Chlordecone Increased Subcellular Distribution of Scavenger Receptor Class B Type II to Murine Hepatic Microsomes without Altering Cytosolic Cholesterol Binding Proteins

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ABSTRACT

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 occurred without changes in hepatic ATP-binding cassette transporter G8 (ABCG8) of the bile canaliculus or scavenger receptor class B type I (SR-BI) of the sinusoidal surface. A variety of tissues express scavenger receptor class B type II (SR-BII) and this protein was identified as a splice variant from the SR-BI gene. Although the function of SR-BII has not been elucidated it may play a role in CH homeostasis and trafficking distinctly different than SR-BI. Western blotting demonstrated that a single dose of CD promoted subcellular distribution of SR-BII to murine hepatic microsomes about 2.2-fold when compared to controls without effect on liver crude membrane SR-BII content. This was consistent with increased vesicular CH trafficking. Relative quantification of hepatic cytosolic proteins in a fraction that sequestered [^{14}C]CH by mass spectrometry (MS) indicated no role for cytosolic CH binding proteins in CD altered CH homeostasis. Western blotting verified no effect of CD on liver fatty acid binding protein (L-FABP) in cytosol. MS detected a statistically significant increase in myosin-9, which was also consistent with increased vesicular trafficking.

INTRODUCTION

Manufacture of the persistent OC pesticide, CD, was banned in the United States in 1977. Literature reviews documented CD was highly hydrophobic, generally extremely resistant to biodegradation and recalcitrant to photodegradation in the atmosphere (ATSDR 1995). The major route of exposure for CD and OC pesticides in general was

trophic transfer through the food web with maximal accumulation in top predators (Christensen et al., 2005). OC pesticides (e.g., CD, dichlorodiphenyltrichloroethane (DDT) and dieldrin) differ from planar polychlorinated biphenyls, the chlorinated furans and dioxins in that they exhibited only slight affinity for the aryl hydrocarbon receptor (Poland and Knutson, 1982). CD exhibited approximately equal potency in activation of the pregnane X receptor (PXR) and estrogen receptor α ($ER_α$). 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 weak agonist for the farnesoid X receptor (FXR) and peroxisome proliferator activated receptor alpha ($PPAR_α$). CD was a strong agonist for the human homologs of PXR and $ER_α$. It was a weak antagonist for liver X receptor α ($LXR_α$), a strong antagonist for $LXR_β$, and inhibited the binding of β-estradiol (E2) to $ER_β$, without agonist activity on $ER_β$ itself. *In vivo,* CD pretreatment was accessed by analyzing hepatic microsomal cytochrome P450 (CYP) protein content and enzyme activities associated with these CYPs. CD induced CYP3A11 (PXR agonist) with little effect on CYP7A1 (LXR/FXR) or CYP4A1 ($PPAR_α$) (Lee el al., 2008b). Since most OC pesticides were PXR agonists and CD was a demonstrated $ER_α$ agonist these results were not surprising (Guzelian, 1982; Coumoul et al., 2002).

CD was unusual in that a large body of toxicological data was available for humans after a well publicized poisoning in chemical workers that occurred in the mid-1970s (Cannon et al., 1978; Guzelian, 1982) Like most OC pesticides, neurotoxicity was a prominent mode of action and tremors were a common symptom in the exposed human population

(Cannon et al., 1978). CD affected ion channels and inhibited Na^+/K^+ ATPases in a variety of tissues (Narahashi et al., 1998; Desaiah, 1982). It was also a liver carcinogen in both rats and mice (Reuber, 1978). CD altered 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). Pretreatment of mice with single doses (5 mg/kg) of CD altered the tissue distribution of ^{14}[C]CD or ^{14}[C]CH delivered in a corn oil bolus (Carpenter and Curtis, 1991; Lee et al., 2008a). Additionally, these single doses of CD altered lipoprotein metabolism in fasted mice (Lee et al., 2008b). 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 in fasted mice (Lee et al., 2008b).The same authors showed CD pretreatment increased the biliary excretion of exogenous ^{14}[C]CH 4 h after administration without effect on hepatic ABCG8 or SR-BI proteins by immunoquantitation (Lee et al., 2008a).

SR-BI mediated selective lipid uptake (SLU) from HDL and reducing its expression increased plasma HDL-CH and decreased biliary CH in rodents (Rhainds and Brissette, 2004; Rigotti et al., 2003). SR-BII was an alternative splice variant of the SR-BI gene that also mediated SLU from HDL but is expressed at much lower levels in liver (Webb et al., 1998). The role it played in lipid homeostasis was not clear.

One possible explanation for the altered lipid homeostasis after CD exposure was alterations in 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). The specifics of these mechanisms are unknown and the non-vesicular transport mechanisms are especially problematic. Candidates for soluble sterol transporters have included, but were not limited to, fatty acid binding proteins (FABPs), caveolins, sterol carrier protein 2 (SCP-2), oxysterol binding proteins (OSBPs) and OSBP-related proteins (ORPs), Niemann Pick type C (NPC) proteins, the steroidogenic acute regulatory protein (StAR) and the StAR related lipid transfer (START) proteins (Ikonen, 2008; Prinz, 2007). A proteomic approach was utilized in our laboratory because of the great potential of that technique for quickly identifying and characterizing relative changes in multiple hepatic cytosolic proteins.

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 CD/kg) CD, or high dose (15 mg CD/kg). They were individually housed in a temperature (22±1ºC) 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. After seven days of acclimatization 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 CD/kg or 15 mg CD/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 (Brinkmann Instruments, Westbury CT) in ice-cold 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 min. 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 min to eliminate mitochondria. This supernatant was centrifuged at 100,000 g for 90 min 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 (SanAntonio, 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 SR-BII and Na^+/K^+ATPase. Microsomes were blotted with SR-BII and CYP4A. 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

Scheri et al., (2008) enriched [^{14}C]CH binding proteins from murine hepatic cytosol by streptomycin precipitation. Hepatic cytosolic proteins from mice that received no [14C]CH were precipitated with streptomycin. These precipitates were resuspended in 8M urea, 10mM potassium phosphate, pH 7.4 and dialyzed overnight at 4ºC against 1M urea, 10mM potassium phosphate. 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. 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 two instruments (details below) 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 was reported 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 was reported 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 was expressed as the mean ± the standard error (SE). Following validation of normality, comparisons between two groups were 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 demonstrated stimulated biliary excretion of exogenous [^{14}C]CH associated with CD pretreatment was not associated with changes in ABCG8 or SR-BI proteins (Lee et al., 2008a). One possible explanation for the altered CH homeostasis observed with CD pretreatment was an increased concentration of soluble hepatic sterol binding protein(s). MS coupled with iTRAQ methodology for relative quantification addressed this issue. Mass spectrometry was limited by dynamic range and the cytosolic proteome was complex. Enrichment of the cytosolic fraction that sequestered [^{14}C]CH was of interest to make the MS more tenable (Scheri et al., 2008).

Approximately 80 proteins were detected with mass spectrometry under the conditions described in the Materials and Methods section (Scheri et al., 2008). Four proteins exhibited mean iTRAQ protein ratios significantly different (p<0.05) than one (Table 1) while the iTRAQ protein ratio for FABP did not change.

CD pretreatment had no effect on cytosolic L-FABP protein content (normalized to β-actin) (Fig. 1).

Although the function of SR-BII was 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). Therefore, the effect of CD pretreatment on SR-BII was analyzed. There was no effect of CD pretreatment on the protein levels or activity of hepatic microsomal CYP4A1, which supported that CD negligibly activated nuclear receptor $PPAR_{\alpha}$ (Lee el al., 2008b). We therefore normalized the microsomal western SR-BII intensities to that of CYP4A (Fig. 2). Hepatic microsomal SR-BII protein content increased up to 2.2-fold ($p<0.05$) after CD pretreatment.

SR-BII was detected on the plasma membrane and underwent rapid endocytosis (Eckhardt et al., 2004). Western blots for SR-BII were performed on hepatic crude membrane fractions. Originally, normalization 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 (Figs. 3 and 4) in hepatic crude membrane preparations. There was no significant effect of CD pretreatment on SR-BII protein content but there was a statistically significant ($p<0.05$) increase in Na^+/K^+ATPase.

DISCUSSION

Streptomycin precipitation of hepatic cytosol from male C57BL/6 mice killed 16 hr after ip administration of [^{14}C]CH/kg in corn oil enriched the label per unit protein approximately 18-fold (when compared with supernatant) and left most of the cytosolic

protein (>85%) in the supernatant (Scheri et al., 2008). Approximately 80 proteins were detected in the streptomycin enriched fraction. The iTRAQ technique utilized peak ratios generated from the CD pretreated samples and controls. Ratios greater or less than one represent treatment dependent increased or decreased protein concentration respectively. Clearly, CD pretreatment had no effect on cytosolic liver FABP (Fig. 1) and similar results were obtained for the streptomycin enriched fraction (Table 1). Statistically significant differences from one were observed for four proteins. Although both SCP-2 and liver FABP were detected, SCP-2 did not meet the minimum criteria for protein identification. α-Enolase, PRX-1, and SBP-1 protein decreased while myosin-9 protein increased. Myosin-9 and SBP-1 played different roles in intracellular vesicular transport (van den Boom et al, 2007; Porat et al, 2000). Since there was no effect on β-actin after CD (Fig. 1), a generalized effect on the cytoskeleton seemed unlikely. While PRX-1 scavenged peroxides and other reactive oxygen species, the α-enolase gene encoded both a glycolytic enzyme and a transcription factor, the c-MYC binding protein (MBP-1) (Egler et al., 2005; Subramanian and Miller, 2000). It is interesting to note that both of these 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 bound the c-MYC promoter and down regulated c-Myc transcription, PRX-1 interacted 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. However, a caveat must be added. Although the observed differences were statistically significant,

the biological significance of these relatively small magnitudes, particularly using the iTRAQ technique, is questionable at best.

Multiple hepatic plasma membrane proteins mediate CH exchange between the liver and blood or bile (Ikonen, 2008; Prinz, 2007). SR-BI transports CH from HDL into liver and the ABCG5/ABCG8 heterodimer effluxes CH into bile. Since no change in either SR-BI or ABCG8 proteins in crude hepatic membrane fractions occurred after CD pretreatment (Lee et al., 2008a), a potential underlying mechanism for the CD stimulated biliary excretion of [^{14}C]CH was increased trafficking via a SR-BII dependent pathway. SR-BII mediated SLU from HDL but at a lower efficiency than SR-BI (Webb et al., 1998). However, SR-BII effluxes about twice as much free CH as SR-BI (Mulcahy et al., 2004). The subcellular distribution and internalization of these receptors also differs. In primary hepatocytes, SR-BI was predominantly located on the plasma membrane although a distinct pool appeared to be in late endosomes and lysosomes (Ahras et al., 2008; Eckhardt et al., 2004). SR-BI underwent endocytosis at a much lower rate than SR-BII in CHO cells (Eckhardt et al., 2004). SLU with SR-BI does not require endocytosis (Nieland et al., 2005; Wustner et al., 2003). Lipids may be transported to different pools in different locations within the cell by endocytosis, and perhaps SR-BII endocytosis played a role in altering the CH content of the endocytotic compartment (Eckhardt et al., 2006). In contrast with SR-BI, SR-BII is located primarily intracellularly and rapidly endocytosed in transferrin containing vesicles in CHO cells (Eckhardt et al., 2004). Hepatic microsomal SR-BII protein increased about 2.2-fold in CD-treated mice compared to controls (Fig. 2). The total amount of SR-BII in a crude membrane fraction

that included plasma membranes was not changed after CD (Fig. 3). Perhaps the increased subcellular distribution to microsomes represented an increased capacity for biliary excretion of exogenous CH in hepatocytes via a vesicular pathway. The earlier experiments with administration of exogenous [^{14}C]CH (Lee et al., 2008a) permitted detection of stimulation of this pathway.

Wustner et al., (2003) proposed involvement of an endocytotic/retroendocytotic pathway in biliary CH excretion. Based on a number of observations (by themselves as well as others) Lopez and McLean (2005) proposed that increases in SR-BII potentially stimulated biliary CH excretion. Our earlier results detected no change in total biliary CH with CD pretreatment in 4 h fasted mice (Lee et al., 2008a). However, there was probably a question of detection sensitivity for changes in total bile CH after CD in fasted mice without administration of exogenous [^{14}C]CH. There was preferential excretion of free CH from HDL in bile, as opposed to *de novo* free CH (Robins and Fasulo, 1997). CD pretreatment stimulated biliary excretion of exogenous [^{14}C]CH (2 μCi/animal, 10 mg CH/kg) and this resulted in bile concentrations of approximately 120 nmol [^{14}C]CH equivalents/ml (Lee et al., 2008a). Average bile CH concentrations in wild type mice range from about 2-5 μmol/ml (Wang et al., 2006; Yu et al., 2005; Tang et al., 2006), so that even with a 2.2-fold increase in SR-BII, a nM change in CH in bile was difficult to detect. It is possible that redistribution of SR-BII from apical/plasma membrane to vesicles occurred upon the administration of an exogenous lipid bolus. Increased turnover of plasma membrane SR-BII is also a possibility. Since endocytosis of HDL appeared

unimportant in SLU, perhaps increased turnover of plasma/apical membrane SR-BII played a role stimulating excretion of [^{14}C]CH into bile.

Na^+/K^+ ATPase protein content increased with CD pretreatment in hepatic crude membrane fractions (Fig. 4). Neurotoxicity was a prominent mode of action for OC pesticides and several studies reported CD inhibited Na^+/K^+ ATPase (reviewed in Desaiah, 1982). The increase observed in this protein was perhaps a compensatory response of this inhibited transport capacity per unit protein.

In summary, CD pretreatment increased the SR-BII protein content of hepatic microsomes but not crude membrane fractions. Increases in myosin-9 were consistent with an increased capacity for vesicular transport. These observations supported a model where SR-BII contributed to CD stimulated biliary excretion of exogenous CH via a vesicular pathway. Decreased SBP-1, α-enolase and PRX-1 in a CH binding protein enriched fraction of cytosol were observed, but the biological significance of such small differences were unclear. Cytosolic CH binding proteins appeared to play no role in CD induced alterations in CH homeostasis under the conditions utilized. Additional MS experiments are needed to adequately address this issue. Overall, these results provided new insights into and partially explain how CD pretreatment induced alterations in CH and lipoprotein homeostasis.

LEGENDS FOR FIGURES

Figure 1. CD pretreatment had no effect on cytosolic L-FABP.

Animals were treated with corn oil or 15 mg CD/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 β-actin proteins, (μg protein/lane was separated by SDS-PAGE and blotted with antibodies against L-FABP and β-actin**);** (B) L-FABP data normalized to β-actin, (error bars are ± SEM, asterisks indicate different than control ($p<0.05$) after a Student's t-test).

Figure 2. Increased hepatic microsomal SR-BII protein by CD pretreatment.

Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg 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 3. No significant effect of CD pretreatment has on SR-BII protein content in the hepatic crude membrane fraction. Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/kg 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 4. Increased hepatic crude membrane fraction Na^+/K^+ATPase protein content after CD pretreatment. Animals were treated with corn oil, 5 mg CD/kg or 15 mg CD/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).

REFERENCES

Ahras, M., Naing, T. and McPherson, R., 2008, Scavenger receptor-BI localizes to a late endosomal compartment. *J. Lipid Res*. In Press.

ATSDR, Agency for Toxic Substances and Disease Registry, Toxicological Profile for Mirex and Chlordecone, (Final Report), Atlanta, GA:ATSDR, Public Health Service, U.S. Department of Health and Human Services, 1995, NTIS Accession No. PB95-264354.

Cannon, C.B., Veazey, J.M.,Jr., Jackson, R.S., Burse, V.W., Hayes, C., Straub, W.E., Landrigan, P.J. and Liddle, J.A., 1978, Epidemic kepone poisoning in chemical workers. *Am. J. Epidemiol*. 107(6), 529-537.

Carpenter, H.M., and Curtis, L.R., 1989, A characterization of chlordecone pretreatment-altered pharmacokinetics in mice. *Drug. Met. and Dist*. 17, 131-138.

Carpenter, H.M., and Curtis, L.R., 1991, Low dose chlordecone pretreatment altered cholesterol disposition without induction of cytochrome P-450. *Drug. Met. and Dist*. 19, 673-678.

Christensen, J.B., Macduffee, M., Macdonald, R.W., Whitcar, M. and Ross, P.S., 2005, Persistent organic pollutants in British Columbia grizzly bears consequence of divergent diets. *Environ. Sci. Technol*. 39, 6952-6960.

Coumoul, X., Diry, M. and Barouki, R., 2002, PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochem. Pharmacol*. 64, 1513-1519.

Desaiah, D., 1982, Biochemical mechanisms of chlordecone toxicity: a review. *Neurotoxicology* 3, 103-110.

Eckhardt, E.R.M, Cai, L., Sun, S., Webb, N.R. and van der Westhuyzen, D.R., 2004, High density lipoprotein uptake by scavenger receptor SR-BII**.** *J. Biol. Chem*. 279, 14372-14381.

Eckhardt, E. R. M., Cai, L., Shetty, S., Zhao, Z., Szanto, A., Webb, N.R. and van der Westhuyzen, D.R., 2006, High density lipoprotein endocytosis by scavenger receptor SR-BII is clathrin-dependent and requires a carboxyl-terminal dileucine motif. *J. Biol. Chem*. 281, 4348-4353.

Egler, R.A., Fernandes, E., Rothermund, K., Sereika, S., de Sousa-Pinto, N., Jaruga, P., Dizdaroglu, M. and Prochownik, 2005, Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. *Oncogene* 24, 8038-8050.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A and Amati, B., 2003, Genomic targets of human c-Myc protein. *Genes Dev*. 17, 1115-1129.

Guzelian, P.S., 1982, Comparative toxicology of chlordecone (kepone) in humans and experimental animals. *Ann. Rev. Toxicol*. 22, 286-292.

Ikonen, E., 2008, Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol*. 9 125-138.

Insightful Corp, Version 8.0, Seattle, WA.

Ishikawa, T., McNeeley, S., Steiner, P.M., Glueck, C.K., Mellies, M., Gartside, P.S. and McMillin, C., 1978, Effects of chlorinated hydrocarbon on plasma alpha-lipoprotein in rats. *Metabolism* 27, 89-96.

Kim, Y.J., Ahn, J.Y, Liang, P., Ip, C., Zhang, Y. and Park, Y.M., 2007, Human prx1 gene is a target for Nrf2 and is upregulated by hypoxia/reoxygenation:implication to tumor biology. Cancer Res. 67, 546-554.

Lee, J., 2002, Alteration of cholesterol disposition by chlordecone is not explained by induction of Cyp7a or Cyb4a1. *M.S. Thesis,* Oregon State University.

Lee, J., Scheri, R.C. and Curtis, L.R., 2008a, Chlordecone altered hepatic disposition of [^{14}C]cholesterol and plasma cholesterol distribution but not SR-BII or ABCG8 proteins in livers of C57BL/6 mice. *Toxicol. Appl. Pharmacol*. 229, 265-272.

Lee, J., Scheri, R. C., Zhang, Y. and Curtis, L. R., 2008b, Chlordecone, a mixed pregnane X receptor (PXR) and estrogen receptor alpha (ER_{α}) agonist, alters cholesterol (CH) homeostasis and lipoprotein metabolism in C57BL/6 mice. *Toxicol. Appl. Pharmacol.* 233, 193-202.

Lopez, D. and McLean, M.P., 2006, Estrogen regulation of the scavenger receptor class B gene: Anti-atherogenic or steroidogenic, is there a priority? *Mol. Cell. Endocrinol*. 247, 22-33.

Matrix Science, Version 2.2, London, UK.

Mulcahy, J.V., Riddell, D.R. and Owen, J. S., 2004, Human scavenger receptor class B type II (SR-BII) and cellular cholesterol efflux. *Biochem. J*. 377, 741-747.

Narahashi, T., Ginsbury, K.S., Nagata, K., Song, J.H. and Tatebayashi, H., 1998, Ion channels as targets for insecticides. *Neurotoxicology* 19, 581-590.

Nieland, T.J.F., Ehrlich, M., Krieger, M. and Kirchhausen, T., 2005, Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI. *Biochim. Biophys. Acta.* 1734, 44-51.

Poland, A. and Knutson, J.C., 1982, 2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons:examination of the mechanism of toxicity. *Ann. Rev. Pharmacol. Toxicol*. 22, 517-554.

Porat, P., Sagiv, Y. and Elazar, Z., 2000, A 56-kDa Selenium-binding protein participates in intra-golgi protein transport**.** *J. Biol. Chem*.275, 14457-14465.

Prinz, W.A., 2007, Non-vesicular sterol transport in cells. *Prog. Lipid Res*. 46, 297-314.

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

Reuber, M.D., 1978, Carcinogenicity of Kepone. *J. Toxicol. Environ. Health* 4, 895-911.

Rhainds, D. and Brissette, L., 2004, The role of scavenger receptor class B type I (SR-BI) in lipid trafficking: Defining the rules for lipid traders. *Intl. J. Biochem. Cell Biol*. 36, 39-77.

Rigotti, A., Miettinen, H.E. and Krieger, M., 2003, The role of the high density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocrine Rev*. 24 357-387.

Robins, S.J. and Fasulo, J.M., 1997, High density lipoproteins, but no other lipoproteins, provide a vehicle for sterol transport to bile. *J. Clin. Invest*. 99, 380-384.

Roth, A., Looser, R., Kaufmann, M., Blaettler, S., Rencurel, F., Huang, W, Moore, D.D. and Meyer, Y.A., 2008, Regulatory crosstalk between drug metabolism and lipid homeostasis: CAR and PXR increase insg-1 expression. *Mol Pharmacol*. 2008 73, 1282-1289.

Scheri, R.C., Junga, L., Barofsky, D.F. and Curtis, L.R, A comparison of relative quantification with isobaric tags on a subset of the murine hepatic proteome using ESI QTOF and MALDI TOF/TOF. *Rapid Comm. Mass Spec*., 2008 Oct;22(20):3137-46.

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

Subramanian A, Miller D.M., 2000, Structural analysis of alpha-enolase; mapping the functional domains involved in down-regulation of the c-myc protooncogene. J Biol Chem. 275, 5958-65.

Tang, W., Ma, Y. and Yu, L., 2006, Plasma cholesterol is hyperesponsive to statin in ABCG5/ABCG8 transgenic mice. Hepatology 44. 1259-1266.

Tirona, R.G. and Kim, R.B., 2005, Nuclear receptors and drug disposition gene regulation. *J. Pharm. Sci*. 94, 1169,1186.

Van den Boom, F., Düssmann, H., Uhlenbrock, K., Abouhamed, M. and Bähler, M., 2007, The myosin IXb motor activity targets the myosin IXb RhoGAP domain as cargo to sites of actin polymerization. *Mol Biol Cell*. 18, 1507–1518.

Wang, J., Einarsson, C., Murphy, C., Parini, P., Bjorkhem, I., Gafvels, M. and Eggertsen, G., 2006, Studies on LXR and FXR mediated effects on cholesterol homeostasis in normal and cholic acid-depleted mice. *J. Lipid Res*. 47, 421-430.

Webb, N.R., Connell, P. M., Graf, G.A., Smart, E.J., de Villiers, W.J.S., de Beer, F.C. and van der Westhuyzen, D.R., 1998, SR-BII, an isoform of the scavenger receptor BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. *J. Biol. Chem*. 273, 15241-15248.

Wustner, D., Mondal, M., Huang, A. and Maxfield, F.R., 2003, Different transport routes for high density lipoprotein and its associated free sterol in polarized hepatic cells. *J. Lipid Res*. 45,427-436.

Yu, L., Gupta, S., Xu, F., Liverman, A.D., Moschetta, A., Mangelsdorf, D.F., Repa, J.J., Hobbs, H.H. and Cohen, J.C., 2005, Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. *J. Biol. Chem*. 280, 8742-8747.

Table 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. Mean iTRAQ ratios represent a relative quantification value when comparing protein expression between treated and control groups Each protein was detected in all four animal pairs. Confidence intervals (CI) were determined using the Student's T-test, $p<0.05$.

Protein	Mean iTRAQ Ratio (Treated/Control)	95% Confidence Interval	p
Alpha-enolase	0.81	0.78 - 0.84	0.000
Fatty acid-binding protein, liver	0.89	0.63 - 1.24	0.334
Myosin-9	1.35	1.03 -1.75	0.037
Peroxiredoxin-1	0.87	0.77 - 0.99	0.044
Selenium-binding protein 1	0.72	0.58 - 0.90	0.019

Figure 1

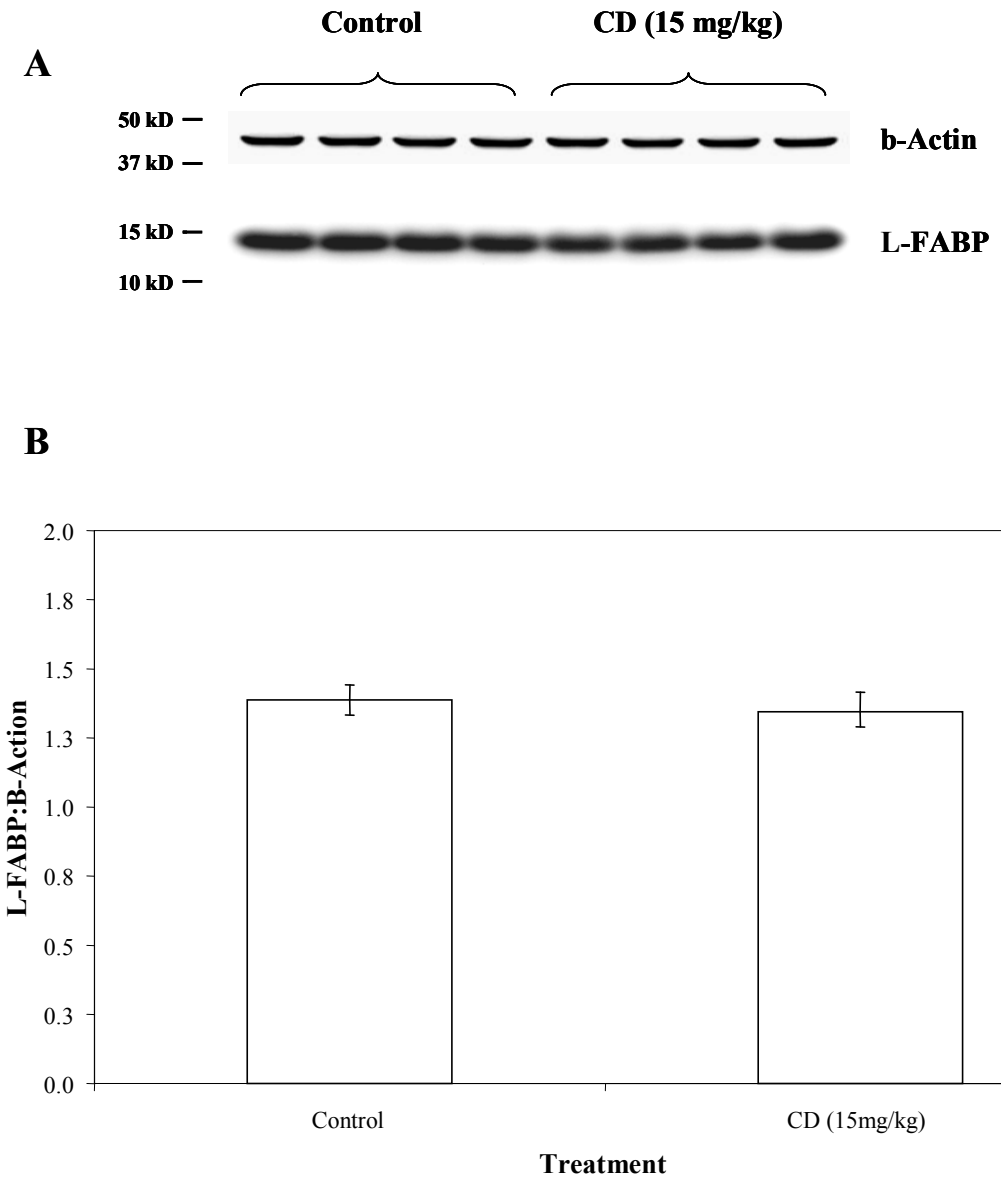

A
Control
CD (15 mg/kg)
50 kD
37 kD
15 kD
10 kD
b-Actin
L-FABP
B
L-FABP:B-Action
2.0
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
Control
CD (15mg/kg)
Treatment

Figure 2

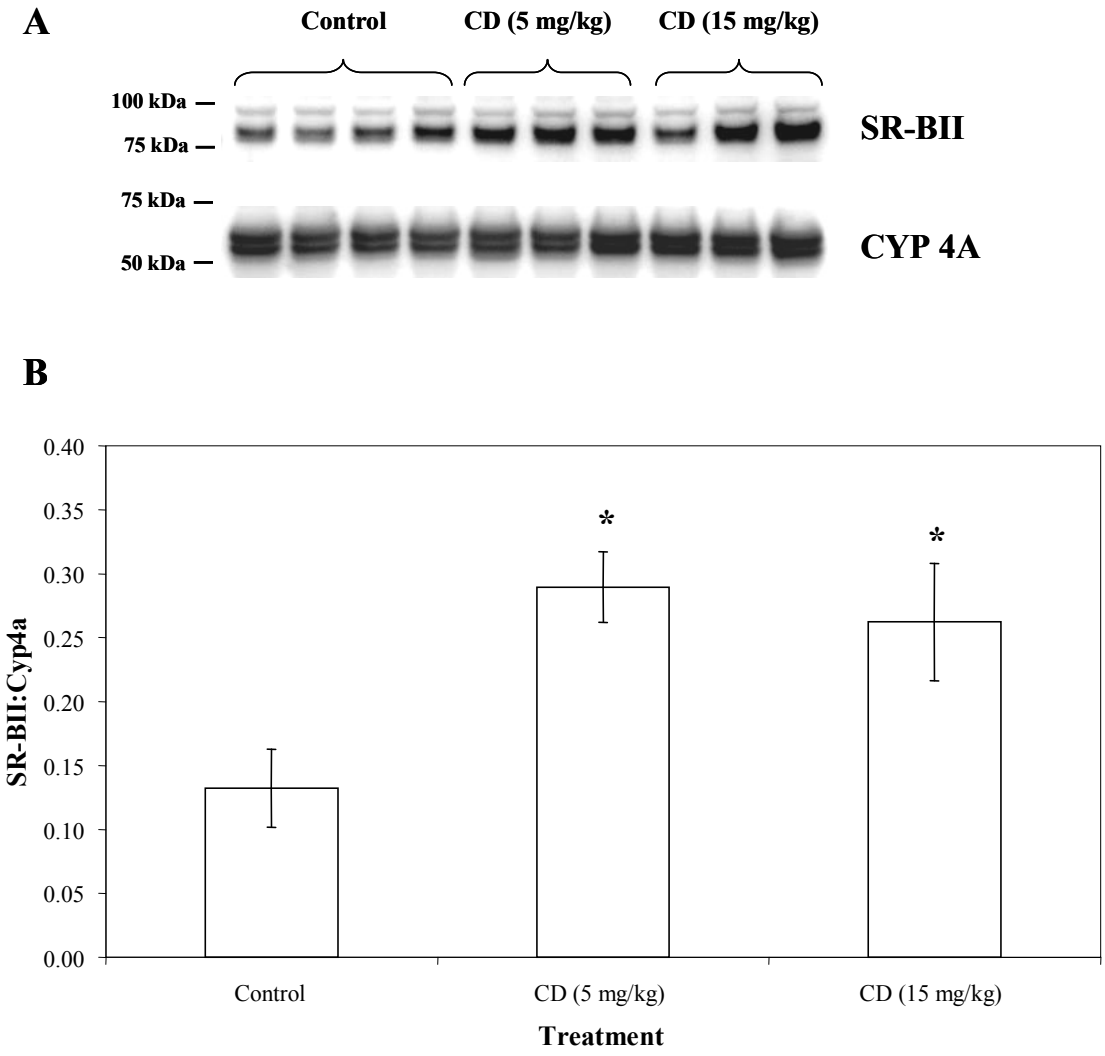

A
Control
CD (5 mg/kg)
CD (15 mg/kg)
100 kDa
75 kDa
75 kDa
50 kDa
SR-BII
CYP 4A
B
SR-BII:Cyp4a
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
Control
CD (5 mg/kg)
CD (15 mg/kg)
Treatment

Figure 3

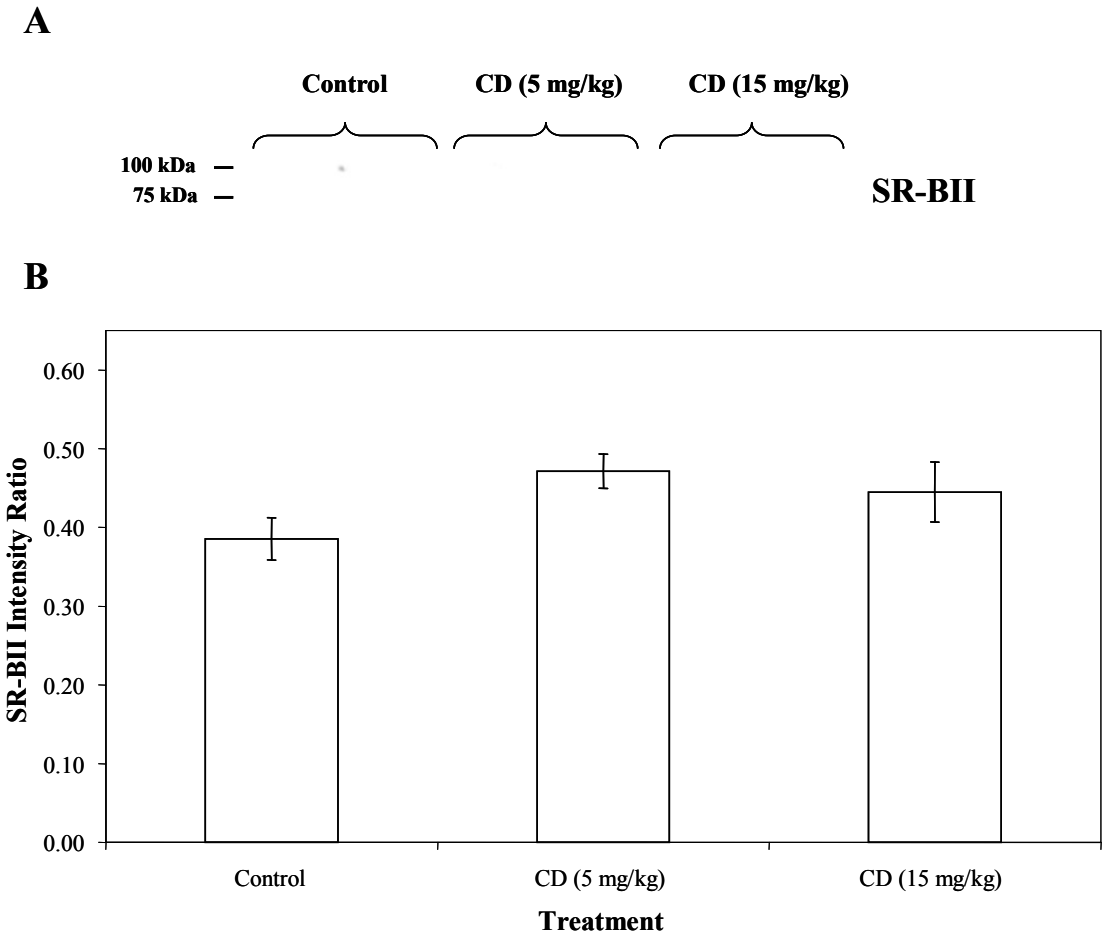
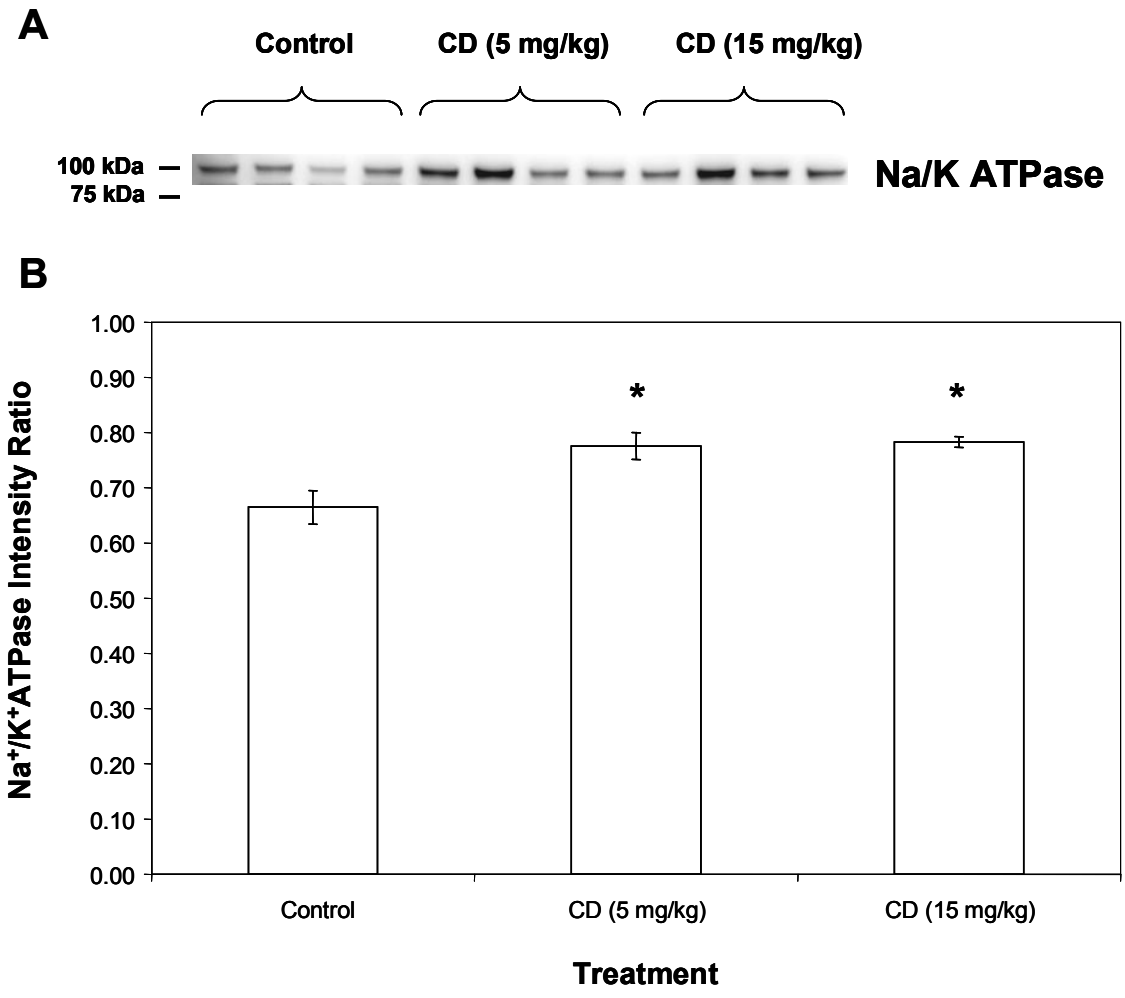

A
Control
CD (5 mg/kg)
CD (15 mg/kg)
100 kDa
75 kDa
SR-BII
B
SR-BII Intensity Ratio
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Control
CD (5 mg/kg)
CD (15 mg/kg)
Treatment

Figure 4


A
Control
CD (5 mg/kg)
CD (15 mg/kg)
100 kDa
75 kDa
Na/K ATPase
B
Na+/K+ATPase Intensity Ratio
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
*
Control
CD (5 mg/kg)
CD (15 mg/kg)
Treatment