AN ABSTRACT OF THE DISSERTATION OF

Richard C. Scheri for the degree of Doctor of Philosophy in Toxicology presented on May 30, 2008
Title: Chlordecone Pretreatment Promoted Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes; Mass Spectrometry Detected Hepatic Soluble Cholesterol Binding Proteins and Comparison of Protein iTRAQ Ratios Using ESI QTOF and MALDI TOF/TOF

Abstract approved:

Lawrence R. Curtis

Chlordecone belongs to the class of persistent organochlorine pesticides that are remarkably resistant to environmental degradation. Even though their use was banned in the United States in 1978, these compounds can still be detected in both humans and wildlife throughout the world. Previous work has shown that the pretreatment of male C57BL/6 mice with low doses of the persistent organochlorine (OC) pesticide, chlordecone (CD) stimulated biliary excretion of exogenous CH up to 3-fold, and further, that increased biliary excretion was not associated with changes in ATP-binding cassette transporter G8 (ABCG8) or scavenger receptor class B type I
(SR-BI). In rodents, hepatic basolateral SR-BI is important in controlling plasma lipoprotein levels and cholesterol (CH) homeostasis, with major roles in reverse CH transport (RCT) and biliary excretion. The hepatic ABCG5/G8 heterodimer is a membrane transporter present on the apical surfaces of hepatocytes, and also plays a key role in biliary CH secretion. Scavenger receptor class B type II (SR-BII) was identified as a splice variant from the SR-BI gene and is expressed in a variety of tissues. Although the function of SR-BII is not clear it was proposed to play a role in CH homeostasis and trafficking that was distinctly different than SR-BI.

In the present study, western blotting was used to show that a single dose of CD promotes subcellular distribution of SR-BII to murine hepatic microsomes while having no effect on liver crude membrane SR-BII. Western blotting also indicated CD pretreatment had no effect on the levels of liver fatty acid binding protein (L-FABP) in cytosol, but an increase in myosin-9 was observed with mass spectrometry. Myosin-9 may play a role in intracellular vesicular transport. This may at least partially explain the previously observed alterations in CH homeostasis produced by CD pretreatment.

Changes in relative protein levels using a CH binding protein enriched fraction prepared from hepatic cytosol were detected with mass spectrometry using isobaric tagging for relative and absolute quantification (iTRAQ) methodology. Many factors can affect the accuracy of quantification with this technique. It has been observed that the low collision energies normally used in ESI QTOF can result in low iTRAQ reporter ion abundances. After evaluating protein changes in response to CD pretreatment, two-way ANOVA was used to compare the mean protein iTRAQ results from mass spectrometers using different collision energies in the same samples. It
appears that iTRAQ analyses performed on an ESI QTOF without any special modifications in instrumental parameters produce essentially the same protein ratios as those obtained on a MALDI TOF/TOF.
Chlordecone Pretreatment Promoted Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes; Mass Spectrometry Detected Hepatic Soluble Cholesterol Binding Proteins and Comparison of Protein iTRAQ Ratios Using ESI QTOF and MALDI TOF/TOF

by
Richard C. Scheri

A DISSERTATION
submitted to
Oregon State University

in partial fulfillment of
the requirement for the
degree of

Doctor of Philosophy

Presented May 30, 2008
Commencement June 2009
ACKNOWLEDGEMENTS

I would like to thank Professors Lawrence Curtis and Douglas Barofsky for their guidance, encouragement and patience during my tenure at Oregon State University. Without their consistency and humor, especially the latter, this sojourn would have been far less rewarding and several orders of magnitude more painful. Many thanks are also due to the individuals that served on my committee: Dr. Michael Schimerlik, Dr. David Williams and Dr. Mark Harmon.

Many thanks to Dr. Dave Williams lab and especially to Beth Siddens for equipment, lab space in which to work, antibodies, patience and advice.

I also need to sincerely thank Lilo Barosky and Brain Arbogast for getting me up to speed with HPLC and mass spectrometry. Thanks to Jeff Morre, Mike Hare and the rest of the mass spectrometry group for their support.

Dr. Sonia Anderson’s lab was extremely helpful and provided equipment and advice and I sincerely thank Dean Malencik for what little I know about protein chemistry.

Thanks also go to Eddie O’Donnell from Dr. Siva Koluri’s lab, and Nathan Lopez from Dr Joseph Beckman’s lab for teaching me how to do westerns and to Dr Samuel Bennett for the use of his imaging equipment and stimulating conversations.

Finally I would like to thank my good friends Jing Wang and Ben Bythell for their friendship and support.
CONTRIBUTION OF AUTHORS

Dr. Junga Lee assisted with sample collection and sample preparation for the work presented Chapter 2 and to a limited extent in Chapter 3.

The author helped with sample preparation and data analysis in the study presented in Appendix A and with sample preparation in the work presented in Appendix B.

Dr. David Mangelsdorf and Dr. Yuan Zhang performed the reporter gene assays presented in Appendix 2.

Brain Arbogast, Lilo Barofsky and Mike Hare, although not coauthors, made technical contributions to the work presented in this dissertation. Brian Arbogast ran the samples and generated the pkl files for all samples run on the QTOF. Lilo Barofsky and Mike Hare performed MALDI TOF/TOF analysis on some of the samples.

Dr. David William’s laboratory generously provided antibodies to CYP4A.

Dr. Samuel Bennett kindly allowed the use of his imaging instrumentation.
# TABLE OF CONTENTS

| Chapter 1 | Introduction .................................................................. 1 |
| Chapter 2 | Chlordecone Pretreatment Promoted Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes ........................................ 13 |
| Chapter 3 | A Comparison of Relative Quantification with Isobaric Tags on a Subset of the Murine Hepatic Proteome using ESI QTOF and MALDI TOF/ ........................................ 39 |
| Chapter 4 | Conclusions ................................................................... 63 |
| Bibliography | ............................................................................. 66 |
| Appendices | ............................................................................. 77 |

**Appendix A** Chlordecone Altered Hepatic Disposition of $[^{14}	ext{C}]$Cholesterol and Plasma Cholesterol Distribution but not SR-BI or ABCG8 Proteins in Livers of C57BL/6 Mice .......................... 78

**Appendix B** Chlordecone, a Mixed Pregnane X Receptor (PXR) and Estrogen Receptor Alpha (ERα) Agonist, Alters Cholesterol Homeostasis and Lipoprotein Metabolism in C57BL/6 Mice .......................... 107
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>10</td>
</tr>
<tr>
<td>1-2</td>
<td>11</td>
</tr>
<tr>
<td>1-3</td>
<td>12</td>
</tr>
<tr>
<td>2-1</td>
<td>33</td>
</tr>
<tr>
<td>2-2</td>
<td>34</td>
</tr>
<tr>
<td>2-3</td>
<td>35</td>
</tr>
<tr>
<td>2-4</td>
<td>36</td>
</tr>
<tr>
<td>2-5</td>
<td>37</td>
</tr>
<tr>
<td>3-1</td>
<td>56</td>
</tr>
<tr>
<td>3-2</td>
<td>57</td>
</tr>
<tr>
<td>3-3</td>
<td>58</td>
</tr>
<tr>
<td>3-4</td>
<td>59</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2-1</td>
<td>Proteins showing a significantly different expression ratio and L-FABP in the CH binding protein enriched fraction</td>
</tr>
<tr>
<td>3-1</td>
<td>Proteins detected in all four animal pairs and with both instruments</td>
</tr>
<tr>
<td>3-2</td>
<td>Mean protein iTRAQ values from both instruments</td>
</tr>
<tr>
<td>3-3</td>
<td>$P$-values associated with the factors sample and instrument</td>
</tr>
</tbody>
</table>
Chlordecone Pretreatment Promoted Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes; Mass Spectrometry Detected Hepatic Soluble Cholesterol Binding Proteins and Comparison of Protein iTRAQ Ratios Using ESI QTOF and MALDI TOF/TOF

Chapter 1

Introduction

Chlordecone (CD), trade name Kepone®, is a member of the persistent organochlorine (OC) pollutants (POPs) that was first synthesized and registered as a pesticide during the early 1950s (ATSDR 1995). The structure of CD is shown in Figure 1-1. Its manufacture was banned in 1976 following the “Kepone episode” in Hopewell, Virginia where poor manufacturing practices resulted in symptoms of CD toxicity in a majority of plant workers and contamination of the nearby James River estuary (Raloff 1976; Guzelin 1982). The majority of CD produced in the United States was exported to West Germany, where it was used in the production of the pesticide Kelevan, which was then exported for use primarily against the banana root borer weevil in Central and South America (Cannon et. al., 1978). Like the other OC pesticides, CD is lipophilic and resistant to bio/photodegradation. Since it is lipophilic and so resistant to degradation, transfer through food webs is the primary route of exposure where it tends to accumulate in the tissues of higher level predators throughout the world.

In addition to concerns about the long term toxicity of CD itself, several studies have shown that low doses perturb lipid homeostasis in a variety of species. Carpenter and Curtis (1989; 1991) showed that pretreatment of mice with an acute,
A nontoxic dose of CD altered the subsequent distribution of an exogenous dose of \([^{14}C]{\text{CD}}\) or \([^{14}C]{\text{cholesterol}}\) (CH). Subsequent studies using CD to perturb lipid homeostasis indicated, consistent with previous results, that acute CD pretreatment (15 mg/kg) resulted in increased biliary excretion of an intraperitoneal (ip) lipid bolus of \([^{14}C]{\text{CH}}\) and reduced deposition of the labeled CH in liver without affecting either scavenger receptor class B type 1 (SR-BI) or ATP cassette binding protein G8 (ABBG8) (Appendix 1). The same study showed that CD pretreatment altered lipoprotein metabolism and, further, that CD (10 \(\mu\)M) strongly suppressed liver X receptor beta (LXR\(\beta\)) activation in cell reporter assays (Appendix 2). Additionally, the same dose of CD was a strong agonist for a human homolog of the pregnane X receptor (PXR), estrogen receptor alpha (ER\(\alpha\)) while suppressing the activation of estrogen receptor beta (ER\(\beta\)) by \(\beta\)-estradiol (E2) (Appendix 2).

The tissue distribution of CD differs from other lipophilic OC pesticides. Its tissue distribution is not proportional to tissue lipid content in both humans and rodents and it tends to concentrate in liver (Cohn et al., 1978; Egle et al., 1978). Other OC pesticides, e.g., dichlorodiphenyltrichloroethane (DDT) and dieldrin, are associated primarily with low density lipoproteins (LDLs) and very low density lipoproteins (VLDLs) in the blood (Micket et al., 1971). In contrast, CD is associated primarily with albumin and high density lipoprotein (HDL), which may account for the different tissue distribution profiles that are observed (Soine et al., 1982). While VLDL and LDL are primarily involved in the delivery of lipids to peripheral tissues, HDL is primarily involved in lipid transport from peripheral tissues to the liver for biliary excretion, a process termed reverse CH transport (RCT) (Lewis and Rader.
2005; Attie 2007). Additionally, CD is refractory to metabolism in mice, therefore the production of metabolites is not an issue in this species (Guzelian 1982).

CH serves many important functions in mammalian cells. It is a constituent of membranes and used for the synthesis of bile acids and steroid hormones. Its association with sphingomyelin allows for the formation of lipid rafts within membranes which may play a role in cell signaling pathways and intracellular transport (Brown and London 2000; Prinz 2007). Although several mechanisms contribute to the carefully controlled intracellular regulation of CH homeostasis, primary regulation is accomplished by the cells ability to monitor sterol levels within the endoplasmic reticulum (ER). This system utilizes sterol controlled transport of the membrane bound transcription factor, sterol response element binding protein (SREBP), from the ER to the Golgi where it is cleaved by proteases. After cleavage, the homodimer migrates to the nucleus where it interacts with sterol response elements in a variety of genes which control CH synthesis and uptake (Brown and Goldstein, 1997). CH is packaged for transport in the plasma as lipoproteins, the levels of which are dictated by a variety of nuclear receptors and the diet (Nomiyama and Breummer 2008; Li and Glass 2004; Wang 2007). Nuclear receptors also mediate the metabolism of CH to bile acids for excretion into the bile, a process that involves the transfer of CH from HDL to SR-BI, at the plasma membrane, the transport of CH through the cell and finally the transfer of CH across the canalicular membrane by the ABCG5/G8 heterodimer (Kosters et. al., 2005). Several studies have indicated that HDL CH is the sole source for biliary CH (Wustner et. al., 2003; Robins and Fasulo 1997). However,
the sequence of events whereby the CH is actually transported from the basolateral to the apical membrane has not yet been fully elucidated.

SR-BI mediates selective lipid uptake (SLU) from HDLs and reducing its expression increases plasma HDL-CH and decreases biliary CH in rodents (Rhainds and Brissette 2004; Rigotti et. al., 2003). Scavenger receptor class B type II (SR-BII) is an alternative splice variant of the SR-BI gene that also mediates SLU from HDL, but the role it plays in lipid homeostasis is not clear (Webb et. al., 1998). In an effort to explain the altered lipid homeostasis resulting from CD pretreatment, earlier work had shown that SR-BI or ABCG8 protein levels were not altered (Appendix 1). The present study examines whether SR-BII protein levels in hepatic plasma membrane or microsomal membrane fractions are affected by CD pretreatment. This work is presented in Chapter 2.

The intracellular transport of CH is complex and both vesicular and non-vesicular transport mechanisms are involved (Ikonen 2008; Prinz 2007). Their relative roles have not been fully elucidated - the non-vesicular transport mechanisms are especially not well understood. Another possible explanation for the altered lipid homeostasis observed with CD pretreatment that does not involve membrane bound transporters might involve soluble (cytosolic) CH binding proteins. A proteomic approach using isobaric tagging for relative and absolute quantification (iTRAQ) methodology (described below) was used in this work because it appeared to have the greatest potential for quickly identifying and characterizing the relative changes in these soluble CH binding proteins. The details of this approach are presented in
Chapter 3, and the changes in protein expression that resulted from CD pretreatment are presented in Chapter 2.

Proteomics is the general term coined for the analysis of the proteome, i.e., the total complement of proteins produced at a given time, under a given set of conditions in a cell. Advances in chromatographic and mass spectrometry techniques over the last decade or so has made proteomics a reality (Abersold and Mann 2003). Although the complete identification of the entire complement of the proteins in a proteome has not yet been achieved, great strides have been made in this direction. A common method of analysis is termed shotgun, or bottom-up proteomics. In this technique, the proteins in a sample are enzymatically or chemically digested into peptides (increasing the complexity of an already complex sample) that are analyzed on a mass spectrometer after chromatographic separation. Because of the aforementioned complexity of the proteome and the limited dynamic range of a mass spectrometer, several means of reducing the complexity of the sample have been developed. Two-dimension gels were first utilized. Gel spots were cut out and the proteins in the spots digested. The resulting mixture of peptides could then be run on an HPLC and analyzed on a mass spectrometer. Inherent problems included variability between gels, their inability to separate membrane proteins or proteins with extreme pIs, and the difficultly of imaging low abundance proteins on a gel (Gorg et. al., 2000; Gygi et. al., 2000). The 2D gel methodologies have slowly given way to multidimensional chromatographic techniques (Washburn et al., 2001; Chen el al., 2006). In this technique the peptide digest is first separated with some type of chromatography; a common first stage chromatographic step uses a strong cation exchange resin. The fractions eluted from
this column are then run on another column, e.g., a reverse phase C18, and the effluent is analyzed on a mass spectrometer.

As a further means of simplifying the cytosolic proteome in this study, enrichment of the soluble CH binding fraction was explored as a means to make the mass spectrometry more tenable. Streptomycin precipitation of cytosol proved to be effective in concentrating $^{14}$C labeled CH while at the same time decreasing the total amount of protein present (Chapter 3).

In order to perform mass spectrometry, biomolecules first have to be ionized into the gas phase. The two most commonly used techniques for ionization of biomolecules are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Yamishita and Fenn 1984; Karas et. al., 1987). Since ESI ionizes molecules directly from the liquid phase by solvent desolvation/evaporation, it can easily be coupled to LC. The peptide ions produced by this technique tend to be multiply charged. In MALDI the analytes are mixed into a matrix that is then crystallized on a sample plate. The dried matrix absorbs photons provided by a laser resulting in the matrix and analytes being transferred to the gas phase and ionized in the process. MALDI tends to produce singly charged ions, which makes the mass spectra easier to interpret. In this study, ion separation in the ESI analyses was accomplished by a quadrupole/ time-of-flight (TOF) (QTOF) and in the MALDI analyses by sequential TOF analyzers (TOF/TOF). Both of these instruments are capable of tandem mass spectrometry (MS/MS), a technique wherein the first analyzer is used to choose a precursor ion; a collision cell located between the analyzers fragments the precursor; and the fragment ions produced are then analyzed.
One of the differences between the two instruments used in this study was the MS/MS fragmentation energies used in the collision cells. The MALDI TOF/TOF fragments precursors at an energy of about 1.0 keV while the ESI QTOF fragments them anywhere from 25-65 eV, depending on the precursor charge state.

Once MS/MS spectra are generated they are searched against a protein database in order to identify the proteins present. Peptides from proteins in the database are fragmented \textit{in silico}, and the resulting theoretical spectra are compared with the experimental spectra. The matches are scored differently by different search engines. The Mascot search engine was used in this study; it uses a probability based implementation of the molecular weight search (MOWSE) algorithm (Matrix Science, UK). It is essentially a probabilistic threshold model that sorts scores by some threshold. The problem is that different search engines have different scoring schemes that result in different thresholds. Additionally with each search engine, several values are usually reported and the question of which value(s) to use or some means of combining this information must be utilized. For example, Mascot reports homology and identity scores, as well as ion scores, but most people just take ions above the identity score as those being significant. This means that results using different search engines cannot be directly compared. Other problems with threshold type algorithms arise because different thresholds may be needed for different charge states, instruments, databases, or sample complexities. Peptide Prophet is an algorithm developed to address these issues (Keller et. al., 2002; Nesvizhskii and Aebersold 2004). It uses Bayesian statistics to compute the true probability that a match is correct. A single discriminant score is calculated for each peptide from all of the threshold
scores from a particular search engine. A histogram is plotted of the number of spectra versus the discriminant score; curves are fit to the bimodal distribution that results (which consists of incorrect and correct distributions); and the probabilities are calculated (Keller et. al., 2002; Nesvizhskii et. al., 2004). The distributions also allow the estimation of errors. Probabilities generated by this software can be compared between search engines, instrument platforms, charge states, etc. The commercial product (Proteome Software, Portland, OR) that incorporates Peptide Prophet is called Scaffold. As mentioned previously, Mascot was used for peptide identification, but the Mascot results were further processed in Scaffold before protein identification (Chapter 3).

The ability to accurately compare tissue protein concentrations under different conditions is vital for understanding the underlying biological process(es). A variety of methods have been developed using isotopic labeling in order to quantify proteins identified with mass spectrometry (Julka and Regnier 2004). These techniques can be MS or MS/MS based depending on the properties of the label itself. A popular approach that is MS based uses an isotope coded affinity tag (ICAT) (Gygi, et. al., 1999) This method uses light and heavy cysteine specific tags (Figure 2) that are linked to a biotin moiety so the cysteine containing peptides can be enriched using affinity chromatography. However, not all proteins contain cysteine, and since only two tags (light and heavy) were available, multiplexing was impossible. An MS/MS methodology, known as iTRAQ was developed by Ross et al., (2004) and commercialized by Applied Biosystems Inc. It was designed to overcome some of the problems associated with the ICAT labeling methodology. The molecule consists of a
charged N-methylpiperazine based reporter group, a carbonyl balance group, and an N-hydroxysuccinimide (NHS) peptide reactive group (Figure 3). The total mass of the reporter and balance group is kept constant at 145.1 Da though use of $^{13}$C, $^{15}$N and $^{18}$O atoms. Currently eight reporters are available so multiplexing up to eight samples, time points, or treatments is possible. After protein digestion, all primary amines in the peptides are labeled. Isobaric tagging implies that peptides from different experimental groups containing different labels elute at the same time and appear in MS spectra at the same $m/z$, significantly simplifying the spectra. Charged reporter ions are then released during collision induced dissociation (CID) with the carbonyl undergoing a neutral loss. Relative quantification is achieved using either the peak area or intensity of the reporter ions.

It has been observed that the low collision energies normally used in ESI QTOF can result in low iTRAQ reporter ion abundances (Wiese et al., 2007). Since I had access to both ESI and MALDI instruments I decided to compare the protein iTRAQ ratios obtained on both instruments. I used two-way analysis of variance (ANOVA) to compare the iTRAQ ratios that were generated on an ESI QTOF and a MALDI TOF/TOF. This work is presented in Chapter 3.
Figure 1-1. Structure of Chlordecone
Figure 1-2. ICAT reagent structure; modified from Liebler, D.C., 2002, Introduction to Proteomics: Tools for the New Biology, Humana Press
Figure 1-3. iTRAQ reagent chemistry: (A) Reporter, mass balance and peptide reactive group of the complete molecule; (B) The iTRAQ molecule bound to a peptide before CID; (C) A example using four iTRAQ reporter tags attached to four of the same peptides from different samples, (modified from Ross, et al., 2004, Mol. Cell Proteomics, 10:1074.)
Chapter 2

Chlordecone Pretreatment Promoted Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes

Richard C. Scheri, Janga Lee, Douglas F. Barofsky and Lawrence R. Curtis
ABSTRACT

The intracellular transport of CH is complex and both vesicular and non-vesicular transport mechanisms are involved. Their relative roles have not been fully elucidated and the non-vesicular transport mechanisms are not well characterized. Previous work has shown that the pretreatment of male C57BL/6 mice with low doses of the persistent organochlorine (OC) pesticide, chlordecone (CD) stimulated biliary excretion of exogenous CH up to 3-fold. Increased biliary excretion was not associated with changes in ATP-binding cassette transporter G8 (ABCG8) or scavenger receptor class B type I (SR-BI). Scavenger receptor class B type II (SR-BII) was identified as a splice variant from the SR-BI gene and is expressed in a variety of tissues. Although the function of SR-BII is not clear it was proposed to play a role in CH homeostasis and trafficking that was distinctly different than SR-BI. Here we use western blotting to show that a single dose of CD promotes subcellular distribution of SR-BII to murine hepatic microsomes about 3 fold while having no effect on liver crude membrane SR-BII. Western blotting also showed CD pretreatment had no effect on the levels of liver fatty acid binding protein (L-FABP) in cytosol, but an increase in myosin-9 was observed with mass spectrometry. This may at least partially explain the previously observed alterations in CH homeostasis produced by CD pretreatment.

INTRODUCTION

CD is a member of the group of persistent OC pesticides whose manufacture in the United States was banned in 1976. Although western manufacture of CD was halted over 30 years ago, it is still present in the biosphere and detected in the tissues of
wildlife and humans throughout the world (Bocquene and Franco 2005; Luellen, et al., 2006). It is highly hydrophobic, generally extremely resistant to biodegradation and undergoes negligible photodegradation in the atmosphere (ATSDR 1995). The major route of exposure for CD and OC pesticides in general is trophic transfer through the food web with maximal accumulations in top predators (Christensen et al., 2005). OC pesticides, e.g., CD, dichlorodiphenyltrichloroethane (DDT) and dieldrin, differ from the polychlorinated biphenyls and the chlorinated furans and dioxins in that they exhibit only slight affinity for the aryl hydrocarbon receptor (Poland and Knutson 1982). CD is unusual in that a large body of toxicological data is available for humans as a result of a well publicized poisoning epidemic in chemical workers that occurred in the mid-seventies (Cannon et al., 1978; Guzelian 1982) Like most OC pesticides, neurotoxicity is a prominent mode of action and tremors were a common symptom in the exposed human population (Cannon et al., 1978). CD has been observed to affect ion channels and inhibit Na\(^+\)/K\(^+\) ATPases in a variety of tissues (Narahashi et al., 1998; Desaiiah 1982). It is also a liver carcinogen in both rats and mice (Reuber 1978). CD has been shown to alter lipid homeostasis in a variety of species. Ishikawa showed single doses of CD and dieldrin decreased total plasma CH and triglycerides in rats (Ishikawa et al., 1978). Several studies showed altered lipid homeostasis and tissue deposition of CD, dieldrin and CH in rainbow trout, rats and mice (Carpenter and Curtis 1989, 1991; Donohoe et. al, 1998; Gilroy et al., 1994). Single doses (5 mg/kg) of CD altered the tissue distribution of an exogenous lipid bolus containing \(^{14}\text{C}\)CD or \(^{14}\text{C}\)CH (Carpenter and Curtis 1991). Pretreatment with single doses of CD altered lipoprotein metabolism in fasted mice (Lee et al., 2007). High density lipoprotein
(HDL) CH was not affected by CD pretreatment, but the non HDL-CH decreased and the HDL-CH:total plasma CH ratio increased (Lee et al., 2007). The same authors showed CD pretreatment increased the biliary excretion of exogenous $^{14}$[C]CH 4 h after administration while having no effect on expression of hepatic ABCG8 or SR-BI (Lee et al., 2008). Whole livers of C57BL/6 mice contained approximately 85 μM CD 16 h after administration of 5 mg CD/kg (Carpenter and Curtis, 1989). Similar concentrations of CD in cell reporter assays indicated that CD was a partial agonist for the farnesoid X receptor (FXR) and peroxisome proliferator activated receptor alpha (PPAR$_\alpha$). CD was also a strong agonist for the human homolog of the pregnane X receptor (PXR), and estrogen receptor alpha (ER$_\alpha$). It was a weak antagonist for liver X receptor alpha (LXR$_\alpha$), a strong antagonist for LXR$_\beta$, and would inhibit the binding of β-estradiol (E2) to ER$_\beta$, but it had no agonist activity on ER$_\beta$ itself. *In vivo* CD exposures increased hepatic microsomal cytochrome P450 (CYP) protein and enzyme activities associated with these nuclear receptors. CD was a strong PXR agonist (CYP3A11) with little effect on CYP7A1 (LXR/FXR) or CYP4A1 (PPAR$_\alpha$) activity. Since most OC pesticides are believed to be PXR agonists and CD has been demonstrated to have estrogenic properties these results were not surprising (Guzelian 1982; Coumoul et al., 2002).

FXR, LXR, PPAR and PXR are members of the nuclear receptor superfamily and can act at several points to influence lipid homeostasis. They are ligand-activated transcription factors that bind to specific response elements after heterodimerization with the retinoid X receptor (RXR) (McKenna et al., 1999; Chalwa et al., 2001).
Physiological ligands for FXR are bile acids and FXR response elements are located in genes involved in bile acid homeostasis (Kalaany and Magelsdorf, 2006). CYP7A1 is the rate limiting step in the neutral bile acid synthesis pathway. Excess bile acids activate FXR, which acts indirectly through small heterodimer partner (SHP) to decrease the expression of CYP7A1 (Kuipers, et al., 2007). Oxysterols are physiological ligands for LXR and high concentrations of oxysterols act through LXR to downregulate CYP7A1 (Kalaany and Magelsdorf, 2006). Similarly, the three isoforms of PPAR play key roles in the synthesis and metabolism of fatty acids, which are their physiological ligands (Smith 2002; Michalik and Wahli 1999). They also regulate the expression of sterol 12α hydroxylase (CYP4A1), a step in bile acid synthesis. A variety of xenobiotics are ligands for PXR, including bile acids that can induce CYP7A1 through this nuclear receptor (Staudinger et. al., 2001a; 2001b).

SR-BI mediates selective lipid uptake (SLU) from HDL and reducing its expression increases plasma HDL-CH and decreases biliary CH in rodents (Rhainds and Brissette 2004; Rigotti et al., 2003). It can also efflux free CH to HDL (Mardones et al., 2001). SR-BII is an alternative splice variant of the SR-BI gene that also mediates SLU from HDL but is expressed at much lower levels in liver (Webb et al., 1998). The role it plays in lipid homeostasis is not clear.

One possible explanation for the altered lipid homeostasis observed with CD pretreatment that does not involve membrane bound transporters might involve changing levels of cytosolic CH binding proteins. The intracellular transport of CH is
complex and both vesicular and non-vesicular transport mechanisms are involved (Ikonen 2008; Prinz 2007). Their relative roles have not been fully elucidated and the non-vesicular transport mechanisms are especially not well understood. Candidates for soluble sterol transporters include, but are not limited to, FABPs, caveolins, sterol carrier protein 2 (SCP-2), oxysterol binding proteins (OSBPs) and OSBP-related proteins (ORPs), Niemann Pick type C (NPC) proteins and the steroidogenic acute regulatory protein (StAR) and the StAR related lipid transfer (START) proteins (Ikonen 2008; Prinz 2007). A proteomic approach was applied here because it had the greatest potential for quickly identifying and characterizing the relative changes in hepatic cytosolic proteins.

In this work, we examined the effect of CD pretreatment on murine hepatic SR-BII, Na⁺/K⁺ATPase, liver fatty acid binding protein (L-FABP), and an enriched cytosolic fraction of CH binding proteins after the intraperitoneal (ip) injection of 5 mL/kg of corn oil containing either 5mg/kg or 15 mg/kg CD. SR-BII was analyzed by western blotting hepatic microsomal fractions from CD treated mice (5 mg/kg and 15 mg/kg) and controls. Na⁺/K⁺ATPase was examined by western blotting crude hepatic membrane fractions from CD treated mice (5 mg/kg and 15 mg/kg) and controls. L-FABP was analyzed by western blotting hepatic cytosol fractions from CD treated mice (5 mg/kg and 15 mg/kg) and controls. Relative quantification of a cytosolic enriched CH binding fraction was performed with mass spectrometry using stable isotope labeling.
Our results demonstrated that CD pretreatment increased hepatic microsomal SR-BII and hepatic crude membrane Na\(^+\)/K\(^+\) ATPase while having no effect on cytosolic L-FABP. An increase in myosin-9 was noted in the CH binding protein enriched fraction, whereas decreases in peroxiredoxin 1 (PRX-1), selenium binding protein 1 (SBP-1), and α-enolase were observed.

**MATERIALS AND METHODS**

*Chemicals*

CD was purchased from Chem Service (West Chester, PA). Purity (99%) was confirmed by gas chromatography-electron ionization/mass spectrometry. Bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce Biotechnology Inc. (Rockford, IL). iTRAQ reagents were purchased from Applied Biosystems (ABI, Foster City, CA). Burdick and Jackson solvents (VWR, West Chester, PA) were used for all high performance liquid chromatography (HPLC). All other chemicals were from Sigma (St. Louis, MO).

*Treatment of mice*

Six-seven week old male C57BL/6 mice were purchased from Simenson Laboratory (Gilroy, CA). They were randomly assigned to one of three groups: control, low dose (5 mg/kg) CD, or high dose (15 mg/kg) CD. They were individually housed in a temperature (22±1°C) and light controlled (12 h light/12 h dark daily) facility with free access to water and fed AIN93 diet (Dyets, Inc., Bethlehem, PA) ad libitum. Seven days were allowed for acclimatization before treatments were initiated. Corn oil was
used as a vehicle (5 mL/kg). Mice received a single dose of either corn oil or CD (5 mg/kg or 15 mg/kg) via intraperitoneal (IP) injection. After 72 h, they were fasted for 4 h and killed by carbon dioxide anesthesia and exsanguination. Livers were harvested. The procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).

**Tissue Preparation**

Liver was homogenized with a Polytron PT 3000 PT (Brinkmann Instruments, Westbury CT) in buffer consisting of 0.01 M potassium phosphate, pH 7.4, 0.15 M potassium chloride (KCl), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM phenylmethlysulfonyl fluoride (PMSF). All centrifugation was performed at 4°C. The homogenate was first centrifuged at 1200 g for 30 minutes. The pellet produced consisted of plasma membrane/cellular debris and was designated the hepatic crude membrane fraction. The supernatant was then centrifuged at 12,000 g for 30 minutes to eliminate mitochondria. The supernatant from this run was centrifuged at 100,000 g for 90 minutes in a Ti 70 rotor. The resulting supernatant and pellet were the cytosolic and microsomal fractions respectively. Microsomes were resuspended in 0.1 M potassium phosphate, pH 7.25, 1.0 mM EDTA, 30% glycerol, 20 μM BHT, 1.0 mM dithiothreitol (DTT), and 0.1 mM PMSF. BCA assay determined protein content.

**PAGE and Western Blot Analysis**
Cytosol, debris and microsome fractions were prepared as described above. Proteins (10 μg) were separated on either 10% or 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) at 100V for 2 h. Proteins were stained with Biosafe Coomassie (Biorad, Hercules, CA) for PAGE gel imaging. For Western blots the unstained proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane using a XCell II Blot module (Invitrogen, Carlsbad, CA) at 30 V for 1 h. Debris, microsome, and cytosol fractions were then blotted with antibodies: Rabbit anti-SR-BII was purchased from Abcam (Cambridge, MA) or GeneTex (San Antonio, TX). Rabbit anti-β-Actin was also purchased from Abcam. Rabbit anti-Na⁺/K⁺ ATPase, and rabbit anti-L-FABP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-CYP4A was a generous gift from the laboratory of Professor David Williams. Debris was blotted with antibodies against SR-BII and Na⁺/K⁺ ATPase. Microsomes were blotted with SR-BII and CYP 4A. Cytosol was blotted with L-FABP and β-Actin. Blots were then probed with either donkey anti-goat or goat anti-rabbit conjugated to horse radish peroxidase (HRP) as the secondary antibody (Santa Cruz Biotechnology, Inc.). Proteins were detected after development with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Images were acquired on a Syngene Chemigenius 2 bioimaging system and analyzed with Genesnap/Genetools software (Syngene, Frederick, MD).

**Tissue Preparation for iTRAQ Experiments**

Cytosolic fractions enriched in CH binding proteins were prepared by streptomycin precipitation (Scheri et al., 2008). The BCA assay determined protein concentrations.
Protein (100 μg) samples were precipitated with six volumes of ice cold acetone and labeled with iTRAQ reagents as detailed below.

**iTRAQ Labeling**

Samples were labeled with iTRAQ reagents according to the manufacturers’ protocol (Figure 2-1). Control samples were labeled with iTRAQ 114.1 or 116.1 m/z, while CD treated samples (15 mg/kg) were labeled with iTRAQ 115.1 or 117.1 m/z. An iTRAQ sample consisted of 100 μg of protein from a control animal pooled with 100 μg of protein from a CD treated animal. One animal pair was used for each iTRAQ sample, and four pairs were analyzed. Two technical replicates were run on each instrument for each sample pair. The iTRAQ ratios from the replicates were then averaged. Mean iTRAQ protein ratios were generated using the average ratio from both instruments. Ratios were then averaged across the biological sample pairs. A complete description this protocol is given elsewhere (Scheri et al., 2008).

**Mass Spectrometry**

After nano high pressure liquid chromatography (HPLC) (Waters nanoAcquity Ultra Performance LC, Milford, MA), mass spectrometry (MS) was performed on a quadrapole orthogonal time-of-flight mass spectrometer (QTOF Ultima Global, Micromass/Waters, Manchester UK) and on an ABI 4700 Proteomics Analyzer (Foster City, CA). The QTOF incorporated an electro-spray ionization (ESI) source, while the 4700 used a matrix assisted laser enhanced desorption ionization (MALDI) source. Tandem mass spectra (MS/MS) were generated with each instrument.
Data was searched against the mammalian Swiss-Prot database (V55.0) using the Mascot (Matrix Science, London, UK) search engine. iTRAQ ratios were generated in Mascot and median normalized. Data sets were further analyzed with Scaffold (Proteome Software, Inc., Portland, OR). Only peptides with a probability of 80% or greater as determined by Scaffold were utilized for quantitative purposes. In the case of duplicate peptides or different charge states, the iTRAQ ratio with the highest Mascot score was used. A minimum of 2 peptides was required for protein identification.

A complete description this protocol is given elsewhere (Scheri et al., 2008).

**Statistical Methods**

Statistical analyses were conducted with either StatGraphics (StatPoint, Herndon, VA) or S-Plus (Insightful Corp, Seattle, WA). Normality of data was accessed with standardized kurtosis, standardized skewness and homogeneity of variance. Western blot data is expressed as the mean ± the standard error (SE). Following validation of normality, comparisons between two groups was performed with a Student’s t-test. Multiple groups were compared with a one-way analysis of variance (ANOVA). In all cases, a 95% confidence level was used as the criterion for significance.

Data for iTRAQ ratios was analyzed in logarithm (natural, ln) space. Means were geometric. The Student’s t-test was used to determine whether the mean iTRAQ ratio for a particular protein was different than 1 (0 in ln space), both within a
biological sample pair and across the four biological sample pairs. In all cases, a 95% confidence level was used as the criterion for significance.

RESULTS

Previous work has shown that the stimulated biliary excretion of exogenous CH associated with CD pretreatment was not associated with changes in ABCG8 or SR-BI (Lee et al., 2008). SR-BII was identified as a splice variant from the SR-BI gene and is expressed in a variety of tissues. Although the function of SR-BII is not clear it was proposed to play a role in CH homeostasis and trafficking that was distinctly different than SR-BI (Eckhardt et al., 2004; 2006). This work suggested that analyzing SR-BII levels after CD pretreatment might offer a partial explanation for the observed alteration in CH homeostasis. As mentioned earlier, CD pretreatment had no effect on the protein levels or activity of hepatic microsomal CYP4A1, which supported their work showing that CD negligibly activated the nuclear receptor PPAR\(\alpha\) (Lee, el. al., 2007). We therefore normalized the microsomal western SR-BII intensities to that of CYP4A (Figure 2-2). SR-BII proteins levels increased at least 2-fold (p<0.05) as a result of CD pretreatment.

SR-BII is present on the plasma membrane and undergoes rapid endocytosis (Eckhardt et al., 2004), therefore, western blots were performed on hepatic crude membrane fractions. Originally, normalization with respect to Na\(^+\)/K\(^+\) ATPase was planned, but the protein content of this enzyme changed with CD pretreatment. Both SR-BII and Na\(^+\)/K\(^+\) ATPase were therefore normalized with respect to lane band intensity (Fig. 3
and 4). CD pretreatment had no significant effect on SR-BII protein levels but resulted in a statistically significant ($p<0.05$) increase in $\text{Na}^+/\text{K}^+$ ATPase.

CD pretreatment had no effect on the levels of cytosolic L-FABP (normalized to $\beta$-actin) (Figure 2-5).

Another possible explanation for the altered lipid homeostasis observed with CD pretreatment was altered concentration of some kind of soluble sterol binding protein(s). Mass spectrometry coupled with iTRAQ methodology for relative quantification addressed this issue. Mass spectrometry was limited by dynamic range, and the cytosolic proteome was complex. An enrichment of the fraction was of interest to simplify matters and make the mass spectrometry more tenable. Original enrichment experiments were performed with [$^{14}$C] CH and streptomycin precipitation of cytosol enriched the label per unit protein and left most of the cytosolic protein (>85%) in the supernatant (Scheri et al., 2008).

Approximately 80 proteins were detected with mass spectrometry under the conditions described in the experimental section (data not shown). Four proteins exhibited mean iTRAQ protein ratios significantly different ($p<0.05$) than one (Table 2-1).

**DISCUSSION**

The underlying mechanisms for the CD pretreatment induced perturbation of CH homeostasis and lipoprotein metabolism remain to be elucidated. As indicated earlier,
no change was observed in either SR-BI or ABCG8 in crude hepatic membrane fractions after CD pretreatment (Lee et. al., 2008b). Although the ABCG5/G8 heterodimer has been shown to be the rate limiting step in biliary CH secretion, its expression does not always correlate with this function which may indicate other processes are involved (Yu el. al., 2005; Geuken et al., 2005).

SR-BI is a promiscuous receptor for a variety of ligands including HDL and modified and unmodified forms of LDL. It mediates selective lipid uptake, which does not require the uptake and degradation of lipoprotein, and CH efflux from cells (Rhaïn ds and Brisette 2004; Rigotti et al., 2003). In rodents, it plays a primary role in controlling plasma lipoprotein levels and CH homeostasis, with major roles in reverse CH transport (RCT) and biliary excretion (Rigotti et al., 2003; Mardones et al., 2001). SR-BII was identified as a splice variant from the SR-BI gene and is expressed in a variety of tissues (Webb et al., 1998). SR-BII also mediates selective lipid uptake from HDL but at a lower efficiency than SR-BI (Webb et al., 1998). However, SR-BII is able to efflux about twice as much free CH as SR-BI (Mulcahy et al., 2004). The cellular distribution and internalization of these receptors also differs. In primary hepatocytes, SR-BI is predominantly located on the plasma membrane although a distinct pool appears to be associated with late endosomes and lysosomes (Ahras et al., 2008; Eckhardt, et., al., 2004). The cytoplasmic tail of SR-BI in hepatic and intestinal tissue interacts with the C-terminal linking and modulating protein (CLAMP), and this association appears to be required for distribution to the plasma membrane because knock-out of CLAMP prevents the surface expression of this receptor (Rhaïnds and
SR-BI undergoes endocytosis but at a much lower rate than SR-BII in CHO cells (Eckhardt et al., 2004). Endocytosis is not required for selective lipid uptake with SR-BI (Nieland et al., 2005; Wustner et al., 2003). The endocytosis of these receptors may serve to transport lipids to different pools in different locations within the cell, and it has been suggested that SR-BII endocytosis plays a role in altering the CH content of the endocytotic compartment (Eckhardt et al., 2006). SR-BII is located primarily intracellularly and is rapidly endocytosed in transferrin containing vesicles in CHO cells (Eckhardt et al., 2004). A dileucine motif in the carboxy terminal appears to be responsible for the rapid endocytosis and incorporation of this motif into SR-BI resulted in endocytosis of SR-BI (Eckhardt et al., 2006). Although mRNA levels of these receptors in murine liver are similar, SR-BII is expressed 10-15% of the levels at which SR-BI is expressed (Webb et al., 1998).

The function of SR-BII is not clear but it was proposed to play a role in CH homeostasis and trafficking that was distinctly different than SR-BI (Eckhardt et al., 2004). Wustner et al. (2003) proposed that an endocytotic/retroendocytotic pathway might be involved in biliary CH secretion. As mentioned previously, SR-BII effluxed free CH at a greater rate than SR-BI (Mulcahy et al., 2004). Based on a number of observations (by themselves as well as others) Lopez and McLean (2005) proposed that increased levels of SR-BII potentially increased excretion of CH in the bile. Our results showed no change in total biliary CH with CD pretreatment in 4h fasted mice (Lee et. al., 2008a). However, this was perhaps a question of limited detection sensitivity of the change in total bile CH after CD pretreatment in fasted mice without
administration of exogenous CH. Free CH from HDL was preferentially excreted in bile, as opposed to *de novo* free CH (Robins and Fasulo, 1997). CD pretreatment stimulated biliary excretion of exogenous [\(^{14}\text{C}\)]CH (2 \(\mu\)Ci/animal, 10 mg CH/kg) and this resulted in bile concentrations of approximately 120 nmol [\(^{14}\text{C}\)]CH equivalents/ml (Lee et al., 2008). Average bile CH concentrations in wild type mice range from about 2-5 \(\mu\)mol/ml (Wang et al, 2006; Yu et al., 2005; Tang et al, 2006), so that even with a 2-fold increase in SR-BII, a change of nmol amounts of CH in bile would not have been detected. The total amount of SR-BII on the plasma membrane was not altered by CD (Figure 2-3) and yet hepatic microsomal content of SR-BII was increased (Figure 2-2). Perhaps this represented an increased capacity for biliary excretion of CH in hepatocytes and upon the administration of exogenous CH, its excretion in the bile was enhanced. The intracellular mechanism for this was unknown. Perhaps a fraction of SR-BII was redistributed to the apical/plasma membrane upon the administration of an exogenous lipid bolus. Increased turnover of plasma membrane SR-BII was also a possibility. Since endocytosis of HDL appeared unimportant in selective lipid uptake, perhaps increased turnover of plasma/apical membrane SR-BII played a role in faster uptake of and excretion of CH into bile.

Apolipoprotein A-I (ApoA-I) is the major lipoprotein found in HDL and CD pretreatment significantly increased its concentration in the hepatic lipoprotein rich fraction and in microsomes (Lee et al., 2007). LXR activation inhibited the synthesis and secretion of apoA-I in Hep3B and HepG2 cells (Huuskonen et al., 2006), but, based on the negligible effects CD had on CYP7A1, the involvement of LXR seems
unlikely. Bachman et al. (2004) showed PXR increased HDL-CH and apoA-I and down regulated ABCA1 in vivo and in vitro in rats. ABCA1 is associated with the transport of free CH to ApoA-1 and lipid poor ApoA1. ABCA1 was located on the plasma membrane, with high concentrations in liver and intestine, and plays an important role in RCT (Attie 2007). PXR agonists down regulated ABCA-1 and SR-BI (Sporstal et al., 2005). The total and free CH concentrations in liver were not changed, but there was a significant decrease in CH ester associated with CD pretreatment (Lee et al., 2007). There was no evidence that SRB-II mobilized CH esters in CHO cells (Mulcahy et al., 2004). Plasma Apo-AI and apolipoprotein B (Apo-B), the latter being the major lipoprotein of VLDL and LDL, were unaffected by CD pretreatment (Lee et. al., 2008a). In humans, estrogen increased biliary lipid output and upregulated both Apo-B and the LDL receptor (Brown and Goldstein 1986; Wang 2007). However, LDL receptors were not upregulated by estrogen in mice (Srivastava et al., 1997). Estrogen decreased SR-BI and upregulated SR-BII in rat liver (Lopez and McLean 2006; Zhang et al., 2007). Additionally, estrogen increased the secretion of apoA-I in HepG2 cells (Lamon-Fava et al., 1999).

In the present study CD pretreatment increased the expression of SR-BII in the microsomal fraction by at least 3-fold (Figure 2-2) but there was no effect on SR-BII expression in the crude membrane fraction (Figure 2-3). Although CD was shown to be an agonist for human ERα, no change in SR-BI we observed and there was no change in total biliary CH after CD pretreatment in fasted mice (Lee et al, 2008a). Elevated CYP3A11 in hepatic microsomes 14 days after treatment with CD was
consistent with PXR signaling, but no increase in HDL-CH or plasma apoA-1 was detected (Lee 2008a). The constitutive androstane receptor (CAR) and PXR increased Insig-1 gene expression in mice (Roth et al., 2008). Increased Insig 1 in the endoplasmic reticulum (ER) would result in increased retention of sterol response element binding protein 1 (SREBP-1) in the ER and the down regulation of target genes including those involved in triglyceride synthesis. Triglycerides were not analyzed in this experiment but an earlier study using the same CD pretreatment regimen detected no changes in plasma triglycerides (Lee 2002). Additionally, toxic doses of CD in humans resulted in no change in plasma triglycerides (Guzelain 1982).

Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf-2) is a transcription factor that binds to antioxidant response elements and regulates genes involved in cellular response to oxidative stress (Tirona and Kim 2005). A significant reduction in prx1 gene expression in Nrf2 knock-outs has been observed (Kim el. al., 2007). The effect of CD pretreatment on the expression of other nuclear receptors, e.g., CAR and Nrf2 was of future interest but the mechanism for the observed increase in SR-BII remained unknown. Never the less, these results were compatible with a role for SR-BII in CD pretreatment induced changes in CH homeostasis.

\( \text{Na}^+/\text{K}^+ \) ATPase protein content increased with CD pretreatment in hepatic crude membrane fractions (Figure 2-4). Neurotoxicity was a prominent mode of action for OC pesticides and several groups showed CD inhibited \( \text{Na}^+/\text{K}^+ \) ATPase (reviewed in Desaiah, 1982). The increase observed was perhaps a compensatory response of this inhibited transport capacity per unit protein.
Mass spectrometry has a limited dynamic range, and the cytosolic proteome is complex. Selective protein enrichment of the fraction of interest, i.e., that containing CH/sterol binding proteins, would help to simplify matters and make the mass spectrometry more tenable. Original enrichment experiments were performed with [14C] CH, and streptomycin precipitation of cytosol proved to be effective in concentrating the label while at the same time decreasing the amount of protein present (RCMS). Clearly, L-FABP protein levels did not change in cytosol with CD pretreatment (Figure 2-5); a similar result was obtained for the streptomycin enriched fraction (Table 2-1).

Approximately 80 proteins were detected (data not shown) in the streptomycin enriched fraction under the conditions described in the methods section. Since the iTRAQ technique was based on a peak ratio between the CD pretreated samples and controls, a ratio different than one indicated a changed protein concentration. Statistically significant differences from one were observed with four proteins. Although both SCP-2 and L-FABP were detected, SCP-2 did not meet the minimum criteria for protein identification. Additionally, with this technique, differences less than 20% were viewed with caution. Decreased expression was observed with α-enolase, PRX-1, and SBP-1. Increased ratio was observed with myosin-9. It has been suggested that myosin-9 and SBP-1 played different roles in intracellular vesicular transport (van den Boom et al., 2007; Porat et al., 2000). While PRX-1 was a peroxide/ROS scavenger, the α-enolase gene encoded both a glycolytic enzyme and a
transcription factor, c-MYC binding protein (MBP-1) (Egler et al., 2005; Subramanian and Miller 2000). An interesting side note was that both proteins appeared to play important regulatory roles for the c-Myc transcription factor, a product of the c-MYC oncogene. The c-MYC oncogene was associated with a variety of cell processes, including apoptosis, cell growth, differentiation and cell cycle progression (Fernandez et al., 2003; Egler et al., 2005). While MBP-1 binds the c-MYC promoter and downregulates c-Myc transcription, prxl interacts with the c-Myc transcription factor itself (Subramanian and Miller 2000; Egler et al., 2005). This was perhaps related to the carcinogenicity of long-term CD exposures in rodents.

In summary, CD pretreatment increased the SR-BII protein content of hepatic microsomes but not crude membrane fractions. Increased myosin-9 was consistent with an increased capacity for vesicular transport. These observations supported a model where SR-BII contributed to CD stimulated biliary excretion of CH. Decreased SBP-1, α-enolase and PRX-1 in a CH enriched fraction of cytosol was observed, but whatever this contributed to CD induced perturbations in CH homeostasis was unclear. There was no evidence for a role of cytosolic binding proteins although additional mass spectrometry experiments were needed to adequately address this issue. Overall, these results provide new insights potentially explained how CD pretreatment induced alterations in CH and lipoprotein homeostasis.
Control (corn oil) Streptomycin Precipitate (100 mg) → Reduction Alkylation Trypsin Digestion → Label with 114 or 116 iTRAQ Pool Samples → SCX Spin Columns - 4 KCl fractions, 75 mM, 150 mM, 350 mM and 700 mM → C18 HPLC followed by MALDI MS/MS and ESI MS/MS. → Data Analysis by GPS Explorer, Mascot and Scaffold

Treated (15 mg/kg CD) Streptomycin Precipitate (100 mg) → Reduction Alkylation Trypsin Digestion → Label with 115 or 117 iTRAQ

Figure 2-1. Process flow chart for isotope labeling and mass spectrometry analysis. A total of four animal pairs were analyzed.
Figure 2-2. Up regulation of hepatic microsomal SR-BII by CD pretreatment. Animals were treated with corn oil, 5 mg/kg or 15 mg/kg CD by ip injection. Hepatic microsomes were prepared from individual animals 3 days after CD pretreatment: (A) western blot of SR-BII and CYP4A proteins, (10 μg protein/lane was separated by SDS-PAGE and blotted with antibodies against SR-BII and CYP4A; (B) variation in SR-BII with CD pretreatment, data normalized to CYP4A, (error bars are ± standard error (SE), asterisks indicate different than control (p<0.05) after one-way ANOVA).
Figure 2-3. No significant effect of CD pretreatment has no effect on SR-BII expression in the hepatic crude membrane fraction. Animals were treated with corn oil, 5 mg/kg or 15 mg/kg CD by ip injection and crude hepatic membrane fractions were prepared from individual animals 3 days after CD pretreatment: (A) western blot of SR-BII, (10 μg protein/lane was separated by SDS-PAGE and blotted with antibodies against SR-BII); (B) Variation in SR-BII with CD pretreatment, data normalized to lane band intensity, (error bars are ± SE).
Figure 2-4. Increased hepatic crude membrane fraction content of Na⁺/K⁺ATPase after CD pretreatment. Animals were treated with corn oil, 5 mg/kg or 15 mg/kg CD by ip injection and crude hepatic membrane fractions were prepared from individual animals 3 days after CD pretreatment: (A) western blot of Na⁺/K⁺ATPase, (10 μg protein/lane was separated by SDS-PAGE and blotted with antibodies against Na⁺/K⁺ATPase); (B) Variation in Na⁺/K⁺ATPase with CD pretreatment, data normalized to lane band intensity, (error bars are ± SE, asterisks indicate different than control (p<0.05) after one-way ANOVA).
Figure 2-5. CD pretreatment had no effect on cytosolic L-FABP.
Animals were treated with corn oil or 15 mg/kg CD by ip injection and hepatic cytosol was prepared from individual animals 3 days after CD pretreatment: (A) western blot of L-FABP and \( \beta \)-actin proteins, ( \( \mu \)g protein/lane was separated by SDS-PAGE and blotted with antibodies against L-FABP and \( \beta \)-actin); (B) L-FABP data normalized to \( \beta \)-actin, (error bars are ± SEM, asterisks indicate different than control (p<0.05) after a Student’s t-test).
### Table 2-1. Proteins showing a significantly different expression ratio and L-FABP in the CH binding protein enriched fraction.

L-FABP was included as an example of a potential sterol carrier protein whose mean iTRAQ ratio was not significantly different than one. Confidence intervals were determined using the Student’s T-test, p<0.05.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of Pairs (n)</th>
<th>Mean iTRAQ Ratio (Treated:Control)</th>
<th>Confidence Interval (95%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-enolase</td>
<td>4</td>
<td>0.81</td>
<td>0.78 - 0.84</td>
<td>0.000</td>
</tr>
<tr>
<td>Fatty acid-binding protein (L-FABP)</td>
<td>4</td>
<td>0.89</td>
<td>0.63 - 1.24</td>
<td>0.334</td>
</tr>
<tr>
<td>Myosin-9</td>
<td>4</td>
<td>1.35</td>
<td>1.03 - 1.75</td>
<td>0.037</td>
</tr>
<tr>
<td>Peroxiredoxin-1</td>
<td>4</td>
<td>0.87</td>
<td>0.77 - 0.99</td>
<td>0.044</td>
</tr>
<tr>
<td>Selenium-binding protein 1</td>
<td>4</td>
<td>0.72</td>
<td>0.58 - 0.90</td>
<td>0.019</td>
</tr>
</tbody>
</table>
Chapter 3

A Comparison of Relative Quantification with Isobaric Tags on a Subset of the Murine Hepatic Proteome using ESI QTOF and MALDI TOF/TOF

Richard C. Scheri¹, Junga Lee¹, Lawrence R. Curtis¹ and Douglas F. Barofsky²
ABSTRACT
The use of isobaric tagging for relative and absolute quantification (iTRAQ) has increased dramatically over the past few years. Many factors can affect the accuracy of quantification. Some of these include the number of biological/technical replicates, sample complexity, instrumentation, method of peptide/protein identification and the statistical techniques used for data analysis. It has been observed that the low collision energies normally used in ESI QTOF can result in low iTRAQ reporter ion abundances. We used two-way analysis of variance (ANOVA) to compare the iTRAQ ratios that were generated on an ESI QTOF and a MALDI TOF/TOF. It appears that iTRAQ analyses performed on an ESI QTOF without any special modifications in instrumental parameters produce essentially the same protein ratios as those obtained on a MALDI TOF/TOF.

INTRODUCTION
The ability to detect protein expression changes is vital for understanding the underlying biology of cellular processes at different times or in different states. For example, a single low dose of the organochlorine pesticide chlordecone (CD) has been shown to alter CH homeostasis in mice (Carpenter and Curtis, 1989;1991). Although membrane transport proteins like ATP-binding cassette protein G8 and scavenger receptor class B type I were not affected, the possibility of alterations in soluble CH binding proteins is currently under investigation (Lee et al, 2008). A great deal of effort has been expended in the development of a variety of stable isotope methods for the relative quantification of changes in protein expression (Julka and Regnier, 2004).
Stable isotopes or isotopically labeled amino acids can be added to media and incorporated into proteins during growth (Kolkman et al., 2005; Ong et al., 2003), but this method is not easily adapted for use outside of the cell culture arena. Chemical labeling methods are required for tissue analysis and many have been developed that react with a variety of functional groups in proteins and peptides (Julka and Regnier, 2004; Schmidt et al., 2005). These techniques can be MS or MS/MS based depending on the properties of the label itself. Isotope coded affinity tag (ICAT) technology is MS based and was first developed by Gygi et al., (1999). It uses light and heavy cysteine specific tags bound to a biotin moiety so the cysteine containing peptides can be enriched using affinity chromatography. Not all proteins contain cysteine however, and with the early versions of the ICAT reagents, the different masses of the light and heavy tags resulted in different chromatographic performance. Additionally, only two tags (light and heavy) were available so multiplexing (running more than two samples at the same time) was impossible. The iTRAQ methodology was developed by Ross et al., (2004), and commercialized by Applied Biosystems Inc. It was designed to overcome some of the liabilities of the ICAT labeling methodology, and its use has increased dramatically over the past few years. The molecule consists of a charged N-methylpiperazine based reporter group, a carbonyl balance group and an N-hydroxysuccinimide (NHS) peptide reactive group. The mass of the reporter and balance groups sum to 145.1 m/z and this is kept constant though use of $^{13}$C, $^{15}$N and $^{18}$O atoms. Originally, only four different reporter groups were available, m/z 114.1, 115.1, 116.1 and 117.1, but the current version is available with eight reporters, which would allow the comparison of up to eight states or time points. The NHS moiety
reacts with primary amines, and labeling is conventionally performed after trypsin
digestion. Consequently, the N-terminal and any lysines present in the peptide are
labeled. Since the tags are isobaric, the peptides from different experimental groups
containing different labels elute at the same time and appear in MS spectra at the same
m/z, significantly simplifying the spectra. N-methylpiperazine is a basic moiety and
accepts protons readily, which enhances the overall ionization efficiency of the
peptides (Ross et al., 2004). iTRAQ is MS/MS based in the sense that the charged
reporter ions are released during collision induced dissociation (CID) with the
carbonyl undergoing a neutral loss. Relative quantification is achieved using either the
peak area or intensity of the reporter ions. Successful use of this technique has been
demonstrated in many organisms and tissues (Ross et al., 2004; Aggarwal et al., 2005;
Hu et al., 2006; Glen et al., 2008; DeSouza et al., 2006; Reinders et al., 2006).

There are numerous factors that can affect the accuracy of iTRAQ quantification.
Some of these include the number of biological/technical replicates, sample
complexity, instrumentation, method of peptide/protein identification and the
statistical techniques used for data analysis. It has been observed that the low collision
energies normally used in ESI QTOF can result in low iTRAQ reporter ion
abundances (Wiese et al., 2007). We used multifactor ANOVA to compare the iTRAQ
ratios that were generated on an ESI QTOF and a MALDI TOF/TOF from a modest
set of samples.

EXPERIMENTAL
**Materials**

Chlordecone (CD) was purchased from Chem Service (West Chester, PA). Purity (99%) was confirmed by gas chromatography-electron ionization/mass spectrometry. Radiolabeled CH ([4-\(^{14}\)C] CH, 55 mCi/mmol) was obtained from Radiolabeled Chemicals, Inc. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce Biotechnology Inc. (Rockford, IL). iTRAQ reagents were purchased from Applied Biosystems (ABI, Foster City, CA). Burdick and Jackson solvents (VWR, West Chester, PA) were used for all high performance liquid chromatography (HPLC). All other chemicals were purchased from Sigma (St. Louis, MO).

**Animals (CH Binding Protein Enrichment)**

Previous dose response and time course experiments (Carpenter and Curtis, 1989, 1991) provided optimal doses and sampling times for CD pretreatment and the exogenously administered lipid bolus containing radiolabeled CH ([\(^{14}\)C]CH). Six-seven week old male C57BL/6 mice were purchased from Simenson Laboratory (Gilroy, CA). They were randomly assigned to one of three groups: Control, Low dose (5 mg/kg) CD, or High dose (15 mg/kg) CD. They were individually housed in a temperature (22±1°C) and light controlled (12 hours light/12 hours dark daily) facility with free access to water and fed AIN93 diet (Dyets, Inc., Bethlehem, PA) ad libitum. Seven days were allowed for acclimatization before treatments were initiated. Corn oil was used as a vehicle (5 mL/kg). Mice received a single dose of either corn oil or CD (5 mg/kg or 15 mg/kg) via intraperitoneal (IP) injection. After 72 hours a lipid bolus
of corn oil (5 mL/kg) containing 10 mg/kg CH (2 μCi/mouse) was administered IP. After injection the mice continued to be housed individually and were allowed free access to water. They were killed 16 hours later by carbon dioxide anesthesia and exsanguination. Livers were removed for analysis. The procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).

Animals (iTRAQ Experiment)
Six-seven week old male C57BL/6 mice were purchased from Simenson Laboratory (Gilroy, CA). They were randomly assigned to one of two groups; 1) Control or 2) High dose (15 mg/kg) CD. They were individually housed in a temperature (22±1ºC) and light controlled (12 hours light/12 hours dark daily) facility with free access to water and fed AIN93 diet (Dyets, Inc., Bethlehem, PA) ad libitum. Seven days were allowed for acclimatization before treatments were initiated. Corn oil was used as a vehicle (5 mL/kg). Mice received a single dose of either corn oil or CD (5 mg/kg or 15 mg/kg) via intraperitoneal (IP) injection. After 72 hours they were fasted for four hours and killed by carbon dioxide anesthesia and exsanguination. Liver, gall bladder and blood were harvested. Here again, the procedures for animal use were approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC).

Tissue Preparation
Liver was homogenized with a Polytron PT 3000 PT (Brinkmann Instruments, Westbury CT) in buffer consisting of 0.01 M potassium phosphate, pH 7.4, 0.15 M potassium chloride (KCL), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM phenylmethlysulfonyl fluoride (PMSF). All centrifugation was performed at 4ºC. The homogenate was first centrifuged at 1200 g for 30 minutes. The pellet produced consisted of plasma membrane/cellular debris. The supernatant was then centrifuged at 12000 g for 30 minutes to eliminate mitochondria. The supernatant from this run was then centrifuged at 100,000 g for 90 minutes in a Ti 70 rotor. The resulting supernatant and pellet were the cytosolic and microsomal fractions respectively. Microsomes were resuspended in 0.1 M potassium phosphate, pH 7.25, 1.0 mM EDTA, 30% glycerol, 20 μM BHT, 1.0 mM dithiothreitol (DTT) and 0.1 mM PMSF. BCA assay was used to determine protein content.

Radioactivity Quantification Assessed Fraction Enrichment of CH Binding Proteins

Subcellular fractions were prepared as described under tissue preparation, but in this experiment, the mice were injected with [14C] CH. Fractionation of [14C] CH was applied in order to evaluate the concentration of CH binding proteins. Fifty μL/mL of 30% streptomycin sulfate was added to cold hepatic cytosol and gently shaken overnight at 4ºC. The resulting precipitate was dissolved in 8.0 M urea, 10 mM potassium phosphate, pH 7.4 and dialyzed overnight in 1.0 M urea, 10 mM potassium phosphate, pH 7.4. Aliquots of the streptomycin precipitated cytosolic fractions
(supernatant and solubilized precipitate) were added to 10 mL of Hionic-Fluor
(Perkin-Elmer, Boston, MA). The samples were mixed and then placed in the dark
overnight to allow the chemiluminescence to decay. Samples were counted in a
Beckman LS7500 liquid scintillation counter. The BCA assay was used to determine
protein concentrations.

**Fluorescent Spectroscopy**

The fluorescent analog NBD CH (22-(N-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino)-
23,24-bisnor-5- cholen-3\(\beta\)-ol) was purchased from Invitrogen/molecular Probes
(Carlsbad, CA). Experiments were performed on a Perkin Elmer LS50 luminescence
spectrometer (Waltham, MA).

**Tissue Preparation for iTRAQ Experiments**

CH enriched fractions were prepared by streptomycin precipitation as outlined above.

BCA assay was used to determined protein concentrations. 100-\(\mu\)g samples of the
streptomycin precipitate fraction were precipitated with six volumes of ice cold
acetone and labeled with iTRAQ reagents as detailed below.

**iTRAQ Labeling**

Following the manufacturer’s protocol, samples were labeled with iTRAQ reagents
according to the scheme shown in Figure 3-1. Briefly, 100-\(\mu\)g samples were dissolved
in 20 \(\mu\)L of 0.5 M tetraethyl ammonium bicarbonate (TEAB), denatured with 0.1%
SDS and reduced with 5 mM tris-(2-carboxyethyl) phosphine (TCEP). After a one hour incubation at 60ºC, cysteines were blocked with 10 mM methyl methane–thiosulfonate (MMTS) in isopropanol, and the samples were incubated at room temperature for 10 minutes. Promega (Madison, WI) sequencing grade trypsin was added in a trypsin:protein ratio of 1:20, and the samples were incubated at 37ºC for 16 hours. The samples were vacuum concentrated, resolubilized in 30 μL TEAB and labeled with 70 μl iTRAQ reagent in isopropanol. Control samples were labeled with iTRAQ 114.1 or 116.1, while CD treated samples (15 mg/kg) were labeled with iTRAQ 115.1 or 117.1 and incubated at room temperature for 2 hours. One control mouse and 1 treated mouse composed one biological sample. Four samples, i.e., a total of eight mice, were prepared. Control and CD treated samples were randomly combined and vacuum concentrated to ~30 μL. Samples were prepared for analysis by chromatography on strong cation exchange (SCX) spin columns (The Nest Group, Inc., Southboro, MA) and eluted with four salt fractions; 75 mM, 150 mM, 350 mM and 750 mM KCl in 25% acetonitrile, 10 mM potassium phosphate monobasic (KH₂PO₄), pH 3.0. The eluted fractions were vacuum concentrated to ~30 μL, and 300 μL of 0.1% trifluoroacetic acid (TFA) was added. Samples were desalted on C-18 spin columns (The Nest Group), eluted with 80% acetonitrile, 0.1% TFA and vacuum concentrated to dryness. Samples were resuspended in 20 μL 0.1% TFA before performing nano HPLC mass spectrometry.

HPLC-Electrospray Ionization (ESI)-MS/MS
Nano HPLC ESI-MS/MS was performed on a nanoAcquity Ultra Performance LC (Waters, Milford, MA) coupled to a quadrupole orthogonal time-of-flight mass spectrometer (QTOF Ultima Global, Micromass/Waters, Manchester UK). Four-μL samples were injected. Peptides were trapped and washed on a Symmetry C18 column (5 μm, 180 μm × 20 mm, Waters) using 2% acetonitrile in 0.1% formic acid at a flow rate of 4 μL/minute for 6 minutes. Peptides were separated using an ethylene bridged hybrid (BEH) column (1.7 μm, 75 μm × 150 mm, Waters) with a binary gradient consisting of 2% acetonitrile in 0.1% forming acid (Solvent A) and acetonitrile in 0.1% formic acid (Solvent B) over 70 minutes at a flow rate of 260 nL/minute.

The ion source was operated with a spray voltage of 2.2 kV in the positive ion mode. Data dependent analysis (DDA) MS/MS mode was utilized with a 0.6 sec survey scan and a 2.4 sec MS/MS scan on the base peak in the MS survey scan. Previously selected m/z values were excluded from DDA for 60 sec. The MS/MS collision energy ranged from 25-65 eV and was based on the precursor charge state selected by the quadrupole. No effort was made to optimize the transmission of low m/z ions through the instrument, i.e., the instrument was run in its standard operating mode. Calibration was performed with [Glu¹]-fibrinopeptide B (MH⁺ 1570.6774 Da, monoisotopic) fragment ions. Lock mass correction was also performed using [Glu¹]-fibrinopeptide B ([M+2H]²⁺ 785.8426 Th) every 30 sec for one scan. Peak list (pkl) files were generated using Protein Lynx Global Server (PLGS, Waters, Manchester, UK).

**HPLC-Matrix Assisted Laser Desorption Ionization (MALDI)-MS/MS**
Peptide separation was performed on a nanoAcquity Ultra Performance LC (Waters, Milford, MA) coupled to a Probot (Dionex/LC Packings, Amsterdam, The Netherlands) MALDI spotting device. Two-μL samples were injected. Peptides were trapped and washed on a Symmetry C18 column (5 μm, 180 μm × 20 mm, Waters) using 0.1% TFA at a flow rate of 4 μl/minute for 6 minutes. Peptides were separated using an Atlantis C18 column (3 μm, 75 μm × 150 mm, Waters) with a binary gradient consisting of 0.1% TFA (Solvent A) and acetonitrile in 0.1% TFA (Solvent B) over 60 minutes at a flow rate of 300 nl/minute.

Column effluent was mixed in a 1:4 ratio with MALDI matrix (3 mg/mL α-cyano-4-hydroxycinnamic acid in water/acetonitrile/TFA/ammonium phosphate dibasic (6 mg/mL), 37.6/56.4/0.1/6, vol:vol) via a tee-junction. Fractions (20 s/spot, 144 spots/plate) were spotted onto an Opti-TOF MALDI plate system (ABI) using stainless steel plates.

MALDI plates were analyzed on an ABI 4700 Proteomics Analyzer equipped with a 200 Hz Nd:YAG laser operating at a wavelength of 355 nm. Data were acquired in the MALDI reflector mode over a mass range of 800-4000 m/z using five spots of ABI calibration mixture as an external standard. ABI calibration mixture consists of the following peptides (monoisotopic mass of the singly protonated ion is given in parenthesis in Da); [Glu¹]-fibrinopeptide B (1570.6774), adrenocorticotropin hormone fragment 18-39 (2465.1989), angiotensin I (1296.6853) and [des-Arg¹]-bradykinin (904.6774). Mass spectra were obtained from each spot using 1000 laser shots per
spectrum. Peaks with a signal:noise (S/N) ratio greater than 30 were selected for MS/MS. Up to 10 MS/MS spectra could be obtained from each spot. MS/MS spectra were acquired with 1500 laser shots, starting with the least intense peak and ending with the most intense peak. Constant laser fluence was used and kept low enough to prevent signal saturation. Ions formed in the source were accelerated to 8 keV. Precursors were selected using timed ion selection (resolving power = 100 full width half maximum [FWHM]) and then decelerated in the collision cell to 1 keV for CID. Air was used as the collision gas at a pressure of about $5.5 \times 10^{-7}$ torr. Fragment ions were accelerated to 14 keV before entering the reflector.

**Data Processing and Database Searching**

MALDI-MS/MS data were processed with GPS Explorer (Applied Biosystems Inc., Framington MA) to create Mascot searchable files. MS/MS peak list files for the four salt fractions, which constituted one biological sample pair (control and treated), were combined using Mascot Daemon (Matrix Science, UK) and searched against the mammalian Swiss-Prot database (V55.0) using the Mascot (Matrix Science, UK) search engine. β–Methylthiolation [methylthiocysteine] was set as a fixed peptide modification. Variable peptide modifications included methionine (M) oxidation and iTRAQ labeling of the N-terminal, lysine (K) and tyrosine (Y). One missed cleavage site was selected with Trypsin/P as the digestion enzyme. ESI-MS/MS precursor and fragment ion mass tolerances were set to $\pm 0.1$ Da and $\pm 0.12$ Da respectively; MALDI-MS/MS precursor and fragment ion mass tolerances were set to $\pm 0.2$ Da and $\pm 0.2$ Da respectively.
iTRAQ ratios were generated in Mascot and median normalized. Tyrosine labeled data was excluded from the iTRAQ ratio calculations. Data sets were further analyzed with Scaffold (Proteome Software, Portland, OR). Only peptides with a probability of 80% or greater as determined by Scaffold were utilized. In the case of duplicate peptides or different charge states, the iTRAQ ratio with the highest Mascot score was used. A minimum of 2 peptides was required for protein identification.

**Statistical Methods**

An iTRAQ sample consisted of 100 μg of protein from a control animal pooled with 100 μg of protein from a CD treated animal. One animal pair was used for each iTRAQ sample, and four pairs in all were analyzed. Each animal pair was analyzed twice on each instrument to generate technical replicates A and B (Figure 3-2A). The iTRAQ ratios from the A and B replicates were averaged for every protein with 2 or more peptides detected in each replicate. If two or more peptides of a protein were detected in only one replicate, that iTRAQ ratio was used in subsequent calculations. Mean iTRAQ protein ratios were generated for each biological pair on the basis of each instrument alone and the average ratios of pairs from both instruments; these mean ratios were then averaged across the biological sample pairs (Figure 3-2B).

Statistical analyses were conducted with either StatGraphics (Statistical Graphics Corp., Herndon, VA) or S-Plus (Insightful Corp., Seattle, WA). Normality of data was accessed based on standardized kurtosis, standardized skewness and homogeneity of
variance. Data for iTRAQ ratios was analyzed in logarithm (natural), ln, space. Means and standard deviations are geometric. The Student’s t-test was used to access whether the mean iTRAQ ratio for a particular protein was different than 1 (0 in ln space), both within a biological sample pair and across the four biological sample pairs. Two-way ANOVA was used to compare the effect of the factors sample (pair) and instrument (ESI QTOF, MALDI TOF/TOF) on the dependent variable, the ln(iTRAQ ratio). In all cases, a 95% confidence level was the criterion for significance. Grubbs' Test (assumes normality) was applied for outlier identification.

RESULTS AND DISCUSSION

NBD CH showed little change (<10%) in emission characteristics (500 – 600 nm) over a range of streptomycin concentrations ranging from 0.05 μM – 15 μM in methanol (data not shown). This indicated little interaction between CH and streptomycin in this solvent.

Mass spectrometry has a limited dynamic range, and the cytosolic proteome is complex. Enrichment of the fraction of interest was explored as a means to simplify matters and make the mass spectrometry more tenable. Original enrichment experiments were performed with [14C]CH as described under methods. Streptomycin precipitation of cytosol proved to be effective in concentrating the label while at the same time decreasing the amount of protein present (Figure 3-3). Clearly the label is enriched in the precipitate (Figure 3-3A), most of the protein remains in the
supernatant (Figure 3-3B), thus reducing the number of proteins that need to be
detected by mass spectrometry.

Approximately 75 proteins were detected with the ESI QTOF and 40 with the MALDI
TOF/TOF under the conditions described in the experimental section (data not shown).
The observed difference between the two techniques was probably due to the
differences in HPLC methodology. The overall relatively low number of proteins
detected probably was due to the limited number (4) of isocratic KCl elutions from the
SCX spin columns.

Wiese et al., (2007) showed that higher collision energies were needed for efficient
liberation of iTRAQ reporter ions from lysine residues on multiply charged peptides.
Peptide VFIEDVSK was chosen to illustrate this effect (Figure 3-4). Their group also
reported a loss of peptide sequence information under the same conditions. They
reported good iTRAQ quantification with single charged peptides and MALDI
MS/MS analysis. Figure 3-4 shows the product ion mass spectra of the iTRAQ labeled
peptide VFIEDVSK generated via ESI (Figure 3-4A) and MALDI (Figure 3-4B). The
higher collision energy of the MALDI instrument resulted in the 115.1 \textit{m/z} reporter
iTRAQ ion being the base peak. The lower collision energy of the ESI instrument
coupled with the fact that the transmission of low \textit{m/z} ions was not optimized through
the instrument resulted in iTRAQ reporter ion intensities well below the base peak (in
this case the \textit{y}_2 ion). Both spectra show extensive \textit{b} and \textit{y} ion ladders that indicated
efficient fragmentation. Intact iTRAQ ions resulting from the cleavage of the amide
bond between the label and the primary amine group without neutral loss of the carbonyl moiety were observed at m/z 145.1.

The question of whether or not iTRAQ data generated using multiply charged peptides and lower collision energies (ESI QTOF) would be comparable to that of singly charged peptides and higher collision energies (MALDI TOF/TOF) (Wiese et al., 2007) was addressed in the present study by performing two-way ANOVA on proteins that were detected in all four biological sample pairs and with both instruments. Specifically, ANOVA was used to determine the extent to which factors sample and instrument affected the ln(iTRAQ ratio). Proteins detected in all four animal pairs and with both instruments are listed in Table 3-1. The mean and standard deviation (SD) of the iTRAQ ratio for each protein in each pair are geometric and were generated from the two technical replicates generated for each pair (Figure 3-2A). The standard deviations could clearly be improved with more technical replicates; however, cost considerations precluded generation of larger data sets for this study.

Table 3-2 shows the mean iTRAQ values and the 95% confidence intervals after the QTOF and TOF/TOF data were combined and then averaged across all four pairs. Myosin 9 was the only protein with an observed mean iTRAQ ratio significantly different than 1. Its possible role in vesicular intracellular transport (van der Boom et al., 2007) will be addressed in future work. Table 3-3 shows the results of performing ANOVA on the data presented in Table 3-1; the p-values associated with each factor for each protein are given at the 95% confidence level. Clearly, the sample has a significant effect on the ln(iTRAQ) ratio for more than half of the proteins. This could
be due to the biological variability inherent in the samples themselves. In contrast, the $p$-values for the instrument factor are greater than 0.05 in all cases, indicating that the instruments had no significant effect on the ln(iTRAQ ratio).

It appears, therefore, that iTRAQ analyses performed on an ESI TOF without any special modifications of the instrumental parameters produce essentially the same protein ratios as obtained on a MALDI TOF/TOF. Although this observation needs to be tested with a larger data set produced with other instruments, it indicates that it should be possible in general to compare iTRAQ data obtained from these two types of instruments.
Control (corn oil) Streptomycin Precipitate (100 μg)

↓

Reduction Alkylation Trypsin Digestion

↓

Label with 114 or 116 iTRAQ

Treated (15 mg/kg CD) Streptomycin Precipitate (100 μg)

↓

Reduction Alkylation Trypsin Digestion

↓

Label with 115 or 117 iTRAQ

Pool Samples

↓

SCX Spin Columns
- 4 KCl fractions, 75 mM, 150 mM, 350 mM and 700 mM

↓

C18 HPLC followed by MALDI MS/MS and ESI MS/MS.

↓

Data Analysis by GPS Explorer, Mascot and Scaffold

Figure 3-1. Flow chart showing the sequence of iTRAQ labeling and mass spectrometry analysis. A total of four animal pairs were analyzed.
Figure 3-2. Flow chart showing how iTRAQ samples were analyzed: (A) breakdown for one biological sample: (B) three procedures used for averaging the iTRAQ protein ratios over all four biological samples.
Figure 3-3. Enrichment of $[^{14}\text{C}] \text{CH}$ and decrease in protein observed in the supernatant and streptomycin precipitate of hepatic cytosol: (A) CH equivalents/mg protein versus chlordecone treatment and streptomycin fraction (n=3 pooled samples, bar heights=mean of three liquid scintillation runs) ± SD; (B) cytosolic protein recovery versus chlordecone treatment and streptomycin fraction n=3 pooled samples, one analysis was done for each pooled sample therefore no error bars are included.)
Figure 3-4. Mass spectra of the iTRAQ labeled peptide VFIEDVSK: (A) spectrum generated on ESI TOF from doubly charged precursor; (B) spectrum generated on MALDI TOF/TOF. The fragment ions were labeled according to the nomenclature of Beimann (1988). The insets in the spectra show enlargements of the reporter ion region.
Table 3-1. Proteins detected in all four animal pairs and with both instruments: means and standard deviations (SDs) are geometric and were generated from the two technical replicates run for each pair; (A) data generated by ESI QTOF; (B) data generated by MALDI TOF/TOF.
<table>
<thead>
<tr>
<th>NCBI Accession Number</th>
<th>Protein</th>
<th>n</th>
<th>Mean</th>
<th>Confidence Interval (95%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>P60710</td>
<td>Actin, cytoplasmic 1</td>
<td>4</td>
<td>0.87</td>
<td>0.48-1.56</td>
<td>0.490</td>
</tr>
<tr>
<td>P00329</td>
<td>Alcohol dehydrogenase 1</td>
<td>4</td>
<td>0.86</td>
<td>0.52-1.42</td>
<td>0.397</td>
</tr>
<tr>
<td>Q91Y97</td>
<td>Fructose-bisphosphate aldolase B</td>
<td>4</td>
<td>0.83</td>
<td>0.66-1.04</td>
<td>0.075</td>
</tr>
<tr>
<td>Q61176</td>
<td>Arginase-1</td>
<td>4</td>
<td>0.91</td>
<td>0.72-1.15</td>
<td>0.295</td>
</tr>
<tr>
<td>P16460</td>
<td>Argininosuccinate synthase</td>
<td>4</td>
<td>1.17</td>
<td>0.89-1.54</td>
<td>0.164</td>
</tr>
<tr>
<td>O35490</td>
<td>Betaine--homocysteine S-methyltransferase 1</td>
<td>4</td>
<td>1.05</td>
<td>0.83-1.33</td>
<td>0.533</td>
</tr>
<tr>
<td>P12710</td>
<td>Fatty acid-binding protein, liver</td>
<td>4</td>
<td>0.89</td>
<td>0.63-1.24</td>
<td>0.334</td>
</tr>
<tr>
<td>P16858</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>4</td>
<td>0.95</td>
<td>0.82-1.11</td>
<td>0.401</td>
</tr>
<tr>
<td>P30115</td>
<td>Glutathione S-transferase A3</td>
<td>4</td>
<td>0.85</td>
<td>0.64-1.13</td>
<td>0.167</td>
</tr>
<tr>
<td>P10649</td>
<td>Glutathione S-transferase Mu 1</td>
<td>4</td>
<td>0.91</td>
<td>0.66-1.25</td>
<td>0.421</td>
</tr>
<tr>
<td>P19157</td>
<td>Glutathione S-transferase P 1</td>
<td>4</td>
<td>0.93</td>
<td>0.49-1.75</td>
<td>0.741</td>
</tr>
<tr>
<td>P01942</td>
<td>Hemoglobin subunit alpha</td>
<td>4</td>
<td>1.20</td>
<td>0.84-1.72</td>
<td>0.205</td>
</tr>
<tr>
<td>Q8VDD5</td>
<td>Myosin-9</td>
<td>4</td>
<td>1.34</td>
<td>1.05-1.70</td>
<td>0.032</td>
</tr>
<tr>
<td>P35700</td>
<td>Peroxiredoxin-1</td>
<td>4</td>
<td>0.90</td>
<td>0.77-0.99</td>
<td>0.044</td>
</tr>
<tr>
<td>P08228</td>
<td>Superoxide dismutase [Cu-Zn]</td>
<td>4</td>
<td>0.96</td>
<td>0.65-1.41</td>
<td>0.738</td>
</tr>
<tr>
<td>P68369</td>
<td>Tubulin alpha-1A chain</td>
<td>4</td>
<td>0.87</td>
<td>0.40-1.88</td>
<td>0.600</td>
</tr>
<tr>
<td>P68372</td>
<td>Tubulin beta chain</td>
<td>4</td>
<td>0.92</td>
<td>0.40-2.08</td>
<td>0.754</td>
</tr>
</tbody>
</table>

Table 3-2. Mean iTRAQ values and the 95% confidence interval after the instrument data were combined and then averaged across all four pairs. Ratios significantly different than one are shown in bold font and (n) is the number of sample pairs used in the calculation of the geometric mean.
### ANOVA (Multifactor)

<table>
<thead>
<tr>
<th>NCBI Accession Number</th>
<th>Protein</th>
<th>p-Value (Sample)</th>
<th>p-Value (Instrument)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P60710</td>
<td>Actin, cytoplasmic 1</td>
<td>0.011*</td>
<td>0.162</td>
</tr>
<tr>
<td>P00329</td>
<td>Alcohol dehydrogenase 1</td>
<td>0.022*</td>
<td>0.261</td>
</tr>
<tr>
<td>Q91Y97</td>
<td>Fructose-bisphosphate aldolase B</td>
<td>0.073</td>
<td>0.060</td>
</tr>
<tr>
<td>Q61176</td>
<td>Arginase-1</td>
<td>0.049*</td>
<td>0.166</td>
</tr>
<tr>
<td>P16460</td>
<td>Argininosuccinate synthase</td>
<td>0.030*</td>
<td>0.747</td>
</tr>
<tr>
<td>O35490</td>
<td>Betaine--homocysteine S-methyltransferase 1</td>
<td>0.025*</td>
<td>0.953</td>
</tr>
<tr>
<td>P12710</td>
<td>Fatty acid-binding protein, liver</td>
<td>0.199</td>
<td>0.715</td>
</tr>
<tr>
<td>P16858</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.195</td>
<td>0.629</td>
</tr>
<tr>
<td>P30115</td>
<td>Glutathione S-transferase A3</td>
<td>0.120</td>
<td>0.550</td>
</tr>
<tr>
<td>P10649</td>
<td>Glutathione S-transferase Mu 1</td>
<td>0.152</td>
<td>0.443</td>
</tr>
<tr>
<td>P19157</td>
<td>Glutathione S-transferase P 1</td>
<td>0.009*</td>
<td>0.157</td>
</tr>
<tr>
<td>P01942</td>
<td>Hemoglobin subunit alpha</td>
<td>0.006*</td>
<td>0.994</td>
</tr>
<tr>
<td>Q8VDD5</td>
<td>Myosin-9</td>
<td>0.019*</td>
<td>0.905</td>
</tr>
<tr>
<td>P35700</td>
<td>Peroxiredoxin-1</td>
<td>0.863</td>
<td>0.707</td>
</tr>
<tr>
<td>P08228</td>
<td>Superoxide dismutase [Cu-Zn]</td>
<td>0.239</td>
<td>0.950</td>
</tr>
<tr>
<td>P68369</td>
<td>Tubulin alpha-1A chain</td>
<td>0.006*</td>
<td>0.556</td>
</tr>
<tr>
<td>P68372</td>
<td>Tubulin beta chain</td>
<td>0.003*</td>
<td>0.355</td>
</tr>
</tbody>
</table>

Table 3-3. *P*-values associated with the factors sample and instrument at the 95% confidence level generated from the ANOVA analysis on the data shown in Table 3-1. The asterisks indicate *p*-values less than 0.050.
Chapter 4

Conclusions

The principle toxicological finding of this study is that CD pretreatment increased the SR-BII protein content of hepatic microsomes but not crude membrane fractions. This increase in microsomal SR-BII may represent an increase in transport capacity for the hepatocytes to handle CH. If this is the case, then CD pretreated animals provided with an exogenous lipid bolus of CH should be able to transport CH through the liver and into the bile more effectively than control animals. The mechanics of this transport remains illusive. SLU with SR-BI occurs without endocytosis and SR-BII is twice as efficient as SR-BI when it comes to CH efflux (Nieland et al., 2005; Mulcahy et al., 2004). Additionally, as mentioned previously, SR-BII is endocytosed much more rapidly than SR-BI. The fact that endocytosis is not needed for SLU excretion into bile provides strong evidence for the involvement of a non-vesicular CH transport system. There was no evidence for a role of cytosolic binding proteins although additional mass spectrometry experiments are needed to adequately address this issue. No changes in plasma membrane SR-BII were observed but it was not possible to distinguish between basolateral and apical membranes. Therefore, if a differential increase and corresponding decrease on one or the other of these membranes is occurring, it would be unobservable. If the number of SR-BII transporter proteins on the cell surface remains constant with CD pretreatment, the increase in microsomal SR-BII may indicate an increased flux between the SR-BII transporters on the cell surface and intracellular membrane. The specific mechanism of SLU has not been elucidated, but it appears to be diffusion driven between the
lipoprotein and the plasma membrane. After the action of hepatic lipase on a lipoprotein, e.g., HDL, the phospholipids on the HDL surface will be broken down to release fatty acids. This would make the CH in that layer less stable and, thereby, easier to transport down the concentration gradient to the plasma membrane of the cell. At this point the CH is moved across the cell by some still unknown mechanism presumably involving non-vesicular transport mechanisms and excreted into the bile. The efficiency of transfer of CH from the HDL to the plasma membrane would depend on how long the concentration gradient near the transporter could be maintained. Each transporter would be expected to be associated with a specific, albeit, transient lipid microdomain. Perhaps rotating transporters out of the plasma membrane rapidly would allow for more efficient CH transfer. The data from this study supports a model wherein SR-BII could contribute to CD induced stimulation of biliary excretion of CH.

A statistically significant increase in the crude membrane content of Na\(^+\)/K\(^+\) ATPase was also observed. Since CD is known to inhibit membrane Na\(^+\)/K\(^+\) ATPase, the observed increase may reflect a compensatory response.

Proteomic analysis of a CH enriched fraction of hepatic cytosol detected significant changes in four proteins. Increased myosin-9 was consistent with an increased capacity for vesicular transport. Decreases in SBP-1, α-enolase, and PRX-1 in a CH enriched fraction of cytosol was observed but how this fits into CD induced perturbations in CH homeostasis is not clear.

The proteomic analysis detected about 80 proteins overall (data not shown). Although both cytosolic sterol carriers L-FABP and SCP-2 were detected, SCP-2 did
not meet the minimum criteria for protein identification. However, many soluble sterol carrier proteins were not detected. Approximately 80 proteins were present in the streptomycin enriched fraction; using more effective separation techniques should allow the detection of many more proteins. Simply increasing the number of salt cuts used in the first chromatographic dimension by a factor of 10 would be a good first step. A more thorough examination of the cytosolic proteome is required before the role of cytosolic transport proteins can be definitively dismissed.

Overall these results may at least partially explain the CD pretreatment induced alterations in CH homeostasis.

The principle bioanalytical finding of this study is that iTRAQ analyses performed on an ESI TOF without any special modifications of the instrumental parameters produce essentially the same protein ratios as obtained on a MALDI TOF/TOF. With the caveat that this observation needs to be tested with a larger data set produced with other instruments, this observation indicates that it should be possible in general to compare iTRAQ data obtained from these two types of instruments.
Bibliography


Applied Biosystems Inc., Version 3.6, Framington, MA.


Insightful Corp, Version 8.0, Seattle, WA.


Lee, J., 2002, Alteration of cholesterol disposition by chlordecone is not explained by induction of Cyp7a or Cyp4a1, *M.S. Thesis*, Oregon State University.

Lee, J., 2007, Chlordecone (CD) A mixed steroid X receptor (SXR) and estrogen receptor alpha (ER$\alpha$) agonist, altered hepatic cholesterol (CH) homeostasis and lipoprotein metabolism, *Ph.D. Dissertation*, Oregon State University.


Proteome Software, Inc., Version 1.6, Portland, OR.


Statistical Graphics Corp., Version 5.1, Herndon, VA.


containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. *J. Biol. Chem.* 273, 15241-15248.


Appendices
Appendix A

Chlordecone Altered Hepatic Disposition of [14C]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 be found in tissues of humans and wildlife throughout the world although they were banned in the United States a few decades ago. Low doses of the OC insecticide chlordecone (CD) alter hepatic disposition of lipophilic xenobiotics and perturb lipid homeostasis in rainbow trout, mice and rats. CD pretreatment altered tissue and hepatic subcellular distribution of exogenous [\(^{14}\)C]cholesterol (CH) equivalents 4 and 16 h after a bolus intraperitoneal (ip) injection of 5 ml corn oil/kg that contained 10 mg CH/kg. CD pretreatment altered tissue distribution of exogenously administered [\(^{14}\)C]CH by decreased hepatic and renal accumulation, and increased biliary excretion up to 300%. Biliary excretion of polar [\(^{14}\)C]CH metabolites was not altered by CD. CD pretreatment decreased subcellular distribution of [\(^{14}\)C]CH equivalents in hepatic cytosol and microsomes 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 h after a bolus lipid dose. CD pretreatment increased plasma non-HDL total CH by 80% 4 h after a bolus lipid dose. 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. Liver membrane contents of SR-BI and ABCG8 proteins were unchanged by CD pretreatment. The data demonstrated that a single dose of CD altered CH homeostasis and lipoprotein metabolism.

Keywords: organochlorines, chlordecone, cholesterol disposition, liver, biliary excretion, lipoproteins.
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. Modes of action for OC insecticides are distinct from that prototypical for chlorinated dioxins and planar PCB congeners in that they exhibit negligible affinity for the aryl hydrocarbon receptor (Poland and Knutson, 1982). As is typical for OC insecticides, neurotoxicity is a prominent mode of action for CD: it is unusual in that there is extensive epidemiologic documentation of a major human poisoning following poor manufacturing and disposal practices (reviewed by Guzelian, 1982). Prominent weight loss despite normal appetite is a common sign of occupational CD poisoning in humans (10 of 23 cases).

The OC insecticides dieldrin and chlordecone (CD) alter hepatic disposition of lipophilic xenobiotics and perturb lipid homeostasis in rainbow trout, mice and rats (Barnhill et al., 2003; Carpenter and Curtis 1989, 1991; Carpenter et al. 1996; Donohoe et al. 1998; Gilroy et al. 1994). These effects of OC insecticides occur at liver concentrations in the 1-75 parts per million (ppm) range. Single, doses of CD and dieldrin decrease plasma triglycerides and total 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, decreasing hepatic disposition and increasing distribution to other tissues (Carpenter and Curtis 1991).

CH is an important constituent of cell membranes and 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 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 indicated that the rate of uptake of high density lipoprotein (HDL)-bound [$^{14}$C]CD or [$^{14}$C]CH was significantly lower in perfused livers from Sprague-Dawley rats pretreated with a single dose of 15 mg/kg CD (Gilroy et al. 1994). Gilroy et al. (1994) reported a significantly increased 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, consistent with in vivo studies of human, rat, and pig plasma (Soine et al. 1984a; 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 CH esters and other lipids from HDL (Hobbs and Rader 1999; Krieger 1999). SR-BI deficiency reduces 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; 2002).
The present studies examined the effect of CD pretreatment on the tissue distribution of exogenous \[^{14}\text{C}]\text{CH}\) 4 and 16 h after intraperitoneal (ip) injection of 5 ml corn oil/kg that contained 10 mg CH/kg. Hepatic subcellular distribution of \[^{14}\text{C}]\text{CH}\) was also determined. Blood plasma, hepatic and biliary total CH were examined in control and CD pretreated mice. This investigation determined whether the reduced disposition of \[^{14}\text{C}]\text{CH}\) to liver was associated with 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 membranes 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}\text{C}]\text{CH}\). CD pretreatment also altered the amount of total CH in blood plasma and gallbladder. The reduced hepatic \[^{14}\text{C}]\text{CH}\) equivalents and increased biliary \[^{14}\text{C}]\text{CH}\) equivalents were not associated with altered hepatic membrane SR-BI or 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-\(^{13}\text{C}\)]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

The CD and lipid bolus including $^{14}$CCH doses and times of sampling were based on previous dose-response and time-course experiments (Carpenter and Curtis, 1989; 1991). 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 mg or 15 mg CD/kg body weight treatment. The animals were housed in a temperature controlled room (22 ± 1°C) with a daily cycle of 12 h of light and 12 h 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.

$^{14}$CCH 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 $^{14}$CCH was administered (10 mg CH/kg body weight, ip). $^{14}$CCH injected mice were housed individually and allowed free access to water. After 4 or 16 h 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 3,000 g for 25 min at 4°C. The 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°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°C for 1.5 – 2 h and decolorized by adding 30% hydrogen peroxide. Plasma and HDL fraction were analyzed by liquid scintillation counting (LSC) without further treatment. Ten ml of Hionic-Fluor (PerkinElmer, Boston, MA) liquid scintillation cocktail was added. After decay of chemiluminescence liquid scintillation counting was conducted using a Beckman LS7500 liquid scintillation counter. Protein concentration was determined by BCA assay (Pierce Biotechnology, Inc. Rockford, IL).

Gallbladders plus bile from mice killed 4 hr after lipid bolus injections were digested,
decolorized, and analyzed by LSC for total $[^{14}\text{C}]\text{CH}$ equivalents as described above. Lipid and polar fractions of gallbladders plus bile from mice killed 16 hr after $[^{14}\text{C}]\text{CH}$ injections were separated by liquid-liquid extraction. Neutral steroids were extracted with slight modifications of the method described by Miettinen et al (1965). A 2.9 ml volume of basic (1.5 N KOH) ethanol/water (2:1) was added to each gallbladder plus bile sample which was heated at 70°C for 30 min. One ml of water was added to cooled samples which were then extracted with 2 ml of hexane. The hexane layer was removed and dried under a nitrogen stream. The residue was resuspended in 1 ml 2-propanol that contained 10% Triton X-100. Duplicate 0.1 ml samples of the ethanol/water phase (polar metabolites) and the hexane residues containing neutral steroids were analyzed by LSC.

**Plasma lipid analyses**

Total plasma CH concentrations were measured enzymatically by using 2 μL of plasma and 200 μL 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 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 h, 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.

**Biliary lipid analyses**

Total CH was determined in the hexane extracts of bile treated with basic ethanol/water for 30 min at 70°C (detailed above). Duplicate subsamples in 2-propanol that contained 10% Triton X-100 were assayed with a CH Quantitation Kit<sup>R</sup> BioVision, Mt. View, CA

**Statistical Methods**

Statistical analyses were conducted with StatGraphics software (StatPoint, Herndon, VA). Normality of data sets were assessed with standardized kurtosis,
standardized skewness, and homogeneity of variance. Data that failed one or more

test for normality were log base 10 transformed. Transformed data were assessed

for normality with the criteria specified above. Data were expressed as means ±

SE. Comparisons among groups validated for normality 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 h after 10 mg [14C]CH/kg in 5 ml corn oil/kg (ip bolus lipid) were similar as

were the liver weights (Table 1-1). No significant differences in gallbladder

weights between control and CD pretreated mice were observed (Table 1).

However, 16 h following ip bolus lipid dose, gallbladder weights were

significantly smaller (~9 to 10 mg) than those at 4 h following ip bolus lipid dose

(~15 to 17 mg) (Table 1).

Plasma [14C]CH equivalents were approximately 3-fold higher at 16 h than at 4 h

after the ip bolus lipid dose (p < 0.05, Fig. 1A, 1B), although plasma total CH

concentration at 16 h was not different from that at 4 h after the ip bolus lipid (Fig.

1C, 1D). Plasma [14C]CH equivalents from CD (5 or 15 mg CD/kg) pretreated

mice significantly decreased 4 h following the bolus lipid dose (31 %, p <

0.05)(Fig. 1A). CD pretreatment (5 or 15 mg CD/kg) significantly decreased

[14C]CH equivalents in the non-HDL fraction compared with controls (45%, p <
On the contrary, CD pretreatment increased total CH in non-HDL fraction 4 h following ip bolus lipid (about 1.8-fold, p < 0.05) (Fig. 1C). At 16 h after the ip bolus lipid, neither $^{14}$CCH equivalents nor total CH concentrations in plasma were altered by CD pretreatment (Fig. 1B, 1D). No significant differences in the $^{14}$CCH equivalents and total CH in the either HDL or non-HDL fractions were observed 16 h following ip bolus lipid (Fig. 1B, 1D). Although the $^{14}$CCH equivalents in the HDL fraction were unchanged, the ratio of $^{14}$CCH equivalents in HDL fraction to that in plasma was mildly but statistically significantly increased by CD pretreatment 4 h after ip bolus lipid in a dose-dependent manner (Fig. 2A).

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. 2B).

Consistent with previous results, CD decreased $^{14}$CCH equivalents distribution to the liver at 16 h after the bolus lipid dose (43 %, p < 0.05) (Fig. 3A). Altered $^{14}$CCH 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 h after the bolus lipid dose, $^{14}$CCH 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 h after ip bolus lipid, $^{14}$CCH 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 redistribution 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 h than at 4 h 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 h after ip bolus lipid, compared with control (about 50%, p < 0.05)(Fig. 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 h after the ip bolus lipid in CD pretreated animals was observed (Fig. 3B).

CD pretreatment increased $[^{14}C]CH$ equivalents of gallbladder 4 and 16 h after the ip bolus lipid dose, in a dose-dependent manner (Fig. 4A). A statistically significant 300% increase total $[^{14}C]CH$ equivalents was observed 4 h after the tracer in 15 mg CD/kg pretreated animals (p < 0.05). A trend for increased $[^{14}C]CH$ equivalents in the neutral steroid fraction from gallbladders at 16 h after the ip bolus lipid in 15 mg CD/kg pretreated animals was observed (p < 0.056, Fig. 4A). Polar metabolites constituted 5.6-10.7% of total biliary $[^{14}C]CH$ equivalents at 16 hr after lipid bolus and there were no significant differences between treatment groups. The absolute polar biliary $[^{14}C]CH$ equivalents for control, 5 and 15 mg CD/kg pretreatments were 1.6, 1.7 and 0.9 nmol/g. CD pretreatment increased total CH concentration in gallbladder 16 h after lipid bolus compared with control (Fig. 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. 4C). Statistically significant
reductions of $[^{14}\text{C}]\text{CH}$ 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, dose of CD alters plasma (Figs. 1, 2), tissue and hepatic subcellular distribution of exogenously administered $[^{14}\text{C}]\text{CH}$ (Figs. 3, 4). There were a number of limitations in interpretation of plasma $[^{14}\text{C}]\text{CH}$ equivalents for explanation of CD-altered disposition of the exogenous lipid bolus. Total plasma $[^{14}\text{C}]\text{CH}$ equivalents included material: (1) newly absorbed from the peritoneal cavity associated with chylomicrons; (2) subjected to intraplastic exchange between lipoprotein classes; and (3) secreted from the liver as nascent HDL or as VLDL. Nonetheless comparisons of data for plasma $[^{14}\text{C}]\text{CH}$ equivalents and plasma total CH in fractions treated with dextran sulfate and magnesium demonstrated CD-dependent differences (Fig. 1A, 1C). CD pretreatment decreased plasma non-HDL $[^{14}\text{C}]\text{CH}$ and increased plasma total non-HDL-CH 4 h after the ip lipid bolus dose (Fig. 1A, 1C). There were at least two mutually nonexclusive explanations. (1) CD delayed $[^{14}\text{C}]\text{CH}$ 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 h after the ip bolus lipid dose about 50 % of plasma $[^{14}\text{C}]\text{CH}$ 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-$[^{14}\text{C}]\text{CH}$. Stimulated plasma clearance of non-HDL-$[^{14}\text{C}]\text{CH}$ seemed unlikely since total non-
HDL-CH was elevated in CD pretreated mice 4 h after the bolus lipid (Fig. 1C). Plasma non-HDL-[\(^{14}\)C]CH equivalents in CD pretreated mice were lower than controls 4 but not 16 h after the bolus lipid dose (Figs. 3A, 3B). This indicated that CD altered a CH exchange pathway or pool that delayed \([^{14}\text{C}]\text{CH}\) incorporation into non-HDL lipoproteins. At 16 h after ip lipid bolus dose \([^{14}\text{C}]\text{CH}\) appeared integrated into the total plasma CH pool (Fig. 1B). In fact, it appeared preferentially retained or secreted in the non-HDL pool since 32 to 35% of \([^{14}\text{C}]\text{CH}\) equivalents were in that fraction compared 18 to 20% of total CH at 16 h. Elevated total plasma non-HDL CH in CD pretreated mice 4 h after the ip lipid bolus (fig. 1C) was of specific interest. This reflected approximately doubled “bad CH” associated with promotion of atherosclerosis (Krieger, 1999). Plasma HDL contained 80-90% of total plasma CH in control mice (fig. 1C, 1D), typical for this species. Since non-HDL lipoproteins predominate in humans, the shift in plasma CH distribution after lipid bolus in CD pretreated mice was not directly applicable to human risk assessment. However, the results suggested work in animals with lipoprotein profiles similar to humans was warranted.

Hepatic \([^{14}\text{C}]\text{CH}\) equivalents were not different between control and CD pretreated mice at 4 h 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 \([^{14}\text{C}]\text{CH}\) in the CD treated perfused rat liver. CD was an agonist for the human pregnane x receptor (PXR) in a reporter gene assay and this was supported by CYP3A protein induction in mouse liver by CD (Lee et al., unpublished data). PXR agonists but
not a selective constitutive androstane receptor agonist increased expression of the apoA-I gene in mice (Bachmann et al. 2004), the principal component of HDL. Sporstol et al. (2005) reported the down regulation of SR-BI by a PXR agonist in vitro. 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. 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 SRBI 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 CH 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 h after the bolus lipid (Fig. 1C). Lower lipoprotein-rich fraction-to-liver homogenate [¹⁴C]CH equivalents ratios in 15 mg CD/kg pretreated mice were consistent with suppression of incorporation of exogenous CH into lipoprotein complexes (Fig. 2-3B). Markedly reduced [¹⁴C]CH lipoprotein-rich fraction-to-homogenate ratio from 4 to 16 h after the bolus lipid dose in both control and CD pretreated mice suggested the secretory phase peaked before 16 h (Fig. 3B). Elevation in plasma non-HDL-total CH at 4 h compared to 16 h after the lipid dose (Figs. 3C, 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 h after the ip lipid bolus. A trend for this persisted after 16 h but was not statistically significant. Since gallbladder weight was lower at 16 than 4 h after the lipid bolus (Table 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 h after the ip lipid bolus. Polar $^{13}$C(CH equivalents in gallbladder bile 16 hr after ip lipid bolus were unchanged (Results). This indicated CD stimulated biliary CH excretion itself, not CH metabolism to bile salts or other polar metabolites. 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 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 h 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 synergistically stimulated an
ABCG5/8-dependent or ABCG5/8-independent pathway of biliary CH secretion.
Another possible explanation was SRBII mediated stimulation of biliary CH
excretion. SR-BII is an alternatively spliced form of SR-BI. SR-BII is expressed
intracellularly 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. Soine et al. (1982) 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.

Chronic exposures of humans and wildlife via trophic transfer through food webs is the most widespread pathway of exposure to persistent organic pollutants, including OC insecticides. Residues occur as complex mixtures (Lordo et al, 1996) and direct extrapolation of results after ip administration to a single compound is tenuous. There is value in elucidation of potential modes of action, however. The ip route of exposure in mice yielded a hepatic CD concentration quite similar to that in rats after dietary exposure. Ten mg CD/kg ip in mice yielded 75 ppm in liver 3 days after dosing (Carpenter and Curtis, 1989). Livers of rats fed a diet that contained 10 pm CD for 15 days accumulated 52 ppm CD (Curtis and Mehendale, 1981). To the extent that liver OC concentration mediates pathophysiological changes, ip administration provides a convenient alternative to long-term feeding studies.

In summary, CD pretreatment altered the plasma, tissue and hepatic subcellular disposition of subsequently administered $^{14}$C]CH. Plasma non-HDL CH almost doubled 4 h after a bolus lipid dose without a significant change in HDL total CH. Our data demonstrated that SR-BI and ABCG8 protein contents in hepatic plasma membranes were unchanged by CD treatment indicating that mechanisms other than SR-BI or ABCG8 may be involved in modulating CD induced CH homeostasis and lipoprotein metabolism.
Acknowledgements:

This work was supported by the Oregon Agricultural Experiment Station (ORE00871) and grant number T32 ES007060 from the National Institutes of Health.
References


Table 1. Body and organ weight of vehicle control and CD pretreated mice that received a challenge dose of \(^{14}\text{C}\)CH.

<table>
<thead>
<tr>
<th></th>
<th>Body mass (g)</th>
<th>Liver (g)</th>
<th>Gallbladder (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hr</td>
<td>16 hr</td>
<td>4 hr</td>
</tr>
<tr>
<td>Control</td>
<td>22.2 ± 0.8</td>
<td>21.9 ± 0.4</td>
<td>1.21 ± 0.03</td>
</tr>
<tr>
<td>5.0 mg CD/kg</td>
<td>22.5 ± 0.7</td>
<td>21.8 ± 0.4</td>
<td>1.23 ± 0.05</td>
</tr>
<tr>
<td>15.0 mg CD/kg</td>
<td>22.9 ± 0.7</td>
<td>22.3 ± 0.3</td>
<td>1.32 ± 0.05</td>
</tr>
</tbody>
</table>

There were 6-7 mice in each group. Values are expressed mean ± SE. *Significantly different from the 4 hr (p < 0.05).
Figure 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. Male C57BL16 mice were pretreated with corn oil, 5, or 15 mg CD/kg. $^{14}$C]CH (10 mg/kg in 5 ml/kg corn oil) was administered 3 days following pretreatment. Determinations were performed 4 and 16 h after ip administration of $^{14}$C]CH. $^{14}$C]CH equivalents of plasma and HDL were 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 h after ip lipid bolus. B. $^{14}$C]CH equivalents of plasma 16 h after ip lipid bolus. C. Plasma total CH 4 h after ip lipid bolus. D. Plasma total CH 16 h after ip lipid bolus. Values are expressed mean ± SE(n=6 or 7). *Significantly different from the 4 h (p < 0.05).
Figure 2. The ratio of [14C]CH equivalents in HDL to that in plasma and hepatic SR-BI contents. Treatments were as described in Figure 1. **A.** The ratio of [14C]CH equivalents in HDL to that in plasma. 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 3. Hepatic disposition of $^{14}$C.CH equivalents of vehicle control and CD pretreated mice that received a challenge dose of $^{14}$C.CH. Treatments were as described in Figure 1. A. Hepatic $^{14}$C.CH equivalents 4 and 16 h after ip bolus lipid of $^{14}$C.CH. B. Hepatic subcellular fraction to homogenate ratios. 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.
Figure 4. $[^{14}C]CH$ equivalents and total CH concentration in gallbladder and hepatic ABCG8 content. Treatments were as described in Figure 1. A. Biliary $[^{14}C]CH$ equivalents 4 and 16 h after ip administration of $[^{14}C]CH$. B, Biliary total CH concentrations were determined 16 h after ip administration of $[^{14}C]CH$ using an enzymatic method. C. Hepatic ABCG8 contents. 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. The asterisk indicates a statistically significant difference ($p < 0.05$) when compared with control. + Indicates a statistically difference ($p<0.1$) when compared with control.
Appendix B

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

Junga Lee, Richard C. Scheri, Yuan Zhang, 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 neurotoxic or carcinogenic. Pretreatment of mice or rats with CD altered tissue distribution of a subsequent dose of $[^{14}\text{C}]{\text{CD}}$ or $[^{14}\text{C}]{\text{ch}}$olesterol (CH). Nuclear receptors regulate expression of genes important in the homeostasis of CH and other lipids. In this study, we report that CD suppresses \textit{in vitro} reporter systems for human liver X receptor (LXRs) and activates those for human farnesoid X receptor (FXR), pregnane X receptor (PXR) and estrogen receptor $\alpha$ (ER$\alpha$) in a concentration-dependent manner (0-50 $\mu$M). Consistent with human PXR activation \textit{in vitro}, three days after a single dose of CD (15 mg/kg) hepatic microsomal CYP3A11 protein increases in C57BL/6 mice. CD decreases hepatic CH 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 significant reduction in non-high density lipoprotein CH but not apolipoprotein B-48/100 (apoB-48/100) in plasma from CD treated mice after a 4 hr fast. At 14 days after 15 mg CD/kg apoA-I and apoB-100 proteins but not CYP3A11 protein in hepatic microsomes are similar to controls. This work indicates altered CH homeostasis is a mode of OC insecticide action of relevance after a single dose. This at least partially explains altered CH tissue distribution in CD-pretreated mice.

Keywords: apolipoproteins, CH, pregnane X receptor, estrogen receptor alpha, liver x receptors, organochlorine insecticides, chlordecone.
INTRODUCTION

Banned organochlorine (OC) insecticides are widespread persistent organic pollutants that bioaccumulate in wildlife and humans. While OC insecticides are structurally diverse, they share a number of modes of toxic action. 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 doses of CD and dieldrin decrease plasma triglycerides and total CH in rats 21 and 60 days after administration, respectively (Ishikawa et al. 1978). Carpenter et al. (1996) report dose-dependent loss of lipid droplets in hepatocytes, but not Ito cells, of mice after 5-40 mg CD/kg. A single dose of 5 mg CD/kg also alters tissue distribution of exogenous $^{14}$C]CH (Carpenter and Curtis 1991; Lee et. al., 2008). 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 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 heterodimerization with
retinoid X receptor (RXR). LXRs respond to elevated sterol concentration and increase 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, respectively. PPARs play a major role as lipid metabolism regulators. They are also suggested as controllers 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). Recent work suggests a potential role of PXR in CH metabolism. Bile acids activate PXR, which reduces their production through repression of the Cyp7a1 gene (Staudinger et al. 2001a; 2001b; Xie et al. 2001). Most banned OC insecticides bind and activate PXR (Goodwin et al. 2002) but exhibit negligible affinity for the aryl hydrocarbon receptor (Poland and Knutson, 1982). Some, including p,o-DDT, CD, and dieldrin are also estrogenic in whole animals or 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 (apoA1) 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.

MATERIALS AND METHODS

Chemicals

Chlordecone (CD, 99 % purity) was purchased from Chem Service (West Chester, PA) and purity was verified by GC-EI/MS. LG268 and T0901317 were acquired from Ligand Pharmaceuticals (San Diego, CA) and Cayman Chemical (Chicago, IL), respectively. [1-14C] Lauric acid (55 mCi/mmol) was obtained from American Radiolabeled Chemicals. Inc. (St. Louis, MO). Other chemicals including 17β-estradiol (E2) 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°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 free access to water. Treatments were initiated after seven days of 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 CO2 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 °C, 95% O₂ and 5% CO₂ in DMEM containing 10% fetal bovine serum. In some experiments HEK293 cells were cotransfected with a plasmid encoding a fusion protein of the Gal4 DNA binding domain and a nuclear receptor ligand-binding domain along with a luciferase reporter plasmid-containing response element. In other experiments HEK293 cells were co-transfected with plasmids encoding full length RXRα with LXRα, LBRβ, or FXR along with a luciferase reporter containing the LXR response element (LXRE) or FXR response element (FXRE). CMX-β-gal was co-transfected as an internal control. Luciferase cotransfection assays were performed as described (Makishima et al. 2002). Nuclear receptor ligand binding domains were from human LXRα, LXRβ, FXR, RXR, ERα and ERβ, or murine PPARα, PPARδ, PPARγ. Cells were treated with vehicle (ethanol) alone or 1 to 50 μM CD and/or 1 nM T0901317 (LXR agonist), 100 nM LG268 (RXR agonist), and 1 nM E2 (ER agonist). Cell morphology by light microscopy and β-galactosidase activity assessed potential cytotoxicity. 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 °C. Plasma was stored at -80 °C. Liver was homogenized in buffer (0.01 M potassium phosphate, pH 7.5; 0.15 M KCl; 1.0 mM ethylenediaminetetraacetic acid (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 °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; 30% glycerol; 0.1 mM PMSF, 1.0 mM DTT (dithiothreitol), and 20 μM BHT). 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 h, proteins were electrophoretically transferred onto PVDF membranes utilizing a Mini trans-blot 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). ApoAI and ApoB antibodies were purchased from Biodesign (Saco, ME). 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. (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 h fast using enzymatic kits (Sigma Diagnostic, St. Louis, MO). Plasma HDL-CH concentrations 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 transfer 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).

**Hepatic CH content**

For extraction of lipids, ~100 mg homogenized liver was incubated in 1.5 N KOH in ethanol/water (2:1) at 70°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 % Triton-X-100 as assay samples. The CH concentration was determined using a CH/Cholesteryl ester Quantitation KitR (BioVision, Mt. View, CA). Hepatic esterified CH concentration was taken as the difference between the total CH and free CH.

**Statistical Methods**

Statistical analyses were conducted with StatGraphics software (StatPoint, Herndon, VA). Normality of data sets were assessed with standardized kurtosis, standardized skewness, and homogeneity of variance. Data that failed one or more tests for normality were log base 10 transformed. Transformed data were assessed for normality with the criteria specified above. Data were expressed as means ± SE. Comparisons among groups validated for normality 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% confidence level was
used as the criterion for significance. For outlier identification, Grubs' Test (assumes normality) was applied

RESULTS
Cell morphology and β-galactosidase activity were not significantly altered by CD (data not shown). A series of reporter gene cotransfection assays in HEK 293 cells characterized potential interactions of CD with nuclear receptors involved with lipid homeostasis. CD activated or inhibited transactivation by several nuclear receptors (Fig. 1). At 10 μM CD there was mild but statistically significant suppression of reporter construct alone and RXRα (about 30 %)(Fig. 1A). CD more substantially inhibited LXRα and strongly suppressed activation for LXRβ. CD modestly but significantly activated (about 1.3-fold) FXR and PPARα and strongly activated PXR about 3.5-fold over background (Fig. 1A, 1B). PPARδ and PPARγ were not affected by CD treatment (Fig. 1B).

Activation of human ERs by CD was also assessed. CD significantly activated a chimera consisting of DNA binding domain of Gal4 and the ligand binding domain of ERα slightly but significantly at 1 μM and almost 10-fold at 10 μM and 50 μM (Fig. 2A). There was no CD significant effect on human ERβ activity (Fig. 2A) but CD repressed E2-induced ERβ activation (40 %) (Fig. 2B). There was no significant difference in activation of ERα by E2 in the presence or absence of 10 μM CD (Fig. 2B). To further characterize CD (Fig. 3A) activation of nuclear receptors involved in lipid homeostasis not previously assessed with regard to OC insecticides, a transient transfection assay using a synthetic LXR or FXR responsive reporter plasmid was assessed. CD alone strongly suppressed activities
of the murine LXRα-RXR and LXRβ-RXR heterodimers in a concentration dependent manner (Fig. 3B and 3C). CD (10 μM) strongly inhibited LXRα-RXR activation by LG268 (RXR agonist) (Fig. 3B), but insignificantly inhibited the receptor activation by T0901317 (LXR agonist) (data not shown). Fifty μM CD moderately but significantly repressed activation of LXRβ-RXR activated by T0901317 (Fig. 3C). CD (50 μM) activated FXR approximately 50 % as effectively as the specific RXR agonist LG268 (Fig. 3D). In the presence of LG268, CD increased FXR transactivation cooperatively (Fig. 3D).

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 for decreased CYP7A1 protein content and its enzyme activity but it was not statistically significant (Fig. 4A, 4B). Interaction with PPARα was assessed by hepatic microsomal CYP4A1 protein immunoquantitation and lauric acid ω-1 hydroxylase activity. There was no evidence for activation of this receptor by the CD doses administered to mice (Fig. 4A, 4B). Finally, interaction with PXR was assessed by hepatic microsomal CYP3A11 protein immunoquantitation. CYP3A11 protein level was highly increased (4-fold) in livers from CD treated animals (Fig. 4A). Increased hepatic microsomal CYP3A11 protein after CD treatment indicated PXR activation.

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 (Fig. 5A). Plasma HDL-CH was unchanged but the
ratio of HDL-CH to plasma CH was mildly but significantly increased (Fig. 5A, 5B).

Immunoblot analysis of plasma revealed no significant difference in apoB-48/100 or apoA-I in CD treated mice (Fig. 5C). The effect of CD on apolipoprotein content was further characterized in liver subcellular fractions (lipoprotein-rich, cytosol and microsomes). Interestingly apoA-I significantly increased in the hepatic lipoprotein-rich and microsomal fractions from CD treated mice (1.5-fold, 1.8-fold, respectively) (Fig. 6A). There was no significant difference in hepatic microsomal apoB-100 between CD treated and control mice (Fig. 6B).

Hepatic total, free and esterified CH content was measured enzymatically. About 90 % of hepatic CH was in the unesterified form (Fig. 7). Hepatic total and free CH contents from CD-treated mice were not different from control mice (Fig.7). However, CD significantly decreased hepatic esterified CH concentration (about 40 % of control) (Fig. 7).

An ancillary experiment examined persistence of CD induced protein changes in liver. Hepatic microsomal CYP3A11 was significantly higher in 15 mg CD/kg treated mice than controls 14 days after injection (Fig. 8). Hepatic microsomal CYP7A1 and CYP 4A1 were not different from controls in the same mice (Fig. 8). Plasma and hepatic microsomal apoA-I were similar to controls 14 days after 15 mg CD/kg (Fig. 8). Hepatic microsomal apoB-100 was not different from controls in the same mice (Fig. 8).
DISCUSSION

Humans and wildlife are exposed to complex mixtures of xenobiotics including 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 a single dose of CD, an OC insecticide, alters tissue distribution of exogenous CH in mice and rats (Carpenter and Curtis 1991; Gilroy et al. 1994; Lee et al. 2008). 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.

Excessive CH 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). A large body of literature shows that nuclear receptors play important roles in the regulation of metabolism of CH and other lipids through a 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. Whole livers of C57BL/6 mice contained 84 μM CD 16 h after 5 mg/kg (Carpenter and Curtis 1989). Hepatic CD residues were persistent in livers with less than a
50% reduction from 3 to 14 days after exposure. Therefore, *in vitro* CD activation
or inhibition of nuclear receptors occurred at concentrations relevant to those
detected in livers of mice treated with doses examined herein. CD significantly
affected transactivation by a chimera consisting of the DNA binding domain of
Gal4 and the ligand-binding domains of LXRα and FXR (Fig. 1A). CD minimally
increased FXR activation and inhibited LXRα to different extents (Fig. 2B, 2C, 2D,
Fig. 1A). CD (50 μM) minimally increased FXR activation (Fig. 1A). Inhibition of
LXRβ was about twice as marked as that of LXRα. Additional *in vitro* experiments
and *in vivo* work further assessed CD interaction with LXRα and FXR (detailed
below).

PPARα, was slightly but significantly activated by 10 μM CD (Fig 1B). PPARδ
and PPARγ were not activated or inhibited by CD treatment (Fig. 1B). The CYP4A
proteins were generally recognized as up-regulated by PPARα agonists (Hunt et.
al., 2000). CD altered neither the CYP4A protein nor lauric acid ω-1 hydroxylase
activity (Fig. 4). Therefore, physiological relevance of PPAR contributions to CD
interaction with lipid homeostasis in mice seemed unlikely.

PXR was identified as a key regulator of CYP3A expression since it was activated
by diverse compounds that induced CYP3A expression and bound xenobiotic
response elements in CYP3A promoters (Xie *et al.* 2000). Because of evolutionary
divergence of the PXR ligand-binding domain in humans and mice, striking
species-specific differences were evident between two species (Kliewer 2003; Xie
*et al.* 2000). The human specific CYP3A inducer, rifampicin, had little effect on
CYP3A gene expression in mice, as did the mouse specific CYP3A inducer, PCN,
in human hepatocytes (Bachmann et al. 2004). Here CD not only transactivated human PXR in an *in vitro* assay (Fig. 1B) but also increased hepatic microsomal CYP3A11 protein in hepatic microsomes from mice (Fig. 4A). The data indicated that CD activated PXR in both species. Recent results suggested that PXR strongly induced the CYP3A4 gene by inhibiting small heterodimer partner (SHP) gene transcription in human livers (Li and Chiang 2006).

Activation of ERα was required for estrogen-mediated protection against vascular injury (Pare et al. 2002). CD was an ERα but not an ERβ agonist in the micromolar range (Fig. 2A). CD activated human ERα cooperatively with E2, although the activity was 10,000 times lower than E2 (Fig. 2B). 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. 2B). A role for ERα in CD altered CH homeostasis was of potential importance since estrogens increased apoB expression (Srivastava et al. 1993; 1997). Plasma non-HDL CH was elevated in CD treated compared to control mice that received 5 ml corn oil plus 10 mg CH/kg 4 hr before sampling (Lee et al. 2008). The reverse was observed in CD treated mice fasted 4 hr before sampling (Fig. 5A). CD depleted lipid droplets and increased putative VLDL particles in mouse liver (Carpenter et al. 1996). Perhaps low hepatic lipid masked CD-stimulated non-HDL secretory capacity in fasted mice and this was reversed when exogenous lipid was provided by ip corn oil. Increased clearance of apoB containing lipoproteins and/or reduced VLDL/LDL-CH secretion potentially contributed to the decreased plasma non-HDL-CH in CD treated fasted mice. E2 treatment increased low density lipoprotein (LDL) receptor protein in livers of rats but not mice. (Srivastava et al. 1993). Activation of LXRs induced hepatic LDL-receptor expression (Masson et al.
2004). Since LXRα activation was inhibited by CD *in vitro* (Fig. 1A, 3B) and LDL receptors were not up-regulated by E2 in mice, reduced VLDL/LDL-CH production was the most plausible explanation for decreased plasma non-HDL-CH concentration in CD treated fasted mice.

Presence or absence of exogenous lipid was a key experimental variable since plasma non-HDL-CH decreased in fasted CD-treated mice compared to fasted controls (Fig. 5A). 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 ubiquitin-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 CH ester, but 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). CD decreased hepatic CH ester, not total or free CH (Fig. 8) but there was no difference in apoB content in the liver microsomal fraction from CD treated animals. Thus, insufficient lipid perhaps increased apoB degradation by ubiquitin-mediated proteasomal degradation and reduced VLDL secretion.

Co-expression of FXR with exogenous RXR resulted in a significant activation enhancement (about 5-fold over background) (Fig. 3D). This finding indicated the requirement of RXR (heterodimer partner) for CD-induced FXR activation. These observations demonstrated that the activation or inhibition required the ligand
binding domain of these receptors. CH 7α-hydroxylase (CYP7A) was the first and a rate limiting enzyme in bile acid synthesis, the major CH elimination pathway (Russell 1999). LXRα and FXR regulated the expression of CH 7α-hydroxylase in opposite ways (Makishima 2003). Therefore, bile acid synthesis was regulated by feed forward and feedback mechanisms. Although CD activated FXR and inhibited LXRα activation in vitro, there was no statistically significant reduction of hepatic CYP7A1 and its enzyme activity by CD treatment in C57BL/6 mice (Fig. 4A, 4B). LXRα induced CYP7A1 by binding to the 5’-flanking region and increased expression of mouse and rat 7α-hydroxylase. FXR repressed the Cyp7a1 gene indirectly through the induction of the transcriptional repressor small heterodimer partner (SHP) (Staudinger et al. 2001a; 2001b). Hepatic content of other proteins in CD treated mice indicated little role for LXRs or FXR in CD-altered CH homeostasis. ATP binding cassette (ABC) G5 and scavenger receptor B1 (SRBI) genes were positively regulated by LXRs (Kalaany and Mangelsdorf, 2006), but hepatic contents of these proteins were not altered by CD (Lee et al., 2008). Activation of FXR negatively regulated expression of apoA1 and apoB genes (Kalaany and Mangelsdorf, 2006). There was no reduction in hepatic microsomal apoB 100 and significantly increased apoA-I in that fraction for CD treated mice (Fig. 6).

LXRβ knockout mice eliminated excess CH as effectively as wild type (Alberti et al. 2001). However, recent studies demonstrated that the LXRβ isoform also contributed to CH homeostasis through activation of ABCAI in macrophages, liver, and intestine (Quinet et al. 2006; Repa et al. 2000). ABCAI was classified as a member of a large family of ABC transporters (Langmann et al. 1999). The
ABCAI transporter increased efflux of intracellular CH to lipid-poor apoA-I to form preβ−HDL or nascent HDL (Wang et al. 2000). While results of the reporter construct assays (Fig. 2A) suggested potential for CD inhibition of LXRβ signaling and disrupted ABCAI activation, there was no reduction in plasma HDL-CH in CD treated mice (Fig. 5A).

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. SRBI, 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 activation 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). While CD was a mixed agonist for human PXR and ERα, HDL-CH and plasma apoA-I contents were not changed in fasted mice (Fig. 5A, 5C). Instead, we observed that CD increased apoA-I content in hepatic lipoprotein-rich and microsomal fractions (Fig. 6A). Gilroy et al. (1994) reported circumstantial evidence that CD stimulated hepatic HDL secretion. CD was strongly associated with HDL rather than other lipoproteins (Guzelian 1982). In situ rat livers were perfused with HDL complexed [14C]CD for one hour, which was then replaced with fresh solution. Efflux of [14C]CD into the perfusate from livers of CD pretreated rats about doubled over one hr. compared to controls. Plasma HDL-to-total [14C]CH ratio increased in CD treated compared to control mice that received
5ml corn oil/kg 4 h prior to sampling (Lee et al., 2008). Therefore, lipid availability was likely a key factor in secretion of apoA-I containing lipoprotein by liver. Since HDL-CH was widely considered as the good CH that provided some protection from heart disease, the potential role of PXR agonists in raising HDL-CH was an important area of current interest. However not all HDL-subclasses were equally protective. Large HDL2b was believed more important for protection from coronary heart disease in clinical observational studies (Pascot et al. 2001). 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). Clearance of $[^{14}C]$CH complexed with HDL from perfusate was reduced in situ liver preparations of CD-treated rats (Gilroy et al., 1994). 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 less than 50% from 3 to 14 days after intraperitoneal 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. 8). Hepatic content of apoA-I and apoB were not elevated at that time (Fig. 8). Perhaps differential adaptation of signaling pathways contributed to this phenomenon.

Even though the United States bans most uses of persistent OC pesticides, they continue to occur in tissues of humans and wildlife. Trophic transfer through food webs that provide dietary fish, meat and dairy products is the principal route of
environmental exposure to these agents. Residues of OC insecticides occur in
tissues of human and wildlife as complex mixtures. In human fat from the United
States, the sum of OC insecticides averages in the nM range (Lordo et al 1996).
Concentrations for wildlife that are top predators are higher. For example, fat of
grizzly bears that feed heavily on Pacific salmon contain 25-fold higher maximum
concentrations (Christensen, et al, 2005) than humans. Since many OC insecticides
or their metabolites are PXR (Goodwin et al, 2002) and ER agonists (Charles et al,
2002; Donohoe and Curtis, 1996) their potential interactions with endocrine
regulation deserves attention (Rajapakse et al 2002). Perturbation of CH
homeostasis by a low dose of CD may represent an environmentally relevant mode
of endocrine disruption. Therefore, it is necessary to continue studies to explain
altered CH and lipid homeostasis by banned OC insecticides. In summary: (1) CD
was a mixed agonist for human PXR and ERα and an effective antagonist for
LXRβ. (2) CD probably modulated lipoprotein metabolism by multiple interactions
with nuclear receptors. (3) This work indicated altered CH homeostasis and
lipoproteins were a mode of OC insecticide action of potential environmental
relevance.
Acknowledgements:

This work was supported by the Oregon Agricultural Experiment Station (ORE00871) and grant number T32 ES007060 from the National Institutes of Health.
References


with hepatic estrogen binding sites. *Aquat Toxicol* 36, 31-52


Srivastava, R. A., Srivastava, N., Averna, M., Lin, R. C., Korach, K. S., Lubahn,


Figure 1. Chlordecone activates Pregnane X Receptor (human homolog) 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 2. 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. Chlordecone inhibits LXRα 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 4. In vivo evidence for chlordecone activation of 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 microsomes. 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).
Figure 5. Chlordecone reduces non-HDL plasma CH 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 HDLCH 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 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 ($p < 0.1$).
Figure 7. Chlordecone reduces hepatic CH 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 8. 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, CYP7A1, CYP4A1 or CYP3A11 antibodies.