genes have not been mapped (Repa *et al.* 2002). The mRNA level of ABCG5/ABCG8 was higher in the liver of mice lacking LXRα/LXRβ (Repa *et al.* 2002). CD moderately inhibited for LXRα activation and strongly suppressed LXRβ activation in a reporter gene assay (Lee et al., unpublished data). We hypothesized that increased basal activity of ABCG5/ABCG8 by CD stimulated biliary CH excretion. Hepatic membrane ABCG8 protein contents were not different between controls and 15 mg CD/kg treatment (Fig. 2-4C). CD pretreatment increased not only [^{14}C]CH equivalents but also total CH in gallbladder 4 and 16 hr after the ip lipid bolus dose, respectively. This indicated that the lipid bolus dose affected biliary CH secretion in CD pretreated mice compared with controls. Gallbladder bile total CH was not increased in CD pretreated mice compared to controls in animals that received no ip lipid bolus dose (Lee et al., unpublished data). Therefore it was possible that CD pretreatment and ip bolus lipid dose somehow synergically stimulated an ABCG5/8-dependent or ABCG5/8-independent pathway of biliary CH secretion. Another possible explanation was SR-BII mediated stimulation of biliary CH excretion. SR-BII is an alternatively spliced form of SR-BI. SR-BII is expressed intracellulary and suggested to mediate the rapid internalization of HDL for biliary excretion (Eckhardt *et al.* 2004). Although HDL was an important source of CH for biliary excretion in rodents, HDL-CH was the preferred substrate for bile acid synthesis. CH is secreted into bile after arrival at the canalicular membrane. An endocytic/retroendocytic pathway of HDL has been

suggested to play a important role for the delivery of CH to the apical membrane for release into the bile (Wustner *et al.* 2004). In addition to membrane transporter, cytoplasmic proteins such as liver fatty acid-binding protein and sterol carrier protein-2 were suggested to play a role in the intracellular transport and biliary CH secretion (Kosters *et al.* 2005). Essentially all CD existed in pig liver cytosol in a protein-bound form (Soine *et al.* 1982). [^{14}C]CH equivalents significantly decreased in the hepatic microsomal (~ 50 %) and cytosolic (~30%) fractions from CD pretreated mice compared to control animals. Sonie et al. suggested that CD and CH shared a common transport pathway in liver cytosol and CD interacted with CH transport and metabolism since isolated-CD binding proteins bind both CD and CH (Soine *et al.* 1984b). Decreased hepatic disposition of [^{14}C]CH equivalents in the microsomal fraction suggested that binding of CD to cytosolic proteins perhaps not only inhibited CH transport to microsomes but also modulated biliary CH secretion. However, a definitive explanation for increased biliary CH excretion in CD pretreated mice remains elusive.

In summary, overtly nontoxic doses of CD pretreatment altered the plasma, tissue and hepatic subcellular disposition of subsequently administered [^{14}C]CH. Our data demonstrated that SR-BI and ABCG8 protein contents in hepatic plasma membrane were unchanged by CD treatment indicating that mechanisms other than SR-BI or ABCG8 may be involved in modulating CD induced cholesterol homeostasis and lipid metabolism.

Table 2-1. Body and organ weight of vehicle control and CD pretreated mice that received a challenge dose of [^{14}C]CH.

	Body mass (g)		Liver (g)		Gallbladder (mg)	
	4 hr	16 hr	4 hr	16 hr	4 hr	16 hr
Control	22.2 ± 0.8	21.9 ± 0.4	1.21 ± 0.03	1.19 ± 0.05	15.0 ± 0.9	9.2 ± 0.5*
5.0 mg CD/kg	22.5 ± 0.7	21.8 ± 0.4	1.23 ± 0.05	1.22 ± 0.04	17.4 ± 0.7	10.7 ± 1.1*
15.0 mg CD/kg	22.9 ± 0.7	22.3 ± 0.3	1.32 ± 0.05	1.22 ± 0.02	16.2 ± 0.7	8.9 ± 0.5*

There were 6-7 mice in each group. Values are expressed mean ± SE. *Significantly different from the 4 hr ($p < 0.05$).

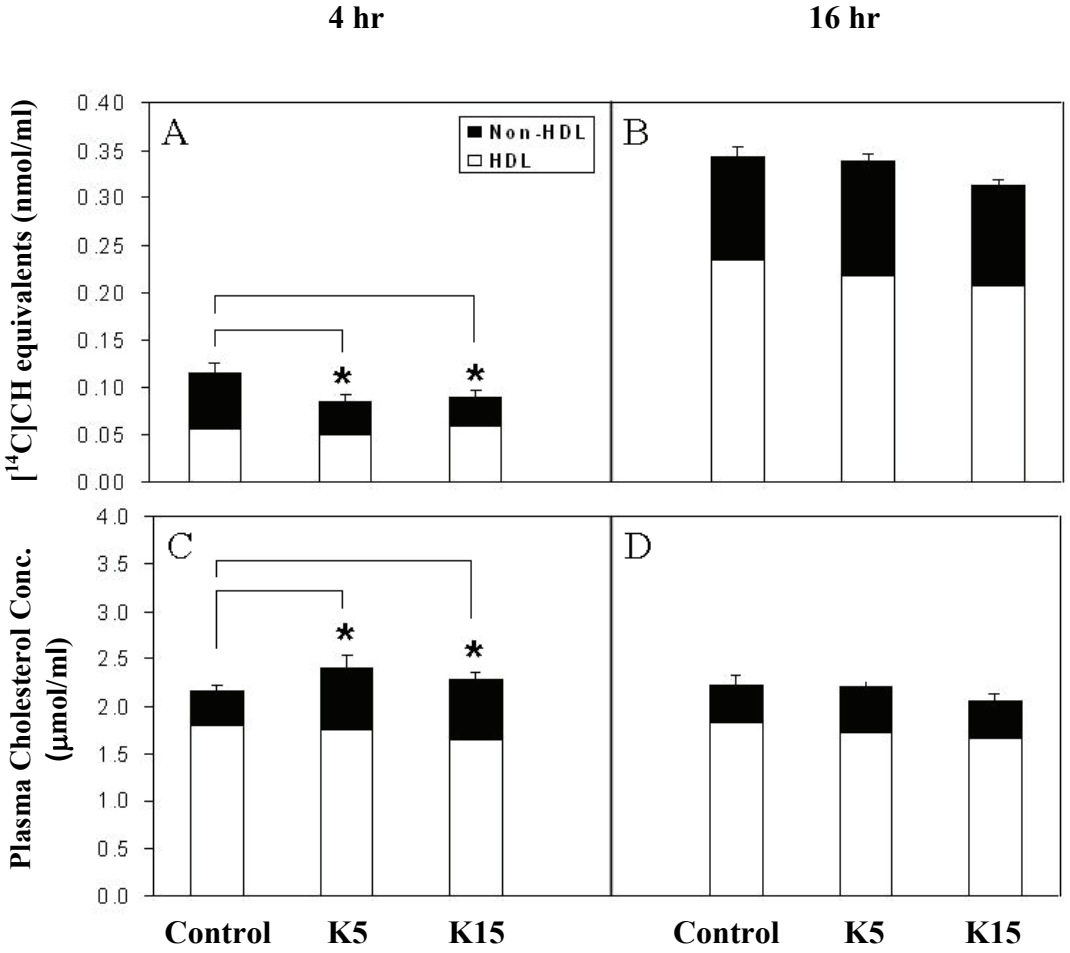
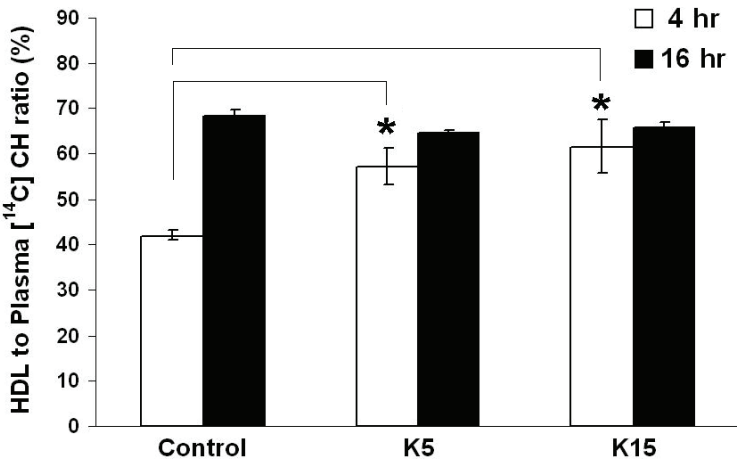


Figure 2-1. [^{14}C]CH equivalents and total CH concentration in plasma of vehicle control and CD pretreated mice that received a challenge dose of [^{14}C]CH. Corn oil, 5, or 15 mg CD/kg was pretreated to C57BL/6 mice. [^{14}C]CH (10 mg/kg in 5 ml/kg corn oil) was subsequently administered 3 days following pretreatment. Determinations were performed 4 and 16 hr after ip administration of [^{14}C]CH. [^{14}C]CH equivalents of plasma and HDL was determined as described under "Material and methods". Plasma total CH and HDL-CH was determined enzymatically as described under "Material and Methods". Non-HDL-CH content was taken as the difference between the total plasma CH and HDL-CH. **A.** [^{14}C]CH equivalents of plasma 4 hr after ip lipid bolus. **B**. [^{14}C]CH equivalents of plasma 16 hr after ip lipid bolus. **C.** Plasma total CH 4 hr after ip lipid bolus. **D.** Plasma total CH 16 hr after ip lipid bolus. Values are expressed mean ± SE. *Significantly different from the 4 hr ($p < 0.05$).

A.



B.

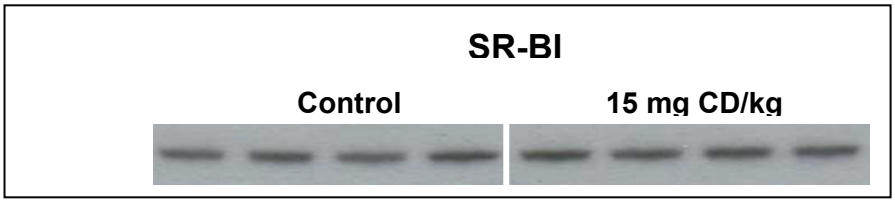
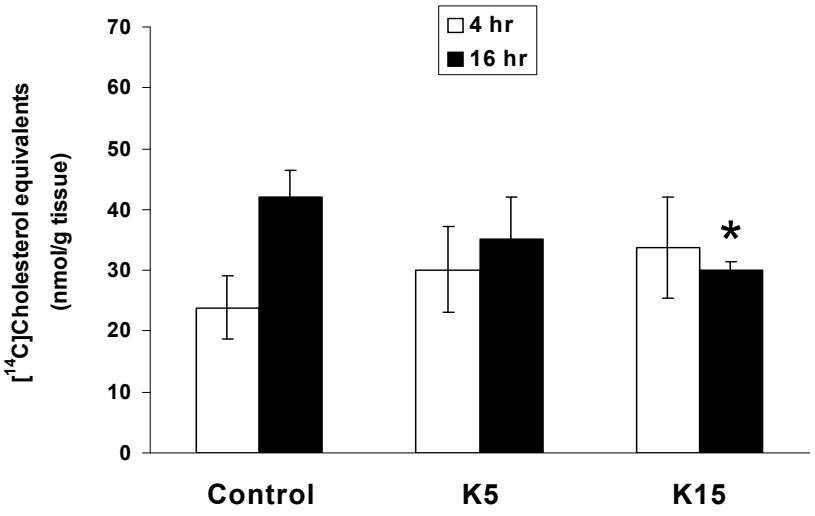


Figure 2-2. The ratio of [^{14}C]CH equivalents in HDL to that in plasma and hepatic SR-BI contents. A. The ratio of [^{14}C]CH equivalents in HDL to that in plasma. Corn oil, 5, or 15 mg CD/kg was pretreated to C57BL/6 mice. [^{14}C]CH (10 mg/kg in 5 ml/kg corn oil) was subsequently administered 3 days following pretreatment. [^{14}C]CH equivalents of plasma and HDL was counted 4 and 16 hr after ip administration of [^{14}C]CH. Values are expressed as mean ± SE. **B.** Hepatic SR-BI contents. Corn oil or 15 mg CD/kg was treated to C57BL/6 mice. Hepatic plasma membrane fractions were prepared from animals (6 mice in each group) as described under "material and methods". Hepatic SR-BI content was measured by western blotting with SR-BI antibody. There were 6-7 mice in each group. * Indicates a statistically significant difference ($p < 0.05$) when compared with control.

Figure 2-3. Hepatic disposition of [^{14}C]CH equivalents of vehicle control and CD pretreated mice that received a challenge dose of [^{14}C]CH. Corn oil, 5, or 15 mg CD/kg was pretreated to C57BL/6 mice. [^{14}C]CH (10 mg/kg in 5 ml/kg corn oil) was subsequently administered 3 days following pretreatment. Determinations were performed 4 and 16 hr after ip administration of [^{14}C]CH. **A.** Hepatic [^{14}C]CH equivalents 4 and 16 hr after ip bolus lipid of [^{14}C]CH. **B.** Hepatic subcellular fraction to homogenate. Hepatic subcellular fractions were prepared and analyzed from animals (6 mice in each group) as described under "material and methods". Data are normalized by the amount of [^{14}C]CH equivalents per mg protein in the liver homogenate. Values are expressed as mean ± SE. There were 6-7 mice in each group. * Indicates a statistically significant difference ($p < 0.05$) when compared with control. † Indicates a statistically difference ($p< 0.1$) when compared with control.

A.


4 hr
16 hr
[14C]Cholesterol equivalents (nmol/g tissue)
70
60
50
40
30
20
10
0
*
Control
K5
K15

B.

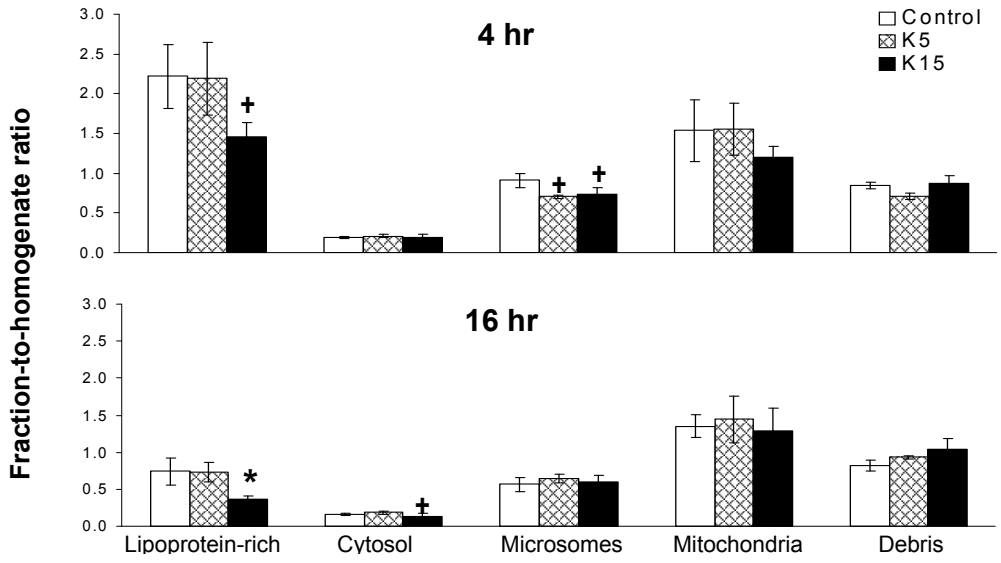
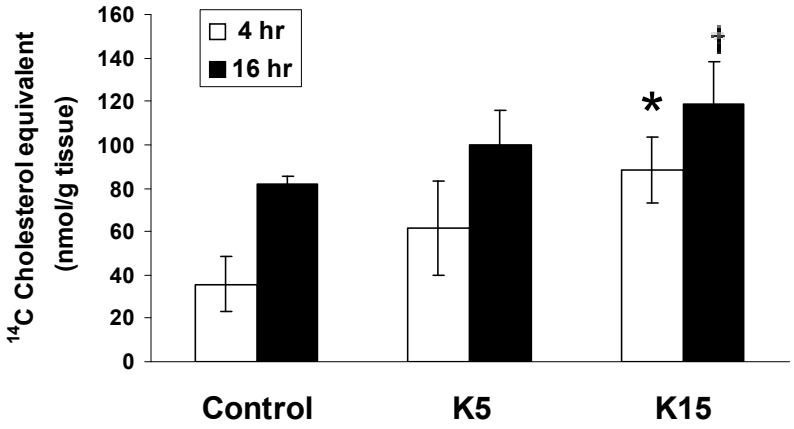
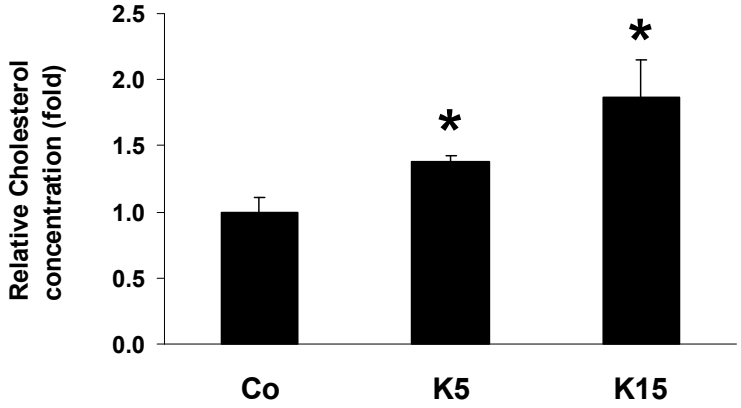

Control
K5
K15
4 hr
16 hr
Fraction-to-homogenate ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
+
*
Lipoprotein-rich
Cytosol
Microsomes
Mitochondria
Debris

Figure 2-4. [^{14}C]CH equivalents and total cholesterol concentration in gallbladder and hepatic ABCG8 content. Corn oil, 5, or 15 mg CD/kg was pretreated to C57BL/6 mice. [^{14}C]CH (10 mg/kg in 5 ml/kg corn oil) was subsequently administered 3 days following pretreatment. **A.** Biliary [^{14}C]CH equivalents 4 and 16 hr after ip administration of [^{14}C]CH. **B.** Biliary total CH concentration was determined 16 hr after ip administration of [^{14}C]CH using an enzymatic method. **C.** Hepatic ABCG8 contents. Corn oil or 15 mg CD/kg was treated to C57BL/6 mice. Hepatic plasma membrane fractions were prepared from animals (6 mice in each group) as described under "material and methods". Hepatic ABCG8 level was measured by western blotting with ABCG8 antibody. Values are expressed as mean ± SE. There were 6-7 mice in each group. * Indicates a statistically significant difference ($p < 0.05$) when compared with control. † Indicates a statistically difference ($p < 0.1$) when compared with control.

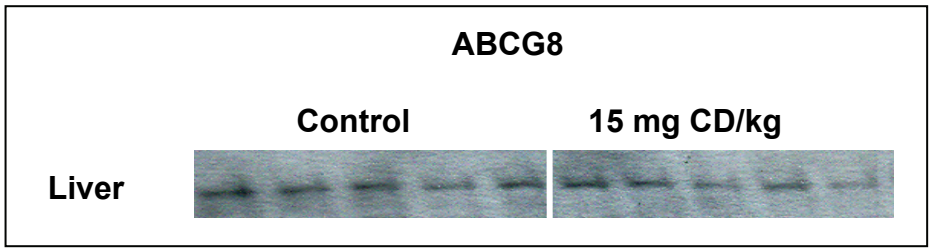
A.


160
140
120
100
80
60
40
20
0
4 hr
16 hr
^{14}C Cholesterol equivalent (nmol/g tissue)
*
†
Control
K5
K15

B.


2.5
2.0
1.5
1.0
0.5
0.0
Relative Cholesterol concentration (fold)
*
*
Co
K5
K15

C.


ABCG8
Control
15 mg CD/kg
Liver

Chapter 3

Chlordecone, a Mixed Steroid X receptor (SXR) and Estrogen Receptor Alpha (ERα) Agonist, Alters Cholesterol Homeostasis and Lipoprotein Metabolism in C57BL/6 Mice

Junga Lee, Richard C. Scheri, Yuan Zhang, David J. Mangelsdorf

and Lawrence R. Curtis

ABSTRACT

Chlordecone (CD) is one of many banned organochlorine (OC) insecticides that are widespread persistent organic pollutants. OC insecticides alter lipid homeostasis in rodents at doses that are not overtly neurotoxic or carcinogenic. Pretreatment of mice or rats with nontoxic doses of CD altered tissue distribution of subsequent dose of [^{14}C]CD or [^{14}C]cholesterol (CH). Nuclear receptors play important roles in the regulation of lipids and CH by regulating the expression of genes which are important in CH and lipid homeostasis. In this study, we report that CD suppresses in vitro reporter systems for LXRs and activates for farnesoid X receptor (FXR), steroid X receptor (SXR, human homolog PXR) and human estrogen receptor α (ERα) in a concentration-dependent manner (0-50 μM). Consistent with SXR activation in vitro, three days after a single low dose of CD (15 mg/kg) hepatic microsomal CYP3A11 protein increases in C57BL/6 mice. CD decreases hepatic cholesteryl ester content without altering total CH concentration. Apolipoprotein A-I (apoA-I) contents of hepatic lipoprotein-rich and microsomal fractions of CD treated mice are higher than controls. There is a trend for decreased apolipoprotein B-48/100 (apoB-48/100) in plasma from CD treated mice. There is a trend for higher apoB-100 contents in hepatic microsomes from CD treated mice compared with controls. More non-spherical and heterogeneous high density lipoprotein (HDL) particles are observed in plasma from CD treated animals compared with controls. At 14 days after 15 mg CD/kg apoA-I and apoB-100 but not CYP3A11 protein in hepatic microsomes is similar to controls. This work indicated altered CH homeostasis was a mode of OC insecticide action of

relevance after a single low dose. This at least partially explains altered CH tissue distribution in CD-pretreated mice.

INTRODUCTION

Banned organochlorine (OC) insecticides are widespread persistent organic pollutants that bioaccumulate in wildlife and humans. They are neurotoxic through their interactions with ion channels (Narahashi *et al.* 1998). Some OC insecticides, chlordecone (CD) for example, are carcinogenic in rodent bioassays (Reuber 1978). Others, such as dieldrin, are tumor promoters in rodent models (Kolaja *et al.* 1998). Alterations in lipid homeostasis occur in rodents at OC insecticide doses that are not overtly neurotoxic or carcinogenic. Single, overtly nontoxic doses of CD and dieldrin decrease plasma triglycerides and total cholesterol (CH) in rats 21 and 60 days after administration, respectively (Ishikawa *et al.* 1978). Carpenter et al. report dose-dependent loss of lipid droplets in hepatocytes, but not Ito cells, of mice after 5-40 mg CD/kg (Carpenter *et al.* 1996). A single dose of 5 mg CD/kg also alters tissue distribution of exogenous [^{14}C]CH (Carpenter and Curtis 1991). The mechanisms that underlie OC insecticide alterations in lipid homeostasis are unclear. The work below employs CD since its toxicities are representative of the OC insecticide class. It is novel in that it is refractory to metabolism in rodents (Guzelian 1982); therefore contributions of metabolites of this OC to altering CH regulatory pathways are highly unlikely.

Liver X receptors (LXRs), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs), and steroid X receptor (SXR)/ pregnane X receptor (PXR) are nuclear receptors which function as ligand activated transcription factors (Makishima *et al.* 1999; Parks *et al.* 1999; Peet *et al.* 1998). These receptors bind specific response elements in 5′ upstream promoter regions of genes after hetero-

dimerization with retinoid X receptor (RXR). LXR responds to elevated sterol concentration and increases the transcription of the Cyp7a1 gene that governs oxidation of CH to bile acids. Physiological concentrations of bile acids activate FXR and thereby repress the transcription of the Cyp7a1 gene (Makishima *et al.* 1999). Therefore, LXR and FXR coordinate CH homeostasis by regulating feed forward and feed back pathways. PPARs play a major role as a lipid metabolism regulator. It is also suggested as a controller of a CH oxidation pathway. PPARα modulates expression of Cyp8b1 (sterol 12α-hydroxylase), a hepatic microsomal enzyme involved in the biosynthesis of bile acids (Hunt *et al.* 2000). Recently a potential role of PXR in CH metabolism is also suggested. Bile acids activate PXR, which reduces their production through repression of the Cyp7a1 gene (Staudinger *et al.* 2001a; Staudinger *et al.* 2001b; Xie *et al.* 2001).

Most banned OC insecticides bind and activate PXR (Goodwin *et al.* 2002). Some, including p,o-DDT, CD, and dieldrin are also estrogenic in whole animals and cell based reporter gene assays (Charles *et al.* 2002; Donohoe and Curtis 1996). CD competes for high affinity estrogen binding sites in rainbow trout liver and increases plasma concentration of the estrogen receptor (ERα)-dependent lipoprotein, vitellogenin (Donohoe and Curtis 1996). Estrogen increases apolipoprotein A-I (apoA-I) secretion in Hep G2 cells, but ERα probably does not directly mediate this (Lamon-Fava *et al.* 1999). Modulating transcription of the apoA-I gene promoter appears more likely. PXR agonists but not a selective constitutive androstane receptor (CAR) agonist increase expression of the apoA-I gene in mice (Bachmann *et al.* 2004). ApoA-I protein is a principal component of high density lipoprotein (HDL). HDL is

the lipoprotein central to CH transport from peripheral tissues to the liver (reverse CH transport)(Lee and Parks 2005). This research addresses the hypothesis that modulation of lipoprotein metabolism at least partially explains CD alterations in CH homeostasis in male C57BL/6 mice.

This study addressed this hypothesis through a combination of in vitro and in vivo experiments. CD activation of reporter constructs for transcription factors important in regulation of CH homeostasis was determined. These experiments were conducted in a concentration range relevant to hepatic CD concentrations measured in C57BL/6 male mice after a single low dose (5 mg/kg) that altered [^{14}C]CH disposition (Carpenter and Curtis 1991). Hepatic and biliary total CH were measured in control and CD treated mice. ApoA-I was quantified by western blotting in whole plasma; lipoprotein-rich fraction, cytosol and microsomes from liver of control and CD pretreated mice. Apolipoprotein B-48/100 (apoB-48/100), a principal component of chylomicrons, VLDL and LDL, was also quantified in plasma and liver microsomes from these mice. Results demonstrated CD was a SXR and ERα agonist, decreased plasma non-HDL-CH level. CD increased hepatic apoA-I content. There was a trend for decreased apoB-100 in hepatic microsomes from CD treated animals. CD decreased hepatic cholesteryl ester concentration without changing total CH concentration. CD treatment also modulated HDL particle size and shape by increasing more non-spherical particles of variable size. At 14 days after 15 mg CD/kg apoA-I and apoB-100 but not CYP3A11 protein in hepatic microsomes is similar to controls. This work indicated altered CH homeostasis was a mode of OC insecticide action of relevance after a single low dose.

MATERIALS AND METHODS

Chemicals

Chlordecone (CD, 99 % purity) was purchased from Chem Service (West Chester, PA). CD purity was verified by GC-EI/MS. LG268 and T0901317 were acquired from Ligand Pharmaceuticals and Tularik Inc., respectively. [1-^{14}C] Lauric acid (55 mCi /mmol) was obtained from American Radiolabeled Chemicals. Inc. (St. Louis, MO). Other chemicals were obtained from Sigma (St. Louis, MO).

Animals

Male C57BL/6 mice, 6 to 7-weeks-old (20-25 g in weight), were obtained from Simeonson Laboratory (Gilroy, CA). The animals were housed in a temperature controlled room (22 ± 1^{o}C) with a daily cycle of 12 hr of light and 12 hr darkness and fed ad libitum with AIN93 diet (Dyets, Inc., Bethlehem, PA) with a free access to water. Treatments were initiated after seven days acclimatization. CD was dissolved in corn oil. Mice received CD (5 or 15 mg/kg body weight) or corn oil alone by intraperitoneal injection (5 ml/kg body weight). After 3 or 14 days, animals were fasted for 4 hr and killed by CO_2 anesthesia and exsanguinations. Blood, liver, and gallbladder were collected. The procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee.

Cell culture and Reporter gene assay

The human embryonic kidney cell line, HEK293, was maintained at 37 ^{o}C, 5 % CO_2 in DMEM containing 10 % fetal bovine serum. Luciferase cotransfection assays were performed as described (Makishima *et al.* 2002). HEK 293 cells were cotransfected with a luciferase reporter plasmid containing response element and expression vector

for murine LXRα, LXRβ, FXR and human ERα and ERβ along with the CMX-β-gal internal control. Cells were treated with vehicle (ethanol) alone or 1 to 50 μM CD and/or 1 μM T0901317 (LXR agonist), 100 nM LG268 (RXR agonist), and E2 (ER agonist). HEK293 cells were cotransfected with a plasmid encoding a fusion protein of Gal4 and the nuclear receptors ligand-binding domain along with a response element linked to a luciferase reporter. Receptors tested were: RXRα (NR2B1), LXRα (NR1H3), LXRβ (NR1H2), PPARα (NR1C1), PPARδ (NR1C2), PPARγ (NR1C3), FXR (NR1H4), SXR (human homolog of pregnane X receptor, NR1I2), human ERα (NR3A1), ERβ (NR3A2). Cells were treated with ethanol vehicle or CD (0.1, 1, 10, or 50 μM). Activation of nuclear receptors was assessed by measuring luciferase activity. Individual assays were repeated at least three times.

Tissue preparation

Blood was collected by cardiac puncture in heparinized syringes and kept on ice until plasma was isolated by centrifugation at 3000 g for 25 min at 4 ^{o}C. Plasma was aliquoted and stored at -80 ^{o}C. Liver was homogenized in buffer (0.01 M potassium phosphate, pH 7.5; 0.15 M KCl; 1.0 mM EDTA; 0.1 mM butylated hydroxytoluene (BHT); 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) with a polytron PT 3000 (Brinkmann, Westbury, NY). Liver homogenate was centrifuged at 12,000 g for 30 min. The supernatant was again centrifuged at 100,000 g for 90 min at 4 ^{o}C with Ti 70 rotor (Beckman, Fullerton, CA). The floating fatty layer in the supernatant was regarded as lipoprotein-rich fraction. The remaining supernatant and resulting pellet were cytosolic and microsomal fractions, respectively. Microsomes were resuspended in microsome resuspension buffer (0.1 M potassium phosphate, pH 7.25; 1.0 mM

EDTA (ethylenediaminetetra aceticacid); 30% glycerol; 0.1 mM PMSF (phenylmethyl sulfonyl fluoride), 1.0 mM DTT (dithiothreitol), and 20 μM BHT (butylated hydroxy-toluene)). Protein concentration was determined by the BCA assay (Pierce Biotechnology, Inc. Rockford, IL).

Western blot analysis

Mouse liver subcellular fractions (lipoprotein-rich, cytosol and microsomes) were prepared from control, 5 or 15 mg CD/kg treated animals as above. Proteins from each preparation were separated on Tris/glycine gels (Bio-Rad, Richmond, CA) under reducing conditions. Following gel electrophoresis at 100 V for 2 hr, proteins were electrophoretically transferred onto PVDF membranes utilizing a Mini trans-blot transfer cell (Bio-Rad, Richmond, CA). Membranes were then blotted with antibodies; Rabbit anti-CYP7A antibody was a generous gift from Professor John Y. L. Chiang (Northwestern Ohio University) or purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-CYP3A antibody was a generous gift from Professor Donald Buhler (Oregon State University). Goat anti-CYP4A antibody was purchased from BD Gentest (Woburn, MA). The blots were then probed with horseradish peroxidase conjugated goat anti-rabbit IgG or rabbit anti-goat IgG as the secondary antibody (Bio-Rad, Richmond, CA). The proteins were detected after development with enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ). Quantification of the intensity of the protein bands was performed with NIH-image software.

Enzyme activities

Liver enzyme activities were analyzed with a HPLC (Waters 2690) and a reverse phase C_{18} HPLC column (4.6 mm × 25 cm, 5 μm, ultrasphere, Beckman). In the assay of CH 7α-hydroxylase, reaction products after the enzymatic conversion of 7α-hydroxylase-CH to 7α-hydroxy-4-cholesten-3-one (7α-HCO) by CH oxidase (Sigma, St. Louis, MO) were detected by a UV spectrophotometer (Chiang 1991). [1-^{14}C] Laurate hydroxylation was measured using slight modifications of the procedure described by Williams et al. (Williams *et al.* 1984). Samples were analyzed using 62 % methanol containing 0.2 % acetic acid to elute ω- and ω-1 hydroxylaurate at a flow rate of 1 ml/min and the mobile phase was switched to 100 % methanol to elute parent compound and detected by Flo-One TR505 radioactivity flow monitor.

Plasma lipid analyses

Total plasma CH concentrations were determined after a 4 hr fast using enzymatic kits (Sigma Diagnostic, St. Louis, MO). Plasma HDL-CH concentration were measured after precipitation of apolipoprotein-B containing particles with dextran sulfate and magnesium (Rachem, San Diego, CA). Plasma contents of apoA-I and apoB-48/100 were quantified by densitometric scanning following electrophoresis on 4-15% Tris/glycine gels (Bio-Rad, Richmond, CA) and transferred onto PVDF membranes (Bio-Rad, Richmond, CA). The membranes were incubated with rabbit anti-apoA-I or rabbit anti-apoB-48/100 polyclonal antibodies (Biodesign, Saco, ME) and probed with horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized by enhanced chemiluminescence (ECL) detection (Amersham, Piscataway, NJ).

Electron microscopy

Negative stain electron microscopy was done using a Philip's CM12 TEM electron microscope (Philip's, Eindhoven). HDL fraction was mixed with an equal volume of 2 % ammonium molybdate on a carbon/formvar-coated copper grid. After excess liquid was blotted, the rest was allowed to dry. The grid was examined within 1 hr.

Hepatic cholesterol content

For extraction of lipids, ~100 mg liver was incubated in KOH/ethanol at 70^{o}C for 30 min. Following incubation, 2 ml hexane was added and the solution was inverted three times and centrifuged for 3 min at 3,000 g. The lipid layer (upper phase) of each sample was collected and dried under nitrogen. Dried lipids were dissolved in 2-propanol containing 10 % Trinton-X-100 as assay samples. The CH concentration was determined using a Cholesterol/Cholesteryl ester Quantitation Kit^{R} (BioVision, Mt. View, CA). Hepatic esterified CH concentration was taken as the difference between the total CH and free CH.

Statistical Methods

Data were expressed as means ± standard error (SEM). Comparisons among groups were submitted to a one-way analysis of variance (ANOVA). For comparison between two groups, student's t-test with equal-variance was applied. In all analyses, a 95% confidential level was used as the criterion for significance. For outlier identification, Grubbs' Test (assumes normality) was applied.

RESULTS

To determine the effect of CD on activation of nuclear receptors involved in lipid homeostasis, a transient transfection assay using a synthetic LXR or FXR responsive reporter plasmid was initially assessed. CD alone strongly suppressed activities of the murine LXRα-RXR and LXRβ-RXR heterodimers in a concentration dependent manner. CD (10 μM) strongly inhibited LXRα-RXR activation by LG268 (RXR agonist)(Fig. 3-1B), but it mildly inhibited the receptor activation by T0901317 (LXR agonist)(data not shown). CD moderately repressed activation of LXRβ-RXR activated by T0901317 (Fig. 3-1C). CD (50 μM) activated FXR approximately 50 % as effectively as the specific RXR agonist LG268 (Fig. 3-1D). In the presence of LG268, CD increased FXR transactivation cooperatively (Fig. 3-1D).

We then performed a series of reporter gene cotransfection assays in HEK 293 cells to characterize the specificity of the CD effect. CD activated or inhibited transactivation by several nuclear receptors (Fig. 3-2). At 10 μM CD there were trends for suppression of reporter construct alone and RXRα (about 30 %)(Fig. 3-2A). CD induced more substantial inhibition for LXRα and strongly suppressed activation for LXRβ. CD modestly increased activation (about 1.3-fold) for FXR and PPARα and strongly activated SXR (human homolog of PXR) about 3.5-fold over background (Fig. 3-2A, 3-2B). PPARδ and PPARγ were not affected by CD treatment (Fig. 3-2B).

The relevance of CD activation of nuclear receptors in vitro was assessed with in vivo assays in C57BL/6 mice. Interactions with LXRα and FXR were assessed by hepatic microsomal CYP7A1 protein immunoquantitation and CH 7α-hydroxylase activity. There was a trend decreased CYP7A1 protein content and its enzyme activity

but it was not statistically significant (Fig. 3-3A, 3-3B). Interaction with PPARα was assessed by hepatic microsomal CYP4A1 protein immunoquantitation and lauric acid ϖ-1 hydroxylase activity. There was no evidence for activation of this receptor (Fig. 3-3A, 3-3B). Finally, interaction with SXR/PXR was assessed by hepatic microsomal CYP3A11 protein immunoquantitation. CYP3A11 protein level was highly increased (4-fold, $p < 0.05$) in livers from CD treated animals (Fig. 3-3A). Increased hepatic microsomal CYP3A11 protein after CD treatment indicated SXR activation.

We also assessed human ERs activation by CD. Ten μM CD activated human ERα (about 2-fold)(Fig. 3-4B) and resulted in a strong activation of a chimera consisting of DNA binding domain of GAL4 and the ligand binding domain of ERα (10-fold)(Fig. 3-4A). CD had minimal effect on human ERβ activity but repressed E2-induced ERβ activation, effectively (40 %)(Fig. 3-4B).

The effect of CD on total plasma CH and HDL-CH concentration were measured using enzymatic analyses. Plasma non-HDL-CH concentration was significantly decreased by CD treatment ($p < 0.05$) (Fig. 3-5A). Plasma HDL-CH was unchanged but the ratio of HDL-CH to plasma CH was mildly increased (10 %, $p < 0.05$) (Fig. 3-5A, 3-5B).

Immunoblot analysis of plasma revealed that there was a trend for decreased apoB-48/100 in CD treated mice without a significant change in apoA-I (Fig. 3-5C). The effect of CD on apolipoprotein content was further characterized in liver subcellular fractions (lipoprotein-rich, cytosol and microsomes). Interestingly apoA-I increased in the hepatic lipoprotein-rich and microsomal fractions from CD treated

mice (1.5-fold, 1.8-fold, respectively, $p < 0.05$) (Fig. 3-6A). There was a trend for increased hepatic microsomal apoB-100 content by CD treatment (Fig. 3-6B).

Negative stain electron microscopic study demonstrated that the HDL particle size from control mice was uniform whereas the HDL particles from CD treated mice were less spherical and more heterogeneous in size (Fig. 3-7). Hepatic total, free and esterified CH content was measured enzymatically. About 90 % of hepatic CH was in the unesterified form (Fig. 3-8). Hepatic total and free CH contents from CD-treated mice were not different from control mice (Fig. 3-8). However, CD decreased hepatic esterified CH concentration (about 40 % of control, $p < 0.05$) (Fig. 3-8).

An ancillary experiment examined persistence of CD induced proteins change in liver. Hepatic microsomal CYP3A11 was 2-fold higher in 15 mg CD/kg treated mice than controls 14 days after injection ($p < 0.05$, Fig. 3-9). Hepatic microsomal CYP7A1 and CYP 4A1 were not different from controls in the same mice (Fig. 3-9). Plasma and hepatic apoA-I were similar to controls 14 days after 15 mg CD/kg (Fig. 9). Hepatic apoB-100 was not different from controls in the same mice (Fig. 3-9).

DISCUSSION

Humans are exposed to complex mixtures of xenobiotics including drugs, pesticides and other environmental contaminants. In the body, xenobiotics, dietary nutrients, and endogenous substances may interact to modulate regulatory pathways for intermediary metabolism. Inhibition or activation of nutrient metabolism or transport pathways may yield beneficial or adverse effects. Prior reports indicate that an overtly non-toxic dose of CD, an OC insecticide, alters tissue distribution of exogenous CH in mice and rats (Carpenter and Curtis 1991; Gilroy *et al.* 1994). Subsequent experiments and other work suggests that CD also modulates other aspects of lipid homeostasis (Carpenter *et al.* 1996; Chetty *et al.* 1993). However, molecular mechanisms underlying these observations are unclear.

CH is an important constituent of cell membranes and necessary for synthesis of bile salts essential for digesting dietary fats. However, CH is not solely beneficial. When present in excessive amounts, it contributes to heart attacks and stroke by promoting atherosclerosis (Krieger 1999). Therefore, CH levels are precisely controlled in the body. The liver is the major organ that controls CH homeostasis in mammals. The role of the liver in CH homeostasis involves coordinate regulation of biosynthesis of CH, its uptake from plasma and catabolism to bile acid (Brown and Goldstein 1999).

Nuclear receptors are ligand activated transcription factors that regulate gene expression through the binding to nuclear receptor response elements. A large body of literature shows that nuclear receptors play important roles in the regulation of metabolism of CH and other lipids through coordinated network of transcriptional

programs (Chawla *et al.* 2001; Lobaccaro *et al.* 2001; Makishima 2003). To increase understanding of how low doses of CD alter CH homeostasis, we assessed CD interactions with several nuclear receptors in vitro and in vivo. Our data demonstrated that CD activated FXR and inhibited LXRs (Fig. 3-1B, 3-1C, 3-1D, Fig. 3-2A). CD (50 μM) minimally increased FXR activation (data not shown) but co-expression of FXR with exogenous RXR resulted in a significant enhancement (about 5-fold over background) (Fig. 3-1D). This finding indicated the requirement of RXR (heterodimer partner) for CD-induced FXR activation. CD affected transactivation by a chimera consisting of the DNA binding domain of GAL4 and the ligand-binding domain of LXRs and FXR (Fig. 3-2). These observations demonstrated that the activation or inhibition required the ligand binding domain of these receptors. CH 7α-hydroxylase (CYP7A) is the first and a rate limiting enzyme in bile acid synthesis, the major CH elimination pathway (Russell 1999). LXRα and FXR regulate the expression of CH 7α-hydroxylase in opposite ways (Makishima 2003). Therefore, bile acid synthesis is regulated by feed forward and feedback mechanisms. Although CD activated FXR and inhibited LXRα activation in vitro assay, there was only a trend for the reduction of hepatic CYP7A1 and its enzyme activity by CD treatment in C57BL/6 mice (Fig. 3-3A, 3-3B). LXRα induces CYP7A1 by binding to the 5'-flanking region and increasing expression of mouse and rat 7α-hydroxlase. FXR represses Cyp7a1 indirectly through the induction of the transcriptional repressor steroid heterodimer partner (SHP)(Staudinger *et al.* 2001a; Staudinger *et al.* 2001b). Recent results suggested that PXR strongly induced the CYP3A4 gene by inhibiting SHP gene transcription in human livers (Li and Chiang 2006).

PXR is regarded as a key regulator of CYP3A expression since PXR is activated by diverse compounds which are known to induce CYP3A expression and binds to xenobiotic response elements in CYP3A promoters (Xie *et al.* 2000). Because of evolutionary divergence of the PXR ligand-binding domain in humans and mice, there are striking species-specific difference between two species (Kliewer 2003; Xie *et al.* 2000). Therefore, the human specific CYP3A inducer, rifampicin, little effected CYP3A gene expression in mice, where as mouse specific CYP3A inducer, PCN, little effected expression of the CYP3A gene in human hepatocytes (Bachmann *et al.* 2004). Here we demonstrated that CD not only activated transactivation by SXR (human homolog PXR) in an in vitro assay but also increased hepatic microsomal CYP3A11 protein level (Fig. 3-3A) in C57BL/6 mice. Our data indicated that CD activated SXR/PXR in both species.

LXRβ knockout mice handle excess cholesterol as effectively as wild type (Alberti *et al.* 2001). However, recent studies demonstrated that LXRβ isoform also can contribute to CH homeostasis through activation of ATP-binding cassette AI (ABCAI) in macrophages, liver, and intestine (Quinet *et al.* 2006; Repa *et al.* 2000).

CD was an ERα but not an ERβ agonist in the micromolar range (Fig. 3-4A). CD activated human ERα cooperatively with E2, although the activity was 10,000 times lower than E2 (Fig. 3-4B). This difference in potency was similar to that reported for competition assays for estrogen binding sites in rainbow trout liver (Donohoe and Curtis 1996). CD inhibited E2-induced ERβ activation effectively (Fig. 3-4B). PPARα, PPARδ and PPARγ were not activated or inhibited by CD treatment (Fig. 3-2B). In the present in vitro study, 10 or 50 μM CD activated or inhibited

transactivation of nuclear receptors involved in CH and lipid metabolism. Whole livers of C57BL/6 mice contained 73 μM CD 3 days after 5 mg/kg (Carpenter and Curtis 1989). Therefore, hepatic protein and enzyme activity response of C57BL/6 mice supported CD activation or inhibition of these nuclear receptors after a single low dose. Among all the nuclear receptors investigated, LXRβ, SXR, and ERα exhibited the highest CD affinity.

Since non-HDL-CH concentration was decreased in CD-treated mice (Fig. 3-5A), increased apoB containing lipoproteins clearance or reduced VLDL/LDL-CH production and/or secretion probably contributed to the decreased plasma CH. There was a trend for higher hepatic microsomal apoB-100 content in 15 mg CD/kg pretreated mice compared to controls suggested the former possibility (Fig. 3-6B). Estrogens enhanced LDL receptor activity and upregulated expression of apoB (Brown and Goldstein 1986; Srivastava *et al.* 1993). LXR activation induced hepatic LDL-receptor expression (Masson *et al.* 2004). Since LXRα activation was inhibited by CD in vitro and LDL receptors were not up-regulated by estrogen in mice (Srivastava *et al.* 1997), reduced VLDL/LDL-CH production was also a plausible explanation for decreased non-HDL-CH concentration. Plasma non-HDL-CH increased in CD pretreated mice compared to controls 4 but not 16 hr after a intraperitoneal (ip) dose of 5 ml/kg corn oil containing 10 mg CH/kg (Lee *et al.* unpublished data).

ApoB is known to be synthesized constitutively and regulated primarily by co and post-transitional mechanisms in the secretory pathway (Avramoglu and Adeli 2004). ApoB secretion is regulated by ubiquidin-mediated proteasomal degradation or

non-proteasomal degradation pathways. There is a positive correlation between hepatic availability of neutral lipids proximal to the site of apoB synthesis and amount of hepatic apoB secretion (Avramoglu and Adeli 2004). Reduced hepatic cholesteryl ester not free CH inhibited VLDL apoB production (Telford *et al.* 2005). Previous work demonstrated that CD decreased cytosolic lipid droplets in hepatocytes in C57BL/6 mice (Carpenter *et al.* 1996). Here we showed that CD decreased hepatic CH ester, not total or free CH (Fig. 3-8) and there was a trend for increased apoB content in the liver microsomal fraction from CD treated animals. Thus, insufficient lipid perhaps increased apoB degradation by ubiquidin-mediated proteasomal degradation and reduced VLDL secretion. However the observation that CD treatment apparently increased number of cytosolic vesicles containing VLDL like structure (Carpenter *et al.* 1996) suggested that CD perhaps increased lipid secretory activity by stimulating VLDL secretion. Increased plasma non-HDL-CH in CD pretreated mice after ip lipid bolus (Lee et al., unpublished data) supported this hypothesis.

HDL is an important source of CH for biliary excretion in rodents. HDL transports CH from peripheral tissues to the liver by the reverse CH transport (RCT) pathway (Srivastava 2003). ApoA-I is a major apolipoprotein in HDL particles. ABCAI is a member of a large family of ABC transporters (Langmann *et al.* 1999). ABCAI transporter increases efflux of intracellular CH to lipid-poor apoA-I to form preβ–HDL or nascent HDL (Wang *et al.* 2000). Scavenger receptor class type I (SR-BI), a physiological HDL receptor, facilitates the selective uptake of HDL-CH esters in the RCT pathway (Rigotti *et al.* 1996; Ueda *et al.* 1999).

PXR increased HDL-CH and expression of apoA-I in vitro and in vivo (Bachmann *et al.* 2004; Sporstol *et al.* 2005). Estrogens were suggested as antiatherogenic since they reduced LDL-CH and elevated HDL-CH (Hargrove *et al.* 1999). Activation of ERα was required for estrogen-mediated protection against vascular injury (Pare *et al.* 2002). While CD was a mixed agonist for human SXR and ERα, HDL-CH and plasma apoA-I contents were not changed in fasted mice (Fig. 3-5A, 3-5C). Instead, we observed that CD increased apoA-I content in hepatic lipoprotein-rich and microsomal fractions (Fig. 3-6A). We also observed that CD increased, although mildly but statistically significantly, the percentage of HDL-CH to plasma-CH (Fig. 3-5B). Since HDL-CH was widely considered as the good CH that provided some protection from heart disease, the potential role of PXR in raising HDL-CH was an important area of current interest. However not all HDL-subclasses were equally protective. Large HDL2b was believed more responsible for protection from coronary heart disease in clinical observational studies (Pascot *et al.* 2001). CD increased less spherical and more heterogeneous HDL particles (Fig. 3-7). Studies demonstrated that PXR regulated expression of ABCAI negatively and apoA-I positively in vitro and in vivo (Bachmann *et al.* 2004; Sporstol *et al.* 2005). These observations indicated that CD perhaps alters lipoprotein metabolism by perturbing the RCT pathway.

Hepatic CD concentrations declined slowly in C57BL/6 mice (Carpenter and Curtis 1989). Liver residues declined 12 -25 % from 3 to 14 days after intraperitoneal injections that yielded doses similar to those used in this study. Elevated CYP3A11 content in hepatic microsomes 14 days after injection was consistent with persistent

PXR signaling (Fig. 3-9). Hepatic content of apoA-I and apoB were not elevated at that time (Fig. 3-9). Perhaps differential adaptation of signaling pathways are involved in this phenomenon.

Even though most uses of persistent OC pesticides were banned they continue to occur in tissues of humans and wildlife. Trophic transfer through food webs that provide dietary fish, meat and dairy product is the principal route of environmental exposure to these agents. Perturbation of CH homeostasis by low dose of CD may be act as an environmentally relevant mode of endocrine disruption. Therefore, it is necessary to continue these studies to explain altered CH and lipid homeostasis by CD.

In summary, 1) CD was a mixed agonist for human SXR and ERα and an effective antagonist for LXRβ. 2) CD modulated lipoprotein metabolism by complex nuclear receptor activations. 3) This work indicated altered CH homeostasis was a mode of OC insecticide action of relevance after a single low dose.

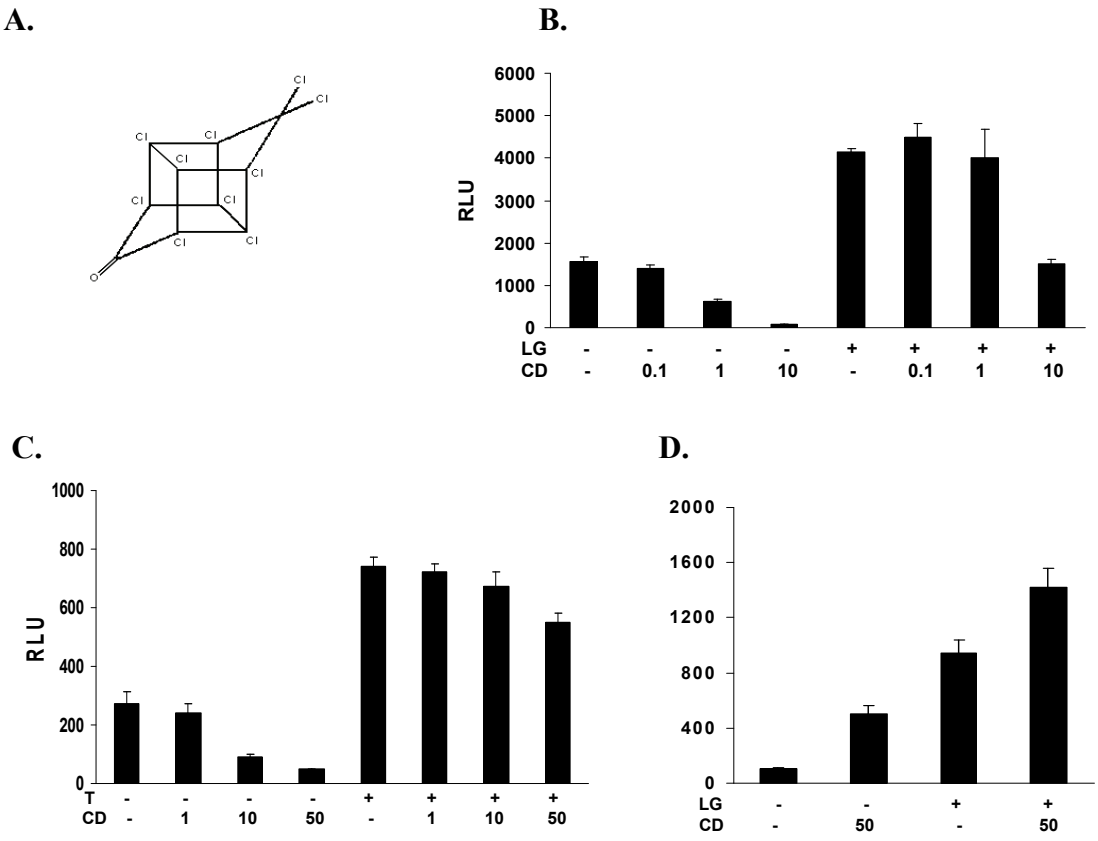


Figure 3-1. Chlordecone inhibits LXRs and activates FXR.
A. Structure of chlordecone (CD). **B.** HEK 293 cells were cotransfected with a luciferase reporter plasmid [$(LXRE)_3$ Tklc] containing three copies of the LXR response element upstream of the thymidine kinase (TK) promoter and expression vector for murine LXRα and RXR along with the CMX-β-gal internal control. **C.** HEK 293 cells were cotransfected as in Fig. 3-1B for LXRβ and RXR. Cells were treated with vehicle (ethanol) alone or 0.1 to 50 μM CD and/or 1 μM T0901317 (LXR agonist) or 100nM LG268 (RXR agonist) as indicated. **D.** HEK 293 cells were cotransfected with a luciferase reporter plasmid [(FXRE) Tklc] construct containing an FXR response element upstream of the TK promoter and expression vector for FXR and RXR. Cells were treated with vehicle (ethanol) alone or 50 μM CD and/or 100 nM LG268 (RXR agonist) as indicated. Values are presented as relative luciferase induction from triplicate assays ± SD.

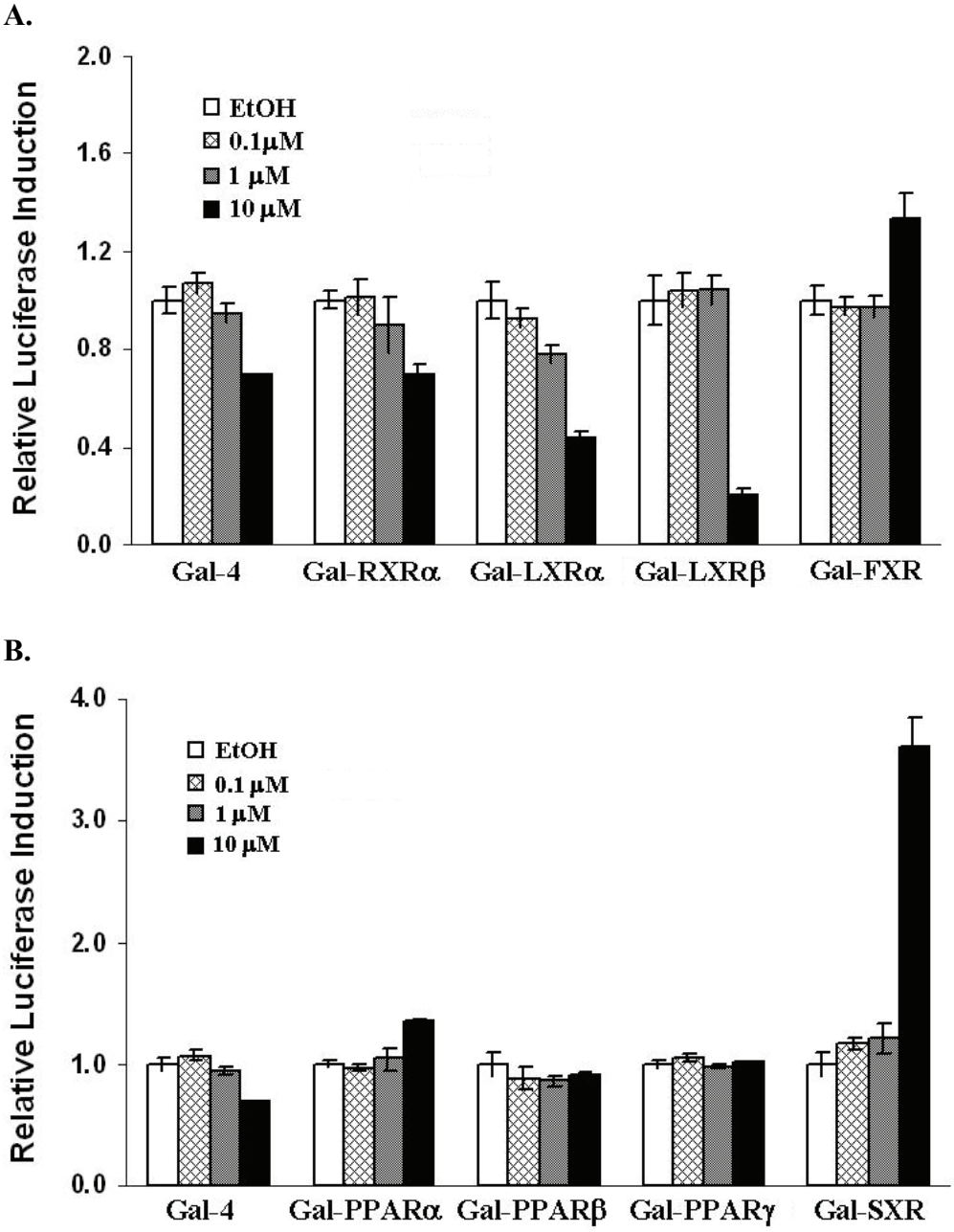
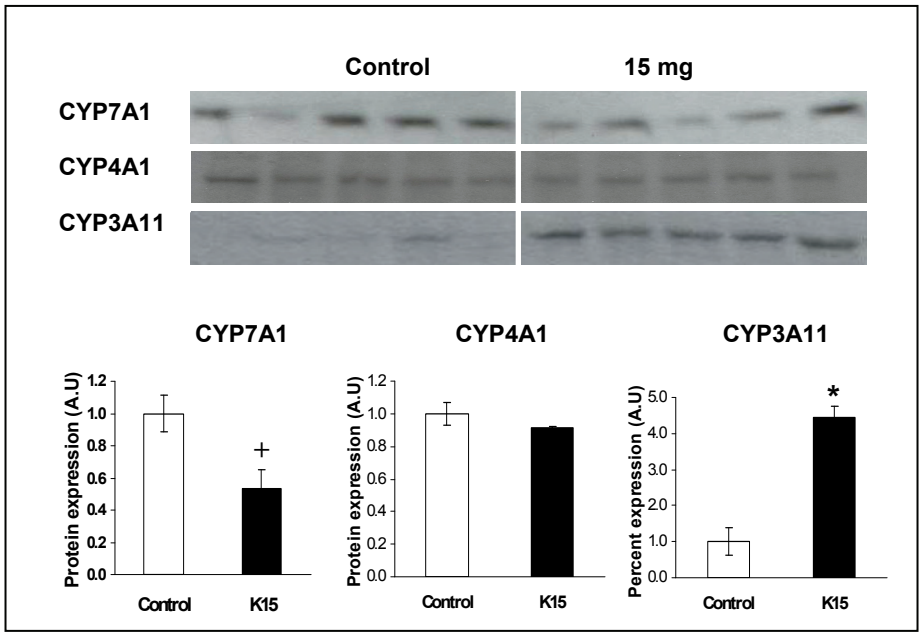


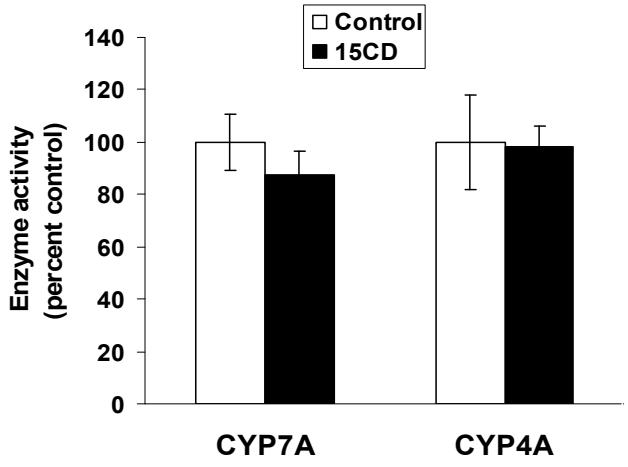
Figure 3-2. Chlordecone activates Steroid X Receptor (human homolog of PXR) HEK 293 cells were cotransfected as in Fig. 3-1B with the Gal 4 luciferase reporter and a series of chimeras in which Gal4 DNA binding domain is fused to the indicated nuclear receptor ligand binding domain. Cells were treated with ethanol or CD (0.1, 1, or 10 μM). Activation of nuclear receptors was assessed by measuring luciferase activity. Values are presented as relative luciferase induction from triplicate assays ± SD.

Figure 3-3. In vivo evidence for chlordecone activation of SXR/PXR.
Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg body weight by ip injection. Hepatic microsomes were prepared from individual animals (5-6 mice in each group) after 3 days as described under "Materials and Methods". **A.** Immunoblot analyses of CYP7A1, CYP4A1, and CYP3A11 proteins in liver microsmes. Total 20 μg protein samples were separated by SDS-PAGE and blotted with antibodies against the specific CYP isoforms as described under "Materials and Methods". Values are expressed as the mean relative protein expression (A.U) ± standard error (SEM) compared with the controls. **B.** Enzyme activity analyses of CH 7 α-hydroxylase for CYP7A1 and lauric acid ω-1 hydroxylase for CYP4A1. Values are expressed as the mean relative enzyme activity (%) ± standard error (SEM) compared with the controls. *Significantly different from the control ($p < 0.05$). $^{+}$Different from the control ($p = 0.056$).

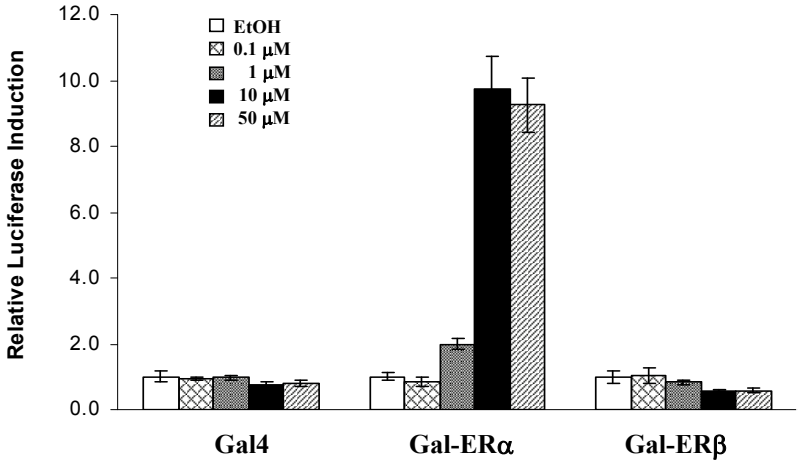
A.


Control
15 mg
CYP7A1
CYP4A1
CYP3A11
CYP7A1
CYP4A1
CYP3A11
Protein expression (A.U)
Protein expression (A.U)
Percent expression (A.U)
Control
K15
Control
K15
Control
K15
+
*

B.


Control
15CD
Enzyme activity
(percent control)
CYP7A
CYP4A

A.



B.

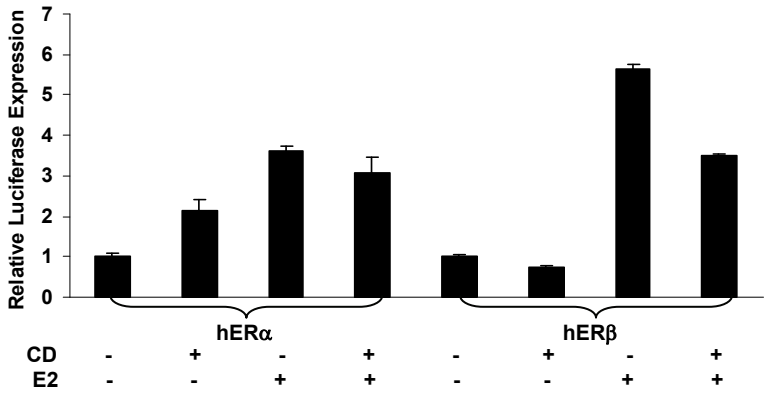


Figure 3-4. Chlordecone activates ERα

A. HEK 293 cells were cotransfected with the Gal 4 luciferase reporter and chimeras in which Gal4 DNA binding domain is fused to the indicated nuclear receptor ligand binding domain. **B.** Expression vectors for human ERα or ERβ were cotransfected with luciferase report construct containing ER response element [ERE(EFP)Tkluc] plasmid along with the CMX-β-gal internal control. Cells were treated with ethanol as vehicle, 10 μM CD or 1 nM E2 (ER agonist). Values are presented as relative luciferase induction from triplicate assays ± SD.

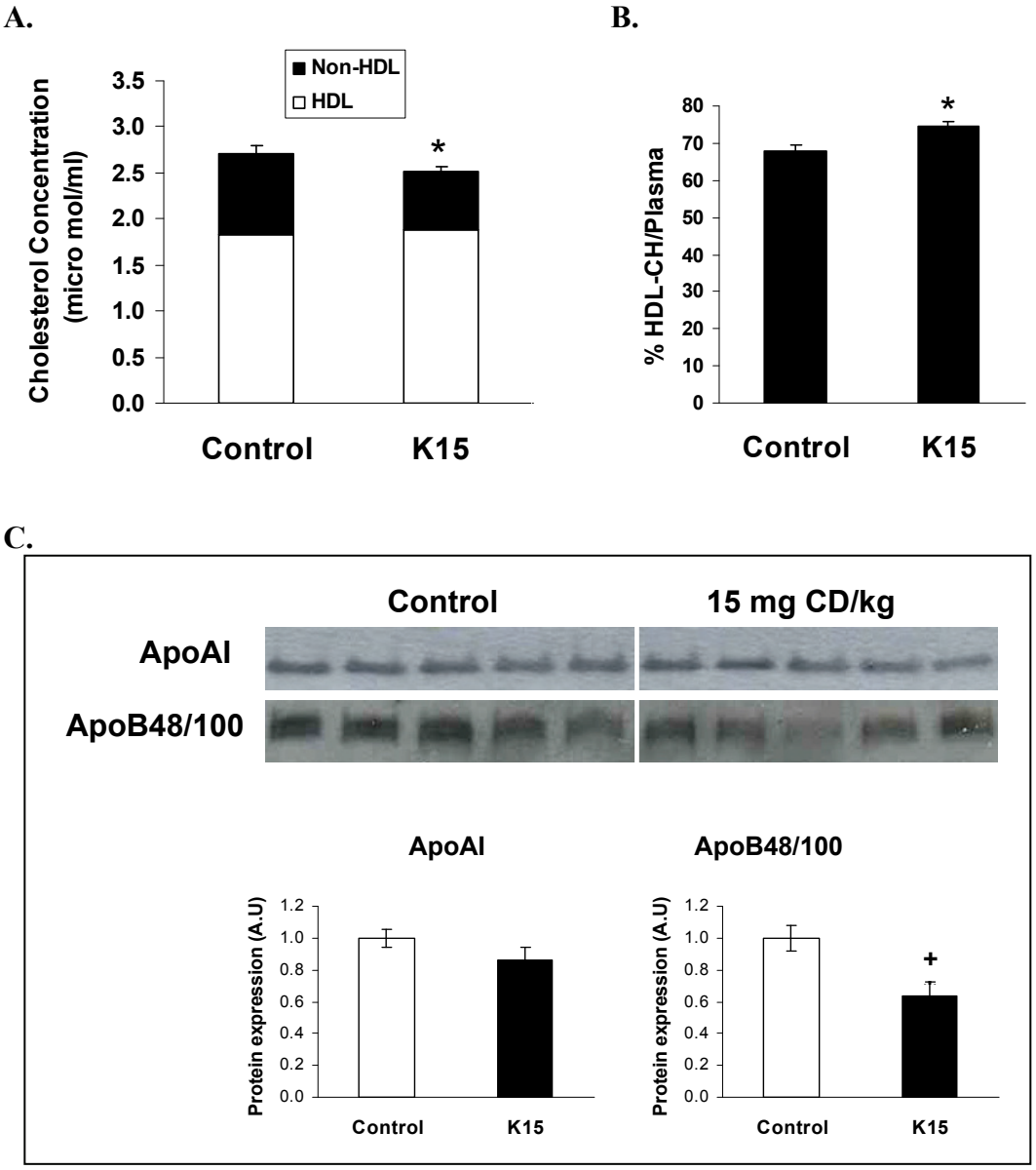
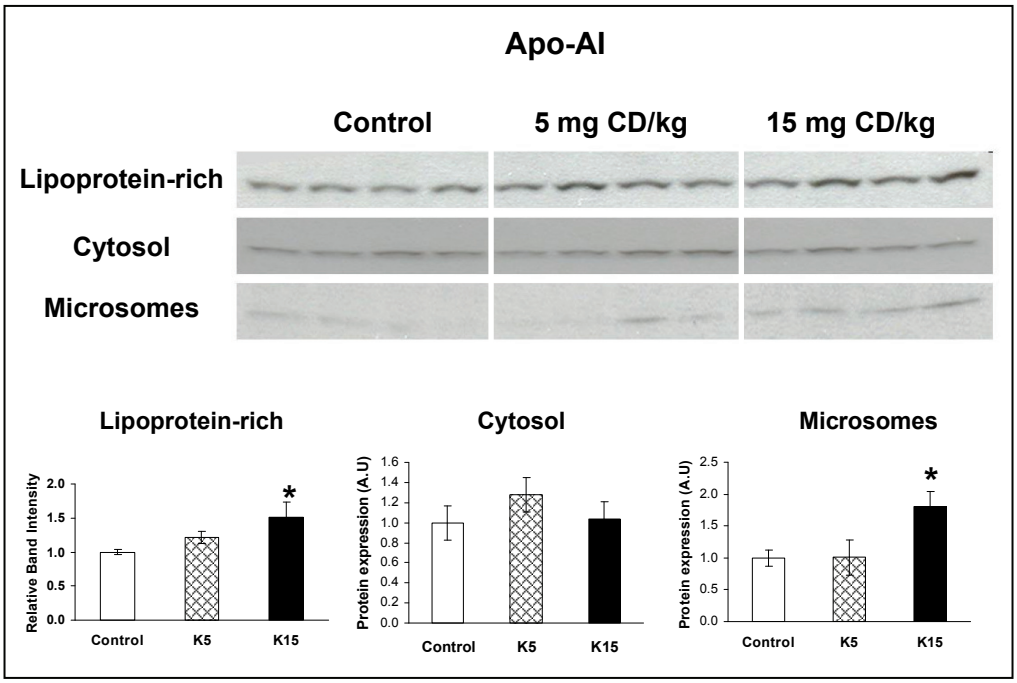


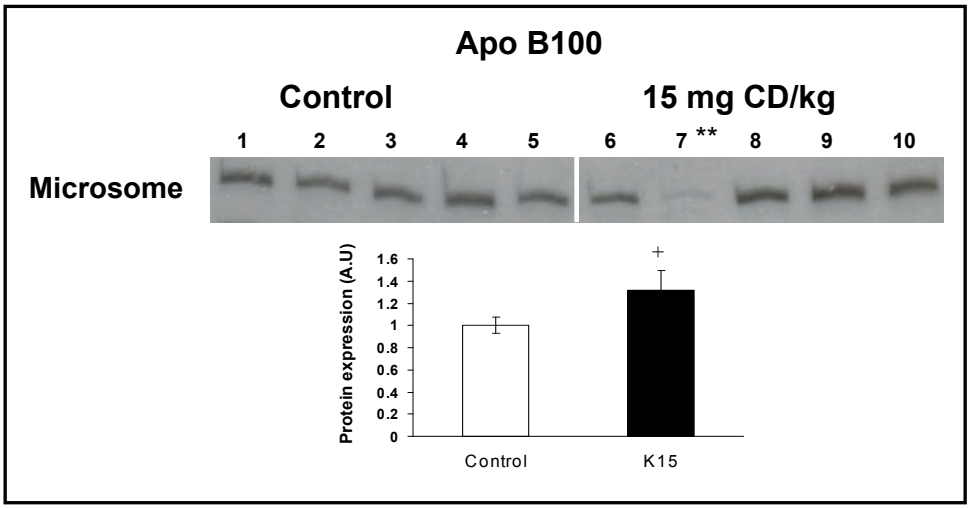
Figure 3-5. Chlordecone reduces non-HDL plasma cholesterol after a 4 hr fast. Individual plasma was collected from animals (5-6 mice in each group) treated with either corn oil or 15 mg/kg body weight by ip injection. **A.** Plasma total CH and HDL-CH was determined enzymatically as described under "Material and Methods". Non-HDL-CH content was taken as the difference between the total plasma total CH and HDL-CH. **B.** Ratio of HDL-CH to plasma CH. **C.** Western blot analyses of plasma apoA-I and apoB48/100 protein. Plasma apolipoprotein content was measured by western blotting with either apoA-I or apoB100 antibodies. Values are expressed as mean ± standard error (SEM). $^{+}$Different from the control ($p = 0.059$).

Figure 3-6. Effect of Chlordecone on hepatic apolipoprotein content.
Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg body weight by ip injection. Hepatic subcellular fractions were prepared from individual animals (5-6 mice in each group) after 3 days as described under "Materials and Methods". **A.** Hepatic apolipoprotein A-I or **B.** apolipoprotein B100 was measured by immunoblotting with either apoA-I or apoB100 antibodies. Values are expressed as the mean relative protein expression (A.U) ± standard error (SEM) compared with the controls. ** Plasma CH concentration was 55.3 mg/dl. Grubbs' test was applied for outlier identification. $^{+}$Different from the control ($p < 0.1$).

A.


Apo-AI
Control
5 mg CD/kg
15 mg CD/kg
Lipoprotein-rich
Cytosol
Microsomes
Lipoprotein-rich
Relative Band Intensity
Control
K5
K15
Cytosol
Protein expression (A.U)
Control
K5
K15
Microsomes
Protein expression (A.U)
Control
K5
K15

B.


Apo B100
Control
15 mg CD/kg
1
2
3
4
5
6
7 **
8
9
10
Microsome
Protein expression (A.U)
Control
K15

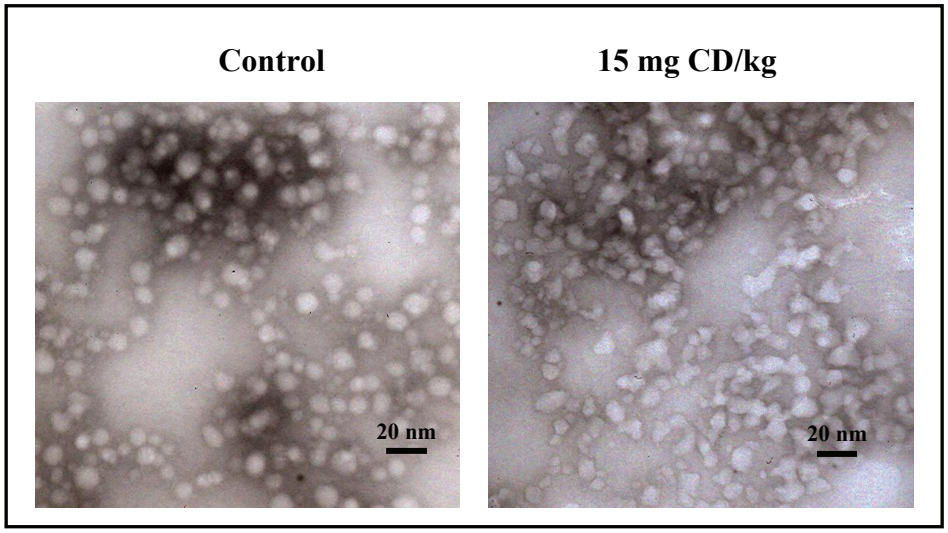


Figure 3-7. Analysis of HDL particles in plasma after a 4 hr fast.
Electron microscopic study of HDL particle size. Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg body weight by ip injection. Plasma was collected from individual animals (5-6 mice in each group) after 3 days. HDL fraction was separated as described under "Materials and Methods" and visualized under an electron microscope.

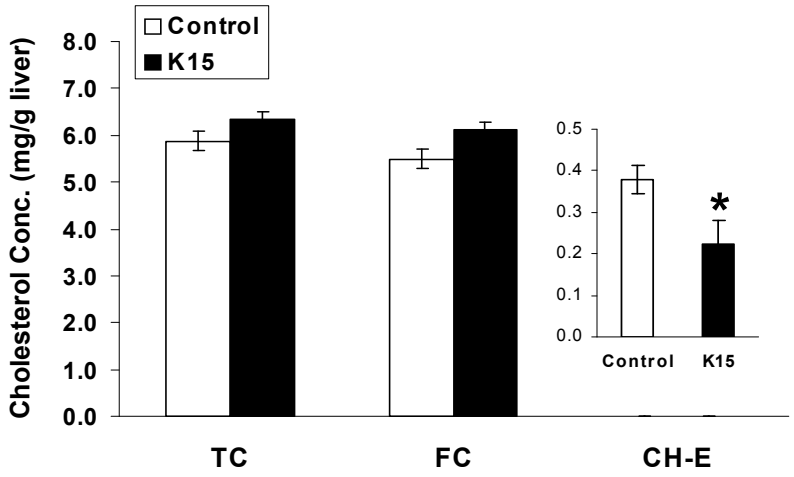


Figure 3-8. Chlordecone reduces hepatic cholesteryl ester content.
Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg body weight by ip injection. Liver was collected from individual animals (5-6 mice in each group) after 3 days. After lipid extraction with hexane, liver total and free CH content were determined using an enzymatic method as in under "Material and Methods. Hepatic esterified CH content was taken as the difference between the total CH and free CH. Values are expressed as mean CH level ± standard error (SEM). *Significantly different from the control ($p < 0.05$).

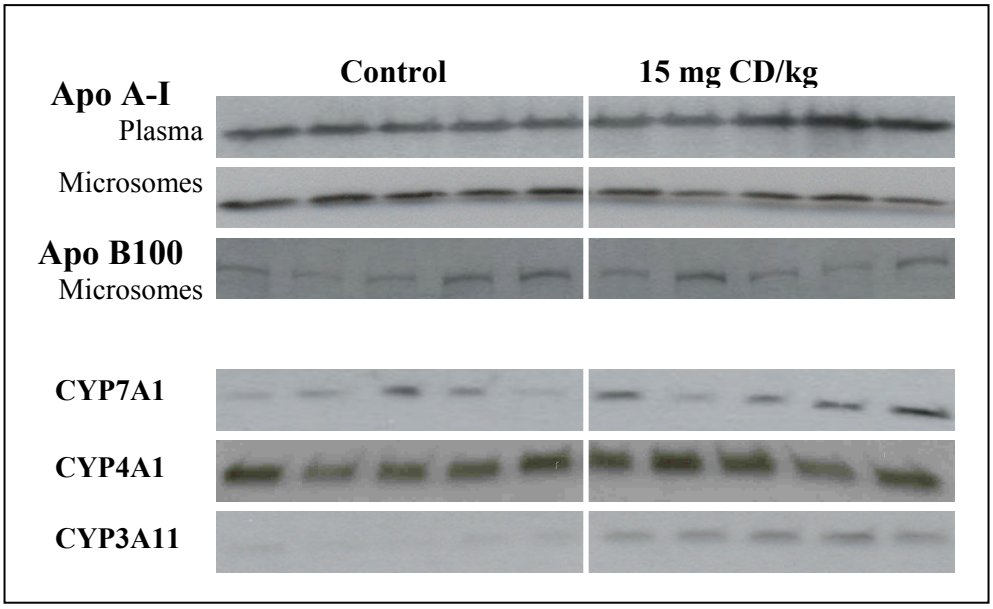


Figure 3-9. Apolipoproteins and cytochrome P450 contents at 14 days after 15 mg CD/kg treatment.
Animals were treated with corn oil or 15 mg CD/kg body weight by ip injection. Plasma and hepatic microsomal fractions were prepared from individual animals (5-6 mice in each group) after 14 days as described under "Materials and Methods". Immunoblotting was performed with apoA-I, apoB100, CYP7A, CYP4A or CYP3A antibodies.

Chapter 4

Conclusion

The present results demonstrate that pretreatment with a single, overtly nontoxic dose of CD alters plasma, tissue and hepatic subcellular distribution of exogenously administered [^{14}C]CH.

CD treatment alone mildly but statistically significantly decreases non-HDL-CH and increases the percentage of HDL-CH in plasma. Subsequent ip lipid bolus injection to CD pretreated animals also reduces [^{14}C]CH equivalents in non-HDL fraction and increases the ratio of [^{14}C]CH equivalents in HDL to that in plasma after 4 hr. However CD pretreatment increases total non-HDL-CH concentration 4 hr after lipid bolus dose.

CD treatment alone decreased hepatic cholesteryl ester concentration but not hepatic total and free CH concentration. Consistent with previous result, subsequent ip lipid bolus injection to CD pretreated animals reduced disposition of [^{14}C]CH to liver. CD pretreatment decreased [^{14}C]CH equivalents in microsomal and cytosolic fractions 4 and 16 hr after ip bolus lipid, respectively and decreased relative distribution of [^{14}C]CH to lipoprotein-rich fraction 16 hr after ip bolus lipid. However CD pretreatment did not change total hepatic total CH and cholesteryl ester concentration 16 hr after lipid bolus dose. Our finding suggests that CD pretreatment increased VLDL production and secretion after ip bolus lipid dose. Present data also suggests that binding of CD to cytosolic proteins perhaps not only inhibited CH transport to microsomes but also modulated biliary CH secretion.

CD treatment dose not change total CH concentration in gallbladder. However, CD pretreatment stimulates biliary excretion of [^{14}C]CH and increases total CH concentration in gallbladder 4 hr and 16 hr after ip lipid bolus, respectively. Reduced disposition of [^{14}C]CH to liver and stimulated biliary CH excretion are not associated with altered hepatic membrane SR-BI and ABCG8 protein contents.

CD (10 μM) strongly suppresses LXRβ activation and increases activation for SXR (human PXR homolog). The same concentration of CD increases activation of human ERα specifically and suppresses E2-mediated ERβ activation effectively. Increased hepatic CYP3A11 protein content by western blotting supports SXR/PXR activation.

CD increases ApoA-I protein content in hepatic lipoprotein-rich and microsomal fractions but not in plasma. There is a trend for increased ApoB100 in hepatic microsomal protein. CD modulates HDL particle size and shape by increasing more non-spherical and heterogeneous particles. This data suggests that CD perhaps alters lipoprotein metabolism by perturbing RCT pathway.

At 14 days after 15 mg CD/kg apoA-I and apoB100 but not CYP3A11 protein in hepatic microsomes is similar to controls. Perhaps differential adaptation of signaling pathways are involved in this phenomenon.

CD is a mixed agonist for human SXR and ERα and an effective ERβ antagonist. CD modulates lipoprotein metabolism by complex nuclear receptor activation. HDL dominates the mouse plasma lipoprotein profile (Peet *et al.* 1998) while LDL is prominent in humans (Famer and Gotto 1997; Genest *et al.* 1999). The ratio of plasma LDL and HDL in Golden Syrian hamsters is similar to that of humans

(Turley *et al.* 1996). Examining CD-modulation of CH homeostasis in this hamster strain is a future interest.

Our data demonstrates that mechanisms other than SR-BI or ABCG8 may be involved in modulating CD induced cholesterol homeostasis and lipid metabolism. However, a definitive explanation for increased biliary CH excretion in CD pretreated mice remains elusive. Figure 4-1 demonstrates working hypothesis based on our data. SR-BII mediated rapid internalization of HDL (Eckhardt *et al.* 2004) or cytoplasmic proteins such as liver fatty acid-binding protein and sterol carrier protein-2 were suggested to play a role in the intracellular transport and biliary CH secretion (Kosters *et al.* 2005). Therefore examining CD-modulation of SR-BII, liver fatty acid-binding protein or sterol carrier protein-2 is also a future interest.

These findings at least partially explain altered CH tissue distribution in CD-pretreated mice. This work indicates altered CH homeostasis was a mode of OC insecticide action of relevance after a single low dose. CD induced alteration of CH homeostasis can be used to develop a model for better understanding atherosclerosis.

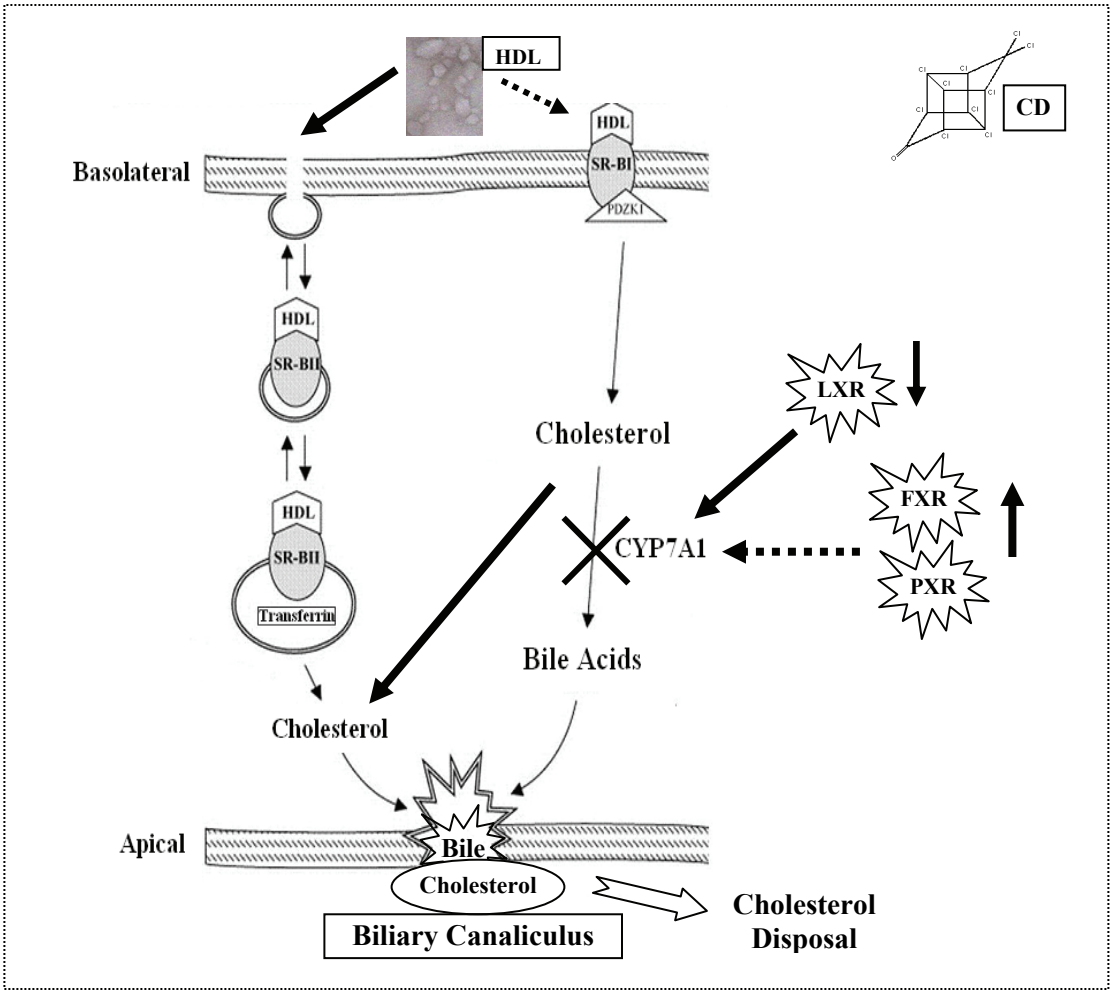


Figure 4-1. Working hypothesis based on present results. SR-BII mediated rapid internalization of HDL or cytoplasmic proteins such as liver fatty acid-binding protein and sterol carrier protein-2 perhaps play a role in the intracellular transport and biliary CH secretion. This figure is modified from hypothetical model for the function of the SR-B isoforms in the liver (Lopez and McLean 2006).

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