An investigation into the mechanisms of the hypocholesterolemic effect of selenium in the Syrian hamster

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ABSTRACT

Supplementation of selenium (Se) has been shown to have important hypocholesterolemic properties in animal experiments and in some human trials; however, underlying mechanisms remain unclear. One possible mechanism may be via the liver X receptor (LXR) pathway that lowers intestinal cholesterol absorption and enhances cholesterol conversion into bile acids. The present thesis studied the effect of Se on LXR ligands (oxysterols) and mRNA abundance of LXR responsive genes, ATP-binding cassette proteins G5 and G8 (*abcg5*, *abcg8*), and Nieman-Pick C1-Like 1 protein (*npc1l1*) in hepatic and jejunal tissues in the Syrian hamster. The first study demonstrated that: (a) hepatic oxysterol concentrations are increased, and plasma cholesterol decreased in hamsters fed supplemental Se; (b) 27-hydroxycholesterol (27-OHC) concentrations are increased in HepG2 cells treated with Se at physiological concentrations in a dose response manner. The second study provided Syrian hamsters supplemental Se in a dose response manner to ascertain if Se: (a) increases hepatic oxysterols through upregulation of specific hepatic P450 enzyme activity and abundance; and (b) decreases plasma and hepatic lipids through LXR pathway upregulation of hepatic *abcg5* and *abcg8* and increased cholesterol secretion into bile. Se lowered plasma LDL-C concentrations and increased hepatic 27-OHC concentrations without effect on specific P450 enzymes, hepatic cholesterol concentrations or *hmgcr*. Se upregulated hepatic *ldlr* and *abcg8* without affecting *abcg5* or cholesterol and bile acid levels in gallbladder bile. The third study looked at the response of the jejunum to dose response Se supplementation in Syrian hamsters in terms of jejunal *abcg5*, *abcg8, ldlr, hmgcr* and *npc1l1*. Similar to liver, Se treatment was associated with increased *abcg8* without showing an effect on *abcg5*. Jejunal *npc1l1* was upregulated with Se treatment suggesting that Se might have diminished enterocyte cholesterol levels via its effect on increasing *abcg8*. As with gallbladder bile, Se did not increase fecal cholesterol concentrations or bile acids. In conclusion, although Se upregulates the hepatic concentrations of oxysterols and increases abundance of hepatic and jejunal *abcg8*, *ldlr* and jejunal *npc1l1*,

the hypocholesterolemic effect of Se does not appear to involve the LXR pathway.

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RESUME

La supplémentation en sélénium (Se) a fait preuve de propriétés importantes hypocholestérolémiques dans des études animaux et quelques études humain, cependant les mécanismes fondamentaux y demeurent vagues. Un mécanisme possible suggère l'activation de la voie du récepteur X du foie (LXR) qui est connu pour sa capacité de baisser l'absorption du cholestérol, d'accélérer sa conversion en acides biliaires. La présente thèse a étudier l'effet du Se sur l'expression des ligands (oxystérols) de LXR, et de l'expression de l'ARNm des gènes cibles de LXR ATP-binding cassette qui sont les protéines G5 et G8 (*abcg5*, *abcg8*) et de la protéine Nieman-Pick C1-Like (*npc1l1*) dans les tissus du foie et de l'intestin du hamster Syrian. La première étude a démontré que ; (a) les concentrations hépatiques des oxysterols sont élevé dans les hamster Syrian nourrit du Se et ce, parallèlement à une diminution au niveau des lipides plasmatiques ; et, (b) les concentrations de l'oxystérol 27-OHC sont élevé dans les cellules HepG2 avec le Se fournis a des concentrations physiologiques de façon dose-réponse. Dans la deuxième étude les hamsters Syrian ont été approvisionnés de façon dose-réponse afin de prouver si le Se : (a) augmente les oxystérols hépatiques par la régulation de l'activité et de l'expression des enzymes P450 spécifiques et s'il (b) diminue les lipides plasmatiques et les concentrations hépatiques du cholesterol par la voie du LXR et ce, par l'augmentation de la régulation des *abcg5* et des *abcg8* hépatiques a fin d'augmenter les taux de cholesterol dans le bile. Le traitement par le Se avait baissé le taux des LDL-C plasmatiques, augmenté les concentrations de 27-OHC hépatiques et n'avait aucun effet ni sur les enzymes P450 spécifiques ni sur les concentrations hépatiques du cholestérol ou *hmgcr*. Le Se avait montré une capacité d'augmenter la régulation des *abcg8* et *ldlr* hépatiques sans avoir aucun effet ni sur des *abcg5* ni sur les taux de cholestérol ou des acides biliares dans la vésicule biliaire. La troisième étude a abordé la réponse du jéjunum face à la supplémentation en Se de facon dose-réponse des *abcg5*, *abcg8*, *ldlr*, *hmgcr* et

de la *npc1l1*. Pareillement au foie, le traitement par le Se était associé à une augmentation des *abcg8* sans avoir aucun effet sur des *abcg5*. Le traitement par Se a augmenter la régulation des *npc1l1* jéjunal qui a suggéré que le Se était peut-être en train d'abaisser les taux de cholestérol dans l'entérocyte par l'entremise de l'effet du Se sur l'augmentation des *abcg8*. Semblable a l'éffet sur les acides biliares dans la vésicule biliaire, le traitement par le Se n'a pas engendré une augmentation au niveau des concentrations fécales du cholestérol ni au niveau des acides biliaires. En conclusion, bien que le Se augmente la concentration hépatique des oxystérols et augmente l'expression hépatique et jéjunal de *abcg8*, *ldlr* et jéjunal *npc1l1*, l'effet du Se sur les lipides plasmatiques ne semble pas inclure la voie du LXR.

PREFACE

This thesis examines the effect of the supplementation of Se on the liver X receptor (LXR) responsive genes ATP-binding cassette transporters G5 and G8 (*abcg5*, *abcg8*), and Nieman-Pick C1-Like 1 protein (*npc1l1*) with the intention of elucidating the mechanism(s) underlying Se's observed hypocholesterolemic effects. More specifically, the effect of supplementation of sodium selenate and selenite on plasma lipid metabolism, liver lipid metabolism, oxysterol levels and tissue messenger ribonucleic acid (mRNA) levels of cholesterol metabolizing enzymes was explored. The response of the liver and intestine, the two organs most involved in the homeostasis of plasma low density lipoprotein cholesterol (LDL-C), to Se supplementation was explored using two feeding trials conducted with the Golden Syrian hamster fed hypercholesterolemic diets.

The present thesis is submitted in the form of original manuscripts. In addition to the appropriate manuscripts, the thesis conforms to all other requirements of the McGill University "Guidelines for Thesis Preparation". Following the thesis table of contents and abstracts (English and French), Chapter 1 provides a general introduction to the thesis in terms of the thesis rationale(s), hypotheses and objectives. In Chapter 2, a comprehensive review of the literature (in addition to that covered in the introduction to each manuscript) applicable to all subject matters of the thesis is presented. Chapter 3 reports the results of two separate experiments. In the first, the influence of Se in addition to α-tocopherol (α-Toc) on hepatic level of oxysterols, lipid metabolism, and oxidative stress was investigated. In the subsequent experiment, the influence of Se on cellular concentrations of 27-hydroxycholesterol (27-OHC) was investigated in a dose related manner in HepG2 cell culture. Chapter 4 reports the results of an in vivo dose related Se supplementation dietary study in male Syrian hamsters on hepatic oxysterols, hepatic and jejunal mRNA of LXR responsive genes and key cholesterol metabolizing enzymes, the activity of hepatic oxysterol generating enzymes, biliary and fecal content of bile acids and cholesterol, and plasma and hepatic concentrations of cholesterol and lipids.

The progression of the thesis is ensured by a continuity of connecting statements providing logical bridges between each of the chapters. The thesis ends with a final conclusion and overall summary as well as a description of the limitations of the thesis and future work (Chapter 5) followed by a comprehensive reference list.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Original contribution to knowledge:

The novel results from the present thesis work have contributed to the knowledge of nutritional sciences by the following:

i) The first direct in vivo evidence that tissue concentrations of an enzymatically generated oxysterol LXR ligand can be related to alterations in cholesterol metabolism as hepatic 27-OHC content was correlated with concentrations of plasma TC and liver TG and hepatic abundance of *cyp7a1* mRNA.

ii) Se is related to metabolism of enzymatically generated oxysterols as enhanced tissue Se status via Se supplementation increases in vitro and in vivo hepatic concentrations of the enzymatically-generated oxysterol, 27-OHC.

iii) Higher hepatic antioxidant content in the form of Se and α-tocopherol induced by supplementation of these antioxidants is associated with increased liver concentrations of enzymatically-generated oxysterols including 24(S)-OHC, 25-OHC and 27-OHC, which suggests antioxidant protection of enzymatically generated oxysterols.

iv) The comprehensive examination of hepatic and jejunal abundance of *abcg5*, *abcg8*, *hmgcr* and *ldlr* showed that genes from these two major cholesterol metabolizing tissues exert similar responses to Se supplementation.

v) Following Se supplementation, there is a specific upregulation of mRNA abundance of the genes, *abcg8, npc1l1* and *ldlr* in the jejunum and *ldlr* and *abcg8* in the liver.

vi) Supplementation of Se has no effect on hepatic mRNA abundance of *cyp7a1* and *cyp27a1* and enzymatic activity of CYP7A1 and CYP27A1.

vii) There is no association in the Syrian hamster between the plasma cholesterol lowering effect of Se supplementation and biliary and fecal levels of bile acids and cholesterol.

viii) Liver cholesterol concentrations and *hmgcr* abundance are not related to the hypocholesterolemic effects of Se supplementation. Modulation of *ldlr* mRNA in Se-supplemented hamsters occurs in the absence of changes in hepatic cholesterol content.

2. Research manuscripts submitted or to be submitted to refereed journals or in preparation:

i) Poirier, J., Cockell, K., Ratnayake, W. M. N., Hidiroglou, N., Gagnon, C., Madere, R., Trick, K., and Kubow, S. Antioxidant Supplements Lower Oxidative Stress and Plasma Lipids in Butterfat-fed Hamsters (to be submitted).

ii) Poirier, J., Cockell, K., Ratnayake, W. M. N., Scoggan, K., Gagnon, C., Rocheleau, H., Gruber, H., Griffin, P., and Kubow, S. Selenium Mediated Plasma Cholesterol Lowering in Hamsters is not Mediated by LXR or Upregulation of Hepatic Hydroxylases (to be submitted).

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS

The present thesis involved the collaboration of Dr. K. Cockell, Nutrition Research Division, Food Directorate, Health Products and Food Branch, Health Canada, the candidate's co-supervisor, Dr. K. Scoggan, Nutrition Research Division, Food Directorate, Health Products and Food Branch, Health Canada and Dr. S. Kubow, the candidate's co-supervisor, School of Dietetics and Human Nutrition, McGill University, MacDonald Campus.

The ideas presented in the thesis resulted from regular meetings between the candidate and the candidate's co-supervisor, Dr. Stan Kubow. The candidate was responsible for the collection of all scientific articles relevant to all manuscripts, and the preparation of the respective tables and the writing of the text. Dr. Kubow provided regular editorial input and written commentaries on all manuscripts until final completion. In addition, Dr. K. Cockell, the candidate's co-supervisor, provided further feedback and written commentaries in the finalization of all work.

The feeding, care, appropriate maintenance and sacrificing of all animals used within the first study were executed by the candidate. The feeding, care, and appropriate maintenance of the animals used in the second study were executed by staff of the Animal Resources Division, Food Directorate of Health Canada.

The candidate performed all of the experimental analyses described within the thesis with the exception of the cell culture oxysterol analysis in the first experiment, and the analysis of *abcg5* mRNA abundance in the second experiment.

The candidate was responsible for the writing of all manuscripts described within the thesis. In regular consultation with Dr. Kubow, the candidate developed all of the relevant experimental designs involved in the animal studies. All pertinent calculations and statistical analysis performed on all collected data were carried out by the candidate.

Dr. Kevin Cockell and Dr. W. M. N. Ratayake provided the candidate with the instrumentation and facilities for the measurement of hepatic oxysterols, CYP7A1 and CYP27A1 activity, and biliary and fecal bile acids and cholesterol. Dr. K. Cockell edited the final drafts of all manuscripts.

Dr. Kylie Scoggan provided the candidate with the analytical method and training, instrumentation and facilities for the measurement of tissue levels of *β-actin*, *gapdh*, *abcg5*, *abcg8*, *npc1l1*, *ldlr*, *hmgcr*, *cyp7a1*, and *cyp27a1* mRNA abundance in Syrian hamster tissue.

Dr. Stan Kubow and Dr. Kevin Cockell, the candidate's co-supervisors, edited all of the manuscripts in the present thesis as well as the final draft of the thesis. Furthermore, Dr. Kubow provided regular meetings with the candidate to provide pertinent feedback on the various projects described within the present thesis and to assess the progress of the candidate's work.

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I would like to acknowledge my two co-supervisors Dr. Stan Kubow and Dr. Kevin Cockell who provided me with constant guidance, support and inspiration during my degree and without whom I could not have accomplished what I set out to do. Dr. Stan Kubow provided me with the needed financial assistance and financial support for all the laboratory work I carried out. He also taught me how to look at things, and how to ask the right questions. Dr. Kevin Cockell generously opened up the doors of Health Canada to me where I was able to do a substantial amount of laboratory work. His keen and scientific insights were of great assistance. At Health Canada Dr. Nimal Ratanyake and Dr. Kylie Scoggan generously permitted me to work in their laboratories. They allowed me the privilege of working with their expert laboratory technicians from whom I learned a great deal. These technicians include Claude Gagnon, Hélène Rocheleau, and Heidi Gruber. Dr. Kevin Cockell's technician Philip Griffin also gave his time and provided helpful answers. At McGill, Dr. Laurie Chan permitted me to work without impedance in his McGill CINE laboratory where Donna Leggee, his technician was always helpful. In Dr. Stan Kubow's laboratory Behnam Azadi also gave his time and was helpful. Dr. Victor Gavino at the University of Montreal opened up his lab to me which further enhanced my scientific knowledge and lab techniques. I am grateful to both McGill and Health Canada for allowing me to pursue my studies and my dreams. Finally I could not have accomplished this without the support of Andre Przybylowski, my significant other, and this thesis is dedicated to him. This thesis is also dedicated to the memory of my late parents Marie-Jeanne Cyr and Lomer Poirier who are always in my thoughts.

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22(R)-OHC	22(R)-hydroxycholesterol
24(S)-EC	24(S)-25-epoxycholesterol
24(S)-OHC	24-hydroxycholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
5'-DI	Type-I 5'-iodothyronine deiodinase
7- Keto	7-Ketocholesterol
7-OHC	7-hydroxycholesterol
abcg5	ATP-binding cassette (ABC) transporter g5 (Animal)
ABCG5	ATP-binding cassette (ABC) transporter g5 (Human)
abcg8	ATP-binding cassette (ABC) transporter g8 (Animal)
ABCG8	ATP-binding cassette (ABC) transporter g8 (Human)
ANOVA	Analysis of variance
CA	Cholic acid
CAD	Coronary artery disease
CDCA	Chenodeoxycholic acid
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
cyp27a1	Sterol 27-hydroxylase (Animal)
CYP27A1	Sterol 27-hydroxylase (Human)
cyp7a1	Cholesterol 7α-hydroxylase (Animal)
CYP7A1	Cholesterol 7α-hydroxylase (Human)
DNA	Deoxyribonucleic acid
FAS	Fatty acid synthase
FC	Free cholesterol
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase

GSHR	Glutathione reductase
GSSG	Oxidized glutathione
HCHS	High cholesterol and/or high saturated fat
HDCA	Hyodeoxycholic acid
HDL-C	High density lipoprotein cholesterol
hmgcr	3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (Animal)
HMGCR	3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (Human)
LCA	Lithocholic acid
LDL-C + VLDL-C	Low density lipoprotein cholesterol + very low density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
ldlr	Low density lipoprotein receptor (Animal)
LDLR	Low density lipoprotein receptor (Human)
LPO	lipid hydroperoxide
LXR	Liver X receptor
LXR/RXR	Liver X receptor/retinoic X receptor heterodimer
LXRE	Liver X Receptor response element
LXRα (NR1H3)	Liver X receptor α
LXRβ	Liver X receptor β
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
non-SeGSH-Px	Non-selenium dependent glutathione peroxidase
Nor-CA	Nor-cholic acid
npc1l1	Nieman-Pick C1-Like 1 protein (Animal)
NPC1L1	Nieman-Pick C1-Like 1 protein (Human)
PPAR	Peroxisome proliferator-activated receptor
RBC	Red blood cell
RNA	Ribonucleic acid

RT-PCR	Reverse transcription polymerase chain reaction
RXR	9-cis retinoic acid receptor
Se	Selenium
SeGSH-Px	Selenium dependent glutathione peroxidase
SREBP	Sterol regulatory element binding protein
SREBP-1a	Sterol responsive element binding protein -1a
SREBP-1c	Sterol responsive element binding protein -1c
SREBP-2	Sterol responsive element binding protein -2
T3	Triiodothyronine
T4	Thyroxine
TBARS	Thiobarbituric acid-reacting substances
TC	Total cholesterol
TG	Triglycerides
UCA	Ursocholanic acid
UDCA	Ursodeoxycholic acid
VLDL-C	Very low density lipoprotein cholesterol
α-Toc	α-Tocopherol

CHAPTER 1 - INTRODUCTION

1.1 Rationale and Statement of Purpose

High cholesterol and/or high saturated fat (HCHS) diets have been shown to result in elevated plasma concentrations of low density lipoprotein cholesterol (LDL-C). A high concentration of plasma LDL-C is a main biochemical risk factor for cardiovascular disease and for this reason the discovery of dietary interventions that favourably modulate plasma LDL-C levels is important. Dietary selenium (Se), supplemented with a HCHS diet or with a normal fat diet and in various forms (selenite, selenate, Se yeast and selenomethionine) has been shown to be associated with decreased plasma levels of total cholesterol (TC), LDL-C, low density lipoprotein cholesterol + very low density lipoprotein cholesterol (LDL-C + VLDL-C) and decreased triglycerides (TG) in various animal models including the hamster, mouse, rat and rabbit (Chidambaram and Barajam, 1995; Crespo et al., 1995; Vinson et al., 1998; Kang et al., 2000; Poirier et al., 2002; Wojcicki et al., 1991; Mehta et al., 2002; Kang et al., 1997; Kang et al., 1998; Dhingra and Bansal, 2005; Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Iizuka et al., 2001). Moreover, the converse involving an increase in plasma TC, TG and in plasma LDL-C concentrations has been associated with Se deficiency (Huang et al., 2002; Qu et al., 2000; Stone et al., 1986; Stone et al., 1988; Stone et al., 1994; Dhingra and Bansal, 2005; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2006d; Mazur et al., 1996; Nassir et al., 1997; Scott et al., 1991; Mueller and Pallauf, 2006). Although there is less evidence in regards to the effect of Se supplementation on human plasma lipids, several studies confirm that the supplementation of Se is effective at lowering LDL-C and raising high density lipoprotein cholesterol (HDL-C) in the human (Djujic et al., 2000; Kauf et al., 1994; Luoma et al., 1984).

Little investigative work has been carried out on the mechanism(s) involved in the hypocholesterolemic properties of Se including when Se is

supplemented with a hypercholesterolemic diet. Recently, investigative work has related the hypocholesterolemic effects of Se in separate rat studies to a lowering action of Se supplementation on hepatic cholesterol content (Iizuka et al., 2001), lowered hepatic 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (*hmgcr)* messenger ribonucleic acid (mRNA) abundance (Dhingra and Bansal, 2006a) and increased hepatic low density lipoprotein receptor (*ldlr)* mRNA abundance (Dhingra and Bansal, 2006b). A lowering of hepatic cholesterol content or *hmgcr* abundance would be expected to increase hepatic *ldlr* mRNA abundance that, in turn, would decrease plasma LDL-C levels; however, the above parameters have not been measured concurrently in relation to Se supplementation and therefore the molecular mechanism(s) are poorly understood.

Although the above recent studies demonstrated a link between Se and some cholesterol metabolizing enzymes, the normal rat was used as the animal model. In regards to the study of Se on plasma lipids, the normal rat is not considered to be the most appropriate model for the study of human sterol synthesis and LDL-C metabolism. The rat, as compared to the Syrian hamster, is resistant to diet-induced hypercholesterolemia due to its ability to: (a) downregulate hepatic activity of HMGCR; and (b) stimulate cholesterol 7α-hydroxylase (*cyp7a1)* gene abundance and thus is highly efficient in the conversion of cholesterol into bile acids (Spady and Dietschy, 1983a; Wilson, 1964). Conversely, in hamsters (Repa and Mangelsdorf, 2000) and humans (Agellon et al., 2002) ingestion of dietary cholesterol does not induce *cyp7a1* abundance and therefore both hamsters and humans more readily develop hypercholesterolemia when fed diets high in cholesterol. Importantly, no studies have explored potential mechanisms of Se on cholesterol metabolism in the Syrian hamster, which is a more appropriate model to study lipid metabolism relative to the human (Spady and Dietschy, 1983a).

The Syrian hamster is also a better animal model for the study of LXR responsive genes as compared to murine models. The mouse is the primary animal model that has been used for the study of liver X receptor (LXR)

responsive genes apart from limited research using the rat (Zelcer and Tontonoz, 2006). Mice are not reliable models with respect to a predictive response of LXR agonists relative to humans since they lack the LXR target gene cholesterol ester transfer protein (CETP) that is central to human lipoprotein metabolism (Tsutsumi et al., 2001). The up-regulation of CETP in humans can result in a more atherogenic profile (Groot et al., 2005). CETP exchanges TG in apoprotein-B containing lipoproteins for high density lipoprotein cholesterol (HDL-C) thereby reducing HDL-C and increasing atherogenic risk (Shah, 2007). In contrast, hamsters (Tsutsumi et al., 2001) and humans (Luo and Tall, 2000) possess a CETP gene. Also, *cyp7a1* is not a LXR target gene in hamsters or humans (Repa and Mangelsdorf, 2000; Agellon et al., 2002), whereas in the mouse *cyp7a1* gene abundance is up-regulated by LXR (Peet et al., 1998). Thus the hamster is a more appropriate animal model than the mouse to study the response of *ldlr* and *hmgcr* mRNA.

Investigators have uncovered two processes most commonly implicated in resistance to dietary cholesterol; (1) a diminished efficiency of cholesterol absorption; and (2) an enhanced capacity to convert cholesterol to bile acids (Spady 1992). Both processes are shown to occur through upregulation of the liver X receptor (LXR) pathway, which results in a return to whole body cholesterol homeostasis after a dietary cholesterol challenge (Peet et al., 1998). The finding that Se is associated with enzymes known to be LXR responsive such as fatty acid sythase (FAS) (Iizuka 2001; Berg 1995) and lipoprotein lipase (LPL) (Chidambaram and Baradarajan, 1995) supports the idea of its involvement with the LXR pathway. Furthermore, Se has been shown to be associated with a decrease in tissue cholesterol levels (Iizuka et al., 2001, Schwenke and Behr, 1998) which might possibly reflect its participation in the LXR pathway through: (1) upregulation of the LXR responsive genes ATP-binding cassette transporters G5 and G8 (*abcg5* and *abcg8)* involved in secreting cholesterol into gallbladder bile and intestinal lumen; and (2) down-regulation of Nieman-Pick C1-Like 1 protein (*npc1l1*) involved in cholesterol absorption.

Naturally occurring oxysterols which are formed enzymatically through the activity of cytochrome P450 enzymes on cholesterol have been found to be ligands of LXR (Janowski et al., 1999; Lehmann et al., 1997; Forman et al., 1997; Spencer et al., 2001). Moreover, elevated levels of dietary cholesterol were shown to result in an increase in hepatic levels of oxysterols (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2003). It is conceivable that the plasma LDL-C lowering effect of Se is mediated through the formation of oxysterols as Se has been shown to be associated with an upregulation of hepatic cytochrome P450 content (Wojicki et al., 1991), which is mostly responsible for production of oxysterols. Relevant P-450 enzymes, in regards to the aims of the present study include CYP7A1 and sterol 27-hydroxylase (CYP27A1), which are involved in the classic and alternative pathways of bile acid synthesis, respectively. These are the two pathways by which the body disposes of excess dietary cholesterol. Additionally, dietary Se has been associated with increases in biliary bile acids in rats (Danik, 1976) and guineau pigs (Asha and Indira, 2004), which suggests that Se acts on increasing activities of CYP7A1 and CYP27A1. Se is an essential component of the cytosolic Se-dependent GSH peroxidase (Se-GSH-Px) and thus acts as an antioxidant. HCHS diets, along with increasing plasma LDL-C concentrations, are also associated with increased oxidative stress as well as impaired antioxidant status (Aviram et al., 1991). Although some oxysterol species result from free radical-based chemical mechanisms, specifically enzymatically generated oxysterols might be depleted by oxidative stress (Zhu et al., 2005). The capability of Se supplements to mitigate against the blood cholesterol elevating effects of HCHS diets by protection of enzymatically generated oxysterols has not been previously studied.

Hence, an improvement in enzymatically-generated oxysterol status as a response to the consumption of Se could conceivably result in an enhanced capacity of the hamster to; (a) secrete cholesterol into bile by increasing the abundance of the hepatic LXR responsive genes *abcg5* and *abcg8*; and (b) lower the absorption of cholesterol at the level of the gut by increasing the abundance of *abcg5* and *abcg8* genes and decreasing abundance of the LXR responsive

gene *npc1l1*. Increased abundance of *abcg5* and *abcg8* in the liver and intestine, and a decreased abundance of *npc1l1* in the intestine would, in turn, lower liver cholesterol. Decreased hepatic cholesterol is associated with a consequent upregulation of the hepatic mRNA abundance of the *ldlr* and *hmgcr* genes and thus would ultimately result in a lowering of plasma LDL-C levels in the cholesterol-fed hamster.

1.2 Project Overall Objectives

The overall objective of the present thesis was to investigate if the hypocholesterolemic effects of Se are mediated through the activation of the LXR pathway using the Syrian hamster model fed a hypercholesterolemic diet. To test the hypothesis that Se is involved in the production of oxysterol LXR ligands, liver tissue from a previous Syrian hamster feeding trial which was included in the candidate's master's thesis was examined for concentrations of hepatic oxysterols and Se. These additional measurements were then related to the previous measurements made and are included in the present thesis. Thus, the efficacy of antioxidant supplementation (Se as selenate at 3.4 ppm, and α-tocopherol (α-Toc) at 67 IU/Kg diet) on increasing hepatic concentrations of oxysterols and decreasing plasma lipids was tested. In that regard, the possible relationship between hepatic Se and α-Toc content with alterations in hepatic oxysterol concentrations was explored. In order to see whether Se exerted a direct effect on hepatic oxysterol content at physiological concentrations, HepG2 cell culture studies were performed to examine a dose response effect of Se in the form of selenite to increase cellular content of oxysterols. As Se exerted an independent treatment effect on concentrations of the hepatic enzymatically generated oxysterol, 27-hydroxycholesterol (27-OHC), these studies provided evidence to proceed with the examination of the effect of Se in the form of selenite on the LXR pathway in the second experiment. The dose response effect of Se (0.15 ppm, 0.85 ppm, 1.7 ppm and 3.4 ppm) on the mRNA

abundance of the LXR responsive genes *abcg5*, *abcg8* was studied in the liver and intestine and on *npc1l1* in the intestine of hamsters. Another objective was to examine the response of hepatic *cyp7a1* and *cyp27a1* mRNA abundance and hepatic activity of the P450 enzymes CYP7A1 and CYP27A1 to Se supplementation in order to explore possible mechanisms by which Se may be associated with an increase in hepatic oxysterol concentrations. In addition, the supplementation of Se was investigated for its effect on; (1) hepatic cholesterol and TG; (2) plasma cholesterol and TG; (3) gallbadder bile acids and cholesterol; and (4) fecal bile acids and cholesterol. Also investigated was the effect of the supplementation of Se on hepatic and jejunal mRNA abundance of *ldlr* and *hmgcr.*

1.3 Specific Objectives

(1) To explore the *in vivo* effects of the supplementation of Se and α-Toc on liver concentrations of LXR oxysterol ligands and examine whether hepatic oxysterol concentrations are related to hepatic levels of Se, α-Toc, glutathione (GSH), glutathione metabolizing enzymes and lipid hydroperoxides (LPO) and to plasma concentrations of lipids.

(2) To examine whether Se treatment at physiological concentrations can increase hepatic oxysterol concentrations in a dose related fashion in cultured HepG2 cells.

(3) To examine the in vivo effects of dose-related Se supplementation on *cyp27a1* and *cyp7a1* mRNA abundance and CYP27A1 and CYP7A1 activity, which are involved in generation of the liver oxysterols 27-OHC and 7-hydroxcholesterol (7- OHC), respectively.

(4) To study possible alterations in hepatic *abcg5*, *abcg8, ldlr* and *hmgcr* mRNA abundance with Se supplementation in relation to hepatic and plasma cholesterol concentrations and hepatic *abcg5*, *abcg8* mRNA abundance in relation to biliary cholesterol concentrations.

(5) To measure the impact of Se supplementation on cholesterol, chenodeoxycholic acid (CDCA), and cholic acid (CA) concentrations in bile and feces and their relationship with hepatic *cyp27a1* and *cyp7a1* mRNA abundance and CYP27A1 and CYP7A1 enzymatic activities.

(6) To determine the relationship between in vivo Se supplementation and changes in mRNA abundance of hepatic and jejunal LXR responsive genes *abcg5* and *abcg8* and jejunal *npc1l1* in order to investigate if tissues respond similarly to the effects of Se on cholesterol secretion and absorption pathways.

(7) To examine the effect of Se on jejunal mRNA abundance of *ldlr* and *hmgcr* and on LXR responsive genes of *abcg5*, *abcg8* and *npc1l1* in relation to hepatic and fecal cholesterol concentrations and plasma total cholesterol and LDL-C concentrations.

1.4 Hypotheses (Figure1.1)

1. Supplementation of Se and α-Toc with a HCHS diet will result in increased hepatic incorporation of Se and α-Toc, which will decrease oxidative stress and result in enhanced liver oxysterol content.

2. Supplementation of a HCHS diet with Se will result in increased hepatic content of Se in a dose related manner and result in increased activity of the liver oxysterol generating enzymes CYP27A1 and CYP7A1, and the

abundance of *cyp27a1* and *cyp7a1* mRNA.

3. Increases in *cyp7a1* and *cyp27a1* mRNA abundance and activity induced by Se supplementation will be associated with increases in liver concentrations of enzymatically generated oxysterols including 27-OHC, 24-(S)-hydroxycholesterol (24(S)-OHC) and 25-hydroxycholesterol (25-OHC) and increased bile acid content in gallbladder bile.

4. Increases in the hepatic oxysterols 24-OHC, 25-OHC and 27-OHC by Se supplements will be associated with an induction of the LXR responsive hepatic genes *abcg5* and *abcg8* mRNA, which will indicate activation of the LXR nuclear receptor.

5. The Se supplement-mediated induction of hepatic *abcg5* and *abcg8* mRNA will be associated with an increase in concentrations of cholesterol in gallbladder bile and a decrease in hepatic concentrations of cholesterol. The decrease in liver cholesterol concentrations will be associated with an increase in mRNA abundance of hepatic *hmgcr* and *ldlr* and a decrease in plasma concentrations of LDL-C.

6. The induction of jejunal *abcg5* and *abcg8* and decreased *npc1l1* mRNA will increase the concentration of cholesterol in feces. The increase in fecal cholesterol content will be associated with a reduction in liver cholesterol ester (CE) levels and increased liver *ldlr* and *hmgcr* mRNA abundance, which will be associated with a reduction in plasma LDL-C concentrations.

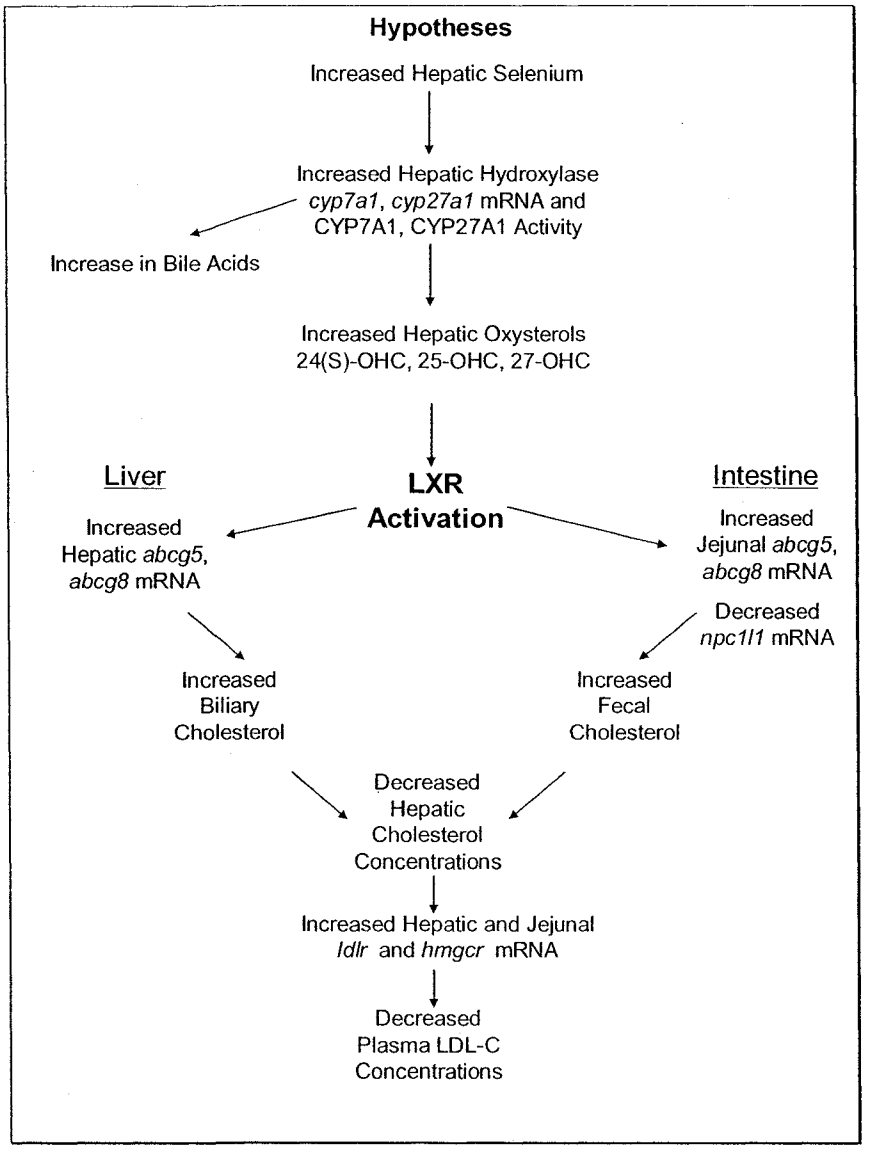

Figure 1.1

Figure 1.1 Supplementation of HCHS diet with Se results in increased incorporation of Se into tissues which is associated with an increase in hydroxylase mRNA and activity which increases oxysterols and bile acids. The increase in oxysterols activates the LXR pathway at the level of the liver and jejunum. Increased abundance of hepatic *abcg5* and *abcg8* at the level of the liver and jejunum and decreased abundance of jejunal *npc1l1* mRNA, is associated with an increase in biliary and fecal cholesterol concentrations which are associated with a decrease in hepatic cholesterol concentrations. The decrease in hepatic cholesterol concentrations is associated with an increased abundance of hepatic and jejunal *ldlr* and *hmgcr* mRNA which in turn is associated with a decrease in plasma LDL-C concentrations.

CHAPTER 2 - The Effects of Se on Lipoprotein Metabolism: A Review of the Human and Animal studies and of the Metabolism of LDL-C

2.1 Abstract

Evidence from animal studies and human trials suggests that selenium (Se) supplements generally exert significant beneficial effects on plasma lipids. The supplementation of Se in diets of a variety of animal models has been shown to exert important hypocholesterolemic effects. The few human intervention trials performed to date have also consistently indicated plasma low density lipoprotein cholesterol (LDL-C) lowering and high density lipoprotein cholesterol (HDL-C) raising effects of Se supplementation. The mechanism(s) by which Se supplements lower plasma lipids have not been extensively explored. Both inhibition of tissue lipid peroxidation and increased thyroid hormone status have been implicated as mechanisms of the hypocholesterolemic action of Se in animal feeding trials. An unexplored hypocholesterolemic mechanism of action of Se supplementation could be mediated via activation of the liver X receptor (LXR) pathway, which merits detailed investigation.

2.2 Introduction

Despite a significant decline in cardiovascular disease (CVD) mortality over the past several decades in the developed world, CVD, mostly in the form of myocardial infarction and stroke, remains the leading cause of death in many developed nations and is an increasing cause of morbidity and mortality in developing nations (Steinberg and Gotto, 1999). The primary lesion characterizing CVD is atherosclerosis, also known as coronary artery disease (CAD) or coronary heart disease (CHD), and which is the slow, progressive narrowing of the three main arteries supplying blood to the heart. The arteries become hardened from the calcification of the gradual accumulation of fatty

deposits in the intima, the innermost tissue layer of the artery which is in contact with the flowing blood. It is known that atherosclerosis begins in childhood and progresses through adolescence and young adulthood progressing to CHD in middle age and later (McGill and McMahan, 2003).

Elevated plasma LDL-C concentrations are a major contributor to atherosclerosis. However, because elevated LDL-C levels do not seem to fully account for the progression of atherosclerosis, it is suspected that the oxidation of LDL-C may play a major role (Steinberg, 1997). Also, at the epidemiological level, depressed HDL-C levels and elevated blood triglyceride (TG) concentrations are shown to correlate positively with CVD risk (Steinberg and Gotto, 1999). Diet has been shown to play a major role in atherogenesis by its influence on blood lipids. For the most part research on dietary factors affecting the risk of CVD in populations has focused on the macronutrient content of the diet showing unequivocally that diets rich in saturated fatty acids elevate plasma cholesterol levels while diets rich in polyunsaturated fatty acids and monounsaturated fatty acids have plasma cholesterol lowering effects (Harris et al., 2003). However, micronutrients including essential trace elements such as Se might also play a role in the aetiology of CVD.

Early in the 20th century, ecological studies revealed the possible association between deficiencies or excesses in the availability of trace elements in soil and water and the prevalence of certain human chronic diseases including CVDs (Masironi, 1979). In particular, the trace element Se was first linked to CVD due to its low levels in the soil of countries showing high rates of CVD, and low serum selenium status (< 45 ug/L) was shown to be a risk factor for CVD (Salonen, 1982). Normal serum selenium concentrations of persons aged nine to more than seventy years as per NHANES III, 1988-1994, range between 95 ± 0.7 ug/L to 163 ± 2 ug/L (Institute of Medicine, 2000). Following the initial ecological studies, Se was studied for its protective effects on CVD risk and CVD risk factors including plasma lipids in order to determine how a low Se status might increase CVD risk. The results of human studies tend to show that Se intake is

associated with decreased plasma lipids, with the strongest evidence obtained from small intervention Se supplementation trials.

The association between Se status and plasma lipids has been investigated in various animal models in the past decade or so. In animal studies, primarily in conjunction with high cholesterol diets, the supplementation of Se was demonstrated to decrease plasma lipids. The mechanism underlying the effect of Se on plasma cholesterol has not been greatly explored; however, Se might function to accelerate catabolism of cholesterol and/or prevent the absorption of cholesterol at the level of the gut. As these two processes are known to be controlled through activation of the liver X receptor (LXR) pathway (Edwards et al., 2002), we have conjectured that Se may be acting to decrease plasma lipids through this latter mechanism. The evidence supporting this contention, however, is indirect as Se has not been previously studied in this regard. The function of the LXR pathway is to decrease whole body cholesterol when challenged with dietary cholesterol (Peet et al., 1998). Activation of the LXR in response to elevated intracellular cholesterol results in the expression of numerous genes involved in cholesterol absorption, efflux transport and excretion (Repa et al., 2002b). For example, over-expression of human ABCG5 and ABCG8 at the level of the liver and intestine was shown to attenuate increases in hepatic cholesterol concentrations resulting from cholesterol feeding (Yu et al., 2002a; Wilund et al., 2004). Conversely, the absence of *abcg5* and *abcg8* which results in a disruption of the genes in both hepatic and intestinal tissues was shown to increase hepatic levels of cholesterol and decrease secretion of cholesterol into bile of *abcg5* and *abcg8* null mice (Yu et al., 2002).

The supplementation of Se has been shown to result in decreased concentrations of tissue cholesterol (liver and aorta) in animals (Iizuka et al., 2001; Schwenke et al., 1998). The ligands of LXR are naturally occurring oxysterols which are produced in the body through the action of P450 hydroxylases on cholesterol. Se has been shown to be associated with an increase in microsomal P450 (Wojicki et al., 1991) and thus we suggest that Se may be participating in the LXR pathway by increasing the formation of naturally

occurring oxysterols. The supplementation of Se has been shown to be protective against free radically formed oxysterols (Huang et al., 2002) and thus we hypothesize that Se is protective of the naturally occurring enzymatically formed oxysterols (Zhu et al., 2005). Finally, Se has been shown to be associated with certain enzymes that are known LXR-responsive genes such as fatty acid synthase (FAS) and lipoprotein lipase (LPL) (Berg et al., 1995; Iizuka et al., 2001; Chidambaram and Baradarajan, 1995). Another pathway of cholesterol catabolism is via the formation of bile acids. In that regard, Se has been shown to be associated with an increase in bile acids (Asha and Indira, 2004; Danik, 1976) and may be lowering total body cholesterol in this manner.

The purpose of this review is to present the evidence linking Se to plasma lipids in both humans and animals and to provide justification for examining the LXR pathway as a mechanism for the plasma lipid lowering effect of Se observed in both humans and animals. The literature review will provide a background on Se and the metabolism of the LDL-C in the LXR pathway. The evidence in the literature supporting a role for Se in lowering plasma lipids in humans and in animals will also be examined. The literature review will focus on the Syrian hamster which is a model more closely resembling humans than the rat and mouse animal models used previously to investigate CVD and LXR pathway.

2.3 Background

2.3.1 Selenium

In parts of the world such as Denmark, Finland, New Zealand, eastern and central Siberia (Russia) and a long belt extending from north-east to south-central China, very low amounts of Se are found in the soil and thus in the food system (Combs, 2000). Thus, studies examining the association between Se status and CVD have been predominantly conducted in these countries, except

for Russia. The earliest studies were conducted in Finland (Salonen et al., 1982; Miettinen et al., 1982; Salonen et al., 1985; Virtamo et al., 1985).

Other areas of the world such as the great plains of the United States of America and Canada, some parts of China and Ireland, Columbia, and Venezuela are seleniferous, meaning they are characterized by high levels of soil Se. Sufficient analytical data are available characterizing the soil Se status of the United Kingdom, European countries, Norway, parts of Australia, Greece, former Yugoslavia, countries of the former Soviet Union, Spain, Turkey, and a few countries in Africa, however, very little is known about the Se content of most of the rest of the world (Combs, 2000). Although being adequate in the soil, the bioavailability of Se may be at risk in areas subject to acid rain or excessive artificial fertilization of soils (Rayman, 1997). In food, the highest Se content is found in fish, mammals and certain plants known to take up Se when grown on Se rich soil such as Brazil nuts, garlic, mushroom, Brussels sprouts, broccoli, asparagus, and wheat (Finley, 2005). Globally, wheat is one of the most important dietary sources of Se as illustrated by the United Kingdom where blood Se levels have declined by around 50% since changing from high-Se Canadian and USA-grown wheat to low-Se European wheat in the mid-1980s (Broadley et al., 2006; Rayman, 1997).

Plants accumulate varying levels of Se and convert it into different chemical forms (Finley, 2005). Selenate is the form primarily absorbed by plants which turn it into selenide. Plant selenide reacts with serine to form selenocysteine which can then be further metabolized into selenomethionine and subsequently be methylated (Finley, 2005). Alternatively, a methylated product of selenocysteine may be formed from the activity of selenocysteine specific methyl transferase enzyme which results in a large accumulation of plant Se (Finley, 2005).

In mammals, the conversion of Se into biologically active forms depends on its chemical form (Gromer et al., 2005). Selenomethionine can be stored in a protein pool when methionine is limited or be catabolized with the release of Se which passes into another pool. Conversely, selenocysteine is not stored but it is

directly catabolized and the resulting selenide goes into a pool to be used later. The inorganic forms of Se (selenate and selenite) go directly into the common pool from which all the Se is used in the synthesis of the specific selenoproteins all of which contain selenocysteine and the excess is excreted.

A total of 25 human selenoprotein genes which express more than 30 selenoproteins have been discovered and are mainly involved in antioxidant defense (Gromer et al., 2005). The functions of the following twelve selenoproteins are still uncertain: an unnamed 15kDa selenoprotein, and selenoprotein-H, -I, -K, -M, -N, -O, -R, -S, -T, -V, and -W. Identified selenoproteins include three deiodinases, which cleave specific iodine carbon bonds in thyroid hormones and thereby regulate their activity. Also characterized are the enzyme selenophosphate synthetase 2, which is required for the formation of monoselenophosphate in the pathway for the formation of selenocysteine (Gromer et al., 2005). Also, a family of three thioredoxin reductases have been characterized which reduce thioredoxin at the expense of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), which can be reoxidized to provide reducing equivalents to various target molecules such as ribonucleotide reductase (Gromer et al., 2005).

Perhaps the best known selenoproteins are members of the family of glutathione peroxidases (GSH-Px-1 to -7), some of which have been associated with CVD risk (Gromer et al., 2005). The GSH-Pxs reduce and thus detoxify different kinds of peroxides to their respective alcohols at the expense of glutathione (GSH) (R-OOH + 2 GSH $\rightarrow$ R-OH + H2O + GSSG). A total of five GSH-Pxs (1-4, and -6) are Se dependent. GSH-Px-1 was first discovered in 1957 and found later to be a selenoprotein (Mills, 1957). GSH-Px-1 is abundant in many tissues including the liver and red blood cells (RBCs). A number of GSH-Px-1 polymorphisms are associated with increased risk of vascular diseases (Hamanishi et al., 2004). GSH-Px-2 is often referred to as gastro-intestinal-GSH-Px due to its presence only in the gastrointestinal system where it is considered to be the first line of defence against ingested organic hydroperoxides. GSH-Px-3 is found extracellularly in the plasma. Its concentration is only exceeded by

selenoprotein P; however, its physiological function is not completely understood. The deficiency of GSH-Px-3 appears to correlate with cardiovascular events (Sarto et al., 1999). GSH-Px-4 exhibits the broadest substrate specificity as it is shown to reduce intact phospholipid hydroperoxides and for this reason is referred to phospholipid-hydroperoxide GSH-Px (Gromer et al., 2005). Its ability to reduce hydroperoxides still integrated in the membranes gives it a role as a universal antioxidant in the protection of biomembranes. The enzyme GSH-Px-6 is suggested to have a role in olfaction. The enzymes GSH-Px-5 and -7 do not contain selenocysteine (Gromer et al., 2005).

Se was first encountered as a toxic element in humans when it was discovered by Berzelius in 1817. It continued to be considered toxic for a long time afterwards and studies remained scarce over a century after its discovery. The first studies conducted in 1916 (Gassmann, 1916) focused on the Se content of bones and teeth (Behne and Kyriakopoulos, 2001). In the early 1930's Se was found to be the cause of the alkali and blind staggers diseases, which developed in cattle grazing on Se accumulator plants growing on the range lands of the north-central United States (Moxon, 1937).

It was only in 1957 that Se was shown to be harmless at lower dietary levels when a German scientist named Schwarz, working in the United States, isolated a fraction in brewers' yeast that contained Se and protected vitamin E deficient rats against liver necrosis (Schwarz and Foltz, 1957). Following this discovery, Se was quickly recognized as being essential in the diets of animals as subsequent studies linked Se deficiency with certain diseases in domestic animals and birds that had previously eluded diagnosis (Muth and Allaway, 1963).

Observations of changes in blood pressure, cardiomyopathy and sudden cardiac death in animals with Se deficiency promoted interest in the role of Se in the aetiology of CVD in humans (Burk, 1978). To date, however, evidence of an association linking Se status and atherosclerosis remains controversial. Although antioxidant combinations including Se have shown a reduction in atherosclerosis in animal models such as rabbits (Leborgne et al., 2005; Schwenke and Behr,

1998; Wojcicki et al., 1991), it has yet to be demonstrated that Se deficiency unequivocally induces atherosclerotic lesions in experimental animals.

Se metabolism was linked with vitamin E and Se was labelled as an antioxidant when subsequent studies suggested that the damaging effects of Se deficiency resulted from the destruction of cellular membranes (Combs et al., 1975). The first evidence that Se exerted antioxidant protection through the cytosolic selenoprotein GSH-Px-1 occurred in 1973 (Rotruck et al., 1973).

The essentiality of Se in humans became apparent in 1979 when a relationship was uncovered between the pathology of Keshan disease and the low soil concentration of Se in the geographical area of Keshan, China (Combs, 2001). In addition, cardiomyopathy in subjects maintained on parenteral nutrition devoid of Se was discovered (Combs, 2001). Another indicator of the essential role of Se in humans was the discovery that a disease of bones and joints called Kaschin-Beck Syndrome is related to low dietary intake of Se. Both Kaschin-Beck Syndrome and Keshan Disease are Se responsive; however, other factors including viral agents have been shown to play a co-morbid role in both diseases (Combs, 2001).

The daily requirement for Se in adults is 55 µg/day (Institute of Medicine, 2000), and is based on maximizing the activity of plasma GSH-Px-3, which reflects the activity of tissue selenoenzymes better than does red blood cell GSH-Px1 activity (Cohen et al., 1985). The Se requirement is readily met by most North Americans; however, a significant proportion of the population of Europe, Asia and parts of Africa have intakes less than the Recommended Daily Allowance. It is estimated that at least a billion people worldwide are Se deficient (Combs, 2000).

A plasma level of 70 – 100 µg Se/l has been deemed to maximize activity of GSH-Px-3, which corresponds to dietary intakes of 40 µg/d. This level has been identified by the World Health Organization (1996) as the average intake level needed to ensure meeting requirements of most healthy adults. However, this level of intake may not be optimal for the full expression of all selenoproteins. Full expression of selenoprotein P requires a greater Se intake than full

expression of plasma GSH-Px-3 and therefore selenoprotein P might be a better indicator of Se nutritional status than GSH-Px-3 (Xia et al., 2005). For adult females and males, the maximum intake recommended by World Health Organization and Dietary Reference Intake Upper Intake Level is 400 µg/d (Institute of Medicine, 2000). Results from future intervention trials are needed to substantiate the argument for increasing Se intake from currently recommended intake.

Although ecological studies for many years have indicated the link between Se deficiency and atherosclerotic disease, the relation between Se and CVD risk has been scarcely studied in an epidemiological context. The level of Se in different foods varies widely due to the dependence on the soil content of Se where the food is grown. Thus, tabulated food composition data for selenium is typically insufficient to accurately estimate Se intake (Holben, 1999). A proper biomarker has yet to be developed, which makes it difficult to conduct epidemiological studies with Se. Regardless, assays of Se in body tissues have been used as a surrogate measure of intake. They are a more direct indicator of Se status than dietary intakes because they take into account variations in absorption and metabolism.

Although a low Se status was shown to be a risk factor for CVD in 1982 (Salonen, 1982), the evidence to date relating Se status in relation to CVD risk in humans remains inconclusive. Recently, a meta-analysis of 25 observational studies and 6 randomized trials demonstrated that a 50% increase in plasma Se concentrations was associated with a 24% decrease in coronary heart disease risk (Flores-Mateo et al., 2006). The authors of the study, however, warned that the validity of the association could be uncertain due to the misleading evidence shown in previous observational trials investigating CVD and other antioxidants such as beta-carotene and vitamin E.

The mechanism by which low Se intake might increase the risk of CVD is still unclear. It is uncertain if low Se increases risk by promoting progression of atherosclerosis or by affecting processes that trigger acute myocardial infarction, which is the most severe result of CAD, and cardiac death in subjects with

atherosclerosis. Studies have been mainly observational rather than interventional in nature. Although small intervention trials have been performed, no large human intervention trials have specifically examined the hypolipidemic effect of Se. Thus, to date, the effect of Se on plasma lipids in humans remains inconclusive. On the other hand, although human studies on the relationship between Se intake and plasma lipid are inconclusive, studies using animal models indicate that the supplementation of Se improves plasma lipid profile and that Se deficiency results in a significant increase in plasma total cholesterol levels.

2.3.2 Human Studies and Se Status

2.3.2.1 Se and CVD Risk

In the early 1980s when Se was first studied in relation to CVD it was studied as a potential independent risk factor. Finland, an area of low Se, had the highest worldwide CHD mortality in 1971, and within Finland, the highest mortality was observed in the eastern province of North Karelia (Keys, 1970). A total of four studies - three case control prospective studies and one prospective cohort study were conducted between the years 1982-1985 in Finland. The first case control (Salonen et al., 1982) found that serum Se concentrations of less than 45 µg/L were associated with an increased relative risk of CVD death, i.e., CHD: 2.9 (95% confidence interval, 1.4 - 6.0); CVD: 2.2 (95% confidence interval, 1.2 - 4.0); and fatal and nonfatal myocardial infarction: 2.1 (95% confidence interval, 1.4 - 3.1). Serum Se concentrations greater than 45 µg/L were not associated with any future risk of CVD. The next three studies were not able to confirm this finding mainly due to an increase in serum selenium concentrations observed in study participants (Miettinen et al., 1982; Salonen et al., 1985; Virtamo et al., 1985).

As TC was a known CVD risk factor, cases were matched with controls by CVD risk factors. The earliest studies did not report the mean levels of cholesterol in study subjects (cases and controls) which precluded an examination of the impact of Se on plasma cholesterol levels. By 1986, however, investigators began to examine if plasma lipids were associated with selenium status (Salonen et al., 1988; Kok et al., 1989; Bor et al., 1999; Kok et al., 1991; Suadicani et al., 1992; Ringstad et al., 1987; Kok et al., 1987; Beaglehole et al., 1990).

2.3.2.2 Se and Plasma Lipids

Several studies conducted after 1986 showed an inverse association between Se status and CVD risk (Kok et al., 1989; Bor et al., 1999; Kok et al., 1991) **(Table 2.1)**, whereas other studies did not (Ringstad and Thelle, 1986; Ringstad et al., 1987; Kok et al., 1987; Beaglehole et al., 1990; Salvini et al., 1995) **(Table 2.2)**. Some cross sectional surveys reported lower plasma selenium concentrations and higher plasma lipid concentrations in cases as compared to controls (Salonen et al., 1988; Kok et al., 1989; Bor et al., 1999; Kok et al., 1991). In some trials, despite significant differences in plasma lipid concentrations between cases and controls, no differences were shown with regards to serum Se concentrations (Ringstad et al., 1987; Kok et al., 1987), which suggested that Se status was not a major modulatory factor in those subjects.

Whereas some observational studies (cross-sectional and prospective cohort) designed to examine the effect of Se on CVD risk factors did not report associations between Se status and any CVD risk factors, including plasma lipids (Robinson et al., 1983; Ellis et al., 1984; Bukkens et al., 1990), most studies have reported Se to be associated with plasma lipids. The studies shown in **Table 2.3** (cross-sectional and one prospective cohort), were designed to investigate the association between Se status and CVD risk factors and reported significant correlations between plasma lipids and Se status **(Table 2.3)**.

Table 2.1 Observational Studies Reporting An Association Between Se Status And CVD Risk[a]

Study and (Design)	Se Status[1] (µg/L)		Sign.	Plasma Lipids (mg/dL)		Sign.
	Cases	**Controls**		**Cases**	**Controls**	
Kok et al., 1989 (CS)	Toenail 0.70 ± 0.02(SD) Plasma 100.8 ± 3.0(SD) RBC 0.54 ± 0.01(SD)	Toenail 0.78 ± 0.02(SD) Plasma 106.8 ± 2.6(SD) RBC 0.59 ± 0.02(SD)	P<0.05 NS P<0.05	TC: 278 ± 58(SD) HDL-C: 43 ±0.77(SD)	TC: 251±46(SD) HDL-C: 50±1.2(SD)	P<0.05 P<0.05
Bor et al., 1999 (CS)	Plasma 63.7±12(SD)	Plasma 82.2±14.6(SD)	P<0.001	TC: 165±46 (SD) HDL-C: 36±9 (SD)	TC: 163±26(SD) HDL-C: 35±8(SD)	NS

Kok et al., 1991 (CS)	Plasma 95.1±21(SD)	Plasma 108.8±29.3(SD)	P<0.01	TC: 286 ± 58(SD) LDL-C: 217±58(SD) HDL-C: 43±12(SD)	TC: 271±50(SD) LDL-C: 197±54(SD) HDL-C: 46±12(SD)	P< 0.05

[a] **ABBREVIATIONS**: CS, cross sectional ; Sign, significance; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; NS, non-significant; RBC, red blood cell; Se, selenium. [1]Toenail, (mg/kg); Plasma (ng/ml); RBC, ug/g haemoglobin.

Table 2.2 Observational Studies Reporting No Association Between Selenium Status And CVD Risk[a]

Study and (Design)	Mean Serum Se (µg/L)		Sign.	Plasma Lipids (mg/dL)		Sign.
	Cases	Controls		Cases	Controls	
Ringstad and Thelle, 1986 (CCP)	131 ± 21.3 (SD)	126.3 ± 16.8 (SD)	NS	NR	NR	NR
Ringstad et al., 1987[b] (CC)	123.9±16.6 (SD)	127.1±21.3(SD)	NS	TC: 292 TG: 205 HDL-C: 55	TC: 251 TG: 151 HDL-C: 58	P<0.001 P<0.001 NS
Kok et al., 1987 (CC)	125.1±3.1(SE)	126.5±2.2(SE)	NS	TC: 256 ± 46(SD)	TC: 232 ± 41(SD)	NS
Beaglehole et al., 1990 (CC)	82.7 (95% CI,80.2-85.2)	88.2 (95% CI,86.8-89.6)	P<0.001	TC: 263 (95% CI,256-270)	TC: 246 (95% CI, 243-250)	P<0.05

Salvini et al., 1995 (Nested CC)	114.4± 15.1 (SD)	113.2±15.7(SD)	NS	NR	NR	NR

[a]**ABBREVIATIONS**: CC, case control; CCP, case control prospective; Sign, significance; NS, non-significant; NR, not reported; CI, confidence interval; Se, selenium; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; SD, standard deviation; SE, standard error.

[b] SD not reported for plasma lipids

Table 2.3 Correlations Between Plasma Lipid Parameters And Se Status Found In Observational Studies Examining The Effect Of Se On CVD Risk Factors[a]

Study and (Design)	Se Status	Study Subjects	Plasma lipid Parameter	Correlation (r)	Significance
Cser et al., 1993	Plasma	Children:	Healthy children:		
(CS)		40 diabetic	TC	0.8535	P<0.01
		38 healthy	HDL-C	0.5696	P<0.001
			TG	0.7669	P<0.001
Spagnolo et al., 1991	Serum	435 females	Males:		
(CS)		486 males	TC	0.2	P<0.001
			Non-HDL-C	0.2	P<0.001
			HDL-C	0.13	P<0.05
			Females :		
			TC	0.17	P<0.01
			HDL-C	0.16	P<0.01

Viegas-Crespo et al., 1994 (CS)	Plasma	60 females 66 males	HDL-C/TC	0.08	P<0.05
Gamez et al., 1997 (CS)	Serum	93 institutionalized elderly	All subjects: TC LDL-C Women: TC LDL-C	 0.2965 0.2765 0.3439 0.3020	 P<0.05 P<0.05 P<0.05 P<0.05
Berr et al., 1998 (CS)	Plasma	815 females 574 males	All subjects: HDL-C TG	 0.15 -0.09	 P<0.001 P<0.05
Navarro-Alarcon et al., 1999 (CS)	Serum	93 institutionalized elderly 103 healthy	All subjects: TG	 0.59	 P<0.05
Lee et al., 2003 (CS)	Serum	142 women of various ages	Women < 40 y: HDL-C	 0.458	 P<0.006

Jossa et al., 1991 (PC)	Serum	380 males	All subjects: TC	0.120	P<0.022

[a] **ABBREVIATIONS:** CS, cross sectional; PC, prospective cohort; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; non-HDL-C, non-high density lipoprotein cholesterol; Se, selenium.

Most large interventional studies (Wei et al., 2004; Stranges et al., 2005) and smaller interventional studies (Korpela et al., 1989; Kuklinski et al., 1994) involving Se supplementation conducted to date did not study whether Se supplementation lowered plasma cholesterol, or when they studied Se in regards to plasma lipids it was done in conjunction with other antioxidants (Hercberg et al., 2005), and thus the extent to which Se influences CVD risk by lowering plasma lipids is unclear.

The strongest evidence supporting an effect of Se supplementation on plasma lipids in humans is derived from three small intervention studies (Luoma et al., 1984; Kauf et al., 1994; Djujic et al., 2000) summarized in **Table 2.4**. Luoma et al. (1984) conducted a double blinded randomized intervention trial involving twenty seven medical students, 18 females and 9 males, aged from 21 to 33 years. Subjects were given either Se-containing yeast tablets (96 µg of Se/day) or control yeast tablets for 2 weeks. Subjects with the lowest plasma Se concentrations had lower serum HDL-C and total cholesterol (TC) levels than subjects with the highest plasma Se concentrations. The supplementation of Se increased serum Se and increased the HDL-C/TC ratio (Luoma et al., 1984). A subsequent study involved individuals with hypothyroidism who had an atherogenic lipid profile of high plasma LDL-C and high LDL-C/HDL-C ratio (Kauf et al., 1994). Thirty seven children aged 9.64 ± 5.09 years, (24 female, 13 male) with congenital hypothyreosis and taking regular hormone medication were supplemented with sodium selenite (115 µg Se/day) for 3 months. Se treatment significant decreased TC and LDL-C concentrations whereas HDL-C concentrations were increased significantly. The LDL-C/HDL-C ratio was also significantly decreased (Kauf et al., 1994). A study investigated Se in the form of L-(+) Selenomethionine (26 µg/day) in wheat using 30 healthy subjects with regards to the association of Se status with CVD risk factors (Djujic et al., 2000). After 6 weeks the mean plasma Se concentration increased by 53%. Se intake also induced a tendency to lower LDL-C (15.1%), TG (14.5%), TC (10.3%) and HDL-C (8.4%) (Djujic et al., 2000).

Summarizing the human studies reviewed here, Se status is shown to have impact on plasma lipids in some studies and not in others. The reason is not clear, however, it is possible that the various forms of Se found in food that are organic in form, i.e., either bound to methionine or cysteine, may exert differential effects on plasma lipids. Epidemiological studies often do not provide information on the dietary form of Se ingested by participants.

Miyazaki et al. 2002 estimated the dietary intake of fish and non-fish Se using a simple food frequency questionnaire and the association of Se status as determined by serum GSH-Px-3 activity with plasma lipids and atherogenic index defined as the ratio of TC-HDL-C/HDL-C as a CHD risk factor (Miyazaki et al. 2002). In all subjects dietary intake of non-fish Se was significantly and negatively associated with the atherogenic index and positively associated with HDL-C. It was also shown that HDL-C was lower and the atherogenic index was higher in the low Se intake groups than in the other groups compared.

Table 2.4 Small Intervention Studies Showing An Effect Of Se Supplementation On Plasma Lipids[a]						
Study	**Study subjects**	**Se treatment**	**Plasma Lipids Measured**		**Se status**	
			Treatment	**Placebo**	**Treatment**	**Placebo**
Luoma et al., 1984	27 Medical Students	Se yeast tablet 96 µg Se/day	At baseline, HDL-C/TC ratio: 0.26±0.04 (SD). After treatment: HDL-C/TC ratio: 0.28±0.05 (SD), P<0.05	No change in plasma lipids	At baseline: 73.7 µg/L ±14.0 (SD) After treatment: 90.8 µg/L±14.1 (SD), P<0.05	No change in Se status
Kauf et al., 1994	37 Hypothyroid children	115 µg Se/day	At baseline, LDL-C: 114 mg/dl ±27(SD); TC: 188 mg/dl ±38 (SD).	None	At baseline, Plasma: 68.7 µg/L ±12.6 (SD).	None

			After treatment: LDL-C: 98 mg/dl ±28 (SD), P<0.01 TC: 176 mg/dl ±32 (SD), P<0.05		After treatment: Plasma: 88.4 µg/L ±20.5 (SD), P<0.001	
Djujic et al., 2000	30 healthy volunteers	Consumed bread containing Se providing average 26 µg/day	After treatment: ↓LDL-C (15.1%) ↓ TG (14.5%) ↓ TC (10.3%) ↓HDL-C (8.4%) Non-significant decreases.	None	↑ Serum Se (53%)	None

[a]**ABBREVIATIONS:** Se, selenium; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol.

2.3.2.3 Conclusion to Human Studies and Se Status

In humans the hypocholesterolemic effects of Se are mainly supported by evidence from small intervention studies. Although numerous observational studies report strong correlations between Se status and plasma lipid parameters in study subjects, they are difficult to interpret as the correlations may not represent causal relationships. The meaning of the correlation between serum Se and serum cholesterol is not clear and requires further study. Previous studies have shown that serum Se, besides being initially transported by serum albumin, is also observed bound to both HDL-C and LDL-C in rats, dogs, mice and humans (McConnell and Levy, 1962; Hirokaa and Galambos, 1966). Thus the observed association may merely be a reflection that Se and cholesterol are found together in plasma. It is also possible that the association is due to the interrelationship of Se and cholesterol with a third unknown variable. For example, the intake of animal protein which is rich in Se and in saturated fat and which is known to increase plasma cholesterol levels could be responsible for the association. It is also possible that Se status may have an influence on plasma cholesterol levels in the human as it is shown to have consistently in various animal models (Chidambaram and Barajam, 1995; Crespo et al., 1995; Vinson et al., 1998; Kang et al., 2000; Poirier et al., 2002; Wojcicki et al., 1991; Mehta et al., 2002; Kang et al., 1997; Kang et al., 1998; Dhingra and Bansal, 2005; Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Iizuka et al., 2001; Huang et al., 2002; Qu et al., 2000; Stone et al., 1986; Stone et al., 1988; Stone et al., 1994; Dhingra and Bansal, 2005; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2006d; Mazur et al., 1996; Nassir et al., 1997; Scott et al., 1991; Mueller and Pallauf, 2006).

Future studies are needed in order to more clearly elucidate the potential association between Se status and plasma lipids in humans. In order to establish that there is a hypolipidemic effect of Se supplementation in humans there needs

to be further clinical research, double blind studies and long-term prospective studies which also examine the effect of Se supplements on plasma lipids.

2.3.3 Animal studies and Se Status

2.3.3.1 Introduction

Two mechanisms have been studied in animal models with regards to Se status and plasma lipid metabolism: a) lipid peroxidation; and b) thyroid status.

Lipid peroxidation is the mechanism most investigated in animal studies to explain the observed association between Se status (both deficiency and supplementation) and plasma lipids (Wojicki et al., 1991; Stone et al., 1994; Kang et al., 1998; Vinson et al., 1998; Qu et al., 2000; Poirier et al., 2002; Mehta et al., 2002; Huang et al., 2002). Most studies show that Se status lowers both plasma lipids and lipid peroxidation; however, some studies report the effect of Se on plasma lipids without showing an effect on lipid peroxidation which suggest that other unmeasured mechanisms might be involved in the plasma lipid lowering effect of Se (Poirier et al., 2002; Stone et al., 1994; Kang et al., 1998). Furthermore, lipid peroxidation may be interacting with other unmeasured factors that, in turn, influence plasma lipids.

Evidence from previous work indicates that lipid peroxidation may affect plasma lipid levels by: a) affecting hepatic cholesterol metabolism, and/or b) accelerating the oxidation of the LDL-C which is not taken up by the low density lipoprotein receptor (LDLr) and thus accumulates in the plasma from which a portion of oxidized LDL-C is taken up by macrophages which express scavenger receptors and which in turn accelerates the development of atherosclerosis. The liver occupies a central role in cholesterol homeostasis (Spady, 1992). Previous in vivo studies have indicated that injury to the liver through increases in lipid peroxidation results in abnormalities in hepatic cholesterol metabolizing enzymes and circulating lipoproteins (Levy et al., 2007; Brunet et al., 1999). An in vitro

study showed that the endoplasmic reticulum, where 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (HMGCR) and cholesterol 7-hydroxylase (CYP7A1) are found, is very sensitive to lipid peroxidation (Brunet et al., 2000). Several studies have shown that LDL-C is more susceptible to oxidation in patients with hypothyroidism and hyperlipidemia (Sudaram et al., 1997; Diekman et al., 1998).Thus, it is believed that the oxidation of LDL-C prevents it from being taken up by LDL receptors and thus LDL-C concentrations accumulate in the plasma which leads to hypercholesterolemia.

The effect of Se on thyroid status which influences LDLr mRNA abundance is another mechanism of the plasma lipid lowering effects of Se. The LDLr mRNA contains a response element for triiodothyronine (T3) (Bakker et al., 1998), and Se increases the selenoprotein type-I iodothyronine deiodinase (5'-DI) activity (Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2005), thereby increasing plasma concentrations of T3, which is hypocholesterolemic (Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2005). Although the animal studies in **(Table 2.7)** show an effect of Se through thyroid hormone, the effects of subclinical hypothyroidism on serum lipid levels in humans remain controversial. A recent meta-analysis on the effect of thyroid therapy for subclinical hypothyroidism on serum lipid levels demonstrated a very limited mean reduction in TC and LDL-C levels and no effect on HDL-C (Danese et al., 2000).

An unexplored mechanism of the hypocholesterolemic effect of Se is the LXR pathway. As Se has not been studied in this regard, the evidence is indirect. We suggest that Se might be associated with the LXR pathway as Se has been associated with an increase in P450 that generates oxysterols (Wojicki et al., 1991) and with increased activity of the enzymes fatty acid synthase (FAS) (Berg et al., 1995; Iizuka et al., 2001) and lipoprotein lipase (LPL) (Chidambaram et al., 1995), which are known LXR-responsive genes (Michael et al., 2005). Also, as supplementation of Se decreases plasma lipids in conjunction with high cholesterol diets (Wojicki et al., 1991; Stone et al., 1994; Kang et al., 1998; Qu et

al., 2000; Poirier et al., 2002; Mehta et al., 2002; Huang et al., 2002; Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2006d; Dhingra and Bansal, 2005) Se might exert its effects through catabolism of cholesterol via formation of oxysterols and bile acids.

Most studies examining the effect of Se on plasma lipids have used normal rats, which are not an appropriate model of human lipid metabolism. Hamsters are a better model of both human lipid metabolism and the study of the LXR pathway and can tolerate higher amounts of Se than the rat (Birt et al., 1983; Jia et al., 2005). Also, in contrast to human studies, where the form of Se in food appeared to influence the effect of Se on plasma lipids (Miyazaki et al., 2002), animal studies show that the use of the inorganic form of Se, i.e., the sodium selenite or sodium selenate forms that do not occur in foods, made no difference in the hypolipidemic effect of Se. The consistent use of inorganic forms of Se might thus explain partly why animal data linking Se with plasma lipid lowering is more consistent than human findings.

2.3.3.2 Evidence for and against Mechanism 1: Lipid Peroxidation

In several studies **(Table 2.5)**, lipid peroxidation was not shown to play a role in the lipid modulating effects of Se status. For example, the supplementation of Se to male Syrian hamsters showed favourable effects on plasma lipids and yet did not impact hepatic and plasma oxidative stress, and thus the findings suggested that antioxidative effects of Se were unlikely to be involved in the hypolipidemic effects of Se (Poirier et al., 2002). Likewise, although Se deficiency was associated with an increase in plasma LDL-C and VLDL-C concentrations in male SHR rats it was not associated with an increase in plasma thiobarbituric acid-reacting substances (TBARS) which suggested that the oxidation of LDL-C was unlikely to be responsible for the increased plasma LDL-C levels associated with Se deficiency (Stone et al., 1994). In a separate

study, male Sprague-Dawley rats fed HCHS diet showed increased serum and hepatic TBARS, which were not decreased by Se supplementation (Kang et al., 1998). In the same study, Se supplementation decreased plasma TC and TG suggesting that lipid peroxidation was not associated with hyperlipidemia (Kang et al., 1998).

On the other hand, other studies **(Table 2.6)** have associated the Se-induced lowering of plasma lipids with its effects on decreasing lipid peroxidation. Serum levels of total lipid, oxidative stress and TC and TG were elevated and serum levels of HDL-C were decreased in male mongrel rabbits consuming HCHS diet as compared to control animals. In support of a role for lipid peroxidation on plasma lipids, Se decreased total lipid, TC and TG levels (Wojcicki et al., 1994). Likewise, the supplementation of Se was shown to reduce plasma TBARS and increase the lag time of low density lipoprotein cholesterol + very low density lipoprotein cholesterol (LDL-C + VLDL-C) oxidation as well as showing reductions in TC and LDL-C + VLDL-C concentrations in male Syrian hamsters (Vinson et al., 1998). Increased levels of malondialdehyde (MDA) were shown in the serum, liver and aorta of male New Zealand white rabbits fed the HCHS diet, which were decreased with the supplementation of Se. Additionally, serum levels of TC and TG were significantly decreased in the Se supplemented group (Mehta et al., 2002). Finally, in Se deficient male Wistar rats, blood Se content and GSH-Px-1 activity were lower and plasma lipid hydroperoxides (LPO) higher. In addition, lower serum HDL-C and higher serum LDL-C concentrations were observed in the same animals (Huang et al., 2002).

Table 2.5 Animal Studies Not Finding An Association Between Se Status And Lipid Peroxidation On Plasma Lipids[a]							
Study (Animal model)	**Se and/or Vit E Status (ppm)**	**Fat Level**	**Se and/or Vit E effect on Lipoproteins**	**Vit E x Se on Lipoproteins**	**Se, Vit E, HCHS effect on Lipid Peroxidation**	**Se, Vit E, HCHS effect on GSH-Px**	**Se and/or Vit E, HCHS, effect on tissue Se and Vit E**
Poirier et al., 2002 (male Syrian hamster)	SSel Suppl: 3.4 Adeq: 0.15 Vit E Suppl: 129 IU/Kg Adeq: 27 IU/Kg	12.9% Fish oil 1.38% Safflower oil	Se suppl ↓TC, LDL-C + VLDL-C ↔ HDL-C, TG Vit E:↔TC, TG HDL-C LDL-C + VLDL-C	Vit E x Se suppl ↔ TC, LDL-C + VLDL-C, HDL-C, TG	Se, Vit E suppl↔ plasma TBARS Se↑ heart LPO	Se, Vit E↔ heart GPx Se ↑ liver GPx	Se not measured Vit E ↑ heart, liver Vit E

Stone et al., 1994 (male Spontaneously Hypertensive rats)	SSe Def: <0.03 Adeq: 0.4 Vit E Def:< 3 mg/Kg Adeq: 50 mg/Kg	Normal Fat 1% Cholesterol	Se def ↑ LDL-C, ↔HDL-C Vit E: N/A	Vit E x Se def ↑ LDL-C, HDL-C	Se def ↔ plasma TBARS	Se↑ plasma, RBC GSH-Px1	Se not measured Vit E def ↓ plasma vit E
Kang et al., 1998 (male Sprague-Dawley rats)	SSe Adeq: Suppl: 1 Vit E: N/A	15% Fat 2% Cholesterol	Se suppl :↓TC, TG vs HCHS Vit E: N/A	N/A	HCHS: ↑ aortic, liver,serum TBARS Se vs HCHS ↓aortic TBARS ↔ serum, liver vs HCHS	HCHS ↑ GSH-Px1 Se ↑ GSH-Px1 vs HCHS	Se not Measured

[a]**ABBREVIATIONS:** SSe, sodium selenite; SSel, sodium selenate; Suppl, supplemented level; Def, deficient level; Adeq, adequate level; N/A, not applicable; RBC, red blood cell; GSH-Px, glutathione peroxidase; Se, selenium; Vit E, vitamin E; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; LDL-C + VLDL-C, low density lipoprotein cholesterol + very low density lipoproptein cholesterol; TG, triglycerides; HCHS, high cholesterol and/or high saturated fat diets; TBARS, thiobarbituric-acid reacting substances; LPO, lipid hydroperoxides.

Table 2.6 Animal Studies Finding An Association Between Se Status And Lipid Peroxidation On Plasma Lipids[a]							
Study (Animal Model)	**Se and/or Vit E Status (ppm)**	**Fat Level**	**Se and/or Vit E Effect on Lipoproteins**	**Vit E x Se on Lipoproteins**	**Se, Vit E, HCHS effect on Lipid Peroxidation**	**Se, Vit E, HCHS Effect on GSH-Px**	**Se and/or Vit E, HCHS, Effect on Tissue Se and Vit E**
Wojcicki et al., 1991 (male mongrel rabbits)	Se: Suppl: 5 ug/Kg Adq: NR Vit E: Suppl: 10 mg/Kg Adq: NR	0.5 HCHS 1 g hydrogenated coconut oil + 3.1% crude fat 0.1 HCHS	Se suppl vs HCHS ↓ TC, TG↔HDL Vit E suppl ↔TC, TG, HDL-C	Vit E x Se suppl vs HCHS↓TC, TG ↔ HDL	HCHS ↑ plasma MDA Se suppl, Vit E suppl, Vit E x Se suppl ↓ plasma MDA vs HCHS	N/A	N/A

Vinson et al., 1998 (male Syrian hamster)	Selenium yeast S: 1, 5;A: 0.2 Vit E: N/A	Normal Fat	Se suppl ↓TC, LDL + VLDL ↔ HDL-C, TG Vit E: N/A	N/A	Se suppl ↓ Plasma TBARS ↑lag time oxLDL	N/A	N/A
Mehta et al., 2002 (male New Zealand white rabbits)	SSe Suppl:1 Adq:0.2 Vit E:N/A	100 g Butterfat 1% Cholesterol	Se suppl↓TG,TC Vit E:N/A	N/A	HCHS ↑ MDA in liver, serum, aorta	N/A	HCHS ↓ Serum S Se ↑ serum Se
Huang et al., 2002 (male weanling Wistar rats)	SSe Def: 0.038± 0.003 Adq: 0.326± 0.041 Vit E: N/A	10% Soybean oil	Se def : ↑ LDL-C, TC, ↓ HDL-C; ↔ TG Vit E: N/A	N/A	Se def:↑ plasma LPO	Se def ↓ Blood GSH-Px1	Se def ↓ Blood Se

[a]**ABBREVIATIONS:** SSe, sodium selenite; Suppl, supplemented level; Def, deficient level; Adq, adequate level; Se, selenium; vit E, vitamin E; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; LDL-C + VLDL-C, low density lipoprotein cholesterol + very low density lipoprotein cholesterol; TG, triglycerides; HCHS, high cholesterol and/or high saturated fat diets; oxLDL, oxidized LDL-C; MDA, malondialdehdye; TBARS, thiobarbituric-acid reacting substances; NR, not reported; GSH-Px1, glutathione peroxidase .

2.3.3.3 Evidence for Mechanism 2: Se Status and Thyroid Status

A series of recent studies **(Table 2.7)** conducted by Dhingra & Bansal have explored the association shown between plasma lipids and the hypothyroid state in regards to Se status and messenger ribonucleic acid (mRNA) abundance of cholesterol metabolizing enzyme *hmgcr* and the low density lipoprotein receptor (*ldlr*) **(Table 2.7)**. An increase in serum concentrations of TC and LDL-C was noted with consumption of HCHS diets (Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2006d; Dhingra and Bansal, 2005) which the supplementation of Se was shown to decrease. The supplementation of Se was also shown to increase serum levels of triiodothyronine (T3) and hepatic activity and mRNA of triiodothyronine deiodinase (5'-DI) and LDLr activity, and hepatic *ldlr* mRNA all of which had been decreased by HCHS feeding. Thus, it was concluded by the authors of these studies that upregulation of selenium dependent 5'-DI expression led to increased T3 levels and up-regulation of LDLr activity and mRNA. In another study, the selenium dependent 5'-DI was also proposed by the authors of the study as being involved in the down-regulation of *hmgcr* mRNA by Se supplementation which was also associated with an increase in 5'-DI activity and mRNA abundance (Dhingra and Bansal, 2006a; Dhingra and Bansal, 2006b; Dhingra and Bansal, 2006c; Dhingra and Bansal, 2005). Conversely, the deficiency of Se was shown to result in decreased hepatic activity and mRNA of selenium dependent 5'-DI along with increases in TC, TG, and LDL-C (Dhingra and Bansal, 2006c; Dhingra and Bansal, 2006d; Dhingra and Bansal, 2005) and decreased LDLr activity and mRNA abundance (Dhingra and Bansal, 2006d; Dhingra and Bansal, 2005). Thus it was proposed by authors of the study that decreased LDL-C removal from blood and consequent hyperlipidemia occurs in Se deficient animals through down-regulation of *ldlr* mRNA abundance.

Table 2.7 Animal Studies Examining The Effect Of Se Status On Thyroid Status And Plasma Lipids[a]					
Study	**Se Status (ppm)**	**Effect of HCHS on Plasma Lipids, Thyroid Status and Cholesterol Metabolizing Enzymes**	**Effect of Se on Plasma Lipids**	**Effect of Se on Thyroid Status**	**Effect of Se on Hepatic mRNA or Activity of Cholesterol Metabolizing Enzymes**
Dhingra and Bansal, 2006d	Def: 0.02 Adq:0.2	↑ TC, TG ↓ T3 ↓LDLr activity ↓*ldlr* mRNA ↓5'-DI activity ↓5'-DI mRNA	Se def ↑ TC, TG	Se def :↓T3↓5'-DI activity↓5'-DI mRNA	Se def :↓ LDLr activity;↓ *ldlr* mRNA
Dhingra and Bansal, 2006a	Suppl: 1 Adq:0.2	↑ TC, LDL-C ↓ T3 ↓5'-DI activity ↓5'-DI mRNA ↓*hmgcr* mRNA	Se suppl ↓ TC, LDL-C	Se suppl : ↑T3 ↑5'-DI activity↑5'-DI mRNA	Se suppl : ↓ *hmgcr* mRNA

Dhingra and Bansal, 2005	Suppl: 1 Def: 0.02 Adq: 0.2	↑ TC, LDL-C ↓ T3 ↓LDLr activity ↓*ldlr* mRNA ↓5'-DI activity ↓5'-DI mRNA	Se def ↑ TC, LDL-C Se suppl ↓ TC, LDL-C	Se def :↓T3↓5'-DI activity↓5'-DI Mrna Se suppl : ↑T3 ↑5'-DI activity↑5'-DI mRNA	Se def :↓ LDLr activity; ↓ *ldlr* mRNA Se suppl :↑LDLr activity; ↑*ldlr* mRNA
Dhingra and Bansal, 2006c	Suppl: 1 Def: 0.02 Adq:0.2	↑ TC, TG ↓ T3 ↓5'-DI activity ↓5'-DI mRNA	Se def ↑ TC, TG Se suppl ↓ TC, TG	Se def :↓T3;↓5'-DI activity;↓5'-DI mRNA Se suppl :↑T3,5'-DI activity, 5'-DI mRNA	None measured

Dhingra and Bansal, 2006b	Suppl: 1 Adq:0.2	↑ TC, LDL-C ↓ T3 ↓LDLr activity ↓*ldlr* mRNA ↓5'-DI activity ↓5'-DI mRNA	Se suppl : ↓ TC, LDL-C	Se suppl : ↑ T3; ↑5'-DI activity; ↑5'-DI mRNA	Se suppl :↑LDLr activity;↑*ldlr* mRNA

[a]**ABBREVIATIONS:** Suppl, supplemented level; Def, deficient level; Adq, adequate level; Se, selenium; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; T4, thyroxine; T3,triiodothyronine ; LDLr, low density lipoprotein receptor; mRNA, messenger ribonucleic acid; 5'-DI,Type-I 5'-iodothyronine deiodinase ; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase ; HCHS, high cholesterol and/or high saturated fat diets. All diets are: 6.67% corn oil (normal) + 2% Cholesterol, selenium as sodium selenite, all animals are male Sprague-Dawley rats. ↓ indicates a decrease; ↑ indicates an increase.

2.3.3.4 Evidence to Suggest Mechanism 3: LXR Pathway and Se

The activation of the LXR is an important in vivo mechanism by which the body rids itself of excess cholesterol (Repa and Mangelsdorf, 2000; Kruit et al., 2006). Although this mechanism has not been studied in relation to Se status, the supplementation of Se has been shown to be involved with various effects that could be associated with the LXR pathway. For example, Se supplementation is associated with a lowering of hepatic concentrations of cholesterol in rats fed the high cholesterol and cholic acid diets (Iizuka et al., 2001). Lowered hepatic levels of cholesterol are associated with over -expression of the LXR responsive genes ABCG5 and ABCG8 that secrete cholesterol into bile and into feces, thereby decreasing hepatic cholesterol levels (Yu et al., 2002a; Wilund et al., 2004). Several other studies have found an association between Se status and known LXR responsive genes **(Table 2.8)** such as FAS (Iizuka et al., 2001; Berg et al., 1995) and LPL (Chidambaram et al., 1995). For example, administration of Se was associated with increased hepatic activity of FAS as in rats (Iizuka et al., 2001, Berg et al., 1995). Moroever, Se supplementation increases hepatic cytochrome P450 content (Wojicki et al., 1991). As certain specific cytochrome P450 hydroxylase enzymes are responsible for the formation of specific oxysterols, Se might increase oxysterol generation via this pathway to influence the LXR pathway. Alternatively, Se may protect enzymatically formed oxysterols from destruction (Huang et al., 2002), thereby enhancing their regulatory effects.

Table 2.8 Effect Of Se On Parameters Associated With LXR Pathway[a]		
Study	**Effect of Se on Measured Parameter**	**Association with LXR pathway**
Wojicki et al., 1991	Increased content of cytochrome P-450 in liver microsomes.	Specific P-450 enzymes synthesize the naturally occurring oxysterols which are ligands of LXR.
Berg et al., 1995	Increased mRNA and activity of Fatty Acid Sythase.	Fatty Acid Synthase is increased by increase in SREBP-1c which is directly upregulated by LXR through LXRE
Chhidambaram and Baradarajan, 1995	Increased activity of total lipases.	LPL is directly upregulated through LXRE
Iizuka et al., 2001	Increased activity of Fatty Acid Sythase.	Fatty Acid Synthase is increased by increase in SREBP-1c which is directly upregulated by LXR through LXRE
[a]**ABBREVIATIONS:** Se, selenium; LXR, liver X receptor; LXRE, liver X receptor response element; SREBP-1c, Sterol regulatory element binding protein 1c; LPL,lipoprotein lipase; mRNA, messenger ribonucleic acid.		

2.4 The Metabolism of LDL-C and Association with the LXR Pathway

2.4.1 Cholesterol Homeostasis

Cholesterol is essential for mammalian life. Low levels of cellular cholesterol are brought back to homeostatic levels by the transcription factor sterol regulatory element binding protein-2 (SREBP-2) which enters the nucleus and binds to a sterol regulatory element in the promoter regions of the genes that encode the LDL receptor and HMG-CoA reductase (Brown and Goldstein, 1997). When levels of cholesterol are sufficient, the presence of cholesterol and oxysterols, which are oxygenated derivatives of cholesterol, suppress the movement of SREBP-2 into the nucleus (Bjorkhem, 2002). Conversely, an excess of cholesterol, that can be harmful to cells and tissues, is lowered with the LXRs which function in a feed forward mechanism to lower levels of cholesterol through its catabolic degradation (Peet et al., 1998; Repa and Mangelsdorf, 2002). The absorption of cholesterol is also limited through activation of the LXR pathway (Yu et al., 2003; Plosch et al., 2004).

Cholesterol enters the intestinal lumen from three sources: diet, bile and intestinal epithelial sloughing. The entire length of the small intestine shows the capability of absorbing cholesterol from the lumen, however, the main sites of absorption are the duodenum and proximal jejunum (Wang, 2007). The absorption of cholesterol is defined as the uptake of cholesterol from the intestinal lumen into the thoracic duct lymph (Wang, 2007). It is a selective process in that plant and shellfish sterols are absorbed poorly if at all (Lammert and Wang, 2005). The cholesterol found in the intestinal lumen is nearly insoluble in the aqueous environment of the intestinal lumen and requires the solubilizing properties of bile salt solutions (Lammert and Wang, 2005). Bile salts are amphipathic detergents which spontaneously form simple micelles (aggregates) when present above a certain threshold concentration (Chiang, 1998). The addition of phospholipids or monoacylglyerols to bile salt solutions

augments the solubility of cholesterol by forming mixed micelles (Chiang, 1998). Cholesterol and bile salts which escape intestinal re-absorption are excreted as fecal neutral and acidic sterols and this represents a major route for sterol elimination from the body.

The hepatobiliary elimination of cholesterol into bile has been shown to be mediated, at least partially, by the hepatic half-transporters ABCG5 and ABCG8 (Yu et al., 2002a; Yu et al., 2002). The transporters *abcg5* and *abcg8* are responsible for secreting cholesterol into the lumen and *npc1l1* is criticial for intestinal cholesterol uptake (Altmann et al., 2004). Over-expression of *abcg5* and *abcg8* has been shown to lower the absorption of cholesterol by increasing fecal concentrations of cholesterol (Wilund et al., 2004). Similarly, treatment of mice with LXR agonists (Duval et al., 2006) or dietary cholesterol (Davis et al., 2004) has been shown to down-regulate the expression of NPC1L1 which also increased the concentrations of cholesterol in the feces. Thus, the combined regulatory effects of NPC1L1, ABCG5 and ABCG8 at the level of the gut may play critical roles in modulating the amount of cholesterol that reaches the lymph from the intestinal lumen. The absorption efficiency of cholesterol is most likely determined by the net effect between efflux and influx of intraluminal cholesterol molecules across the brush border of the enterocyte (Lammert and Wang, 2005).

2.4.2 The Liver X Receptor, LXR

The liver X receptor (LXR) is a transcription factor important in the regulation of cholesterol metabolism (Kruit et al., 2006). Two LXR isoforms have been identified, LXRα and LXRβ. LXRα is the best characterized and is expressed in high amounts in tissues which are known to play important roles in lipid metabolism (Repa and Mangelsdorf, 2000). The highest levels are found in the liver, with lower but significant levels present in the small intestine, macrophage, kidney, spleen, and adipose tissue (Repa and Mangelsdorf, 2000). LXRβ is ubiquitously expressed, including the liver and brain.

The creation of the LXRα-null mouse was critical for understanding the role of LXR in cholesterol homeostasis. The genes identified as being involved with LXR point to a role of LXR in reverse cholesterol transport which leads to the lowering of total body cholesterol (Peet et al., 1998). Mice carrying a targeted disruption of *Lxrα* failed to induce transcription of *cyp7a1* in response to dietary cholesterol, implicating LXRα in the control of bile acid synthesis (Peet et al., 1998). In the same study mice lacking LXRα were shown to be deficient in expression of lipid metabolizing genes including FAS and SREBP-1c suggesting a role for LXRα in lipogenesis. Also, loss of LXRα was shown to lead to increases in hepatic cholesterol concentrations and plasma LDL-C as compared to control mice (Peet et al., 1998). Thus, it was shown that interventions which accelerate the conversion of cholesterol to bile salts reduce plasma LDL-C concentrations. The cholesterol which is diverted into bile acid synthesis depletes the free cholesterol (FC) within the hepatocyte and triggers a compensatory increase in de novo cholesterol synthesis and induction of the LDL-C receptor pathway which leads to an enhanced clearance of LDL-C from plasma (Spady, 1992).

Thus, murine *cyp7a1* was the first LXRα responsive gene to be identified. LXRα agonists however are not expected to promote dietary cholesterol induced changes in bile acid synthesis in the human as the LXR-response element (LXRE) as found in the murine model is not conserved in the promoter of the human CYP7A1 gene and it shows only subtle sterol-mediated up-regulation (Taniguchi et al., 1994). The hamster *cyp7a1* gene also shows resistance to LXR-mediated up-regulation which is due to unknown factors (Repa and Mangelsdorf, 2000)

A liver X responsive element (LXRE) has been identified in the 5'-flanking region of the following genes which are directly up-regulated by LXR: LXRα, CYP7a1 (murine), ATP-binding cassette transporter A1, ATP-binding cassette transporter G1, Cholesteryl ester transfer protein (CETP), phospholipid transfer protein, apolipoprotein E, SREBP-1c, and lipoprotein lipase (LPL) (Michael et al., 2005). The ABCG5 and ABCG8 genes are shown to be up-regulated by LXR

agonists and cholesterol feeding without identification of a LXRE in their 5'-flanking region and so the mechanism of regulation is unknown (Michael et al., 2005). Also, a LXRE in the promoter of NPC1L1 has not yet been identified (Huff et al., 2006) although the gene has been shown to be down-regulated by LXR agonists and cholesterol feeding (Davis et al., 2004; Duval et al., 2006). Recently, a LXRE was identified in the human LDLR gene (Ishimoto et al., 2006). However, the rodent *ldlr* LXRE has not yet been identified and there may be species differences between human and rodents.

LXR binds with 9-cis retinoic acid receptor (RXR) and forms an obligate heterodimer which enables it to bind to a ligand. The LXR/RXR-ligand moves into the nucleus where it recognizes a specific LXR/RXR response element (LXRE) in the promoter of a target gene (Repa and Mangelsdorf, 2000). Known LXR ligands include the naturally occurring oxysterols which are oxygenated derivatives of cholesterol formed enzymatically.

2.4.3 Oxysterols

The role of naturally occurring oxysterols in the control of cholesterol homeostasis is suggested to occur through a feed back effect via down regulation of cholesterol synthesis (Bjorkhem, 2002) and most importantly, through a feed forward effect as ligands of LXR (Janowski et al., 1999; Lehmann et al., 1997; Forman et al., 1997; Spencer et al., 2001) the activation of which induces enzymes important in the removal of cholesterol from the body (Bjorkhem, 2002; Edwards et al., 2002). Reported mean levels of oxysterols of biomedical interest as found in plasma in normal human subjects range between 0.010 - 0.100 μM (Schroepfer, 2000).

The naturally occurring oxysterols 24(S), 25-epoxycholesterol (24,25-EC), 22(R)-hydroxycholesterol (22(R)-OHC) and 24(S)-hydroxycholesterol (24(S)-OH) were demonstrated to be the most potent in vitro ligands for LXRS (Janowski et al., 1996; Lehmann et al., 1997; Forman et al., 1997; Spencer et al., 2001),

whereas 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) were considered to be weaker ligands of LXR (Lehmann et al., 1997; Forman et al., 1997; Spencer et al., 2001). In contrast, 7-ketocholesterol (7-Keto) and 7-hydroxycholesterol (7-OHC) were not found to bind with high affinity to LXR in vitro which suggested that they do not regulate LXR in vivo (Janowski et al., 1996; Lehmann et al., 1997; Forman et al., 1997).

Cholesterol was also tested and found not to bind to LXR (Janowski et al., 1996; Lehmann et al., 1997; Forman et al., 1997). Regardless, elevated levels of dietary cholesterol are shown to result in an increased expression of LXR responsive genes (Berge et al., 2000; Repa et al., 2002; Yu et al., 2002a; Plosch et al., 2006). Thus it has been suggested that cholesterol is metabolized in various tissues to oxysterols that subsequently activate LXR, resulting in increased expression of LXR-responsive genes (Edwards et al., 2002). In support, several studies have demonstrated an increase in hepatic formation of oxysterols with animals fed cholesterol (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2003). Levels of hepatic 7-OHC, 24(S)-OHC, and 27-OHC were shown to increase in livers of rats fed cholesterol as compared to control rats (Lund et al., 1992; Saucier et al., 1989). A 3-fold increase in 24(S), 25-EC, 4-fold increase in 24-OHC, 10-fold increase in 25-OHC and no change in 27-OHC was shown to result in the liver of rats fed an atherogenic diet (Zhang et al., 2001).

Although oxysterols have been shown to be increased in vivo with cholesterol feeding, the association between oxysterols and LXR responsive genes has not been greatly studied in animal models. Only one study showed an association between increased hepatic oxysterol concentrations of 22(R)-OHC, 24(S)-OHC, 25-OHC and 27-OHC and increased expression of hepatic expression of ABCA1 and LPL mRNA (Xu et al., 2003). In another study Syrian hamsters were fed 27-OHC without examining for its association with LXR responsive genes (Souidi et al., 2003).

There is more evidence in cell culture studies linking oxysterols and LXR-responsive genes, however oxysterols were added to the medium and not

measured for subsequent to cholesterol loading (Sparrow et al., 2002; Venkateswaran et al., 2000; Fu et al., 2001; Wong et al., 2007; Rowe et al., 2003) **(Table 2.9)**. Only one study examined for the endogenous synthesis of oxysterols after cholesterol loading. In that study, cholesterol loading, in human macrophages was shown to result in a dose response increase in the formation of 27-OHC with an absence of 20-OHC, 22-OHC and 24(S), 25-EC (Fu et al., 2001). In the same study endogenously formed 27-OHC was a potent activator of LXR target genes (Fu et al., 2001) which suggests that 27-OHC could be a potent LXR ligand in vivo.

Table 2.9 In Vitro Studies Relating An Oxysterol With An LXR Responsive Gene[a]				
Study	**Cell Assayed**	**Oxysterol**	**LXR responsive gene studied**	**Effect of oxysterol on LXR-responsive gene**
Sparrow et al., 2002	THP-1 macrophages	22-OHC added to the medium.	abca1	22-OHC increased abca1 levels but not as much as synthetic ligand.
Sparrow et al., 2002	Human Macrophages	22-OHC, 25-OHC and 27-OHC added to medium.	abca1, abcg1, srebp-1c	Oxysterols were not as effective as synthetic ligand. 22-OHC was more effective than 27-OHC.
Venkateswaran et al., 2000	Murine macrophage	22(R)-OHC,22(S)-OHC,25-OHC,20(S)-OHC added to medium.	*abc8*	22(S)-OHC = 5 fold increase in abc8. 20(S)-OHC, 22(R)-OHC, and 25-OHC = 15 fold increase in abc8.
Venkateswaran et al., 2000	Human macrophage	22(R)-OHC,22(S)-OHC,25-OHC,20(S)-OHC added to medium.	Mammalian White[b]	22(S)-OHC= no effect, all others were stimulatory on white mRNA.

Fu et al., 2001	Human embryonic kidney cells	22-OHC and 27-OHC added to medium.	abca1, abcg1,srebp1-c	22(S)-OHC better than 27-OHC, but both increase levels of all genes vs control.
Fu et al., 2001	Human monocyte derived macrophages	Cells were cholesterol loaded and oxysterols measured: 27-OHC found, but no 20(S)-OHC, 22(R)-OHC, or 24(S), 25-EC.	abca1, abcg1	Both abca1, and abcg1 were increased by cholesterol and 27-OHC levels.
Wong et al., 2007	Chinese Hamster Ovary -7	24(S), 25-EC was added to medium. When cells were loaded with cholesterol, there was no 24(S), 25-EC found.	abca1	24(S), 25-EC increased abca1.
Rowe et al., 2003	J774A.1 macrophages	24(S), 25-EC was added to medium.	srebp-1c , abca1, abcg1 mRNA	24(S), 25-EC increased all genes vs control.

[a]**ABBREVIATIONS:** abca1, ATP-binding cassette (ABC) transporter a1; abcg1, ATP-binding cassette (ABC) transporter g1; abc8, ATP-binding cassette (ABC) transporter 8; SREBP-1c, sterol regulatory binding protein 1c; mRNA, messenger ribonucleic acid; 24(S), 25-EC, 24 (S),25 epoxycholesterol; 22-OHC, 22-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol. [b] A member of the ATP-binding cassette (ABC) transporter family.

2.4.3.1 Conclusion to Oxysterols

Although the naturally occurring oxysterols 22-OHC, 24(S)-OHC and 24(S), 25-EC were considered to be the most potent LXR ligands (Janowski et al., 1999; Lehmann et al., 1997; Forman et al., 1997; Spencer et al., 2001), the oxysterol 24(S), 25-EC is not derived from cholesterol but is formed de novo in the mevalonate pathway which simultaneously synthesizes cholesterol (Nelson et al., 1981). Thus, an increase in cholesterol accumulation which occurs with a high cholesterol diet would not be expected to lead to an increase in 24(S),25-EC levels (Bjorkhem and Diczfalusy, 2004). In support, cholesterol loading was shown to inhibit 24(S), 25-EC synthesis (Wong et al., 2007). Also, the supplementation of cholesterol was shown to result in a significant increase in hepatic oxysterol concentrations of 22(R)-OHC, 24(S)-OHC, 25-OHC and 27-OHC without showing an effect on 24(S), 25-EC in comparison to control diet fed rats (Xu et al., 2003). Additionally, in regards to the other strong LXR ligand 22(R)-OHC, which is formed as a transient intermediate in steroid hormone synthesis, its distribution is limited to the organs producing steroid hormones (Burstein, 1971). Duan et al. (2004) did not find an effect of feeding 22(R)-OHC or its isoform 22(S)-OHC on abundance of *lxrα*, *lxrβ*, *abcg5* or *abcg8* in the small intestines of either AKR and C57L mice whereas an effect on the genes was shown with consumption of a synthetic LXR ligand (Duan et al., 2004).

The weaker in vitro LXR ligands 25-OHC and 27-OHC have also been studied and the evidence supporting a role for them as in vivo ligands remains inconclusive. For example, in a previous work, the measurement of oxysterols in hepatic subcellular compartments found both 24(S),25-EC and 24(S)-OHC in the nuclei whereas 27-OHC and 25-OHC were not, suggesting that the latter oxysterols may not be important as in vivo ligands of LXR (Zhang et al., 2001). In that study, however, animals were not fed a high cholesterol diet. Hepatic levels of oxysterols which have been raised by cholesterol feeding in animals make the oxysterols measured the leading candidates as physiological regulators

of LXR in the liver. More recently, triple-knockout mice which do not synthesize 24(S)-OHC, 25-OHC and 27-OHC were fed a high cholesterol diet and were shown to fail to induce hepatic mRNA abundance of LPL, *abcg5*, and *abcg8* which implicates these three oxysterols as in vivo ligands for LXR in conjunction with a HCHS diet (Chen et al., 2007).

2.4.4 Reverse Cholesterol Transport and Bile Acids

Reverse cholesterol transport, which is the process by which excess cholesterol in peripheral tissues is returned to the liver for excretion, occurs in three major steps (Groen et al., 2004). Firstly, the free cholesterol (FC) which is taken up by tissue-based macrophages or that which is found in peripheral cells is moved to HDL-C through the transporters ABCA1 and ABCG1. In the presence of apolipoprotein A-I, a portion of the FC is esterified by lysolecithin cholesterol acyltransferase and moves into the hydrophobic core of the HDL particle (Oram and Yokoyama, 1996). In exchange for TG, CETP mediates the transfer of CE found in the HDL-C particle to the VLDL-C remnant and LDL-C which are ultimately cleared by the liver through the LDL receptor (Oliveira et al., 1997). CETP which is found in primates and hamsters, but not in rats or mice (Tsutsumi et al., 2001; Ha and Barter, 1982), is secreted by the liver and circulates in the plasma bound principally to the HDL-C (Barter, 2000). Secondly, HDL-C is taken up by the liver mainly through scavenger receptor-BI (Tontonoz and Mangelsdorf, 2003). The final step in the reverse cholesterol transport pathway includes the secretion of cholesterol from the liver into bile, either as cholesterol itself or as the cholesterol metabolic breakdown product, bile acid, and subsequent delivery to the intestine for excretion in feces.

The conversion of cholesterol into bile acids occurs via either the classic or one of three alternative pathways (Bjorkhem et al., 1999). In the classic pathway, the hydroxylation of cholesterol is initiated by the microsomal cytochrome P-450 enzyme cholesterol 7α-hydroxylase (CYP7A1) which is the

initial and rate-limiting enzyme in the major bile salt biosynthetic pathway and is specific to the liver (Chiang, 1998). The activity of CYP7A1 produces 7-OHC. The major bile acids produced from this pathway are cholic acid (CA) and chenodeoxycholesterololic acid (CDCA) which are formed in the liver in a sequence of 14 enzymatic reactions (Chiang, 2002). The three alternative pathways of bile acid biosynthesis involve the conversion of cholesterol first to oxysterols by specific hydroxylases (Pikuleva, 2006). In peripheral tissues oxysterols are able to pass lipophilic membranes much more quickly than does cholesterol itself which permits their degradation and excretion (Bjorkhem, 2002). In the liver and extrahepatic tissues two hydroxylases CYP27A1 and 25-hydroxylase convert cholesterol into 27-OHC or 25-OHC respectively. The 25-hydroxylase enzyme is not a cytochrome P-450 enzyme but belongs to a smaller family of non-heme iron-containing proteins (Bjorkhem, 2002). In the brain the 24(S)-hydroxylase converts cholesterol into 24(S)-OHC (Pikuleva, 2006). As a consequence, there is a continuous flow of oxysterols from extrahepatic organs to the liver where the metabolites are further metabolized into bile acids. The mechanism of secretion of oxysterols from peripheral tissues can be regarded as an alternative to the classical reverse cholesterol transport mediated by ABCA1 and HDL-C (Pikuleva, 2006). The predominant oxysterols in the circulation are 7-OHC, 24(S)-OHC and 27-OHC which make up 75% of oxysterol concentration in the healthy human (Bjorkhem et al., 1999). The oxysterol 25-OHC is present in very low concentrations in the circulation and in tissues (Bjorkhem et al., 1999).

Due to the broad distribution of CYP27A1 in various tissues, 27-OHC has been proposed to be involved in the regulation of lipid metabolism (Bjorkhem, 2002), either by direct intracellular modulation of cholesterol biosynthesis or by acting as ligands for the LXR (Fu et al., 2001). The oxysterol 27-OHC is transported with LDL-C but mainly with HDL-C (Diczfalusy et al., 1996). The oxysterols formed in peripheral tissues are secreted into the circulation and end up in the liver where they are hydroxylated into either cholic acid (CA) or chenodeoxycholic acid (CDCA) (Chiang, 2002).

2.4.5 ABCG5/ABCG8, and NPC1L1 - In Vivo Studies

Complete mutation in either ABCG5 or ABCG8 alone is sufficient to cause a disease in humans called sitosterolemia which is characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia, and premature atherosclerosis (Berge et al., 2000). Individuals affected with sitosterolemia show mutations in either ABCG5 or ABCG8 but not both and based on this fact, the genes are hypothesized to function as obligate heterodimers. Furthermore, each is a half-transporter made up of six transmembrane domains and not twelve which is the number of transmembranes found in the classical functional ATP-binding Cassette (ABC) transporter. However, no functional assay has been developed to demonstrate whether or not the two half-transporters function as homo- or heterodimers and whether they selectively pump sterols.

A study which used human tissues to determine the localization of ABCG5 and ABCG8 in liver, gall bladder and intestine confirmed that ABCG5 and ABCG8 may have functions independent of each other (Klett et al., 2004a). Although the distribution of ABCG5 and ABCG8 was compatible with both proteins forming a heterodimer, in fractional studies, the authors noted some fractions where only one of the proteins and not the other was detected which supported the notion that these proteins may have independent functions. A subsequent study in animals confirmed these findings as *abcg5* was shown to be apically expressed despite the absence of *abcg8* in *abcg8* -/- knock out mice (Klett et al., 2004). Also, residual cholesterol secretion into bile is still observed in complete knockout mice (*abcg5/abcg8-/-*), and diosgenin-induced hypersecretion of cholesterol into bile in mice occurs in the absence of *abcg5/abcg8* induction. Thus, it appears that hepatobiliary cholesterol secretion can occur by both *abcg5/abcg8*-dependent and –independent routes. The findings of a study supported the notion that *abcg5/abcg8* has a rate-controlling function for the majority of hepatobiliary cholesterol transport in mice under basal conditions

however, under stressed conditions such as cholesterol feeding, cholesterol may reach the bile via a *abcg5/abcg8* independent route (Plosch et al., 2006).

Cholesterol absorption was shown to be mainly regulated by the jejunal and ileal *abcg5* and *abcg8* expression in mice (Duan et al., 2004). Six murine strains were examined for relative mRNA for duodenal, jejunal and ileal *abcg5* and *abcg8* and efficiency of cholesterol absorption. Cholesterol absorption was determined by the fecal dual-isotope ratio method which involves the investigation of the relationship between food intake, fecal output and fecal excretion of radioisotopes after a single dose of radioisotopes administered by gavage. Marked differences were observed among mouse strains with respect to intestinal cholesterol absorption efficiency. The strains showing the lowest cholesterol absorption showed the highest abundance of the jejunal and ileal *abcg5* and *abcg8*, and the strains showing the highest cholesterol absorption showed the lowest abundance of the transporters in the jejunum and ileum. Feeding 0.5% or higher dietary cholesterol significantly increased abundance of *abcg5* and *abcg8* in the jejunum and ileum but not in the duodenum which was associated with increased fecal neutral sterol excretion. Feeding 22(R)-OHC and its isoform 22(S)-OHC did not influence relative mRNA abundance for *lxrα*, *abcg5* or *abcg8* in the small intestines of these mice strains.

In several studies, cholesterol feeding was shown to result in coordinate increases in duodenal, jejunal and hepatic expression of murine *abcg5* and *abcg8* mRNA (Berge et al., 2000; Repa et al., 2002; Yu et al., 2002a). Regulation of the expression of the genes through LXR was confirmed with the use of an LXR null mouse treated with an LXR agonist (Repa et al., 2002). In a separate study cholesterol was identified as the primary transport substrate of ABCG5/ABCG8 (Yu et al., 2002a). The purpose of the next study was to characterize the expression and regulation of *abcg5* and *abcg8* in male and female Sprague-Dawley rats and compare their expression patterns to those observed in the murine model used the most, to date, to characterize the LXR genes active in cholesterol homeostasis (Dieter et al., 2004). The highest expression of *abcg5* and *abcg8* was shown to occur in the small intestine

(duodenum=jejunum> ileum) and liver of the rat as compared to other tissues which showed minimal or undetectable expression. Unexpectedly, in the rat liver, cholesterol feeding decreased *abcg5* mRNA levels approximately by 75% and *abcg8* mRNA was repressed by approximately 90%. The experiment was repeated with mice and cholesterol was found to cause an increase in liver mRNA for both *abcg5* and *abcg8* in this animal model. Furthermore, only minor changes in *abcg5* and *abcg8* were shown in the small intestine of the mouse after cholesterol feeding. The findings of the study thus pointed to a variation between rats and mice in regulatory mechanisms controlling *abcg5* and *abcg8* expression, and which might explain the differences in lipid metabolism observed between these two species of rodents.

Similar to humans, in mice, the disruption of both *abcg5* and *abcg8* in liver and intestine was associated with a reduction of biliary cholesterol, increase in hepatic levels of cholesterol and a 2.4 fold increase in plasma cholesterol levels (Yu et al., 2002). Conversely, the over-expression of human ABCG5 and ABCG8 in mice which is expressed in both liver and intestine was able to attenuate the increases in hepatic cholesterol when the animals were fed a high cholesterol diet (Yu et al., 2002a). On the other hand, over-expression of human ABCG5 and ABCG8 in only the liver was not enough to facilitate the removal of cholesterol from the liver over that of control mice (Wu et al., 2004). Thus, hepatic cholesterol concentrations remained unchanged and compensatory changes in liver enzymes or in hepatic receptors were not observed and plasma lipids were not changed. It was shown in the study that the increased biliary cholesterol secreted by the liver due to the over-expression of human ABCG5 and ABCG8 was reabsorbed by the intestine rather than excreted in the feces. In conclusion, the findings of the study identified distinct roles for human liver and intestinal ABCG5 and ABCG8 in modulation of sterol murine metabolism.

The aim of the subsequent study was to determine whether over-expression of human ABCG5 and ABCG8 in both the liver and intestine could protect *ldlr* null mice against dietary hypercholesterolemia (Wilund et al., 2004). Plasma cholesterol concentrations were 45% lower and liver cholesterol levels

were 30% lower in the *ldlr* -/- mice over-expressing human ABCG5 and ABCG8 compared with *ldlr* -/- mice. A significant reduction in fractional absorption of cholesterol and increase in fecal neutral sterol levels was noted in Western fed mice over-expressing human ABCG5 and ABCG8. Finally, biliary cholesterol levels were also shown to be increased in the bile of the Western fed mice with over-expression of the genes. Thus, in summary, the findings of this study indicated that over-expression of human ABCG5 and ABCG8 in both liver and intestine may attenuate diet-induced hypercholesterolemia. In a similar vein, the relative contribution of hepatic vs intestinal ABCG5/G8 on sterol excretion (Basso et al., 2007) was studied. In order to assess whether an increase in biliary cholesterol secretion and also a decreased intestinal absorption are required for hepatic ABCG5/G8 to decrease liver cholesterol concentrations and to trigger compensatory mechanisms which reduce plasma cholesterol levels, mice over-expressing human ABCG5/G8 in liver (ABCG5/G8-Transgenic) were crossed with *ldlr* knock out mice which generated ABCG5/G8-transgenic X *ldlr*-knock outs which were compared in the study with *ldlr*-knock out mice. Mice were fed a Western diet and treated or not with ezetimibe which prevents cholesterol absorption by blocking the activity of the NPC1L1 transporter. Overall, the findings of the study indicated that in addition to enhanced biliary secretion, a reduction in intestinal cholesterol absorption is required for ABCG5/ABCG8 to have an antiatherogenic effect. The blocking of reabsorption of biliary cholesterol secreted in the ABCG5/ABCG8 over-expressing mice resulted in more fecal cholesterol excretion and a negative hepatic cholesterol balance as demonstrated by significant decreases in plasma lipids in *ldlr*-knock out mice over-expressing ABCG5/ABCG8.

Evidence in support of an in vivo role for NPC1L1 for cholesterol absorption was first acquired from animal studies using the *npc1l1* null (-/-) mouse. It was shown that *npc1l1* null mice fed either a normal fat diet or a diet supplemented with 0.1% cholic acid showed 70 % less absorption of cholesterol than wild-type mice fed the same diet (Altmann et al. 2004). Also, in the same study, ezetimibe had no effect in *npc1l1* knockout mice, suggesting that *npc1l1*

resides in an ezetimibe-sensitive pathway responsible for intestinal cholesterol absorption. Thus, the findings of this study indicated that *npc1l1* is critical for the uptake of cholesterol across the plasma membrane of the intestinal enterocyte. Despite reduced cholesterol absorption rate, the *npc1l1* knock out mice showed comparable plasma lipid levels to the wild type mice.

In a separate study *npc1l1* was also shown to be important for the uptake of plant sterols as *npc1l1* null mice fed chow diet showed a 90% reduction in phytosterol absorption as compared to wild-type mice fed the same diet (Davis et al., 2004). Also, in the same study, the absence of *npc1l1* was shown to prevent the elevation of VLDL-C and LDL-C in the plasma of *npc1l1* null mice as compared to wild type mice fed a similar diet. Feeding a HCHS diet caused a 7-fold increase in hepatic CE content compared to chow fed wild-type mice which was shown to be significantly decreased in the livers of *npc1l1* null mice. In this study, the absence of *npc1l1* was shown to prevent the elevation of hepatic cholesteryl ester content and plasma VLDL-C and LDL-C in mice consuming a 1% cholesterol and 0.5% cholic acid diet for 7 days as compared to wild type mice consuming the same diet. In summary, the study showed that *npc1l1* is required for intestinal uptake of both cholesterol and phytosterols and plays a major role in cholesterol homeostasis.

The location and tissue expression of NPC1L1 in human and *npc1l1* in mouse tissues were determined (Davies et al., 2005). It was found that in human tissues NPC1L1 is predominantly expressed in liver with detectable levels in lung, heart, brain, pancreas, and kidney. Human NPC1L1 is also expressed in the small intestine at 1-4% of the levels expressed in the liver. The expression of NPC1L1 was also measured in HepG2 (hepatic), and Caco-2 (intestinal) cell lines, and was shown to be in agreement with results of tissue expression. NPC1L1 was expressed at higher levels in the HepG2 cells than in the Caco-2 cells. Additionally, growth of HepG2 cells in the presence of LDL-C caused an increase in the expression of NPC1L1. In order to determine where in the cells NPC1L1 is located, staining of HepG2 cells showed that NPC1L1, rather than residing at the plasma membrane, was predominantly found to reside in internal

membranes. In contrast, mouse *npc1l1* was predominantly expressed in the small intestine and was not highly expressed in the liver. Expression of *npc1l1* was also noted in other tissues such as pancreas, kidney, lung and heart, indicating that *npc1l1* does not only function in the small intestine. To further characterize the role of *npc1l1* in lipid transport *npc1l1* null mice were generated and mouse fibroblasts isolated from them. Studies revealed marked defects in the transport of a number of lipids including cholesterol and sphingolipids in cells immortalized from *npc1l1* null mice.

In the same study, both wild-type and *npc1l1* null mice were fed a high cholesterol diet and their plasma analyzed for lipid levels. Wild-type mice consuming the high cholesterol diet showed an increase in serum TC, LDL-C and HDL-C and a decrease in TG as compared to *npc1l1* null mice fed the high cholesterol diet. Unexpectedly, histochemical analysis of liver tissues from wild-type mice on the high fat diet showed that these animals had fat-laden livers whereas livers from *npc1l1* null mice had resisted the diet-induced fatty liver. Also, gallbladders from wild-type and *npc1l1* null mice were dramatically different with wild-type mice gallbladders showing they were cholestatic whereas *npc1l1* null mice gallbladders appeared normal. Thus, the results of the study revealed that lack of *npc1l1* exerted a protective effect against diet-induced hyperlipidemia.

Duval et al. (2006) investigated the effect of peroxisome proliferator-activated receptor alpha (PPARα) or LXR activation on *npc1l1* gene expression in the intestine of female Apolipoprotein E2-knock out mice (Duval et al., 2006). Mice fed a Western diet high in fat and cholesterol for 2 months were randomly assigned to receive an LXR agonist (n=4) by oral gavage or vehicle(n=5) during 5 days after which time plasma was collected for the analysis of plasma lipids, and duodenum was isolated for measurement of mRNA of *npc1l1* expression. Results showed a strongly decreased gene expression of duodenal *npc1l1* after treatment with the LXR agonist. The expression of FAS mRNA in liver was also increased by LXR agonist treatment. Plasma concentrations of TC were decreased and TG increased. The gene expression of *npc1l1* was not regulated

by the PPARα agonist. In conclusion, the study showed that *npc1l1* was an LXR target in the mouse which further supported the crucial role LXR has in intestinal cholesterol homeostasis.

2.4.7 The Syrian Hamster as a Model for Human Sterol Synthesis and In Vivo LDL-C Metabolism

In recent years the Syrian hamster has been used as a model for human sterol synthesis and LDL-C metabolism (Spady et al., 1985). Detailed information has been acquired regarding how dietary cholesterol and fatty acids interact *in vivo* to affect cholesterol synthesis and LDL-C metabolism using the Syrian hamster. In these studies it was found that the hepatic cholesterol synthesis rate of hamsters is similar to humans. Hamsters display a low rate of cholesterol synthesis and thus don't maintain hepatic cholesterol balance by decreasing de novo synthesis when faced with a high fat and cholesterol diet (Spady, 1992). Instead, hamsters when challenged with a high cholesterol diet, decrease the synthesis of LDL-C receptors. Thus, as most LDL-C uptake in the hamster occurs in the liver (around eighty percent) with more than ninety percent occurring through LDL-C receptors, high plasma LDL-C concentrations are without exception associated with a decreased clearance of LDL-C.

Most investigators examining for the effect of Se on plasma lipids have used the normal rat model which is resistant to the development of atherosclerosis and is not a suitable subject for cardiovascular research (Russell and Proctor, 2006). Unlike hamsters, rats are resistant to diet-induced hypercholesterolemia, due to a high rate of hepatic cholesterol synthesis which is decreased with consumption of a high cholesterol diet (Horton et al., 1995). Furthermore, unlike the hamster and human which both fail to induce CYP7A1 expression when challenged with a high cholesterol diet, rats stimulate CYP7A1 activity which increases bile acid synthesis (Xu et al., 2000; Horton et al., 1995; Taniguchi et al., 1994). Likewise, the murine model is also quite resistant to diet

induced hypercholesterolemia due to its capacity to induce CYP7A1 expression (Peet et al., 1998).

In regards to the examination of the effect of cholesterol and/or synthetic LXR agonists on LXR responsive genes, the murine model has been predominantly used. The mouse, similar to the rat, lacks the LXR target gene CETP which is central to human lipoprotein metabolism, and thus neither model is a reliable predictive model of the response of LXR agonists in humans. On the other hand, the hamster, similarly to the human, possesses a CETP gene, the up-regulation of which could result in a more atherogenic profile as CETP exchanges TG in apo-B containing lipoproteins for HDL-C which reduces HDL-C. Therefore the hamster is expected to be a much more representative model in regards to the human response to LXR agonists and to Se in this regard.

Finally, unlike the rat or mouse, both of which carry most of their cholesterol in the HDL-C particle, the hamster transports most of its cholesterol in the form of LDL-C, like the human (Spady and Dietschy, 1983a). Therefore, the hamster when fed a high fat diet, which also contains cholesterol, shows a human-like increase in LDL-C, VLDL-C and HDL-C thus presenting a more human-like lipoprotein profile than observed in other rodent species (Spady and Dietschy, 1988).

In studies with hamsters fed 0.12 percent cholesterol, the consumption of 20 percent saturated fatty acid as hydrogenated coconut oil was shown to increase LDL-C more than 200 percent (%) by suppressing LDL-C receptor activity to around 88% as compared to the consumption of 20% polyunsaturated fatty acid as safflower oil (Spady and Dietschy, 1988). Also, increasing levels of dietary cholesterol were shown to raise LDL-C in a dose-dependent manner when fed with a diet rich in saturated fatty acids but not with polyunsaturated fatty acids (Spady and Dietschy, 1985). Increasing amounts of dietary cholesterol progressively elevate the level of CE in the hepatocyte (Spady, 1992). In vivo studies with the hamster have shown that the LDL-C receptor number and HMGCR activity vary inversely with the CE content of the hepatocyte. An increased concentration of CE is associated with a decrease in the activity of

HMGCR and a decrease in the number of LDL-C receptors expressed on the liver plasma membrane and results in increased levels of plasma LDL-C. Levels of LDL-C in plasma are therefore regulated by the flux of cholesterol across the hepatocyte (Spady, 1992).

2.4.7.1 Syrian Hamster and LXR Pathway

The following study investigated the Syrian hamster response to two synthetic LXR agonists (Groot et al., 2005). Although the LXR is shown to be beneficial in regards to CVD, LXR activation also increases TG synthesis by stimulating lipogenesis through upregulation of SREBP-1c, an LXR responsive gene, and this makes LXR a less attractive anti-atherosclerosis target. Also, CETP, found in both hamsters and humans is a direct LXR target which could pose an additional liability for LXR agonists as CETP exchanges TG in apoB-containing lipoproteins for HDL-C which reduces HDL-C and results in a more atherogenic lipoprotein profile.

The study compared the effect of two LXR agonists on the expression of hepatic and intestinal *abcg5*, *abcg8* expression in the Syrian hamster. Expression was found to be more strongly upregulated in the small intestine as compared to the liver. In the liver, effects on *abcg5* and *abcg8* were very small and therefore not considered reliable. Additionally, hepatic *ldlr* mRNA abundance was not affected by LXR agonist treatment. In regards to plasma lipids, concentrations of LDL-C and TG were greatly enhanced by LXR agonist treatment, whereas HDL-C concentrations were slightly decreased and TC concentrations were not changed. Hepatic levels of TG were increased with one LXR agonist, but not with the other. More recently, increased expression levels of *abcg5* but only a tendency to higher levels of *abcg8* were observed in the liver of hamsters fed a hypercholesterolemic diet in comparison to untreated controls whereas the proximal small intestine did not respond (Jia et al., 2007).

van Heek et al. (2001) assessed the effect of ezetimibe which blocks *npc1l1* activity on the combined hypercholesterolemia and hypertriglyceridemia in an obese hyperinsulinemic Syrian hamster model induced by high-fat diets (van Heek et al., 2001). Ezetimbe was shown to completely block the accumulation of both hepatic CE and FC that was observed with high fat diet consumption. Also ezetimibe treatment normalized the diet-induced hypertriglyceridemia. In regards to plasma lipids, the presence of ezetimibe dramatically reduced serum cholesterol in all but the chow fed hamsters, and reduced LDL-C to chow-fed levels or below, under all dietary conditions studied. HDL-C levels were also reduced by ezetimibe but remained above chow-fed levels. In summary, the data in the study suggested that the hypercholesterolemia and hypertriglyceridemia induced by modest cholesterol and high-fat diets may be ameliorated by blocking cholesterol absorption.

Field et al. (2004) studied the Syrian hamster in order to examine if dietary cholesterol demonstrated an effect on *abcg5/8* and *npc1l1* mRNA abundance in the Syrian hamster (Field et al., 2004). Adding cholesterol to the diet increased plasma cholesterol levels by 2.5-fold (276 mg/dl) and cholesterol concentrations in intestinal cells of hamsters, notably in the jejunal and ileal cells.

The effects of the addition of cholesterol to the diet on *abcg5* and *abcg8* were modest and at times non-existent in some sections of the intestine. Adding cholesterol to the diet significantly increased mRNA levels of *abcg5* but not *abcg8* in the jejunum and ileum, and *abcg8* in the duodenum. Basal expression of *npc1l1* mRNA levels was shown to be the highest in the jejunum and lowest in the duodenum. Findings suggested that this gene is regulated differently from the ABC transporters in the hamster intestine as levels of npc1l1 were not changed by cholesterol feeding. In a separate study, hamsters demonstrated significantly lower expression of *npc1l1* in the duodenum and jejunum of hamsters fed the fish oil diets compared to animals fed the control diet or the olive oil diet (Mathur et al., 2007). Examination of basal levels of *npc1l1* showed highest levels in the ileum vs duodenum with intermediate jejunum levels. Intestinal cells treated with a PPAR-d agonist showed a modest decrease in *npc1l1* mRNA levels which was

suggested by the authors of the study to have been activated by docosahexanoic acid levels a known ligand of the PPAR-d receptor.

2.4.7.2 Se and the Syrian Hamster

The 1995 National Research Council requirement for Se in hamsters has been set at 0.15 mg/kg diet (National Research Council, 1995). Hamsters are more tolerant to Se toxicity compared to other animals. Hadjimarkos, (1970) studied the effects of chronic Se intake in weanling hamsters by administering 6, 9, or 12 ppm of selenite-Se (Hadjimarkos, 1970). After one year all hamsters were alive. The groups given 9 and 12 ppm had a ten percent weight loss compared to the control group.

Birt et al. (1983) found that the Syrian hamster tolerated a wide range of dietary Se (0.05 ppm to 5.0 ppm range) for twenty-five weeks without apparent adverse effects (Birt et al., 1983). In the same study, increased dietary Se caused a consistent rise in blood and tissue Se in the Syrian hamster. In another study, Julius et al. (1983), evaluating the level (0.25 ppm to 80 ppm range), and dietary source of dietary Se needed to produce toxicity, compared sodium selenite and selenomethionine (Julius et al., 1983). Dietary Se levels of 10 ppm and above were found to be toxic to hamsters resulting in reduced growth rate, food consumption and liver atrophy. In the same study it was found that the liver content of Se increased with increasing dietary intake regardless of source.

2.4.8 Conclusion to the Metabolism of LDL-C and Association with the LXR Pathway

In summary, in the above-mentioned studies it was demonstrated that the expression of *abcg5* and *abcg8* is up-regulated by both cholesterol feeding and LXR agonists in the mouse, which supports the notion that the genes are LXR

regulated (Berge et al., 2000; Repa et al., 2002). Additionally, cholesterol was identified as the primary transport substrate of *abcg5/abcg8* in both chow fed and cholesterol fed mice (Yu et al., 2002a). Furthermore, in the same study, the over expression of human ABCG5/ABCG8 was shown able to counter the effect of a high cholesterol diet on increasing liver cholesterol levels (Yu et al., 2002a). Another study showed that in abcg5/abcg8 null mice, a decrease in biliary cholesterol concentrations was associated with an increase in hepatic cholesterol concentrations after cholesterol feeding (Yu et al., 2002a).

Also, it was shown that an overexpression of human ABCG5 and ABCG8 in murine liver only is not sufficient to impact both liver and plasma lipid concentrations (Wu et al., 2004). A subsequent study indicated that the overexpression of both human ABCG5 and ABCG8 in both liver and intestine were necessary in order to impact both liver and plasma cholesterol levels in *ldlr* knock out mice (Wilund et al., 2004). These findings were supported by another study which showed that both an increase in biliary cholesterol and a decrease in intestinal absorption of cholesterol were shown to be required in *ldlr* knock out mice overexpressing human hepatic ABCG5 and ABCG8 and fed ezetimibe (Basso et al., 2007). Finally, at the level of the murine intestine, it was shown that jejunal and and ileal *abcg5* and *abcg8* were responsible for regulating cholesterol absorption in six strains of mice (Duan et al., 2004).

Although, the previous work showed the importance of both *abcg5* and *abcg8* in secretion of cholesterol into bile, some studies showed that it is not necessary that *abcg5* and *abcg8* function as an obligate heterodimer in order to secrete cholesterol into bile (Klett et al., 2004). Along with showing that they function as a heterodimer they were also shown to have independent effects (Klett et al., 2004a). Additionally, the findings of another study suggested that perhaps other independent pathways of cholesterol secretion into bile exist, and that abcg5/abcg8 may function under basal conditions but with high cholesterol diets, dietary cholesterol may reach bile through an independent route (Plosch et al., 2006).

The studies showed species- and tissue-specific responses of expression of *abcg5* and *abcg8* to cholesterol and LXR agonists. Cholesterol feeding was shown to be associated with different effects on the expression of *abcg5* and *abcg8* in the rat liver and intestine as compared to the mouse (Dieter et al., 2004). Most studies examining the effect of cholesterol on *abcg5* and *abcg8* have used the mouse. Little is known regarding the hamster and LXR responsive genes. One study examined the effect of two synthetic LXR agonists on hepatic *abcg5* and *abcg8* and observed no response whereas the small intestine of the same hamsters responded with higher expression levels (Groot et al., 2005). More recently, increased expression levels of *abcg5* but only a tendency to higher levels of *abcg8* were observed in the liver of hamsters fed a hypercholesterolemic diet in comparison to untreated controls whereas the proximal small intestine did not respond (Jia et al., 2007). Yet in another study, cholesterol feeding was shown to have a differential effect on the expression of *abcg5* and *abcg8* in the hamster small intestine which showed *abcg5* to be upregulated in the jejunum whereas *abcg8* was not (Field et al., 2004).

In mice, expression of *npc1l1* was shown to be down regulated *in vivo* by cholesterol feeding (Davies et al., 2005) and LXR agonist treatment (Duval et al., 2006). In the hamster, *npc1l1* was found to be expressed the highest in jejunum and lowest in the duodenum (Field et al., 2004) yet in another study was found to be expressed the highest in the ileum vs duodenum and expression levels of jejunum were in between (Mathur et al., 2007). NPC1L1 was shown to be directly involved in the absorption of cholesterol and was also shown to be involved in the absorption of phytosterols (Altmann et al., 2004; Davis et al., 2004). One study showed that *npc1l1* is expressed in tissues other than the intestine (Davies et al., 2005), and in contrast to the mouse which shows a greater expression in the intestine vs the liver, in humans NPC1L1 is found in the highest amounts in the liver vs the intestine which suggests involvement in more than cholesterol absorption. Also, in the same study it was found that a lack of *npc1l1* exerted a protective effect against diet-induced hyperlipidemia (Davies et al., 2005). This finding was consistent with the findings of a separate study conducted in the

hamster, which showed that both hypercholesterolemia and hypertriglyceridemia were ameliorated by blocking *npc1l1* through the use of ezetimibe (van Heek et al., 2001).

Similarly to expression of *abcg5* and *abcg8, npc1l1* responses were shown to be species and tissue specific. A study examining the Syrian hamster small intestine showed that *npc1l1* gene expression was not regulated by cholesterol feeding (Field et al., 2004). It was shown to be regulated by fish oil in a separate study using the hamster, which was associated with a decrease in expression of *npc1l1* (Mathur et al., 2007). The Syrian hamster is a more representative animal model of the human response to LXR agonist treatment than the mouse or rat, due to expression of CETP, and thus merits further study in this regard.

The studies reviewed above also point to the multiple regulators shown to be active in the in vivo studies on expression levels of genes involved in lipid metabolism and transport. Although the LXR pathway has been emphasized in the control of *abcg5* and *abcg8* expression other pathways do exist. Although most studies to date have examined the effect of cholesterol on expression of these LXR regulated genes, only one study has examined the effect of exogenous treatment with 22-OHC on intestinal expression of *abcg5* and *abcg8* showing no effect (Duan et al., 2004).

Most importantly, in the studies which have been reviewed, both the *abcg5/abcg8* transporters and *npc1l1* were shown to be involved in cholesterol homeostasis as they showed protective effects against diet-induced increases in liver cholesterol (Yu et al., 2002a), and their absence increased hepatic (Yu et al., 2002) and plasma cholesterol concentrations (Yu et al., 2002). A clear effect on plasma lipids was noted with increased expression of both hepatic and intestinal expression of human ABCG5 and human ABCG8 (Wilund et al., 2004). Additionally, both the over-expression of hepatic ABCG5/G8 and the absence of *npc1l1* activity through ezetimibe treatment exerted a protective effect against diet-induced increases in hepatic and plasma lipids (Basso et al., 2007).

CONNECTING STATEMENT

As described in the literature review of Chapter 2, numerous studies have demonstrated important hypocholesterolemic effects of Se in various animal models including in relation to HCHS diets. The actual mechanisms responsible for the lipid lowering effects of Se are not yet well described. Several enzymatically generated oxysterols have been shown to be important in vitro ligands of the liver X receptors (LXR) and were also shown to be increased with cholesterol feeding in in vivo studies. Additionally, animal feeding trials have demonstrated that hypercholesterolemia induced by HCHS diets can increase production of oxygen free radicals leading to increased tissue lipid peroxidation and elevated tissue concentrations of free-radical generated oxysterols that are implicated in cardiovascular disease. Thus, in the following chapter, we investigated the effect of Se and α-tocopherol on hepatic concentrations of enzymatically and free-radical generated oxysterols as well as on plasma lipid concentrations.

Thus, the following chapter refers to specific objectives 1 and 2 and hypothesis 1 described in Sections 1.3 and 1.4 of Chapter 1.

Chapter 3 - Antioxidant Supplements Lower Oxidative Stress and Plasma Lipids in Butterfat-fed Hamsters

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3.1 ABSTRACT

High cholesterol and/or high saturated fat (HCHS) diets are associated with elevated total blood cholesterol (TC), depressed antioxidant status and increased oxidative stress. In Experiment 1, effects of supplemental selenium (Se; 3.4 mg Se/kg diet) and α-tocopherol (α-Toc; 67 IU all-racemic α-tocopheryl acetate/kg diet) on blood lipids, hepatic oxysterols, antioxidant and oxidative stress status was studied using male Golden Syrian hamsters. Four groups of hamsters (n = 6 – 8) were fed semi-purified diets containing 0.46% cholesterol and 14.3% added fat (8% saturated fat): 1) HCHS; 2) HCHS + α-Toc; 3) HCHS + Se; and 4) HCHS + α-Toc + Se. Se and α-Toc supplemented hamsters exhibited decreased plasma thiobarbituric acid reactive substances and lipid hydroperoxides in heart and liver. Increased hepatic and cardiac concentrations of glutathione and higher hepatic Se-dependent glutathione peroxidase (SeGSH-Px) activity were observed in Se-supplemented hamsters. Hamsters supplemented with α-Toc showed elevated cardiac glutathione. Se and α-Toc supplements significantly lowered plasma TC. Higher hepatic 24(S)-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol concentrations were observed with Se supplementation. In Experiment 2, Se supplementation induced 27-hydroxycholesterol and total GSH-Px activity in HepG2 cell cultures in a dose dependent manner. The present study indicates Se and α-Toc supplements: (1) effectively improve antioxidant status and diminish oxidative stress associated with HCHS diets; and (2) elevate hepatic levels of cholesterol-modulating oxysterols.

3.2 INTRODUCTION

Numerous human and animal studies have demonstrated that intake of high cholesterol and/or high saturated fat (HCHS) diets results in elevated plasma concentrations of low density lipoprotein cholesterol (LDL-C), a major risk factor

for cardiovascular disease (Khosla and Sundram, 1996; Kris-Etherton and Yu, 1997). HCHS diets can adversely influence cholesterol homeostasis via down-regulation of low density lipoprotein receptors (LDLr) and by modulation of key enzymes and proteins involved in cholesterol synthesis and degradation pathways (Kris-Etherton and Yu, 1997). Rodent feeding trials typically utilize HCHS diets to test modulatory factors of diet-induced hypercholesterolemia (Kang et al., 1997; Schwenke and Behr, 1998; Wojicki et al., 1991). HCHS diets are also associated with increased oxidative stress and impaired antioxidant capacity, particularly in terms of status of glutathione (GSH) and GSH-dependent antioxidant enzymes (Hyekyeong et al., 2003; Swagell et al., 2005; Lu and Chiang, 2001; Aviram et al., 1991; Meynier et al., 2002). For example, rats fed high saturated fat diets showed lower hepatic activities of a number of key GSH-dependent enzymes including GSH reductase, GSH transferase and GSH peroxidase (GSH-Px) relative to rats fed polyunsaturated fatty acid-enriched diets (Hyekyeong et al., 2003). Cultured hepatic cells exposed to palmitate have demonstrated induction of an oxidative stress response leading to down regulated genes for key enzymes involved with cellular GSH maintenance (Swagell et al., 2005). Similarly, cholesterol feeding in experimental animal studies was shown to decrease liver GSH-Px activity (Lu and Chiang, 2001). Additionally, animal feeding trials have demonstrated that hypercholesterolemia induced by HCHS diets can increase production of oxygen free radicals leading to increased tissue lipid peroxidation (Aviram et al., 1991) and elevated tissue concentrations of free-radical generated oxysterols that are implicated in cardiovascular disease (Meynier et al., 2002). Also, cholesterol feeding has been shown to increase hepatic levels of enzymatically formed oxysterols (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2003). Interestingly, tissue levels of selenium (Se), which is an essential component of cytosolic Se-dependent GSH peroxidase (SeGSH-Px), are depleted by HCHS feeding but can be repleted by Se supplementation (Dhingra and Bansal, 2006). The capability of Se supplementation, however, to enhance GSH-associated enzymes in association with HCHS diets has not been determined.

The importance of Se and α-Toc as antioxidants has been well demonstrated; however, surprisingly little is known regarding their antioxidant efficacy to limit oxidative stress associated with HCHS diets. SeGSH-Px plays a key role in inhibiting lipid peroxidation by reducing lipid hydroperoxides (LPO) to their corresponding alcohols. There is also non-Se-dependent cytosolic GSH peroxidase (non-SeGSH-Px) activity that constitutes the activity of GSH S-transferases, which catalyzes reactions of organic peroxides but not hydrogen peroxide with GSH to form oxidized glutathione (GSSG) and alcohols. Increased dietary intake of Se is also associated with higher hepatic concentrations of GSH (LeBoeuf et al., 1985). Vitamin α-Toc is a well-characterized chain-breaking antioxidant that has a sparing effect on requirements of Se (Combs and Scott, 1974). Although α-Toc supplements are only partially effective at inhibiting in vivo lipid peroxidation associated with a high saturated fat intake (Stein et al., 1996), supplemental combination of Se and α-Toc could be more effective since these nutrients are known to support each other in their antioxidative effects (Packer, 1991).

Supplementation of Se has demonstrated beneficial hypocholesterolemic effects both in humans (Djujic et al., 2000; Luoma et al., 1984; Kauf et al., 1994) and rodents (Kang et al., 1997; Schwenke and Behr, 1998; Wojcicki et al., 1991); however, the capability of Se supplements to mitigate against the blood cholesterol elevating effects of HCHS diets has not been studied. The cholesterol lowering mechanisms of Se are not well understood; however, recently, several enzymatically generated oxysterols found in human plasma including 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) have been implicated as important modulators of cholesterol and bile acid metabolism via regulation of two families of transcription factors, sterol regulatory element binding proteins (SREBP) and liver X receptors (LXR) (Bjorkhem, 2002). It is possible that the cholesterol lowering effects of Se are partly mediated via an increased production of these oxysterols, as they are mostly generated by cytochrome P450 enzymes and Se supplementation increases hepatic cytochrome P450 content (Swagell et al., 2005).

Supplementation of α-Toc has also been associated with plasma cholesterol lowering in a variety of animal models in the non-vitamin E deficient state (Pal et al., 2003). Although some studies have shown plasma cholesterol lowering in subjects supplemented with α-Toc (Stephens et al., 1996), most human trials have not shown significant hypocholesterolemic action (Pal et al., 2003). Combined Se and α-Toc supplementation, however, may exert synergistic effects on plasma cholesterol-lowering via enhancement of hepatic cholesterol-modulating oxysterol content since this antioxidant combination increased hepatic cytochrome P450 content that was lowered by HCHS feeding (Wojicki et al., 1991).

There is limited knowledge regarding the effectiveness of Se and α-Toc supplements to lower oxidative stress induced by HCHS feeding and the efficacy of antioxidant supplements to modulate enzymatically generated cholesterol-modulating oxysterols has not been studied. In the present study, two experiments were performed. Experiment 1 was conducted to examine in male Syrian hamsters the effects of Se and α-Toc supplements in concert with HCHS (high cholesterol and high saturated fat) feeding on: (i) plasma lipid concentrations; (ii) GSH content, SeGSH-Px and non-Se dependent GSH-Px activities; (iii) in vivo lipid peroxidation; and (iv) endogenously produced oxysterols. The plasma, heart, and liver were studied to investigate whether these tissues respond similarly to the effects of the Se and α-Toc supplements. In Experiment 2, HepG2 cells were treated for seven days with graded levels of Se in order to test whether hepatic tissue would show a dose-related response in the generation of 27-OHC.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Design

3.3.1.1 Experiment 1: Animals and Diets

Care and handling of the hamsters conformed to the guidelines of the McGill University Animal Care Committee and Canadian Council of Animal Care (Canadian Council on Animal Care, 1984) and the protocols for the animal experiments were approved by the McGill University Animal Care Committee. Thirty two male Syrian hamsters weighing on average 112 ± 3 g at 10 weeks of age were obtained from Charles River Laboratories (St-Constant, Quebec). Upon arrival, hamsters were provided with a 4-wk acclimatization period during which they were fed standard laboratory nonpurified chow diet to enable them to adapt to their new surroundings. Hamsters were housed individually in a room of controlled light (light 0700-1900 h) in plastic bottom cages and fed diets and tap water ad libitum. Fresh diets were fed to the hamsters daily and uneaten food was discarded. Food consumption was measured daily and body weights were recorded weekly. After being acclimatized, eight animals were randomly assigned to each one of the four dietary treatments **(Table 1)** and the experimental diets were fed for three wk. Hamsters were fed one of 4 diets which consisted of: (i) HCHS containing formulated National Research Council (National Research Council, 1995) basal requirements of vitamin E and selenium; ii) HCHS + α-tocopherol (α-Toc; 67 IU all-racemic α-tocopheryl acetate/kg diet); iii) HCHS + Se (3.4 mg Se/kg diet); iv) HCHS + α-Toc (67 IU all-racemic α-tocopheryl acetate/kg diet) + Se (3.4 mg Se/kg diet). The diet supplied 143 g added fat/kg diet (31% energy as fat) and contained 83 g saturated fat/kg providing 17% total energy as saturated fat. Separate additions of Se and α-Toc were made for the final formulations of all diets. Selenate was added to the diets to provide the stated Se levels and α-tocopheryl acetate which provided the stated IU of vitamin E. Hence, the basal Se diet contained 0.1932 mg of Se/kg diet (0.00046% Se w/w), whereas the supplemental Se diet provided 3.4 mg of

Se/kg diet (0.0083 % Se w/w). The basal α-Toc diet contained 27 IU all-racemic α-tocopheryl acetate/kg diet (0.00052% w/w) whereas the supplemental α-Toc diet provided 67 IU all-racemic α-tocopheryl acetate/kg diet (0.0013 % w/w) (10 tocopherol equivalents = 10 mg all-racemic α-tocopherol = 15 IU). Suitable vitamin and mineral mixes (excluding selenium and vitamin E, which were added separately) for hamster needs were developed following the National Research Council requirements (National Research Council, 1995) and were given at 1.3 times the published requirement levels in all diets to ensure at least an adequate intake of those nutrients. Hamsters began consuming their respective diets in a staggered manner. The staggered procedure involved the inclusion of one hamster randomly chosen from each diet group so that four hamsters began consuming their respective diets, one hamster from each diet group per day. The staggered design was necessary in order to allow for equal sampling across treatment groups over several days at the end of the experiment when animals were killed in a similarly staggered manner. Diets were prepared in pellet form and vacuum packed by Dyets, Inc. (Bethlehem, PA). Diets were kept refrigerated at 4°C. One bag was opened every wk to feed the hamsters for the upcoming wk.

3.3.1.2 Experiment 2: Cell Culture

Monolayer cultures of HepG2 (HB-8065; American Type Culture Collection cells) were grown and maintained in minimum essential medium (MEM) containing Earle's salts with L-glutamine and non-essential amino acids (Gibco 41500-067) containing 10% (v/v) fetal bovine serum (Invitrogen 10082-147), 1% medium essential medium with sodium pyruvate (Gibco 11360-070), 1.5% sodium bicarbonate (Gibco 25080-094), and penicillin-streptomycin (Gibco 15140-122) (100 µg/mL) as recommended by American Type Culture Collection for maximal growth of HepG2 cell cultures. Frozen cells were received from American Type Culture Collection and dimethyl sulfoxide washed out immediately upon thawing. HepG2 cells were grown until reaching 80% confluence after 7 days. The HepG2 cells were then passaged twice before

treatment with sodium selenite. A solution using sodium selenite (ICN 10102-18-8) was prepared at a concentration of 1 nmol Se/µL using the above-mentioned medium. For dose response, medium (30 mL per flask) containing the following levels of Se was used per treatment (n=5 flasks per treatment): 0, 0.4 nmol/ml, 1 nmol/ml, 2 nmol/ml , 4 nmol/ml, and 8 nmol/ml. Cells were cultured in 150 cm^2 flasks at 37°C in an atmosphere containing 5% carbon dioxide /95% air for a total of 7 days and grown to 80% confluence. The medium was changed as required.

3.3.2 Tissue Sample Preparation.

3.3.2.1 Experiment 1: Animals

At the end of the feeding period, the hamsters were fasted overnight. They were anesthetized with carbon dioxide and killed by exsanguination after cardiac puncture. Blood was drawn into vacutainer tubes containing ethylene diamine tetraacetic acid. The plasma was immediately isolated by centrifugation at 1500 x g, 4°C, for 10 min and aliquoted into micro-centrifuge tubes. Liver and heart were immediately removed, rinsed in ice cold physiological saline solution and rapidly frozen in liquid nitrogen. Plasma and tissues were subsequently stored at – 80°C until analysis.

3.3.2.2 Experiment 2: Cell Culture

Cells were harvested with trypsin- ethylene diamine tetraacetic acid (Gibco 25200-072), counted using a hemocytometer, and washed in phosphate buffered saline (Gibco 14190-144). For the analysis of oxysterols, cells from three flasks were pooled and re-suspended in phosphate buffered saline. The washed cells were divided into equal aliquots and frozen in phosphate buffered saline.

3.3.3 Plasma Cholesterol and Triglyceride (TG) Analysis

Automated enzymatic methodology using the Abbott VP Super System (Abbott, Irving, TX) with Abbott enzymatic reagent kits (Irving, Texas) was used to measure plasma triglyceride (TG) (Abbott Kit #6097), total cholesterol (TC) (Abbott Kit #6095) and HDL-C (Abbott Kit #6039). Non-HDL-C [LDL cholesterol + intermediate density lipoprotein (IDL) + VLDL cholesterol] was calculated from the difference between TC and HDL-C.

3.3.4 Tissue Lipid Peroxidation Analysis

The extent of lipid peroxidation in plasma was determined as thiobarbituric acid reactant substances (TBARS) using a modified method of Asakawa and Matsushita (Asakawa and Matsushita, 1980) and Wong et al. (Wong et al., 1987) as previously described (Poirier et al., 2002). Lipid extracts of heart and liver tissues obtained via the Folch method (Folch et al., 1957) were analyzed for lipid hydroperoxides (LPO) using a kit assay (LPO kit assay, Kamiya Biomedical, Thousand Oaks, CA) based on the modification of the method of Oshishi et al. (Oshishi et al., 1985). The absorbance of the samples was determined at 620 nm using the series 750 microtiter plate reader (Cambridge Technology, Inc, Watertown, MA).

3.3.5 Total GSH-Px, SeGSH-Px and non-SeGSH-Px (GSH transferase) Activities Analysis

Tissue supernatants were assayed for total GSH-Px and SeGSH-Px activity in Experiment 1 and cultured cells were assayed for total GSH-Px in Experiment 2, using an automated modification (L'Abbé et al., 1991) of the coupled assay of Paglia and Valentine (Paglia and Valentine, 1967) as described previously (Poirier et al., 2002). The assay mixture was prepared fresh daily and contained: 150 mM potasssium phosphate buffer (pH 7.0); potassium phosphate, monobasic (Sigma P 5379), potassium phosphate, dibasic (P-5504), 5 mM ethylene diamine tetraacetic acid (disodium salt), (Sigma E-5134), 0.5 mM

sodium azide (Sigma S-2002), 2 mM GSH (reduced, crystalline free acid) (Sigma G 6654), 0.24 mM nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (Sigma N6505) and 1 U/mL of glutathione reductase (GSSGRase, EC 1.6.4.2) (Sigma G-3664). Total GSH-Px activity and SeGSH-Px activity were determined using 1.2 mM and 0.3 mM t-butylperoxide (Sigma B-2633) as substrates, respectively. The blank activity was significantly lower with 0.3 mM t-butylperoxide than with hydrogen peroxide, and for this reason t-butylperoxide was used instead. The ability to detect only SeGSH-Px activity using a concentration of 0.3 mM *t*-butylperoxide has been confirmed previously (Prohaska, 1991). Non-SeGSH-Px activity due to glutathione transferases was determined from the difference between total GSH-Px activity and SeGSH-Px activity. Reactions were carried out using the Abbott VP Super System (Abbott, Irving, TX) at 37°C with a 340/380 filter. The sample cuvette contained 250 µL of the assay mixture and 5 µL of sample. The reaction was initiated by the addition of substrate. One unit of GSH-Px activity catalyzes the oxidation of 1.0 µmol of reduced NADPH per min, and was expressed per mg of protein.

3.3.6 Tissue GSH and GSSG Analysis

The detection of GSH and GSSG was done using a Cayman chemical kit (Ann Arbor, MI, GSH Assay Kit Cat #703002). The kit protocol was modified as tissues were homogenized in a homogenizing buffer made up of 5% sulfosalicylic acid (w/v) at pH 1.0 in order to maintain the ratio of GSH/GSSG, which is subject to change without the acidic pH (Meister and Anderson, 1983). Protein content was removed by adding metaphosphoric acid (Aldrich 23,927-5) and centrifuging at 3,000 x g for 5 min. Supernatant was then stored at -80°C for later glutathione analyses. For the final analysis, the supernatant was adjusted to pH 7.0 with the addition of triethanolamine (Aldrich T5, 830-0) and diluted so that absorbance of the sample fell within the absorbance of the standard curve values specified by the kit protocol. GSSG was measured after derivatizing GSH with 2-vinylpyridine. The ELX 8081U Ultra Microplate reader (Bio-TEK, Inc, Vermont, USA) was used

along with the KC4 Kineticalc software for Windows version 2.6 RevXX (Bio-Tek Instruments Inc) using a 405 nm filter.

3.3.7 Tissue α-Toc and Se Analysis

The concentrations of α-Toc in heart and liver were analyzed by High Performance Liquid Chromatography as described previously (Poirier et al., 2002). Tissue samples were homogenized in water, diluted 1:1 with ethanol to de-proteinize and the tocopherols were extracted with heptane. Samples of 100 µL volume were injected for High Performance Liquid Chromatographic separation on a silica column (25 cm x 4.6 mm ID, 5 µm particle size, Supelcosil LC-SI, Supelco Canada Ltd., Oakville, Ontario, Canada) using a Waters 717+ autosampler (Waters Scientific Ltd., Mississauga, Ontario, Canada), Waters model 510 pump and Perkin-Elmer 650 fluorescence detector. The mobile phase (1% 2-propanol in hexane) was degassed by sonication and run at a flow rate of 2 mL/min at ambient temperature. Detector settings were: excitation wavelength 290 nm and slit 10 nm, emission wavelength 320 nm and slit 12 nm. Three-fold higher detector sensitivity was used for measurement of α-Toc in heart because of the lower levels of α-Toc compared to liver. For liver Se analysis, hepatic tissue was digested with nitric acid and the Se content was measured using flame atomic absorption spectrophotometry (Hitachi, Polarized Zeeman AAS, Z-8200, Mississauga, Canada).

3.3.8 Liver Oxysterol Analysis

Oxysterol determination in hepatic tissue in Experiment 1 and in cultured HepG2 cells in Experiment 2 was performed by Gas Chromatograph/ Mass Spectrometer as described previously (Cockell et al., 2005). Briefly, 19-hydroxycholesterol was added to the samples as an internal standard before lipid extraction. Artifactual oxidation of cholesterol was minimized by incorporation of L-ascorbic acid and sodium acetate to scavenge oxygen and acidic species,

respectively. The lipid extract was saponified, unsaponified lipids were extracted with diethyl ether and free fatty acids were removed by potassium hydroxide. Bulk cholesterol was removed by solid-phase extraction and oxysterols were eluted with 2-propanol in hexane. Samples were evaporated at room temperature under nitrogen and converted to trimethylsilyl ethers for Gas Chromatograph/ Mass Spectrometer analysis (Agilent 6890 Gas chromatograph System with 5973 Mass Selective Detector, Agilent Technologies, Wilmington, DE, USA) using a J & W DB-1 capillary column with flow rate of helium carrier gas of 1.0 mL/min. The injector was operated in splitless mode and with an initial temperature of 290° C. After injection, oven temperature began at 80° C, and then programmed at a rate of 30° C/min to a final temperature 215° C, held for 2 min, followed by a rate of 2° C/min to a final temperature of 280° C held for 10 min. A volume of 1 µL per sample was injected. Oxysterol analysis was carried out using selected ion monitoring. The multiple ion detector was focused on m/z 145, 353, and 366 for 19-OHC; m/z 367 and 472 for 7-Keto; 145, 413, 456 for 24-OHC; 131, 327, 456 for 25-OHC; and m/z 129, 417, and 456 for 27-OHC.

3.3.9 Tissue Protein Determination

Protein concentrations of all supernatants were determined using the biuret assay (Abbott Total Protein Kit # LN5A13-26) with bovine serum albumin as a standard. Homogenates were centrifuged for 5 min at 12,000 x *g* and supernatants assayed for protein using the Abbott VP Super System.

3.3.10 Statistical Analysis

Results are presented as means ± standard error of the means. Data were tested using the mixed model procedure using SAS software version 9.1 (SAS Institute Inc., Cary, NC). For Experiment 1, to determine whether the α-Toc and Se treatments had independent or interactive effects on the dependent variables tested, a fixed effect, 2 x 2 ANOVA factorial design was used. Data was

assessed for normality (univariate) and homogeneity of variance using a mixed model procedure. Blocking was included in the statistical analysis because it was an integral part of the experimental design due to the staggered feeding which occurred in distinct blocks over time and included one hamster from each dietary group to make a total of four hamsters per block. Blocking was found to be not statistically significant in the analysis of heart GSH/GSSG ratio and liver oxysterols. The food intake and final body weight were considered in the statistical model as co-variables. Food intake was found to significantly affect the results for liver 27-OHC and SeGSH-Px and was kept in the model to analyze these dependent variables. In the heart, food intake was used in the model to analyze GSH-Px activities and final weight was used in the model to analyze heart GSH and GSSG levels. Covariates were found to be insignificant when analyzing all other dependent variables and were omitted from the model. Analysis of variance (ANOVA) was used to determine the effect of treatment and differences between treatment means were identified by least square means. *P* values are given as the least square means of the interaction effects (α-Toc x Se) and the main effects (α-Toc, Se) with pooled standard errors of the means. Main effects include the effect of basal α-Toc vs. supplemental α-Toc, and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se. Correlations between tissue Se and α-Toc levels and biochemical measurements were examined by using Spearman's correlation coefficient by rank. For Experiment 2, a fixed effect ANOVA factorial design was used to determine whether Se treatment had an effect on HepG2 concentrations of total GSH-Px activity. Treatment effects and differences between treatments were considered significant when $P < 0.05$. ANOVA was not used to determine whether Se treatment had an effect on HepG2 concentrations of 27-OHC as only one measurement was taken per level of Se used per treatment.

3.4 RESULTS

3.4.1 Experiment 1

3.4.1.1 Average Daily Food Intake, Body and Organ Weights

A significant main effect of Se treatment ($P < 0.05$) on average daily food intake was observed as hamsters fed the diets supplemented with Se consumed less diet than hamsters fed the non-Se supplemented diets **(Table 3.2)**. Neither α-Toc nor Se treatment was associated with any significant effect on initial weight, final weight, change in weight, liver weight or heart weight.

3.4.1.2 Plasma TBARS Concentrations

A significant main effect of Se treatment ($P < 0.05$) on plasma TBARS concentrations was observed **(Table 3.3)** as hamsters consuming Se supplemented diets showed approximately 19% lower plasma TBARS concentrations as compared to hamsters consuming HCHS diets. In addition, α-Toc supplemented hamsters had significantly lower plasma TBARS concentrations as compared to the hamsters fed the HCHS diet. Plasma TBARS showed strong positive correlations with plasma TC and non-HDL-C ($r^2 = 0.50$; $P < 0.005$ and $r^2 = 0.54$; $P < 0.005$, respectively) and negatively correlated with hepatic activities of SeGSHPX ($r^2 = -0.44$; $P < 0.05$) and non-SeGSH-Px ($r^2 = -0.40$; $P < 0.05$).

3.4.1.3 Plasma Cholesterol and TG Concentrations

As shown in **(Table 3.3)**, a significant main effect of Se treatment was observed with respect to plasma concentrations of TC ($P < 0.05$), non- HDL-C ($P < 0.005$) and the ratio of potentially atherogenic non-HDL-C/antiatherogenic HDL-C ($P < 0.005$), which were 18%, 32% and 22% lower, respectively, than the

control diet-fed hamsters. The hamster treatment group receiving α-Toc supplements also had significantly ($P < 0.05$) lower plasma concentrations of TC and non-HDL-C concentrations **(Table 3.3).** Significant interactive effects of α-Toc x Se on plasma HDL-C concentrations were observed ($P < 0.05$) **(Table 3.3)**. The hamsters fed the α-Toc and Se supplemented diet had significantly ($P < 0.05$) higher plasma HDL-C concentrations in comparison to the α-Toc-supplemented hamsters. In addition, hamsters receiving the combined supplements of α-Toc and Se showed a significantly lower ratio of non-HDL-C/HDL-C in comparison to all other treatment groups. Plasma TG concentrations did not differ among treatment groups **(Table 3.3)**. A strong positive correlation was noted between plasma TC and hepatic LPO content ($r^2 = 0.51$; $P < 0.005$). Conversely, plasma TC was strongly negatively correlated with hepatic GSSG content ($r^2 = -0.60$; $P < 0.005$). Negative correlations were also observed between plasma TC and hepatic activities of both SeGSH-Px ($r^2 = -0.52$; $P < 0.005$) and non-SeGSH-Px ($r^2 = -0.33$; $P < 0.05$). A strong positive correlation was seen between plasma non-HDL-C concentrations and hepatic LPO content ($r^2 = 0.59$; $P < 0.005$). Plasma non-HDL-C concentrations were negatively correlated with hepatic SeGSH-Px activity ($r^2 = -0.51$; $P < 0.005$) and with both hepatic content of GSH ($r^2 = -0.34$; $P < 0.05$) and GSSG ($r^2 = -0.53$; $P < 0.005$). A negative correlation was noted between 7-ketocholesterol (7-Keto) and plasma TG ($r^2 = -0.34$; $P < 0.05$). A significant negative correlation was observed between plasma non-HDL-C and hepatic Se content ($r^2 = -0.29$; $P = 0.05$).

3.4.1.4 Tissue LPO concentrations

A significant main effect of Se treatment on concentrations of LPO was observed in liver ($P < 0.05$) **(Table 3.4)** and heart ($P < 0.05$) **(Table 3.5).** Se supplementation resulted in a significant ($P < 0.05$) lowering of LPO concentrations of approximately 45% in liver tissue and a 33% in heart relative to hamsters fed the HCHS diet. In addition, hamsters fed the HCHS + α-Toc or HCHS + α-Toc + Se diets had significantly lower ($P < 0.05$) liver and heart LPO

concentrations relative to the HCHS-fed hamsters. Liver LPO concentrations were negatively correlated with hepatic Se content (r^2 = -0.37; P = 0.05).

3.4.1.5 Tissue GSH and GSSG Concentrations

Significant main effects of Se supplementation were seen with respect to hepatic concentrations of GSH (P < 0.005) **(Table 3.4)** and heart GSH concentrations (P < 0.005) **(Table 3.5)**. Hamsters receiving diets containing Se supplements had significantly higher hepatic concentrations of GSH relative to non-Se supplemented dietary treatments **(Table 3.4)**. The Se-supplemented hamsters showed significantly higher cardiac GSH concentrations as compared to the hamsters fed the CT diet **(Table 3.5)**. A significant main effect of α-Toc (P < 0.005) was seen with respect to heart GSH concentrations **(Table 3.5)** and hamsters fed the combined α-Toc + Se supplement showed higher cardiac concentrations of GSH relative to α-Toc and HCHS diet **(Table 3.5)**. In the liver, a main effect of Se supplementation was observed with respect to hepatic GSSG concentrations (P < 0.05) **(Table 3.4)**. In the heart, the supplementation of Se had no effect on cardiac concentrations of GSSG **(Table 3.5)**. Supplementation of α-Toc did not affect hepatic or cardiac concentrations of GSSG **(Tables 3.4 and 3.5)**. Both hepatic and cardiac GSH/GSSG ratios were unaffected by dietary treatments **(Tables 3.4 and 3.5)**. A positive correlation between hepatic GSH content and hepatic concentrations of 24-hydroxycholesterol (24(S)-OHC) was observed (r^2 = 0.42; P < 0.05). Strong positive correlations were observed between liver GSH concentrations and both hepatic Se content (r^2 = 0.54; P < 0.005) and hepatic SeGSH-Px activity (r^2 = 0.50; P < 0.005). Hepatic GSSG concentrations were positively correlated with: (a) liver 25-OHC content (r^2 = 0.45; P < 0.05); (b) hepatic activity of SeGSH-Px (r^2 = 0.33; P < 0.05) and non-SeGSH-Px (r^2 = 0.33; P < 0.05); (c) hepatic α-Toc concentrations (r^2 = 0.39; P < 0.05); and (d) hepatic Se content (r^2 = 0.41; P < 0.05).

3.4.1.6 Tissue SeGSH-Px and Non-SeGSH-Px (GSH Transferase) Activities

The hepatic SeGSH-Px activity was significantly higher ($P < 0.05$) in association with Se supplementation as the HCHS + Se and the HCHS + α-Toc + Se groups had higher activity relative to the HCHS group **(Table 3.4)**. Cardiac activity of SeGSH-Px was unaffected by Se supplementation **(Table 3.5)**. Neither cardiac nor hepatic SeGSH-Px activity was affected by supplemental α-Toc **(Tables 3.4 and 3.5)**. Non-SeGSH-Px activity was unaffected by the dietary treatments in both heart and liver **(Tables 3.4 and 3.5)**.

3.4.1.7 Tissue α-Toc and Se Content

As expected, significant ($P < 0.005$) main effects of α-Toc treatment on α-Toc concentrations were observed in liver **(Table 3.4)** and heart **(Table 3.5)** with α-Toc-supplemented hamsters showing 2.3- and 1.7-fold increases in α-Toc content in heart and liver tissues, respectively, relative to the HCHS-fed hamsters. In both liver and heart, the HCHS + α-Toc and the HCHS + α-Toc + Se treatment groups had significantly higher ($P < 0.05$) α-Toc concentrations relative to the other diet groups. Similarly, a significant main effect of Se treatment ($P < 0.005$) on liver Se concentrations was observed **(Table 3.4)** as consumption of Se was associated with a significant 209% increase ($P < 0.005$) in liver Se content in hamsters fed the HCHS + Se diet in comparison to the liver Se content of hamsters fed the HCHS diet alone. A significant main effect of α-Toc ($P < 0.005$) and a significant interactive effect of α-Toc x Se ($P < 0.05$) on liver Se content were observed **(Table 3.4)**. Hamsters fed the HCHS + α-Toc + Se diet showed significantly higher liver concentrations of Se as compared to hamsters consuming the HCHS ($P < 0.005$), HCHS + α-Toc ($P < 0.005$) and HCHS + Se ($P < 0.005$) diets **(Table 3.4)**. Hepatic content of α-Toc and Se were positively correlated ($r^2 = 0.38$; $P < 0.05$). Liver Se content was positively correlated with both hepatic content of both 24(S)-OHC ($r^2 = 0.33$; $P < 0.05$) and 25-hydroxycholesterol (25-OHC) ($r^2 = 0.37$; $P < 0.05$). Similarly, hepatic α-Toc was

positively related to 27-hydroxycholesterol (27-OHC) (r^2 = 0.33; P < 0.05) and showed a marginally significant positive correlation with 25-OHC (r^2 = 0.31; P = 0.05).

3.4.1.8 Liver Oxysterol Content

No significant effect of the dietary treatments on hepatic concentrations of 7-Keto was observed **(Table 3.6)**. A significant main treatment effect of Se supplementation on hepatic concentrations of 24(S)-OHC (P < 0.05), 25-OHC (P < 0.05) and 27-OHC (P < 0.05) was observed **(Table 3.6)**. The livers of hamsters consuming the HCHS + α-Toc + Se diet had a significantly higher (P < 0.05) content of 24(S)-OHC as compared to the HCHS + α-Toc-fed hamsters. In terms of the liver content of 25-OHC, the HCHS + Se and the HCHS + α-Toc + Se groups had higher (P < 0.05) concentrations in comparison to the HCHS and the HCHS + α-Toc treatment groups. Both Se-supplemented diet groups had a significant (P < 0.05) increase of approximately 200% in the liver content of 27-OHC as compared to livers of hamsters consuming HCHS alone. Positive correlations were observed between 7-Keto and 24(S)-OHC (r^2 = 0.45; P < 0.05), 25-OHC (r^2 = 0.32; P < 0.05) and 27-OHC (r^2 = 0.38; P < 0.05). Similarly, 24S-OHC was positively correlated with 25-OHC (r^2 = 0.54; P < 0.05) and 25-OHC was strongly correlated with 27-OHC (r^2 = 0.61; P < 0.005).

3.4.2 Experiment 2

3.4.2.1 Cell Culture Growth

HepG2 cells showed an increase in growth for seven days for all treatments except for sodium selenite treatments 4 nmol/mL and 8 nmol/mL, where a sharp decrease in cell number was noted **(Table 3.7)**.

3.4.2.2 Cell Total GSH-Px Activity

Se supplementation was effective in inducing GSH-Px activity in the cells in a significant ($P < 0.05$) dose related manner **(Table 3.7)**. The activity of GSH-Px was shown to be unaffected by 4 nM Se treatment as compared to untreated cells, whereas no activity was shown with 8 nM treatment.

3.4.2.3 Cell 27-OHC Concentrations

HepG2 cells showed a dose-dependent increase in 27-OHC concentrations/cell with increasing selenium supplementation as sodium selenite added to the medium **(Table 3.7)**.

3.5 DISCUSSION

The present study shows that, similar to the well demonstrated efficacy of Se and α-Toc supplements in diminishing lipid peroxidation induced by polyunsaturated fatty acid intake (Poirier and Kubow, 2006), high dose supplements of Se and α-Toc also effectively decrease lipid peroxidation in plasma, heart and liver associated with the HCHS diet **(Tables 3.3, 3.4 and 3.5)**. The antioxidant efficacy of Se in the heart and liver tissues likely involves the observed enhancement of cardiac and hepatic GSH content **(Tables 3.4 and 3.5)**, as induction of tissue GSH has been well characterized to decrease tissue lipid peroxidation (Comporti, 1987). Supplementation of Se has consistently been shown to increase hepatic concentrations of GSH in a variety of species (Le Boeuf et al., 1985). Elevated concentrations of GSH and GSSG in liver tissue induced by Se treatment have been related to increased hepatic activities of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH biosynthesis (Chung and Maines, 1981). The lower lipid peroxidation in heart tissue observed in the Se-supplemented hamsters **(Table 3.5)** is in concert with previous findings showing decreased cardiac TBARS concentrations in Golden Syrian hamsters

receiving Se supplementation (Jamall et al., 1987). The activity of non-SeGSH-Px enzymes was not influenced by Se supplementation **(Tables 3.4 and 3.5)**, which is likely due to the relatively high tissue abundance of non-Se-GSH-Px enzymes and their lower sensitivity to induction in comparison to other antioxidant enzymes (Maral et al., 1977). On the other hand, SeGSH-Px activity was increased in the livers but not in the hearts of Se-supplemented hamsters, which coincides with our earlier work showing that hepatic SeGSH-Px activity is more sensitive to induction by Se supplementation than is the activity of SeGSH-Px in heart tissue (Poirer et al., 2002). It thus appears that high dose Se supplementation can at least partly overcome the suppression of hepatic GSH-Px activity and GSH content consistently noted with HCHS diets (Hyekyeong et al., 2003; Lu and Chiang, 2001). The antioxidant efficacy of α-Toc supplementation against hepatic lipid peroxidation found with the HCHS diet concurs with previous work demonstrating that rats fed saturated fat-based diets supplemented with 300 IU α-Toc/kg diet had significantly reduced hepatic levels of lipid peroxidation (Pulla Reddy and Lokesh, 1994). The present results indicate that α-Toc supplementation can also effectively decrease plasma and cardiac lipid peroxidation associated with HCHS feeding. The hepatic and cardiac activities of SeGSH-Px and non-SeGSH-Px were unaffected by the α-Toc supplementation **(Tables 3.4 and 3.5)**. Similar observations were noted in hepatic tissues of rats fed high dose supplemental α-Toc with high saturated fat lard-based diets (Eder et al., 2002). Likewise, daily subcutaneous injections of α-Toc in hamsters showed no effect on cardiac SeGSH-Px and non-SeGSH-Px activities (Li et al., 1997).

Free radical-mediated mechanisms are involved in the production of some oxysterols, such as 7-Keto and 7β-hydroxycholesterol. Although the Se and α-Toc supplemented hamsters showed significantly reduced tissue lipid peroxidation, no effect on hepatic concentration of 7-Keto was observed. This latter observation is in agreement with previous findings that supranutritional α-Toc supplements did not affect the liver and plasma concentrations of 7β-hydroxycholesterol in rats (Keller et al., 2004) or the hepatic oxysterol content in

guinea pigs fed oxidized fat (Ringseis and Eder, 2002), despite greatly enhanced tissue α-Toc content. In contrast, long term α-Toc supplementation in humans was associated with decreased plasma concentrations of free radical generated oxysterols (Porkkala-Sarataho et al., 2000). The α-Toc supplement used in the present study provided 2.5-fold higher α-Toc intake (67 IU/kg diet) than the National Research Council recommendation for the hamster (27 IU/Kg diet) (National Research Council, 1995), which was based on maintenance of hepatic α-Toc levels and prevention of vitamin E deficiency symptoms with high polyunsaturated fatty acid intake (Farwer et al., 1994). The α-Toc supplementation, however, resulted in hepatic α-Toc concentrations of 34 nmol/g protein that were only 1.7 times higher than the non-supplemented hamsters **(Table 3.4)** and only 2.5-fold higher than hepatic α-Toc concentrations shown in hamsters fed α-Toc deficient diets (4.5 IU/kg diet) (McGuire et al., 1997). Thus, it is possible that the hepatic α-Toc content induced by α-Toc supplementation in the present study was insufficient to provide significant antioxidant protection against the free radical generation of oxysterols. The occurrence of relatively low hepatic α-Toc concentrations despite the high α-Toc supplementation might have been due to negative effects on α-Toc status mediated by the HCHS diet. For example, rats fed 20% lard diets showed a significantly lower hepatic α-Toc content than those fed fish oil diets following a 10-wk feeding period (Tijburg et al., 1997). Similarly, a 24-fold increase in dietary α-Toc intake led to only two- to nine-fold increases in α-Toc concentrations in liver and LDL-C, respectively, in rats fed a highly saturated 10% coconut fat-based diet (Ringseis and Eder, 2002). In contrast, fish oil-fed rats receiving equivalent dietary α-Toc concentrations showed 12- and 100-fold increases in liver and LDL concentrations, respectively (Ringseis and Eder, 2002). There is indication that intestinal uptake of α-Toc is enhanced by dietary intake of polyunsaturated fatty acids in comparison to saturated fatty acids (Tijburg et al., 1997).

The major decrease in concentrations of plasma TC and non-HDL-C observed with Se supplementation in the context of HCHS feeding extends previous Syrian hamster studies demonstrating hypolipidemic effects of Se

supplements with low-fat standard laboratory diets (Vinson et al., 1998) and cholesterol-supplemented fish oil-based semi-purified diets (Poirier et al., 2002) and HCHS diets in other rodent studies (Dhingra and Bansal, 2006). Conversely, no effect is shown on TC concentrations in rats supplemented with Se and fed normal fat diets (Crespo et al., 1995; Chidambaram and Baradarajan, 1995). Species differences or the absence of cholesterol in the diets used might account for differences among studies. The lack of significant plasma TG lowering with Se supplementation in the present work contrasts with previous studies showing significant decreases in plasma TG concentrations in humans (Djujic et al., 2002) and rodents in conjunction with HCHS diets (Wojicki et al., 1991; Iizuka et al., 2001) and low fat diets (Chidambarm and Baradarajan, 1995) but concurs with previous observations in Se-supplemented hamsters (Vinson et al., 1998; Poirier et al., 2002) and rats (Kang et al., 1997) and rabbits (Schwenke and Behr, 1998).

In concert with previous findings noting α-Toc-mediated lowering effects on plasma TC and non-HDL-C concentrations involving hamsters (Kubow et al., 1996) and other species such as rabbits (Phonpanichrasamee et al., 1990), α-Toc supplementation was associated with the lowering of plasma TC and non-HDL-C concentrations **(Table 3.3)**. There is contradictory evidence on the cholesterol-modulating impact of α-Toc supplementation in hamster feeding trials. Short-term Golden Syrian hamster feeding trials of 3-4 wk involving high dose α-Toc supplements have not generally shown plasma non-HDL-C lowering effects in combination with high cholesterol feeding (ranging from 0.4-2%), regardless of whether butterfat (de Oliveira et al., 2000), corn oil (Parker et al., 1995) or fish oil (Poirier et al., 2002) based diets were utilized. On the other hand, plasma non-HDL-C lowering with HCHS diets was observed in hamsters fed for more prolonged feeding periods of 16 wks or longer (Stein et al., 1996; Xu et al., 1998), which might suggest the need for accumulation of sufficiently high hepatic α-Toc concentrations to exert hypolipidemic effects. When provided at high doses, α-Toc may exert pharmacological effects on lipid metabolism independent of its antioxidant function. For example, increased activity of the liver-specific enzyme, CYP7A1, that converts cholesterol to 7α-

hydroxycholesterol resulting in enhanced bile salt formation has been noted (Phonpanichrasamee et al., 1990) following prolonged high level α-Toc supplementation in rabbits fed HCHS diets. In addition, recent HepG2 cell culture studies have shown that depending on dose, either up-regulation or down-regulation of LDL receptor and cholesterol synthesis can be observed with α-Toc treatment (Pal et al., 2003). The possible mechanisms involved in the α-Toc-mediated modulation in bile salt and low density lipoprotein receptor formation are not clear; however, the positive correlations between hepatic α-Toc concentrations and 27-OHC seen in the present results suggests that enhanced oxysterol production could play a role.

A novel finding of this study is that Se supplements or combined Se and α-Toc supplementation in hamsters increased hepatic 24(S)-OHC, 25-OHC and 27-OHC content, which provides the first evidence that antioxidants can enhance tissue concentrations of these enzymatically generated oxysterols **(Table 3.6)**. Similarly, Se supplementation was effective in inducing 27-OHC and GSH-Px activity concentrations in HepG2 cells in a dose response manner up to the 2 nM dose. The 4 nm and 8 nM doses appeared to be toxic to HepG2 cells as the number of cells were shown to decrease as compared to control levels **(Table 3.7)**. The lack of measurable free radical-induced oxysterols in our cell culture experiment indicates that our methodology was not confounded via autoxidation of cholesterol during sample purification, which has been observed in previous cell culture studies involving oxysterol measurements (van Reyk et al., 2006). Although it is unclear how the antioxidant supplementation resulted in an increase in tissue oxysterols, antioxidants might protect oxysterols from destruction through oxidative stress. In that regard, previous work has shown that cultured human umbilical vein endothelial cells exposed to oxidative stress for 24 h via addition of oxidized LDL-C had cellular 27-OHC concentrations lowered by 78% (Zhu et al., 2005). The positive correlations found in the present study between hepatic levels of Se and α-Toc and the hepatic oxysterol content provides support to the concepts that enhanced antioxidant protection and/or increased synthesis of oxysterols afforded by Se and α-Toc supplementation

helped maintain hepatic oxysterol concentrations. Increased hepatic oxysterol concentrations might play a role in the hypocholesterolemic effects of Se, as in vitro studies have shown physiological concentrations of oxysterols to be important activators for liver X receptor (LXR) (Janowski et al., 1996; Lehmann et al. 1997; Forman et al. 1997; Spencer et al., 2001). Activation of the LXR pathway results in the lowering of whole body levels of cholesterol that, in turn, can lead to hypocholesterolemic effects (Peet et al., 1998).

An interesting observation was the enhancement of hepatic Se concentrations from the combined supplements of Se and α-Toc. This finding is supported by earlier observations that platelet Se concentrations in diabetic rats were increased by the combined supplementation of Se and α-Toc vs. Se supplements alone (Douillet et al., 1997). Previous work by Cagyill et al. (Cagyill et al., 1973) has shown that tissue Se concentrations are affected by the dietary content of α-Toc, which is likely due to a sparing effect of α-Toc on tissue Se concentrations due to their combined antioxidant function. A similar sparing effect of α-Toc has been noted previously with tissue concentrations of other antioxidants such as ascorbic acid (Keller et al., 2004).

In conclusion, the present study indicates Se and α-Toc supplements were effective in diminishing oxidative stress and improving antioxidant status in association with HCHS diets. An enhancement of hepatic cholesterol-modulating oxysterols via antioxidant supplementation of Se and α-Toc was also observed, which could play a role in their hypocholesterolemic effects. Further studies are needed to determine whether the hyperlipidemia associated with HCHS feeding is partly mediated by the depressed production of enzymatically generated oxysterols involved in cholesterol synthesis and metabolism. We are currently conducting studies to examine the effect of Se supplementation on hepatic cholesterol metabolizing and LXR responsive genes and on bile acid metabolism to examine the possible mechanism of hypocholesterolemic action involving oxysterols.

Table 3.1. Composition Of Experimental Diets (g/kg)[a]				
Ingredients	**HCHS[b]**	**HCHS[b] + α-Toc**	**HCHS[b] + Se**	**HCHS[b] + α-Toc + Se**
Casein, vitamin-free	159.1	159.1	159.1	159.1
Cornstarch	285.44	285.34	284.66	284.56
Sucrose	175.3	175.3	175.3	175.3
Dextrose	99	99	99	99
Cellulose	43	43	43	43
Butterfat[c]	157	157	157	157
Safflower oil[d]	13.76	13.76	13.76	13.76
Cholesterol, USP[e]	4.5	4.5	4.5	4.5
Mineral mix[f]	43	43	43	43
Vitamin mix[g]	8.6	8.6	8.6	8.6
Choline bitartrate	11.2	11.2	11.2	11.2
Sodium selenate	0.046	0.046	0.8229	0.8229
Vitamin E acetate	0.052	0.134	0.052	0.134
Metabolizable energy, MJ/Kg	18.3	18.3	18.3	18.3

[a]HCHS = Butterfat ; HCHS + α-Toc = control + α-tocopherol; HCHS + Se = control + selenium; HCHS + α-Toc + Se = control + α-tocopherol + selenium. All diets were formulated at McGill University and prepared in pellet form by Dyets Inc. (Bethlehem, Penn)

[b]Fatty acid composition of HCHS is as follows (% by weight) as provided by Dyets: C4:0, 3.4; C6:0, 2.0; C8:0, 1.2; C10:0, 2.7; C12:0, 3.0; C14:0, 10.7; C14:1, 1.6; C16:0, 28.0; C16:1, 2.5; C18:0, 13.0; C18:1, 26.8; C18:2, 2.5; C18:3, 1.5; C20:0, 1.1.

[c]Butterfat contains 18% H_2O and therefore 157 g fat /kg diet provided 129 g fat/kg diet.

[d]Safflower oil was added to prevent essential fatty acid deficiency. α-Toc concentration of SAFF is 350 ppm of α-tocopherol, 180 ppm of other tocopherols. Fatty acid profile of safflower oil included (% by weight): 14:0, trace; 16:0, 6.9; 16:1, trace; 18:0, 2.9; 18:1, 12.2; 18:2, 78.0; 18:3, trace.

[e]Cholesterol USP was added to butterfat 4.5 g/kg

[f]The mineral mix was free of Se and was composed of (g/kg): calcium carbonate 336.4;calcium phosphate, monobasic 285.0; magnesium oxide 2.985; potassium iodate (10 mg KI/g) 0.76; potassium phosphate, dibasic 40.76; sodium chloride 11.45; cupric carbonate 0.084; cobalt chloride 0.133; sodium fluoride 0.002; ferric citrate 25.45; manganous carbonate 0.229; ammonium paramolybdate 0.008; zinc carbonate 0.53; sucrose 296.209. Sodium selenate (10 mg/g sodium selenate) was added separately to make the diets; for basal Se diets 0.046; for high Se diets 0.8229.

[g]The vitamin mix was free of α-Toc and was composed of (g/kg): vitamin A palmitate (500,000 IU/g) 0.4263; vitamin D3 (400,000 IU/g) 0.9315; vitamin K1 premix (10 mg/g) 110.0; biotin 0.03; folic acid 0.3; niacin 13.5; pantothenate (Ca) 1.5; riboflavin 2.25; thiamin HCl 3.0; pyridoxine HCl 0.9; vitamin B12 (0.1%) 1.5; sucrose 865.6622. α-Toc acetate (500 IU/g) was added separately to make the diets; for basal α-Toc diets, 0.052; for high α-Toc, 0.129.

Table 3.2. ANOVA Of The Effects Of Dietary A-Toc And Se Supplementation On Average Daily Feed Intake, Body Weight Gain, Liver Weight And Heart Weight Of Adult Male Syrian Hamsters Fed HCHS Diet For 3 Wk[1]

	Dietary treatment				Main and interaction effects[2]		
Variable	**HCHS**	**HCHS + α-Toc**	**HCHS + Se**	**HCHS + α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
Average daily intake (g/d)	7.7± 0.2[a]	7.5± 0.2[a]	6.9± 0.2[b]	7.0± 0.2[b]	NS	$P<0.05$	NS
Initial weight (g)	115 ±2.7	117 ±2.7	113 ±2.5	114 ±2.1	NS	NS	NS
Final weight (g)[3]	121 ±1.5	122 ± 1.4	121 ± 1.4	124 ± 1.4	NS	NS	NS
Change in weight (g)	10 ±2.6	7.5 ±0.84	4.2 ±0.96	5.8 ±1.5	NS	NS	NS
Liver weight (g)[4]	6.8± 0.1	6.8± 0.1	6.7± 0.1	7.1± 0.1	NS	NS	NS
Heart weight (g)[4]	0.49±0.01	0.51±0.01	0.49±0.01	0.49±0.01	NS	NS	NS

[1]Values are mean ± SEM (n=8). Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 3.1. HCHS = High cholesterol and/or high saturated fat diet.

[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.
[3]Average daily intake used as covariate in the statistical model.
[4]Average daily intake and final body weight used as covariates in the statistical model.

Table 3.3. ANOVA Of The Effects Of Dietary A-Toc And Se Supplementation On Plasma TBARS And Plasma Lipid Concentrations (TC, Non-HDL-C, HDL-C, Non-HDL-C/HDL-C Ratio And TG) Of Adult Male Syrian Hamsters Fed HCHS Diets For 3 Wk[1]

	Dietary treatment				Main and interaction effects[2]		
Variable	**HCHS**	**HCHS+ α-Toc**	**HCHS + Se**	**HCHS+ α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
TBARS (μmol/L)	1.08±0.06[a]	0.93±0.06[b]	0.87±0.06[b]	0.87±0.06[b]	NS	*P*<0.05	NS
TC (mmol/L)	5.8± 0.2[a]	4.9± 0.2[b]	4.8± 0.2[b]	4.9± 0.2[b]	NS	*P*<0.05	NS
Non-HDL-C (mmol/L)	2.6± 0.2[a]	2.1± 0.2[b]	1.8± 0.2[b]	1.6± 0.2[b]	NS	*P*<0.005	NS
HDL-C (mmol/L)	3.2± 0.2[ab]	2.8± 0.2[a]	3 ± 0.2[ab]	3.3± 0.2[b]	NS	NS	*P*<0.05
Non-HDL-C/ HDL-C	0.83±0.08[a]	0.84±0.08[a]	0.65±0.08[b]	0.50±0.08[c]	NS	*P<0.005*	NS
TG (mmol/L)	4.4± 0.4	4.1± 0.4	3.7± 0.4	4.1± 0.4	NS	NS	NS

[1]Values are mean ± SEM, (n = 8). Means within rows without a common superscript letter differ, $P < 0.05$. Diets and abbreviations are as indicated in Table 1. HCHS = High cholesterol and/or high saturated fat diet.

[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.

Table 3.4. ANOVA Of The Effects Of Dietary A-Toc And Se Supplementation On Liver LPO, GSH, GSSG, GSH/GSSG Ratios, SeGSH-Px Activity, Non-SeGSH-Px Activity, A-Toc And Se Of Adult Male Syrian Hamsters Fed HCHS Diets For 3 Wk[1]

	Dietary treatment				Main and interaction effects[2]		
Variable	**HCHS**	**HCHS+ α-Toc**	**HCHS + Se**	**HCHS + α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
LPO (μmol/g protein)	14.8±1.5[a]	10.4± 1.5[b]	8.2± 1.5[b]	8.5± 1.5[b]	NS	$P<0.05$	NS
GSH (μmol/g protein)	[4]18±2.4[a]	16± 2.0[a]	27± 2.0[b]	30± 2.0[b]	NS	$P<0.005$	NS
GSSG (μmol/g protein)	[3]8±1.0[a]	9± 0.9[a]	11± 0.9[b]	11± 0.9[b]	NS	$P<0.05$	NS
GSH/GSSG	[4]3±0.55	2± 0.55	3± 0.45	3± 0.45	NS	NS	NS
SeGSH-Px (units/mg protein)	208±9[a]	198± 9[a]	238± 8[b]	232± 9[b]	NS	$P<0.05$	NS
Non-SeGSH-Px (units/mg protein)	54±5	41± 5	50± 5	49± 5	NS	NS	NS
α-Toc (nmol/g protein)	[3]21± 2[a]	[3]34±2[b]	[3]24± 2[a]	36±2[b]	$P<0.005$	NS	NS

Se (nmol/g wet wt)	12±2[a]	13±2[a]	24± 2[b]	34± 2[c]	*P*<0.005	*P*<0.005	*P*<0.05
[1]Values are mean ± SEM (n=8) except where noted, [3]n=7, [4]n=6. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 1. HCHS = High cholesterol and/or high saturated fat diet.							
[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.							

Table 3.5. ANOVA Of The Effects Of Dietary Vit E And Se Supplementation On Heart LPO, GSH, GSSG, GSH/GSSG Ratios, SeGSH-Px Activity, Non-SeGSH-Px Activity And A-Toc Of Adult Male Syrian Hamsters Fed HCHS Diets For 3 Wk[1]

	Dietary Treatment				Main and interaction effects[2]		
Variable	**HCHS**	**HCHS + α-Toc**	**HCHS + Se**	**HCHS + α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
LPO (μmol/g protein)	6.5 ± 0.4[a]	4.8± 0.4[b]	4.4± 0.4[b]	5.0± 0.4[b]	NS	$P<0.05$	NS
GSH (μmol/g protein)	7± 2[a]	12± 2[b]	22± 2[bc]	26± 2[c]	$P<0.05$	$P<0.005$	NS
GSSG (μmol/g protein)	9± 1	12± 1	[3]11± 1.5	[3]11± 1.5	NS	NS	NS
GSH/GSSG	3± 0.7	3± 0.7	[3]3± 0.8	[3]2± 0.8	NS	NS	NS
SeGSH-Px (units/mg protein)	52± 2	[3]45± 3	51± 2	[3]53± 3	NS	NS	NS
Non-SeGSH-Px (units/mg protein)	10± 1	[3]9± 1.1	[4]9± 1.4	[3]12± 1.1	NS	NS	NS
α-Toc (nmol/g protein)	4± 1[a]	10± 1[b]	5± 1[a]	10± 1[b]	$P<0.005$	NS	NS

[1]Values are mean ± SEM (n = 8) except where noted, [3]n = 7, [4]n = 6. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 1. HCHS = High cholesterol and/or high saturated fat diet.
[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.

Table 3.6. ANOVA Of The Effects Of Dietary A-Toc And Se Supplementation On Liver 7-Keto, 24(S)-OHC, 25-OHC And 27-OHC Of Adult Male Syrian Hamsters Fed HCHS Diets For 3 Wk[1]

	Dietary treatment				Main and interaction effects[2]		
Variable	**HCHS**	**HCHS+ α-Toc**	**HCHS + Se**	**HCHS + α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
7-Keto (pmol/g wet wt)	35±15	47±20	25±13	30±15	NS	NS	NS
24(S)-OHC (pmol/g wet wt)	$49±12^{ab}$	$34±9^{a}$	$63±16^{ab}$	$81±21^{bc}$	NS	$P<0.05$	NS
25-OHC (pmol/g wet wt)	$10±2.5^{a}$	$10±2.5^{a}$	$17±2.5^{b}$	$17±2.5^{b}$	NS	$P<0.05$	NS
27-OHC (pmol/g wet wt)	$^{3}104±25^{a}$	$184±40^{ab}$	$221±45^{b}$	$216±47^{b}$	NS	$P<0.05$	NS

[1]Values are mean ± SEM (n = 8) except where noted, [3]n = 7. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 1. HCHS = High cholesterol and/or high saturated fat diet.

[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.

Table 3.7. Effects Of Se Treatment On Concentrations Of 27-OHC And Total GSH-Px In HepG2 Cells[1]							
	Cell Culture Treatment with Se (nmol/mL)[2]						
Variable	**0**	**0.4**	**1**	**2**	**4**	**8**	**Main Effect[5]**
Average total cell numbers (10^6)[3]	68.8	67.6	69.7	79.3	18.2	3.6	N/A
Cells Assayed (10^6)	64.6	73	70	87	73	18	N/A
27-OHC (pmol)[4]	353	789	1,006	1,127	1,505	825	N/A
27-OHC (pmol)/cell[4]	5.5	10.8	14.4	13.0	20.6	45.8	N/A
Total GSH-Px activity/mg protein	38.7 ± 2^{a}	58.3 ± 1.2^{bc}	168.1 ± 0.7^{d}	85.0 ± 0.9^{c}	52.6 ± 7.4^{b}	ND	$P<0.005$

[1]Values are mean ± SEM (n = 3) for total GSH-Px activity. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). [2]Selenium was added as sodium selenite to medium. [3]Average of 5 flasks.

[4]Values are for n=1, calculated error for each analysis is ±20% as analysis includes one single Gas Chromatograph/Mass Spectrometer injection.

[5]N/A indicates ANOVA is non-applicable as ANOVA procedure was not run due to n=1.

CONNECTING STATEMENT

The previous two experiments in the last chapter demonstrated that the in vivo supplementation of selenium (Se) increased hepatic concentrations of 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC) and Se treatment in HepG2 cells enhanced 27-OHC concentrations. Both antioxidants were shown to be protective against oxidative stress and lowered plasma lipids. The positive relationship between hepatic content of Se and α-tocopherol (α-Toc) with liver oxysterol content suggests that hepatic levels of oxysterols may have been protected against oxidative destruction. Furthermore, the enhanced hepatic levels of oxysterols could play a role in the hypocholesterolemic effects of Se through the activation of the liver X receptor (LXR) pathway.

In the following chapter we further confirmed the effect of Se on hepatic concentrations of oxysterols and examined possible mechanisms by which Se was shown to increase oxysterols. Thus, we examined the effect of Se supplementation on messenger ribonucleic acid (mRNA) abundance of cholesterol 7-hydroxylase (*cyp7a1)* and sterol 27-hydroxylase (*cyp27a1*), activity of CYP7A1 and CYP27A1, increases in hepatic levels of 27-OHC and 7-hydroxcholesterol (7-OHC) and increases in chenodeoxycholic acid (CDCA) and cholic acid (CA) in gallbladder bile. As 27-OHC along with 24(S)-OHC and 25-OHC, known in vitro ligands of LXR, were shown to be increased with Se supplementation, we examined the liver and jejunum for mRNA abundance of ATP-binding cassette (ABC) transporter g5 (*abcg5)* and ATP-binding cassette (ABC) transporter g8 (*abcg8)* and jejunum for mRNA abundance of LXR responsive gene Nieman-Pick C1-Like 1 protein (*npc1l1).* Additionally, increased biliary and fecal concentrations of cholesterol were examined in order to explore a possible role for Se-induced decrease in plasma lipids through the LXR pathway. Also, we examined for hepatic and jejunal mRNA abundance of 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (*hmgcr)* and low density lipoprotein receptor (*ldlr)* and the plasma and liver for cholesterol and triglyceride

(TG) concentrations. The following chapter thus refers to the specific objectives 3 - 7, and hypotheses 2 to 6 described in Sections 1.3 and 1.4 of Chapter 1.

CHAPTER 4 - Selenium Mediated Plasma Cholesterol Lowering in Hamsters is not Mediated by LXR or Upregulation of Hepatic Hydroxylases

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4.1 ABSTRACT

Selenium (Se) supplementation has been demonstrated to have important hypocholesterolemic effects yet the underlying mechanisms are unclear. The molecular basis for the lipid lowering action of Se may be mediated via the liver X receptor (LXR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. A dose response study involving dietary supplementation of Se was carried out to examine activation of the LXR pathway as assessed by: (a) hepatic oxysterol, cholesterol and triglyceride content and sterol 27-hydroxylase (CYP27A1) and cholesterol 7α-hydroxylase (CYP7A1) activity; (b) Hepatic messenger ribonucleic acid (mRNA) abundance of *cyp7a1, cyp27a1*; (c) Hepatic and jenunal 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase *(hmgcr)*, low density lipoprotein receptor *(ldlr),* ATP-binding cassette (ABC) transporter g5 *(abcg5),* ATP-binding cassette (ABC) transporter g8 *(abcg8);* (d) Nieman-Pick C1-Like 1 protein *(npc1l1)* and (e) bile acid and cholesterol content in bile and feces. Male Syrian hamsters (4 groups, n=10 per group) were fed semi-purified diets containing 0.1% cholesterol and 15% added fat: 1) Control (HCHS; 0.15 ppm Se); 2) HCHS + 0.85 ppm Se; 3) HCHS + 1.7 ppm Se; and 4) HCHS + 3.4 ppm Se. All Se supplemented dietary treatments were associated with increased mRNA abundance of hepatic and jejunal *abcg8* and *ldlr* but *abcg5 and hmgcr* were unaffected. No significant effect of Se was observed on activity of hepatic CYP7A1 or CYP27A1 and hepatic mRNA abundance of *cyp7a1* and *cyp27a1* was unaffected. The 1.7 and 3.4 ppm Se supplemented treatment groups had lower plasma LDL-C and increased hepatic 27-hydroxycholesterol (27-OHC) content and mRNA abundance of *abcg8* and *ldlr*. The absence of effect of Se on *abcg5* abundance suggests that Se-induced *abcg8* expression is not LXR mediated. Unexpectedly, jejunal *npc1l1* was shown to be upregulated which further suggests Se does not modulate expression of *npc1l1* through LXR activation. Fecal bile acids and cholesterol and hepatic levels of cholesterol were not significantly affected by Se supplementation. The present study demonstrates that increased hepatic 27-OHC content and lowered plasma LDL-

C concentrations induced by Se supplementation are not modulated by either upregulation of hepatic hydroxylases or the LXR pathway.

4.2 INTRODUCTION

Selenium (Se) supplements lower plasma low density lipoprotein cholesterol (LDL-C) in both human (Djujic et al., 2000; Kauf et al., 1994; Luoma et al., 1984) and rodent studies (Wojicki et al. 1991; Dhingra and Bansal, 2006; Dhingra and Bansal, 2005; Iizuka et al. 2001; Dhingra and Bansal, 2006a) including when these supplements are provided in concert with high fat, high cholesterol diets (HCHS) (Poirier et al., unpublished data). Although the mechanism(s) underlying the effect of Se on plasma lipids have not been clearly defined, the plasma cholesterol lowering action of Se supplementation has been partly explained in separate rat studies by lowered hepatic 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase (*hmgcr*) messenger ribonucleic acid (mRNA) abundance and increased hepatic low density lipoprotein receptor (*ldlr*) mRNA abundance (Dhingra and Bansal, 2006b). A lowering of *hmgcr* mRNA abundance would be expected to increase hepatic *ldlr* mRNA abundance that, in turn, would decrease plasma levels of LDL-C, however, these parameters have not been measured concurrently in relation Se supplementation and therefore the molecular mechanism(s) are poorly understood.

Se has also been shown to be associated with a decrease in tissue cholesterol concentrations (Iizuka et al., 2001, Schwenke and Behr, 1998) including hepatic cholesterol concentrations. The effect of Se on hepatic cholesterol concentrations might possibly reflect its effects on bile acid secretion as Se supplementation has been shown to accelerate the secretion of bile acids into bile in the rat (Danik, 1976). Diversion of cholesterol into bile acid synthesis depletes the free cholesterol (FC) within hepatocytes, which triggers a compensatory increase in de novo cholesterol synthesis and induction of the

LDL-C receptor pathway leading to an enhanced plasma clearance of LDL-C (Peet et al., 1998).

Reductions in plasma cholesterol concentrations normally occur when the two major sources of body cholesterol, hepatic synthesis and intestinal absorption are concurrently inhibited (Yu et al., 2002a; Basso et al., 2007). Three LXR responsive genes known to play critical roles in cholesterol absorption include Nieman-Pick C1-like 1 protein (*npc1l1*), and ATP binding cassette (ABC) transporter *g5* (*abcg5*) and ATP-binding cassette (ABC) *g8* (*abcg8*). NPC1l1 plays a key role in intestinal and whole body cholesterol homeostasis and is required for uptake of cholesterol and phytosterols (Altmann et al., 2004; Davis et al., 2004). At the apical membrane are the ATP-binding cassette transporters g5 and g8 (*abcg5* and *abcg8)* that promote partial efflux of cholesterol from the enterocyte into the intestinal lumen. Thus, the combined regulatory effects of *npc1l1*, *abcg5* and *abcg8* may play critical roles in modulating the amount of cholesterol that reaches the lymph from the intestinal lumen.

At the level of the liver, activation of the LXR is shown to increase mRNA abundance of murine hepatic *abcg5* and *abcg8* (Repa et al., 2002) involved in secreting cholesterol into gallbladder bile and thus decreasing hepatic levels. Previous work has shown that disruption of the *abcg5/abcg8* genes in both hepatic and intestinal tissues in mice results in a 2.4 fold increase in plasma cholesterol levels in conjunction with an increase in hepatic cholesterol levels in *abcg5/abcg8* null mice when fed a 2% cholesterol diet as compared to wild type mice fed the same diet (Yu et al., 2002). Conversely, *ldlr* null mice which showed an overexpression of human ABCG5/ABCG8 in both the liver and intestine, showed lower liver and plasma cholesterol concentrations as compared to *ldlr* null mice not overexpressing human ABCG5/G8 when fed a HCHS diet (Wilund et al., 2004).

In the previous chapter the supplementation of Se was associated with an increase in vivo concentrations of hepatic oxysterols, naturally occurring ligands of liver X receptor (LXR) which were associated with lowered plasma lipid levels (Poirier et al., unpublished observations in chapter 3). Excess cholesterol, over

and above the needs of the cell, is converted to regulatory oxysterols which are naturally occurring ligands of the liver X receptor (LXR) (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2003).This latter finding further suggests that Se may act to lower lipids by promoting ligand activation of the LXR pathway.

Although Se was shown to be associated with an increase in hepatic oxysterols it was not clear how Se increased oxysterols (Poirier et al., unpublished observations in chapter 3). The lowering of oxidative stress by Se shown in the previous work indicated that Se might be protecting hepatic oxysterols derived from dietary cholesterol from degradation, through decreasing oxidative stress. Alternatively, Se might increase hepatic oxysterol concentrations via an increase in cytochrome P450 hydroxylase activities, which are induced by Se supplementation (Wojicki et al., 1991). Two important hydroxylases involved in cholesterol catabolism are cholesterol 7-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1). CYP7A1 is a liver specific enzyme that catalyzes the initial and rate limiting step in the major bile acid synthetic pathway. Activation of LXR has been shown to upregulate CYP7A1 in the rat but not in the hamster or human (Javitt, 2002). CYP27A1 is a ubiquitous mitochondrial enzyme that is expressed in both the liver and extrahepatic tissues. CYP27A1, by acting on excess cholesterol in peripheral cells, is part of the alternative pathway of bile acid biosynthesis whereby peripheral tissues lower cellular cholesterol content via secretion of 24(S)-OHC, 25-OHC and 27-OHC into plasma for transport to the liver and subsequent bile acid formation (Bjorkhem, 1999). The alternative pathways are postulated to be an alternative or complementary mechanism to high density lipoprotein cholesterol (HDL-C)-dependent reverse cholesterol transport.

Although activation of LXR by oxysterols is associated with increased in vitro expression of LXR responsive genes (Sparrow et al., 2002; Venkateswaran et al., 2000; Fu et al., 2001; Wong et al., 2007; Rowe et al., 2003) many in vivo studies have used synthetic LXR agonists (Duval et al., 2006; Groot et al., 2005) and studied the effect of dietary cholesterol on LXR responsive genes (Berge et

al., 2000; Repa et al., 2000; Yu et al., 2002a; Dieter et al., 2004; Duan et al., 2004; Field et al., 2004). Apart from one study which examined the relationship between hepatic oxysterol concentrations and hepatic LXR-responsive genes (Xu et al., 2003), no studies to our knowledge have examined the association between known ligands of the LXR in the form of endogenously produced oxysterols with LXR responsive genes involved in cholesterol absorption. Thus we studied the association of Se-modulated mRNA levels of sterol metabolizing proteins with hepatic levels of endogenously produced oxysterols that are ligands of LXR.

Also, although the small intestine also has an important role in plasma lipid homeostasis in the human (Kesaniemi and Miettinen, 1987), surprisingly no studies have examined the effect of Se supplementation on cholesterol absorption at the level of the gut. The aim of the present study was to examine in the Syrian hamster, the effect of a Se-supplemented HCHS (high cholesterol and high fat) diet on hepatic and jejunal abundance of *abcg5*, *abcg8*, *hmgcr* and *ldlr* mRNA and jejunal abundance of *npc1l1* and their association with hepatic lipids, plasma lipids and biliary and fecal levels of bile acids and cholesterol. Upregulation of both hepatic and jejunal *abcg5* and *abcg8* and downregulation of jejunal *npc1l1* by Se in the hamster small intestine would support an involvement of Se with the LXR pathway. Additionally, we studied whether Se supplements can increase hepatic oxysterol concentrations by upregulation of the activities of hepatic cytochrome P450 hydroxylases CYP7A1 and CYP27A1 and liver mRNA abundance of *cyp7a1* and *cyp27a1*.

4.3 MATERIALS AND METHODS

4.3.1 Animals and Diets

Forty Syrian male hamsters, aged 9-10 wks (approximate weight 110-120 g) were purchased from Charles River Laboratories (St-Constant, QC). Hamsters

were housed in stainless steel wire-bottom cages and acclimatized to laboratory conditions for 10 days while being fed a standard commercial chow diet. At the end of the acclimatization period hamsters were weighed and randomized to four groups of ten animals each and fed their respective test diets for four weeks. The dietary levels of Se were adjusted to four different levels which included: 1) Control (HCHS; 0.15 ppm Se); 2) HCHS + 0.85 ppm Se; 3) HCHS + 1.7 ppm Se; and 4) HCHS + 3.4 ppm Se. The minimal level of Se of 0.15 ppm in the basal diet conformed to National Research Council guidelines of 0.1 ppm (National Research Council, 1995). The highest supplemental level of Se selenite (3.4 ppm) was chosen on the basis of a previous study (Birt et al., 1983), which demonstrated that hamsters can safely tolerate this level of Se supplementation. The semi-purified diets provided 19.2 megajoules of metabolizable energy per kg diet and cholesterol was added at 0.1% weight/weight **(Table 4.1)**. The diets were formulated to provide as calories 14% protein, 33% fat, and 53% carbohydrate. Diets were obtained from Dyets, Inc. (Bethlehem, PA) in pellet form in vacuum-packed bags, which were stored at -20°C to prevent autoxidation of lipids. Approximately 15 ± 1 g of feed were provided on a daily basis and hamsters had free access to tap water. Hamsters were weighed three times per week for the initial two weeks of feeding and thereafter body weight was recorded on a weekly basis.

4.3.2 Tissue Collection

At the end of the feeding period, hamsters were fasted overnight and sacrificed within 2 days in a treatment-blocked randomized order. Under isoflurane anesthesia, blood was drawn by cardiac puncture and plasma was prepared with ethylene diamine tetraacetic acid. After surgical exposure of liver, bile was aspirated from the gallbladder by tuberculin syringe, transferred to sample tubes, mixed by gentle inversion and stored at -80°C. Immediately after removal, the liver pieces were frozen in liquid nitrogen. The intestine was

dissected out, rinsed with filtered phosphate buffered saline solution before separating into sections and freezing in liquid nitrogen. Both liver and intestine were stored at -80°C until further use. During dietary treatment, and near the end of the feeding phase, feces was collected on three consecutive days and stored at -20°C until further use.

4.3.3 EXPERIMENTAL PROCEDURES

4.3.3.1 Plasma Lipids Analysis

Measurement of plasma lipids: total cholesterol (TC), LDL-C, HDL-C, and triglycerides (TG) were carried out according to manufacturer's instructions using commercially available kit assays. Randox enzymatic reagent kits (Randox Laboratories Ltd., Antrim, United Kingdom) were used for measurement of plasma TC (Randox CHOL enzymatic endpoint method, Kit CH200), plasma HDL-C (Randox HDL-C Precipitant Method, Kit 203), and plasma TG (Randox TG GPO-PAP Method, Kit TR 210). Plasma LDL-C was assessed using Wako L-Type LDL-C kit assay (Reagents 1 and 2, Kit 993-0040, and 999-00504, respectively, with the LDL-C Calibrator, 991-00302, Wako Pure Chemical Industries, Ltd, Osaka, Japan).

4.3.3.2 Hepatic Lipids Analysis

Hepatic total cholesterol (TC), free cholesterol (FC), esterified cholesterol (CE), and triglycerides (TG) were determined from lipid extracts via a method developed by Carr et al. (1993) using commercially available enzymatic assay kits. Hepatic TC was determined using cholesterol E reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan, 439-17501). Hepatic FC was measured using free cholesterol C Enzymatic colorimetric Method (COD-PAP) (Wako Pure

Chemical Industries, Ltd., Osaka, Japan, 274 - 47109E). The hepatic CE concentration was calculated by the difference between liver TC and FC. Hepatic TG concentrations were determined using Wako L-Type TG H Reagents 1 & 2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan, 998-40391 & 994-40491, respectively).

Briefly, approximately 200 mg of liver tissue (wet weight) was weighed and subsequently tissue lipids were extracted using 30 mL chloroform: methanol (2:1) according to the method of Folch et al. (Folch et al., 1957). Phases were separated by the addition of 6 mL 0.05% sulphuric acid and the lower chloroform phase volume was extracted and measured. For lipid solubilization, 1 mL of lipid extract was aliquoted into 16 x 100 mm screw capped tubes in which 1 mL of 1% Triton X-100 solution was then added. The mixture was dried under a stream of nitrogen, 500 µL of nanopure water was then added and all tubes were capped and placed in a shaking water bath for 15 min at 37°C to solubilize all tissue lipids. Tubes were then removed from the water bath, vortexed, and made ready for enzymatic determination of hepatic tissue lipids.

For the enzymatic determination of hepatic tissue lipids, all assays were performed using 50 µL aliquots of sample and standard using 96-well microtiter plates. Separate microtiter plates were used for hepatic TC, FC, and TG determination. For the standard solution preparations, soybean oil (SIGMA S7381) was used as the primary TG standard and cholesterol purchased from Sigma Chemical Co. (St. Louis, MO, USA) was used as the standard for both TC and FC determinations.

Standard solutions for total and free hepatic cholesterol were made to the following concentrations: 10, 20, 50, 100, and 200 µg/mL chloroform, and standard solutions for total hepatic TG were made to the following concentrations: 20, 40, 100, 200, and, 400 µg/mL. A blank containing 1% Triton-X was also made.

For all lipid assessments, assays were carried out at room temperature (25°C) and were read using a multifilter microtiter plate reader (Titertek Multiskan Plus MKII, ICN Biochemicals, Cleveland, OH).

4.3.3.3 Hepatic Se Analysis

For liver Se analysis, hepatic tissue was digested with nitric acid and the Se content was measured using flame atomic absorption spectrophotometry (Hitachi, Polarized Zeeman AAS, Z-8200 Mississauga, Canada).

4.3.3.4 Hepatic Oxysterol Analysis

Oxysterol determination in hepatic tissue was performed by gas chromatograph/mass spectrometer as described previously (Cockell et al., 2005). Briefly, 19-hydroxycholesterol (19-OHC) was added to the samples as an internal standard before lipid extraction. Artifactual oxidation of cholesterol was minimized by incorporation of L-ascorbic acid and sodium acetate to scavenge oxygen and acidic species, respectively. The lipid extract was saponified and unsaponified lipids were extracted with diethyl ether and free fatty acids were removed by potassium hydroxide. Bulk cholesterol was removed by solid-phase extraction and oxysterols were eluted with 2-propanol in hexane. Samples were evaporated at room temperature under nitrogen and converted to trimethylsilyl ethers for gas chromatograph/mass spectrometer analysis (Agilent 6890 GC System with 5973 Mass Selective Detector, Agilent Technologies, Wilmington, DE, USA) using a J & W DB-1 capillary column with flow rate of helium carrier gas of 1.0 mL/min. The injector was operated in splitless mode and with an initial temperature of 290° C. After injection, oven temperature began at 80° C, and then programmed at a rate of 30° C/min to a final temperature 215° C, held for 2 min, followed by a rate of 2° C /min to a final temperature of 280° C held for 10 min. A volume of 1 µL per sample was injected. Oxysterol analysis was carried out using selected ion monitoring. The multiple ion detector was focused on m/z 145, 353, and 366 for 19-OHC; m/z 367 and 472 for 7-ketocholesterol (7-Keto);

129, 441, and 456 for 7-hydroxycholesterol (7-OHC); 145, 413, 456 for 24(S)-OHC; 131, 327, 456 for 25-OHC; and m/z 129, 417, and 456 for 27-OHC.

4.3.3.5 Hepatic CYP7A1 and CYP27A1 Activity Analysis

Preparation of liver subcellular fractions: Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation (Petrack and Latario, 1993).

The activity of CYP7A1 and CYP27A2 was measured by a new method based on a combination of two methods (Petrack and Latario, 1993; Honda et al., 1999). For both CYP7A1 and CYP27A1 assays, the reaction mixture of a total volume of 200 µL consisted of 100 mM phosphate buffer, pH 7.4, containing 5 mM dithiothreitol, 0.2 mM ethylene diamine tetraacetic acid was added to a silanized test tube placed in water bath at 37°C. For CYP7A1 reaction, Triton X-100 solution was added at 30 µL 2.5% (w/v) per sample in order to solubilize microsome in the reaction mixture. Following this, 200 µL of assay buffer containing 0.5 to 1 mg microsomal protein for CYP7α1, or 0.2 to 0.4 mg mitochondrial protein for CYP27A1 was added to the test tube. The reaction was initiated with the addition of isocitrate (5 mM) (Sigma-Aldrich I1252), isocitrate dehydrogenase (0.2 units) (Sigma-Aldrich I2516) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (final 1.2 mM) in 100 µL and continued for 30 min (final volume 0.5 mL). The reaction was stopped with 1 mL 1 N ethanolic potassium hydroxyide, 5 µg butylated hydroxytoluene, and 100 ng 19-OHC as internal recovery standard. Controls had 1 mL 1 N ethanolic potassium hydroxide, 5 µg of butylated hydroxytoluene, and 100 ng of 19-OHC added at the beginning of assay. After saponification at 37° C for 1 h, 0.5 mL nanopure water was added and 7-OHC or 27-OHC were extracted three times with 1 mL hexane, and the extracts were pooled and evaporated to dryness under nitrogen. The residue was dissolved in 1 mL toluene and applied to a 1 mL Superclean Solid Phase Extraction cartridge (Supelco, Bellefonte, PA,

504041) pre-conditioned with 2 mL hexane. After washing with 4.5 mL 0.5% propanol in hexane, 7-OHC or 27-OHC was eluted with 7 mL 30% propanol in hexane, and derivatized with N,O-Bis(trimethylsily)trifluoroacetimide (Sigma, T6381). Gas chromatograph/mass spectrometer analysis with selected ion monitoring was performed using an Agilent 6890 Gas chromatographic System with 5973 Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA) as described for oxysterol analysis, above. The multiple ion detector was focused on m/z 145, 353, and 366 for 19-OHC, 129, 441, and 456 for 7-OHC, and m/z 129, 417, and 456 for 27-OHC.

4.3.3.6 Hepatic and Jejunal mRNA Analysis

Estimation of mRNA levels by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR): Total ribonucleic acid (RNA) was extracted from tissues, of individual hamsters, flash frozen at time of tissue harvest and kept frozen at -80°C until Trizol reagent use (Invitrogen) which proceeded according to the manufacturer's instructions. The quality of crude RNA quality was monitored by running on formaldehyde gel. Chosen crude RNA samples were cleaned of contaminating genomic deoxyribonucleic acid (DNA) by being subjected to DNase digestion using Qiagen Rneasy Mini Kit and run on a formaldehyde gel and verified for RNA band integrity. A band intensity ratio of 2:1 between 28S and 18S rRNA band was indicative of an absence of degradation in the RNA isolation process. Subsequently, cleaned RNA was quantitated using Ribogreen RNA Quantitation Reagent and Kit (Invitrogen R-11491, and R-11490). Cleaned RNA (2 µg) per sample was transcribed to complementary deoxyribonucleic acid for QPCR by RETROscript Reverse Transcription for RT-PCR (Ambion) using Oligo dT at 65°C for 3 min and 42 °C for 1 h and finally at 95 °C for 10 min to denature the RNA-dependent DNA polymerase (reverse transcriptase). RT-PCR exploits a characteristic of mature mRNAs known as the polyadenylated region, commonly called the poly (A) tail, as a common binding

site for poly (T) DNA primers. These primers anneal to the 3' end of every mRNA in the solution and allow a 5'→3' synthesis of complementary DNA by the reverse transcriptase enzyme.

The primers were designed using PrimerQuest software. **Table 4.11** provides the sequences and accession number of the primers used for each gene. Sequences were chosen in consideration of their length, GC% content, primer temperature (Tm), hairpin and self dimerization. The sequences were analyzed by BLAST (National Center for Biotechnology Information, National Institutes of Health) to verify that the primers were specific for each gene. Primers on receipt were re-suspended in tris-ethylenediamine tetraacetic acid buffer, diluted and stored at -20°C. Primer pairs before being tested with samples were optimized for concentration of primer and magnesium in final mix and tested for optimal serial dilution for standard curve using an appropriate hamster sample complementary deoxyribonucleic acid as control.

The quantitation of genes of interest was carried out using Sybergreen Core reagents kits (Stratagene). To each microtiter plate well a 5 µL volume of cDNA was added per reaction up to 50 µL final volume per well. Final primer concentration equalled 250 nM, magnesium chloride at 2.5 mM. Duplicate samples were run and averaged. A standard curve was used as a method of quantitation and therefore one standard curve was run with every gene of interest. For every primer set 2 types of control were used: 1) No-template control in which water was added instead of complementary deoxyribonucleic acid template; and 2) No-RT Control, in which the RT enzyme was omitted in the complementary deoxyribonucleic acid synthesis reaction. The values were normalized using glyceraldehyde-3-phosphate dehydrogenase (*gapdh)* mRNA as an endogenous internal standard. A serial dilution of a sample and standard was run on each microtiter plate for each mRNA and used to calculate the relative levels of mRNA.

Real time quantitative PCR was performed in a model Mx4000 Stratagene detection system using MX4000 software. The thermal cycler parameters were as follows: 95°C for 10 min 1 cycle to activate SureStart Taq, 40 cycles of

denaturation (95°C 30 sec), Annealing (60°C 45 sec) and Extension (72°C 1 min), 1 cycle for denaturation of amplicons (95°C 1 min), 81 cycles for dissociation curve (55 °C to 95 °C, 10 sec, 0.5 sec/cycle), 1 cycle for end of assay (25 °C, hold).

4.3.3.7 Gallbladder Bile Acid and Cholesterol Analysis

The analysis of the bile acids contained in bile was carried out using a combination of two methods (Chijiiwa and Nakayama, 1988; Batta et al., 1998). Briefly, 5 µL of bile was added to 5 mL volumetric flask containing 2 mL ethanol and heated until boiling point in a water bath and allowed to cool to room temperature. Ethanol was added to make 5 mL volume and was transferred to a 10 mL centrifuge tube with cap. To precipitate protein the tube was centrifuged at 400 x g for 10 min and 2 mL of the ethanol extract was taken and transferred to 10 mL centrifuge tube for the determination of cholesterol and each bile acid. Nor-cholic acid (23-NOR-5β-cholanic acid-3α, 7α, 12α -triol) Nor-CA (Steraloids N2450) and Cholestane (5α-cholestane) (Steraloids C3300-000) were added as standards at 10 µg of each in 100 µL of ethanol and the 2mL sample containing the standards was evaporated under nitrogen. Following evaporation of ethanol the conjugated bile acids were subjected to hydrolysis by the addition of a 4 mL solution containing the following: 1 mL acetate buffer, pH 5.6, 1 mL 1.86% ethylenediamine tetraacetic acid (Sigma E5134), 1 mL 0.87% mercaptoethanol (Sigma M6250), 1 mL of acetate buffer pH 5.6, containing the enzymes 0.1 mg cholyglycine hydrolase (Sigma C-4018 from Clostridium perfringens) + 0.1 mg β-glucuronidase (Sigma G-8192 from E. coli). The test tube was incubated in dry bath at 37° C for 18 h. After hydrolysis of bile acids the solution was acidified by adding concentrated hydrocholic acid and pH of solution was brought to pH 1. The cholesterol and bile acids were extracted using 5 mL of ethyl acetate (Sigma 154857) 3 times (3 x 5 mL). Ethyl acetate was pooled into a new tube and evaporated. To the test tube, 200 µL butanol (Aldrich, 154679) and 20 µL

hydrocholic acid was added and tube was placed in dry bath at 60°C for 4 h. Contents of the tube were evaporated by passing under nitrogen gas and 100 µL Sil-Prep was added to the tube and vortexed. Tube was heated in dry bath for 30 min at 55°C and evaporated under nitrogen gas. Hexane (100 µL) was added and vortexed and was transferred to a sample vial and capped. 1 µL of hexane was injected into a gas chromatograph for analysis in 20:1 split-mode.

Standards containing Nor-CA and α-cholestane as internal standards were made up using the following bile acids purchased from Steraloids : Chenodeoxycholic acid (3α, 7α, -Dihydroxy-5β-cholanoic acid) CDCA (C0940-000), Cholic acid (3α, 7α, 12α-Trihydroxy-5β-cholanoic acid) CA (C1900-000), Lithocholic acid (3α- Hydroxy-5β-cholanoic acid) LCA (C1420-000), Deoxycholic acid (3α, 12α-Dihydroxy-5β-cholanoic acid) DCA (C1070-000), Ursocholanic acid (5β-cholanic) (UCA) (C0800-000), Ursodeoxycholic acid(3α, 7β-Dihydroxy-5β-cholanoic acid) UDCA (C1020-000), Hyodeoxycholic acid (3α, 6α-Dihydroxy- 5β-cholanoic acid) HDCA (C0860-000), and Cholesterol (5-cholesten-3β-ol) (C6760-000). Standard concentrations ranged between 0.02 and 0.2 µg bile acid per 1 uL hexane injected and were subjected to the same treatment as delineated above.

Gas Chromatography: Identification and quantification of bile acids were achieved using a Hewlett-Packard moded 6890 gas chromatograph equipped with a flame ionization detector and injector with a split/splitless device for capillary columns. The chromatographic column used was a J&W 122-1031 capillary column (30 m x 0.250 mm I.D.). Helium was used as the carrier gas. The gas chromatograph operating conditions were as follows: injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 150°C for 1 min, then programmed at a rate of 7°C/min to a final temperature of 272°C.

4.3.3.8 Fecal Bile Acid and Cholesterol Analysis

Quantification of fecal bile acids and cholesterol was carried out as a modification of the method of Batta et al. (2002). Stool from 1 day of collection was freeze-dried and ground to a fine powder. To a weighed test tube, 10 mg freeze-dried stool (exactly weighed), was added. In order to quantitate bile acids an internal standard was added (20 µg of nor-cholic acid (Steraloids, Newport, Rhode Island, USA 2450-000) in 100 µl *n*-butanol (Aldrich 154679) to the test tube. In order to quantitate cholesterol concentrations in each sample, a separate internal standard was added (20 µg 5α-cholestane (Steraloids, C3300-000), in 100 µl *n*-butanol), to the same test tube. To a total volume of 200 µl n-butanol containing standards, a volume of 20 µl concentrated hydrochloric acid (HCl) (Sigma H1758) was added to allow *n*-butyl ester formation, and the tube was vortexed. The test tube was heated at 60 °C in a dry bath for 4 h after which solvent was evaporated under N_2 and the esterified product was directly subjected to trimethylsilylation with the addition to the tube of 200 µl Sil-Prep (Alltech 18013). The capped test tube was heated in dry bath for 30 min at 55°C and evaporated under nitrogen gas. A volume equal to 200 µl of hexane (Sigma 139386) was added to the tube which was vortexed and then centrifuged at 1000 rpm for 15 min to separate stool debris. Clear supernatant (100 µl) was transferred to a sample vial and capped. 1 µl was injected into gas chromatograph for analysis in the 20:1 split mode. Standards were made as mentioned above in section 4.3.3.7. Identification and quantification of fecal bile acids were achieved as described above in section 4.3.3.7.

4.3.3.9 Hepatic Protein Determination

Protein concentrations of liver enzymatic assays and liver lipids were determined using the Bradford reagent (Sigma St. Louis, Missouri, USA, B6916). Bovine serum albumin stock in saline was used as standard. Bovine serum

albumin standards and unknowns were prepared in the same buffer. The standards were created by serially diluting either 2 mg/mL or 1 mg/mL bovine serum albumin stock solution. Protein standards ranged from 0.25 to 1.4 mg/mL. Unknowns were diluted to fall between 0.5 and 1 mg/mL.

4.3.3.10 Statistical Analysis

Statistical analyses were performed using the mixed model procedure (MIXED) for all analyses of SAS 9.1 (SAS USA release 9.1, 1989-1996, by SAS Institute Inc., Cary, NC.). A fixed effect of one factor with 4 levels factorial design was used. The statistical significances of the differences between least square means were determined using LSMeans test. The statistical model was as follows:

$Y_{ij} = \mu + Se_i + block_{ij} + e_{ij}$

Y_{ij}= plasma TC, HDL-C, LDL-C, plasma TG, liver TC, liver CE, liver FC, liver TG, liver CYP7A1 enzyme activiy, liver CYP27A1 enzyme activity, liver oxysterols, liver and jejunal mRNA, biliary bile acids, biliary cholesterol, fecal bile acids, fecal cholesterol of the ith hamster receiving the ith Se level from the jth cage.

μ= the overall mean of the sampled population.

Se= fixed effect representing the differences between Se levels, where i=1, 2, 3, 4.

Block= 4 blocks of 10 hamsters, containing an equal number of hamsters from each of four dietary groups, or microtiter plate used as block. Blocking was included in the model for the analysis of hepatic cholesterol ($P < 0.05$), CE ($P < 0.005$), TG ($P < 0.05$), CYP27A1 activity, hepatic *cyp7a1* mRNA abundance *($P < 0.05$)*, hepatic *abcg5 ($P < 0.005$* and *abcg8 ($P < 0.05$)*, 7-Keto ($P < 0.05$) and, 24-OHC ($P < 0.05$) concentrations. For assays using microtiter plates, such as mRNA analysis, plasma lipids, and liver lipids, the plate was also considered to be a block. Plate was included in the model for the analysis of hepatic *hmgcr*

(P<0.005), hepatic *ldlr (P <0.005)*, hepatic *cyp7a1 (P<0.05),* and jejunal *abcg8* ($P < 0.05$) mRNA concentrations.

The following measured variables were used as covariates in the model when they were found to be significant: feed intake, initial body weight, final body weight, and liver weight. Feed intake was included in the model for the analysis of hepatic concentrations of *cyp27a1* mRNA (P <0.005), jejunal *abcg8* ($P <$ 0.005), and change in weight (P <0.05). Initial body weight was included in the model for the analysis of average daily intake ($P < 0.05$). Final body weight was included in the analysis of bile CA ($P < 0.005$), and liver weight ($P < 0.005$). Final weight was included in the analysis of jejunal *ldlr* (P <0.005), and jejunal *abcg8* (P <0.005).

4.4 RESULTS

4.4.1 Average Daily Food Intake, Body and Organ Weights

No effect of Se treatment was observed on initial body weight, final body weight, liver weight or liver weight/ final body weight ratio **(Table 4.2)**. A significant effect of Se was noted on average daily feed intake ($P < 0.05$). Hamsters consuming the HCHS + Se (0.15 ppm) and HCHS + Se (3.4 ppm) diets showed a higher intake than hamsters consuming the HCHS + Se (0.85 ppm) diet ($P < 0.05$). A significant effect of Se treatment was observed on change in weight ($P < 0.05$). Hamsters consuming HCHS + Se (0.85 ppm), HCHS + Se (1.7 ppm) and HCHS + Se (3.4 ppm) diets showed a significantly greater change in weight as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet ($P < 0.05$). Feed intake showed strong positive correlations with final body weight, change in weight and liver weight (r^2=0.74; $P < 0.005$, r^2= 0.6; $P < 0.005$, r^2= 0.6; $P < 0.005$, respectively). Final body weight was positively correlated with liver weight (r^2= 0.7; $P < 0.005$).

4.4.2 Plasma TC and TG Concentrations

As shown in **(Table 4.3)**, a significant main effect of Se treatment was observed on plasma concentrations of TC ($P < 0.05$), HDL-C ($P < 0.05$), TG ($P < 0.05$), and LDL-C/HDL-C ratio ($P < 0.05$).

Hamsters consuming the HCHS + Se (3.4 ppm) and HCHS + Se (0.85 ppm) diets as compared to the HCHS + Se (0.15 ppm) diet showed lower concentrations of TC in their plasma ($P < 0.05$). No change in concentrations of TC were noted in the plasma of hamsters consuming the HCHS + 1.7 ppm diet as compared to hamsters consuming HCHS + Se (0.15 ppm) diet.

Hamsters consuming the HCHS + Se (3.4 ppm) and HCHS + 1.7 ppm diets showed no change in plasma HDL-C concentrations as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet. Hamsters consuming the HCHS + Se (0.85 ppm) diet had significantly lowered concentrations of HDL-C in their plasma as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet ($P < 0.05$). Hamsters consuming the HCHS + Se (1.7 ppm) and HCHS + Se (3.4 ppm) diets showed a higher concentration of HDL-C in their plasma as compared to hamsters consuming the HCHS + Se (0.85 ppm) diet ($P < 0.05$).

Hamsters consuming the HCHS + Se (3.4 ppm) and HCHS + Se (0.85) diets showed no difference in plasma concentrations of TG as compared to hamsters consuming the HCHS + Se (0.15) diet. A significant increase in TG in the plasma of hamsters consuming the HCHS + Se 1.7 ppm diet was noted as compared to concentrations found in the plasma of hamsters consuming the HCHS + Se (0.15 ppm) diet ($P < 0.05$). A significant decrease in TG in the plasma of hamsters consuming the HCHS + Se 3.4 ppm diet was observed as compared to concentrations of TG observed in the plasma of hamsters consuming the HCHS + Se (1.7 ppm) diets ($P < 0.05$).

Hamsters consuming the HCHS + Se (3.4 ppm) and HCHS + Se (1.7 ppm) diets showed no change in LDL-C/HDL-C ratios as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet. Hamsters consuming the HCHS + Se (0.85 ppm) diet showed significantly higher LDL-C/HDL-C ratios as compared

to hamsters consuming the HCHS + Se (0.15 ppm) diet P <0 .05). A lower LDL-C/HDL-C ratio was noted in hamsters consuming the HCHS + Se (3.4 ppm) and HCHS + Se (1.7 ppm) diets as compared to hamsters consuming the HCHS + Se (0.85 ppm) diet ($P < 0.05$).

The effect of Se on plasma LDL-C was marginally significant ($P = 0.05$). Hamsters consuming the HCHS + Se (3.4 ppm) diet showed a lower concentration of LDL-C in their plasma as compared to concentrations observed in the plasma of hamsters consuming the HCHS + Se (0.15 ppm) and HCHS + Se (0.85 ppm) diets ($P < 0.05$). Hamsters consuming the HCHS + Se (1.7 ppm) diet showed significantly lower concentration of LDL-C in their plasma as compared to hamsters consuming HCHS + Se (0.15 ppm) diet ($P < 0.05$).

4.4.3 Hepatic CYP7A1 and CYP27A1 Enzymatic Activity

No significant effects of Se treatment were noted on hepatic activity of CYP7A1 and CYP27A1 **(Table 4.4)**. Hepatic CYP27A1 activity was positively correlated with hepatic levels of Se ($r^2 = 0.6$; $P < 0.05$).

4.4.4 Hepatic Lipids and Se Concentrations

No significant effect of Se was noted on concentrations of hepatic TC, CE, or FC in the liver **(Table 4.5)**.

As shown in **(Table 4.5)**, a significant effect of Se treatment was observed with respect to liver concentrations of TG ($P<0.05$). The hamster group consuming the HCHS + Se (3.4 ppm) diet showed a significantly greater concentration of hepatic TG as compared to levels observed in the livers of hamsters consuming HCHS + Se (0.15 ppm) diet ($P < 0.05$), and hamsters consuming HCHS + Se (0.85 ppm) diet ($P < 0.05$).

As expected, a significant ($P < 0.005$) effect of Se treatment was noted on Se concentrations in the liver **(Table 4.5)**. The livers of hamsters responded in a dose response way to the supplementation of Se. Hamsters fed HCHS + Se (0.85) showed 122%, HCHS + Se (1.7 ppm) 160%, and HCHS + Se (3.4 ppm) 203% increase in hepatic levels of Se as compared to levels found in livers of hamsters fed HCHS + Se (0.15 ppm) diet. Hamsters consuming the HCHS + Se (0.85 ppm) ($P < 0.05$), HCHS + Se (1.7 ppm) ($P < 0.005$) and HCHS + Se (3.4 ppm) ($P < 0.005$) diets showed a significantly higher concentration of Se in their livers as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet. Hamsters consuming the HCHS + Se (3.4 ppm) diet showed a higher concentration of Se in their livers as compared to hamsters consuming the HCHS + Se (1.7 ppm) diet ($P < 0.005$).

Hepatic Se concentrations were positively correlated with hepatic *ldlr* mRNA abundance ($r^2 = 0.4$; $P < 0.05$).

4.4.5 Hepatic Oxysterol Concentrations

No significant effect of Se treatments on hepatic concentrations of 7-Keto, 24(S)-OHC and 25-OHC was observed **(Table 4.6)**.

The effect of Se on levels of 7-OHC showed a tendency towards significance ($P = 0.08$). Hamsters consuming HCHS + Se (3.4 ppm) showed a tendency towards greater levels of 7-OHC in the liver as compared to hamsters fed HCHS + Se (0.15 ppm) ($P = 0.05$) and a significant increase in levels of 7-OHC in the liver as compared to livers of hamsters fed HCHS + Se (1.7 ppm)($P < 0.05$).

The effect of Se on levels of 27-OHC showed a tendency towards significance ($P = 0.09$). Hamsters consuming HCHS + Se (3.4 ppm) showed a tendency to increased levels of 27-OHC in the liver as compared to livers of hamsters fed the HCHS + Se (0.15 ppm) ($P = 0.05$) diet. Hamsters fed the HCHS + Se (1.7 ppm) diet showed a significant increase in hepatic levels of 27-OHC as

compared to levels observed in the livers of hamsters fed the HCHS + Se (0.15 ppm) diet ($P < 0.05$).

Hepatic 27-OHC concentrations were positively correlated with plasma TC (r^2 = 0.4; $P < 0.05$), and were negatively correlated with *cyp7a1* mRNA abundance (r^2 = -0.6; $P < 0.005$). Hepatic 27-OHC concentrations showed a tendency to be correlated with *abcg8* mRNA abundance (r^2 = 0.3, P = 0.10).

4.4.6 Hepatic mRNA Concentrations

No significant effect of Se was observed on abundance of *cyp7a1, abcg5, cyp27a1, and hmgcr* mRNA **(Table 4.7)**.

A significant effect of Se treatment was observed on *abcg8* mRNA ($P <$ 0.005). Hamsters consuming HCHS + Se (0.85 ppm) ($P < 0.05$), HCHS + Se (1.7 ppm) ($P < 0.05$) and HCHS + Se (3.4 ppm) ($P < 0.05$) diets showed a significantly greater abundance of *abcg8* mRNA in their livers as compared to hamsters consuming HCHS + Se (0.15 ppm) diet.

A significant effect of Se treatment was noted on hepatic abundance of *ldlr* mRNA ($P < 0.05$). Hamsters consuming HCHS + Se (3.4 ppm) showed higher abundance of *ldlr* mRNA in their livers as compared to the livers of hamsters fed the HCHS + Se (0.15 ppm) ($P < 0.005$) and HCHS + Se (0.85 ppm) diets ($P <$ 0.005). Hamsters consuming HCHS + Se (0.85 ppm) and HCHS + Se (1.7 ppm) showed higher abundance of *ldlr* in their livers as compared to hamsters consuming HCHS + Se (0.15 ppm) diet.

Abundance of *abcg8* mRNA was positively correlated with *ldlr* mRNA (r^2 = 0.5; $P < 0.005$) abundance. Abundance of *ldlr* mRNA was positively correlated with mRNA abundance of *hmgcr* (r^2 = 0.6; $P < 0.005$).

4.4.7 Jejunal mRNA Concentrations

No significant effect of Se was observed on the abundance of *abcg5*, and *hmgcr* mRNA **(Table 4.8)**. A significant effect of Se treatment was noted on the abundance of *npc1l1* mRNA ($P < 0.05$). Hamsters consuming HCHS + Se (3.4 ppm) showed a higher abundance of *npc1l1* mRNA in their jejunum as compared to the jejunum of hamsters fed the HCHS + Se (0.15 ppm) ($P < 0.0001$), HCHS + Se (0.85 ppm) ($P < 0.005$) and HCHS + Se (1.7 ppm) ($P < 0.05$) diets **(Table 4.8)**.

A significant effect of Se treatment was noted on jejunal abundance of *abcg8* mRNA ($P < 0.05$). Hamsters consuming HCHS + Se (3.4 ppm) showed a higher abundance of *abcg8* mRNA in their jejunum as compared to the jejunum of hamsters fed the HCHS + Se (0.15 ppm) ($P < 0.05$), HCHS + Se (0.85 ppm) ($P < 0.005$) and HCHS + Se (1.7 ppm) ($P < 0.05$) diets.

A significant effect of Se treatment was noted on jejunal abundance of *ldlr* mRNA ($P < 0.05$) **(Table 4.8)**. Hamsters consuming HCHS + Se (3.4 ppm) showed a higher abundance of *ldlr* mRNA in their jejunum as compared to the jejunum of hamsters fed the HCHS + Se (0.15 ppm) ($P < 0.0001$), and the HCHS + Se (0.85 ppm) diets ($P < 0.05$).

The expression levels of *ldlr* mRNA in the jejunum of hamsters consuming HCHS + Se (0.15 ppm) showed a tendency to be decreased as compared to hamsters consuming HCHS + Se (0.85 ppm) diet ($P = 0.05$), and was significantly decreased as compared to hamsters consuming the HCHS + Se (1.7 ppm) diet ($P < 0.005$).

The expression levels of *npc1l1* mRNA were positively correlated with liver TG concentrations ($r^2 = 0.5$; $P < 0.05$), and liver Se concentrations ($r^2 = 0.9$; $P < 0.005$), and were negatively correlated with liver free cholesterol concentrations ($r^2 = -0.5$; $P < 0.005$), and jejunal *hmgcr* mRNA abundance ($r^2 = -0.4$; $P < 0.05$). The expression levels of *ldlr* mRNA were positively correlated with liver Se concentrations ($r^2 = 0.7$; $P < 0.005$) and with abundance of *hmgcr* mRNA ($r^2 = 0.4$; $P < 0.05$), and *abcg8* ($r^2 = 0.4$; $P < 0.05$).

4.4.8 Gallbladder Bile Acids and Cholesterol Concentrations

No significant effect of Se treatments on bile concentrations of CA, DCA, UDCA, UCA, or cholesterol was observed **(Table 4.9)**.

A significant effect of Se on concentrations of HDCA was observed ($P < 0.05$). Hamsters consuming the HCHS + Se (0.85 ppm) and HCHS + Se (3.4 ppm) showed a lower concentration of HDCA in their bile as compared to hamsters consuming the HCHS + Se (0.15 ppm)($P < 0.05$). Hamsters consuming HCHS + Se (1.7 ppm) showed a significantly higher level of LCA in their bile as compared to hamsters consuming the HCHS + Se (0.15 ppm) diet ($P < 0.05$). Hamsters fed the HCHS + Se (1.7 ppm) showed an increase in total bile acids in their bile as compared to level of total bile acid observed in bile of hamsters consuming the HCHS + Se (0.15 ppm) diet ($P < 0.05$).

Most bile acids showed a strong correlation with bile cholesterol; UCA ($r^2 = 0.9$; $P < 0.005$), UDCA ($r^2 = 0.8$; $P < 0.005$), LCA ($r^2 = 0.6$; $P < 0.05$), CDCA ($r^2 = 0.6$; $P < 0.05$), HDCA ($r^2 = 0.5$; $P < 0.05$). Plasma HDL-C was correlated with bile cholesterol ($r^2 = 0.4$; $P < 0.05$) and CDCA ($r^2=0.3$; $P < 0.05$).

4.4.9 Fecal Bile Acid and Cholesterol Concentrations

No significant effect of Se was observed on concentrations of fecal cholesterol, LA, DCA, ursocholic acid, CDCA or CA concentrations **(Table 4.10)**. Concentrations of HDCA and UDCA were not detected.

Fecal concentrations of CA were positively correlated with *abcg5* mRNA abundance ($r^2 = 0.5$, $P < 0.01$) and *abcg8* mRNA abundance ($r^2 = 0.5$, $P < 0.01$). Fecal concentrations of LCA were positively correlated with *abcg5* mRNA abundance ($r^2 = 0.5$, $P < 0.006$) and *abcg8* mRNA abundance ($r^2 = 0.5$, $P < 0.01$). Total fecal bile acid concentrations were positively correlated with *abcg5* mRNA abundance ($r^2 = 0.6$, $P < 0.006$) and *abcg8* mRNA abundance ($r^2 = 0.5$, $P < 0.02$).

4.4.10 Fecal Steroid Excretion Data

No significant effect of Se was observed on average daily cholesterol intake, total fecal steroids, cholesterol balance or cholesterol balance on a 100 g body weight basis **(Table 4.12)**. Average daily cholesterol intake on a 100 g body weight basis was significantly greater in the 3.4 ppm diet as compared to control ($P < 0.05$).

4.5 DISCUSSION

The primary objective of the present study was to determine if the supplementation of Se acts through activation of the LXR to decrease hepatic cholesterol concentrations and plasma LDL-C concentrations. The hepatic and jejunal transporters *abcg5* and *abcg8* and jejunal *npc1l1* were examined for their association with Se. At the level of the liver all doses of Se (0.85, 1.7 and 3.4 ppm) were associated with an increase in abundance of *abcg8* as compared to control ($P < 0.05$) without showing an effect on abcg5 mRNA abundance **(Table 4.7)**. Similarly, in the jejunum high selenite intake (3.4 ppm) was associated with increased abundance of *abcg8* mRNA ($P < 0.05$) without showing an effect on the abundance of *abcg5* mRNA **(Table 4.8)**.

It is not clear why Se showed an effect on *abcg8* and not *abcg5* in both tissues. Se was positively correlated with liver concentrations of 27-OHC ($r^2 = 0.4$; $P < 0.05$) that, in turn, showed a tendency to be correlated with hepatic *abcg8* ($r^2 = 0.3$, $P = 0.10$) but not *abcg5* mRNA abundance. Thus the absence of tendency for a correlation between *abcg5* and 27-OHC suggests that *abcg5* is regulated differently from *abcg8* in hamster liver. Both transporters are shown to be similarly regulated in the murine model (Repa et al., 2005), however appear to be differentially regulated in the hamster liver (Jia et al., 2007) and intestine (Field et al., 2004).

The differential effect of Se on *abcg5* and *abcg8* shown in the present study could also indicate that Se does not increase abundance of *abcg8* through activation of the LXR. In support of this contention, at the level of the jejunum the highest dose of Se (3.4 ppm) was associated with a significant increase in expression levels of *npc1l1* mRNA ($P < 0.05$) **(Table 4.8)**. Expression of *npc1l1* is normally shown to be decreased by LXR agonists in other rodent models (Davis et al., 2004), and thus the mechanism by which Se was associated with increased abundance of *npc1l1* mRNA does not appear to be acting through the LXR pathway. Both *abcg8* and *npc1l1* have been shown to be regulated by other nuclear receptors such as PPAR (van der Veen et al., 2005). Se in the form of selenate increases expression of PPARγ (Mueller and Pallauf, 2006) and thus may also influence other forms of PPAR associated with modulation of the expression of *abcg8* and *npc1l1* mRNA.

Previous work has demonstrated that depletion of cholesterol or over expression of SREBP2 causes an increase in NPC1L1 expression levels in Caco-2 cells (Alrefai et al., 2006). In the present study the increased expression levels of jejunal *abcg8* by Se may have contributed to lowered levels of enterocyte cholesterol which could partly explain the increase in jejunal *npc1l1* mRNA abundance. This is supported by a significant and positive correlation noted between jejunal *abcg8* and jejunal *ldlr* mRNA abundance ($r^2 = 0.42$; $P < 0.02$), which might indicate increased secretion of cholesterol into lumen by enhanced jejunal *abcg8* activity in hamsters consuming the 3.4 ppm diet and which leads to a compensatory increase in *npc1l1*-mediated cholesterol absorption. The converse of increased abundance of *npc1l1* mRNA as resulting in increased abundance of *abcg8* is unlikely as abundance of the *abcg8* transporter is unchanged by cholesterol feeding in the hamster (Field et al., 2004). The possible lowering of enterocyte cholesterol levels by Se supplementation is further supported by a borderline significant positive correlation between jejunal *npc1l1* and *ldlr* mRNA abundance ($r^2 = 0.3$, $P = 0.06$) since *ldlr* mRNA abundance is upregulated by cellular cholesterol depletion. The significant positive correlation between jejunal abundance of *ldlr* mRNA and

hmgcr mRNA (r^2 = 0.4, P < 0.05) also supports a generalized response to decreased enterocyte cholesterol concentrations.

The efficiency of cholesterol absorption may depend on the net effect between influx and efflux of intraluminal cholesterol molecules crossing the brush border of the enterocyte. The concept of compensatory intestinal transporter activities was observed previously in aging mice fed a low cholesterol diet that showed increased *npc1l1* mRNA abundance and cholesterol absorption that was counteracted by tendency for decreased *abcg5* and *abcg8* mRNA abundance (Duan et al., 2005). In the present study, hepatic levels of cholesterol, which are a surrogate marker for cholesterol absorption (Schwarz et al., 2001), showed only a tendency to increase in hamsters supplemented the 3.4 ppm dose despite a significant upregulation of *npc1l1*. The significant increased expression levels of *abcg8* observed with Se supplementation at the same dose (3.4 ppm) might explain why. In that regard, the significant negative correlation between *npc1l1* mRNA abundance and hepatic free cholesterol (FC) concentrations (r^2 = -0.5; P < 0.05) suggests that enhanced *abcg8* transporter activity was compensated for by increased *npc1l1* activity.

The absence of effect of Se on abundance of hepatic *abcg5* may explain why the livers of hamsters fed the 0.85 and 1.7 ppm doses showed a tendency towards a decrease in hepatic cholesterol concentrations rather than a significant decrease. Expression of both *abcg5* and *abcg8* is needed in mice in order to secrete cholesterol into bile (Yu et al., 2003). The lack of effect of Se on liver cholesterol levels agrees with previous work, which showed no significant difference in hepatic sterols of rats fed a Se deficient diet (Nassir et al., 1997). In contrast, others have shown a significant lowering effect of Se administration on hepatic cholesterol levels was observed in rats fed a 1% cholesterol diet and 0.5% CA for 10 weeks (Iizuka et al., 2001). The addition of dietary CA likely led to the differences in hepatic cholesterol levels among studies since CA has been shown to stimulate the *abcg5*/*abcg8* pathway to increase biliary cholesterol (Xu et al., 2003).

The tendency for Se to increase biliary and fecal bile acids in the present study may have influenced jejunal expression levels *abcg8* which were correlated to levels of CA (r^2 = 0.5, P < 0.05) and LCA (r^2 = 0.5, P < 0.05) in the feces. These latter relationships are in concert with previous mouse studies that showed that feeding CA with high cholesterol diet enhanced intestinal *abcg8* expression levels (Duan et al., 2004; Yu et al., 2005; Xu et al., 2003). Gene expression levels of *abcg8* are up-regulated by feeding mice CA but not DCA (Wang et al., 2003). Conversely, bile duct ligation has been demonstrated to decrease both hepatic and intestinal expression levels of *abcg8* (Kamisako and Ogawa, 2006). The mechanisms by which bile acids are associated with *abcg8* expression levels are unclear, particularly as cholesterol is absorbed at the jejunum whereas specific bile acid transporters are located in the ileum (Chiang, 1998).

The highest dose of Se was associated with an increase in hepatic TG concentrations (P < 0.05) **(Table 4.5)**, and *npc1l1* expression levels were positively correlated with liver TG (r^2 = 0.5, P < 0.05), which suggests that the higher abundance of *npc1l1* shown with Se treatment contributed to higher hepatic concentrations of TG. Although *npc1l1* has not been generally regarded to play a role in lipid absorption (Davis et al., 2004), some authors have recently indicated an important role for *npc1l1* in lipid transport and homeostasis (Davies et al., 2005). For example, ezetimibe, which blocks *npc1l1* activity exerts hepatic TG-lowering action in hamsters fed HCHS diets (Van Heek et al., 2001) and lowers hepatic TG enrichment in *ldlr* null mice fed a high cholesterol diet (Repa et al., 2005). Additionally, a significant positive correlation was observed between levels of liver TG and hepatic 27-OHC (r^2 = 0.4, P < 0.05), which suggests that the increased hepatic concentrations of 27-OHC and TG might be associated through 27-OHC activation of LXR. In that regard, Groot et al. (2005) demonstrated increases in hepatic and plasma TG in male Syrian hamsters administered with an LXR agonist along with a dose dependent increase in hepatic mRNA for sterol responsive element binding protein 1 c (SREBP-1c) and fatty acid synthase (FAS) (Groot et al., 2005). Thus, it is not clear if Se acted

through the LXR pathway or not as the elevated hepatic levels of TG with Se supplementation is consistent with an association between Se and the LXR responsive gene SREBP1-c. On the other hand, it is also conceivable that concentrations of TG were increased in the liver due to the effect of Se on PPARy expression which is known to increase hepatic TG levels (Yu et al., 2003).

Se supplementation at the two highest doses (1.7 and 3.4 ppm) was associated with an increase in liver *ldlr* mRNA abundance **(Table 4.7)** and decreased plasma LDL-C concentrations as compared to control levels **(Table 4.3)**. Moreover, significant decreases in plasma cholesterol were associated with Se intake at 0.85 ppm and 3.4 ppm relative to controls ($P < 0.05$) **(Table 4.3)**. These latter results are in agreement with the findings of a previous work, which showed hepatic levels of *ldlr* mRNA and plasma LDL-C concentrations respond to the supplementation of Se in rats fed with a high cholesterol (2%) diet (Dhingra and Bansal., 2005). Dhingra and Bansal (2005), however, did not assess hepatic levels of cholesterol and thus it was unclear whether hepatic cholesterol levels were altered in concert with the up-regulation of the *ldlr* mRNA (Dhingra and Bansal, 2005). In the present study, although Se was shown to decrease plasma LDL-C in the two highest doses, it appears that the plasma LDL-C lowering effect of Se is beneficial to tissues only with the 1.7 ppm dose which showed a tendency to decrease hepatic levels of cholesterol. In contrast, the 3.4 ppm dose showed a tendency to increase hepatic concentrations of TC, possibly due to the effect of Se on jejunal *npc1l1* abundance.

Few studies have examined transcription of rodent liver *ldlr* in relation to changes in liver cholesterol levels. In one murine study, *ldlr* expression was not repressed despite an 18-fold increase in hepatic cholesterol levels resulting in persistent hepatic LDLr function and efficient clearance of plasma lipoproteins (Yu et al., 2002). In a previous study which examined the rate of uptake of LDL-C by the liver and rates of hepatic cholesterol synthesis in the Syrian hamster, a dissociation of hepatic sterol synthesis from hepatic LDL-C clearance suggested that the regulation of these two processes is not tightly coupled in the intact liver

of this species (Spady et al., 1983). The upregulating effect of Se on *ldlr* mRNA shown in the present study may thus be hepatic sterol independent and is supported by previous work demonstrating sterol-independent gene regulation of hepatic enzymes responsible for cholesterol homeostasis (Kojima et al., 2004). Increased expression of *ldlr* through activation of sterol responsive element binding protein 2 (SREBP-2) is normally associated with depressed hepatic levels of cholesterol (Goldstein and Brown, 1990). Thus it is unlikely that the supplementation of Se increased *ldlr* mRNA abundance through increased SREBP-2 levels in the present study.

The absence of an effect of Se to alter abundance of hepatic and jejunal *hmgcr* mRNA in the present study contrasts with a previous study, which showed decreased expression of *hmgcr* mRNA in livers of rats fed 1 ppm Se and a high cholesterol diet (Dhingra and Bansal, 2006a). Despite the decreased expression of *hmgcr* mRNA with Se supplementation, Dhingra and Bansal in a separate study, also showed increased liver *ldlr* mRNA abundance (Dhingra and Bansal, 2006b). These latter findings differ strongly from the present study results showing a strong correlation between abundance of *hmgcr* mRNA and *ldlr* mRNA in the liver ($r^2 = 0.6$, $P < 0.005$) and in the jejunum ($r^2 = 0.4$, $P < 0.05$). Differences in the effect of Se supplementation on *hmgcr* mRNA and and *ldlr* mRNA may have to do with species differences in regulation of the *hmgcr* and *ldlr* genes (Spady and Dietschy, 1983a).

The oxysterol, 27-OHC showed a tendency to be correlated with hepatic *ldlr* ($r^2 = 0.3$, $P = 0.10$). Thus, the lowering of plasma LDL-C concentrations by Se supplementation may have resulted from Se-induced upregulation of hepatic LDLr mRNA abundance mediated via increased hepatic 27-OHC concentrations which was increased through an unmeasured pathway. The specific upregulation of *abcg8* mRNA and *ldlr* mRNA by Se and the significant and positive correlation noted between *abcg8* mRNA and *ldlr* mRNA abundance ($r^2 = 0.5$; $P < 0.005$) provide support to the concept that the increased expression of these genes occurred through a common pathway different from activation of LXR which is

shown to have no effect on *ldlr* mNRA expression in the hamster liver (Groot et al., 2005).

The two highest doses of Se supplementation (1.7 and 3.4 ppm) were shown to be without effect on plasma HDL-C as compared to the control group **(Table 4.3)** and agrees with the findings of a previous study in hamsters showing no effect of Se on plasma HDL-C levels (Vinson et al., 1998). The findings are however in contrast to a previous work which showed an effect of LXR agonist on decreasing plasma HDL-C concentrations in the Syrian hamster (Groot et al., 2005) but which further supports that the notion that the two highest doses of Se supplementation in the present study did not act through LXR activation of CETP which would be expected to decrease concentration of HDL-C. In regards to the 0.85 ppm dose, the supplementation of Se was shown to significantly decrease plasma concentrations of HDL-C which points to a possible activation of LXR at the lowest dose. In the present study, most bile acids were significantly and positively correlated with biliary cholesterol concentrations supporting cholesterol as their source. The source of bile acids is believed to be cholesterol derived from HDL-C arriving from the periphery and in the present study, this notion was supported by a positive correlation noted between HDL-C and bile cholesterol ($r^2 = 0.4$; $P < 0.05$).

A secondary objective of the study was to examine whether Se supplements can increase hepatic oxysterol concentrations by upregulation of the activities of hepatic hydroxylases CYP7A1 and CYP27A1 and liver mRNA abundance of *cyp7a1* and *cyp27a1*. The supplementation of Se was not associated with increased abundance of *cyp7a1* and *cyp27a1* mRNA or CYP7A1 and CYP27A1 activity **(Tables 4.4 and 4.7)**. Hepatic 27-OHC content was negatively correlated with *cyp7a1* abundance, which is consistent with the known feedback inhibition of 27-OHC on this rate limiting enzyme involved in bile acid synthesis (Souidi et al., 2003). A negative correlation between CYP7a1 activity and *hmgcr* abundance was observed, which is consistent with previous studies showing such an inverse relationship in lead nitrate-treated male rats (Kojima et al., 2004). The positive correlation between *cyp7a1* and *hmgcr*

mRNA abundance is in concert with the concept that expression of *cyp7a1* enhances the abundance of LDL receptors (Spady et al., 1995; Pandak et al., 2001).

Importantly, the lack of Se-mediated hepatic upregulation of CYP7A1 activity and *cyp7a1* mRNA and the unchanged bile acid and cholesterol content in bile **(Table 4.9)** indicates that increased biliary secretion in bile is not a key blood cholesterol lowering mechanism of Se supplementation in the hamster. Likewise, the upregulation of *abcg8* mRNA abundance without affecting *abcg5* mRNA abundance by Se supplementation appeared to be insufficient to increase biliary concentrations of cholesterol. On the other hand, an analysis of the effect of Se on cholesterol balance **(Table 4.12)** shows a tendency for an increase in total fecal steroid concentrations suggests that Se may influence blood cholesterol levels through an unknown mechanism.

Despite the absence of effect of Se on *cyp27a1*, increased concentrations of 27-OHC were shown in the livers of hamsters consuming diets supplemented with Se (1.7 ppm, $P < 0.05$) and (3.4 ppm, $P = 0.05$) as compared to controls. The results of the present study are in agreement with previous work which demonstrated a dose response increase in 27-OHC levels without seeing an increase in CYP27A1 levels in the cholesterol loaded macrophage (Fu et al., 2001). Taken together, these findings suggest that Se acted in extra-hepatic tissues to increase hepatic concentrations of 27-OHC which is transported to the liver from the periphery via LDL-C and HDL-C (Diczfalusy et al., 1996). Plasma concentrations of 27-OHC were not measured, however, the influx of 27-OHC from peripheral cells is supported by the positive correlation noted between hepatic 27-OHC and plasma TC concentrations ($r^2 = 0.4$, $P < 0.05$). It is also conceivable that the hepatic antioxidant protection exerted via Se supplementation could lead to decreased breakdown of enzymatically-generated oxysterols via oxidative stress. Hepatic levels of 27-OHC are susceptible to oxidative stress as shown by a 78% lowering of cellular 27-OHC concentrations within 24 h following oxidative stress treatment of cultured human umbilical vein endothelial cells via addition of oxidized LDL-C (Zhu et al., 2005).

Additionally, hepatic concentrations of the product of CYP7A1 activity, 7-OHC, were greater in hamsters consuming HCHS + 3.4 (ppm) diet relative to hamsters fed HCHS + Se (1.7 ppm) ($P < 0.05$) and control diet ($P = 0.05$) **(Table 4.6)**. Both 7-OHC and 27-OHC are substrates for sulfotransferases (Javitt et al. 2000). It is thus possible that Se supplementation may have decreased catabolism of oxysterols as mediated by the cholesterol sulfotransferase 2B1b, which is found expressed in tissues and has been shown to catabolize 24-OHC, 25-OHC and 27-OHC in vitro (Fuda et al., 2007; Chen et al., 2007). Selenate but not selenite is shown to compete with sulphate for incorporation into ATP sulphurylase (Shaw and Anderson, 1972), thus the similarity of Se and sulphur metabolism might allow Se to replace sulphur as an adenylyl sulfate 3'-phosphate donor and possibly decrease activity of cholesterol sulfotransfereas 2B1b resulting in an enhancement tissue oxysterol concentrations. This might partially explain the significant effect of Se on increasing 24-OHC and 25-OHC hepatic concentrations shown in the first study and which used selenate as compared to only a tendency to increase the same oxysterols noted in the second study and which used selenite at the same dose (3.4 ppm). Selenium has been shown to regulate estrogen sulfotransferase (Yang and Christensen, 1998) and thus may also have an effect on cholesterol sulfotransferase 2B1b.

In the present study, the highest dose of Se (3.4 ppm) showed no effect on plasma TG levels as compared to control which agrees with previous work with hamsters fed comparable levels of fat and cholesterol (Poirier et al., 2002), and in hamsters fed comparable levels of Se in conjunction with a standard rodent diet (Vinson et al., 1998). In contrast, previous studies have shown significant decreases in plasma TG concentrations in humans (Djujic et al., 2000), rats (Crespo et al., 1995), and mice (Mueller and Pallauf, 2006). Differences in results may have to do with species, dosage, length of time of feeding or form of Se used.

In summary, the main objective of the study was to examine if Se acted through activation of the LXR to decrease plasma LDL-C concentrations. The findings of the association of Se with increased hepatic concentrations of 27-

OHC and *abcg8* mRNA abundance and the tendency for a correlation between 27-OHC and hepatic abcg8 expression levels implicates an up-regulation of the abundance of *abcg8* through 27-OHC activation of LXR. In previous work, triple knockout mice not expressing the 24-, 25-, and 27-hydroxylases and therefore not synthesizing 24(S)-OHC, 25-OHC and 27-OHC were shown to fail to induce hepatic mRNA abundance of lipoprotein lipase (LPL), ABCG5, and ABCG8 when fed a high cholesterol diet implicating these oxysterols as *in vivo* hepatic ligands for LXR (Chen et al., 2007). On the other hand, although Se was associated with increased hepatic *abcg8* mRNA abundance, it did not show an effect on hepatic abundance of *abcg5* which has been shown to be upregulated by cholesterol feeding in a previous study examining the Syrian hamster (Jia et al., 2007). Similarly, at the level of the jejunum, Se treatment was shown to have an effect on *abcg8* and not *abcg5* which has been shown to be upregulated by cholesterol feeding in a previous study examining the Syrian hamster and thus is considered to be LXR responsive (Field et al., 2004). In the same study, jejunal *abcg8* was not upregulated by cholesterol (Field et al., 2004). In the present study, at the level of the jejunum, the highest dose of Se (3.4 ppm) was associated with an increase in expression levels of *npc1l1* mRNA which is normally down-regulated by LXR activation and cholesterol feeding. Thus, taken together these results suggest that Se-mediated lowering of plasma LDL-C concentrations might not be mediated via activation of LXR through 27-OHC. Limitations to this hypothesis, however include the Se-induced increase in hepatic TG levels and the positive correlation noted between hepatic TG and 27-OHC noted in the present study, which suggests that the increased hepatic concentrations of 27-OHC and TG might be associated through 27-OHC activation of LXR. On the other hand, it is also conceivable that concentrations of TG were increased in the liver due to the effect of Se on PPARγ expression which is known to increase hepatic TG levels (Yu et al., 2003). Also, in the present study the lowest dose of Se (0.85 ppm) was shown to be associated with a significant decrease in plasma HDL-C concentrations which suggests that Se at this concentration may have acted to increase concentrations of CETP a direct LXR target in hamsters and humans.

In the present study, as levels of enterocyte cholesterol were not measured it is unclear if enhanced expression levels of *npc1l1* resulted from Se-induced decreased levels of cholesterol. The mechanism by which Se intake may regulate abundance of the *abcg8* and *npc1l1* genes in the jejunum remains to be explored. In a previous study, intestinal *npc1l1* was downregulated in mice fed a cholesterol diet (Davis et al., 2004) which is consistent with a sterol-regulated element within the *npc1l1* promoter, however to date a LXRE has not been identified in the *npc1l1* promoter (Huff et al., 2006). Alrefai et al. (2007) recently identified two putative sterol regulatory cis-elements in the human NPC1L1 promoter and demonstrated the involvement of these elements in mediating the modulation of human NPC1L1 (Alrefai et al., 2007). It is possible, for example, that the observed increase of *npc1l1* mRNA in the present study reflects subcellular levels of *npc1l1* protein which are not active in cholesterol absorption (Yu et al., 2006). Further research studying the protein abundance or activity of these intestinal sterol transporters would be needed to explore the possibility of posttranscriptional regulation of these proteins by Se.

Thus it is conceivable that the increase in *abcg8* with Se treatment occurred through a LXR-independent pathway. The specific upregulation of *abcg8* mRNA and *ldlr* mRNA by Se and the significant and positive correlation noted between *abcg8* and *ldlr* mRNA provide support to the concept that the increased abundance of these genes occurred through a common pathway different from activation of LXR which is shown to have no effect on *ldlr* mNRA expression in the hamster liver (Groot et al., 2005). In regards to plasma lipids, Se was associated with a decrease in LDL-C and no effect on plasma HDL-C and TG which contradicts earlier findings of the response of the Syrian hamster plasma lipids to treatment with LXR agonists (Groot et al., 2005). The two LXR responsive genes, *abcg8* and *npc1l1* have been shown to be regulated by other nuclear receptors such as PPAR (van der Veen et al., 2005). Se in the form of selenate increases expression of PPARγ (Mueller and Pallauf, 2006) and thus may also influence other forms of PPAR associated with modulation of the expression of *abcg8* and *npc1l1* mRNA. Further studies are needed to clarify

possible mechanisms for the Se-induced increases in hepatic and jejunal *ldlr* and *abcg8* and jejunal npc1l1 mRNA expression levels.

In conclusion, we have shown that Se supplementation regulates in the hamster jejunum three genes involved in cholesterol metabolism in the form of *npc1l1*, *ldlr*, and *abcg8.* The mechanism by which Se intake may influence plasma lipids does not appear to be through the LXR pathway as plasma concentrations of LDL-C and hepatic cholesterol concentrations were not affected by altered *npc1l1* abundance nor by increased fecal cholesterol concentrations via activation of *abcg8.* An analysis of cholesterol balance indicated that Se in the lower doses appears to modulate cholesterol absorption favourably in the two lower doses (0.85 and 1.7 ppm) and thus, it appears that the intestine is an important site of the cholesterol-lowering effects of Se supplementation through an unmeasured mechanism. Clarification of the precise mechanism(s) by which Se supplementation modulates plasma concentrations of cholesterol still remains to be determined.

Table 4.1 Composition Of Experimental Diets (g/kg)[a]				
Ingredients	**HCHS (0.15 ppm)**	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**
Casein, vitamin-free	160	160	160	160
Cornstarch	244.679	244.526	244.34	243.967
Sucrose	200	200	200	200
Dextrose	100	100	100	100
Cellulose	50	50	50	50
BF[b]	150	150	150	150
Safflower Oil[c]	16	16	16	16
Cholesterol, USP[d]	0.938	0.938	0.938	0.938
Mineral Mix[e]	50	50	50	50
Vitamin Mix[f]	10	10	10	10
Choline Bitartrate	13	13	13	13
Sodium Selenite	0.033	0.186	0.372	0.745
Vit. A Palmitate	4.45	4.45	4.45	4.45
Vit. E Acetate	0.090	0.090	0.090	0.090
Metabolizable energy, MJ/Kg	19.2	19.2	19.2	19.2

[a]HCHS= butter fat; Se = Se. All diets were formulated at McGill University and prepared in pellet form by Dyets Inc. (Bethlehem, Penn)

[b] Butterfat . Fatty acid composition of BF is as follows (% by weight) as per Dyets inspection report: C4:0, 3.4; C6:0, 2.0; C8:0, 1.2; C10:0, 2.7; C12:0, 3.0; C14:0, 10.7, C14:1, 1.6; C16:0, 28.0; C16:1, 2.5; C18:0, 13.0; C18:1, 26.8; C18:2, 2.5, C18:3, 1.5, C20:0, 1.1.

[c] Safflower oil was added to prevent essential fatty acid deficiency. α-Toc concentration of SAFF is 350 ppm of α-tocopherol, 180 ppm of other tocopherols. Fatty acid profile of safflower oil included (% by weight): 14:0, trace; 16:0, 6.9; 16:1,trace; 18:0, 2.9; 18:1, 12.2; 18:2, 78.0; 18:3, trace.

[d] Cholesterol USP was added to butterfat 4.193 g/kg

[e]The mineral mix was free of Se and was composed of (g/kg): calcium carbonate 336.4;calcium phosphate, monobasic 285.0; magnesium oxide 2.985; potassium iodate (10 mg KI/g) 0.76; potassium phosphate, dibasic 40.76; sodium chloride 11.45; cupric carbonate 0.084; cobalt chloride 0.133; sodium fluoride 0.002; ferric citrate 25.45; manganous carbonate 0.229; ammonium paramolybdate 0.008; zinc carbonate 0.53; sucrose 296.209. Sodium selenite (10 mg/g sodium selenite) was added separately to make the diets; for HCHS + Se 0.15 ppm, 0.033; for HCHS + Se 0.85 ppm, 0.186; For HCHS + Se 1.7 ppm, 0.372; for HCHS + Se 3.4 ppm, 0.745.

[f]The vitamin mix was free of α-Toc and Vitamin A and was composed of (g/kg):vitamin D3 (400,000 IU/g) 0.9315; vitamin K1 premix (10 mg/g) 110.0; biotin 0.03; folic acid 0.3; niacin 13.5; pantothenate (Ca) 1.5; riboflavin 2.25; thiamin HCl 3.0; pyridoxine HCl 0.9; vitamin B12 (0.1%) 1.5; sucrose 866.0885; vitamin A palmitate (500 IU/g) was added separately to make the diets; for all diets 4.45; α-Toc acetate (500 IU/g) was added separately to make the diets; for all diets, 0.090.

Table 4.2 ANOVA Of The Effects Of Dietary Se Supplementation On Average Daily Intake, Final Weight, Change In Weight And Liver Weight Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]

Variable	Dietary Treatment				P value
	HCHS (0.15 ppm)	HCHS + Se (0.85 ppm)	HCHS + Se (1.7 ppm)	HCHS + Se (3.4 ppm)	Se
Average daily intake(g/d)[2]	7.3±0.15[a]	6.5±0.15[b]	6.9±0.26[ab]	7.2±0.15[a]	P <0.05
Initial weight (g)	120±2	123±2	124±2	122±2	NS
Final weight (g)	116±3	109±3	111±3	109±3	NS
Change in weight (g)[3]	-6±1.8[a]	-12±2.1[b]	-12±2.5[b]	-14±2.6[b]	P <0.05
Liver weight(g)[4]	4.9±.12	5.1±.12	5.0±.12	5.1±.12	NS
Liver weight/final weight	0.044±0.001	0.045±0.001	0.045±0.001	0.046±0.001	NS

[1]Values are mean ± SEM (n=10) Means within rows with no common superscript roman letter differ significantly (P<0.05). Diets and abbreviations are as indicated in Table 4.1. [2] Initial weight significant ($P < 0.05$) in statistical model.
[3] Intake significant ($P < 0.05$) in statistical model. [4] Final weight significant ($P < 0.005$) in statistical model.

Table 4.3 ANOVA Of The Effects Of Dietary Se Supplementation On Plasma TC, HDL-C, LDL-C, TG, And LDL-C/HDL-C Ratio Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]					
Variable	**Dietary Treatment**				**P value**
	HCHS (0.15 ppm)	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
TC (mmol/L)	[3]4.7± 0.10[a]	[2]4.2± 0.23[b]	4.5± 0.21[ab]	[3]4.3± 0.13[b]	*P* <0.05
HDL-C (mmol/L)	1.7± 0.18[a]	1.3± 0.08[b]	1.7± 0.15[a]	1.6± 0.15[a]	*P* <0.05
LDL-C (mmol/L)	[2]1.4± 0.04[a]	[2]1.3± 0.04[a]	[3]1.3 ± 0.03[b]	[2]1.2± 0.04[b]	*P=0.05*[4]
TG (mmol/L)	2.4± 0.31[a]	[3]3± 0.36[ab]	[2]3± 0.33[b]	2.3± 0.31[a]	*P* <0.05
LDL-C/HDL-C	[3]0.75± 0.08[a]	[2]1.1± 0.08[b]	[3]0.82± 0.08[a]	[3]0.72± 0.08[a]	*P*<0.05

[1]Values are mean ± SEM (n=10) except where noted, [2] n= 9, [3] n=8. Means within rows with no common superscript roman letter differ significantly ($P<0.05$). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: TC, Total cholesterol; HDL-C, High Density Lipoprotein cholesterol; LDL-C, Low Density Lipoprotein cholesterol; TG, Triglycerides; LDL-C/HDL-C, Low Density Lipoprotein cholesterol/High Density Lipoprotein cholesterol ratio.

[4] ANOVA was marginally significant at P=0.05.

Table 4.4 ANOVA Of The Effects Of Dietary Se Supplementation On Liver CYP27A1 And CYP7A1 Enzyme Activity Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1,2]

Variable	Dietary Treatment				P value
	HCHS (0.15 ppm)	**HCHS+ Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
CYP27A1 (pmol/mg/min^2)	16± 9	35±13	22± 7	51±27	NS
CYP7A1 (pmol/mg/min^2)	95± 29	83± 29	76± 29	79± 29	NS

[1]Values are mean ± SEM (n=5). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: CYP27A1, Sterol 27-hydroxylase; CYP7A1, 7α-hydroxylase.

[2] Activity : pmol of 7-OHC for CYP7A1 or 27-OHC for CYP27A1 formed per mg protein assayed per minute.

Table 4.5 ANOVA Of The Effects Of Dietary Se Supplementation On Liver Se, TC, CE, FC, And TG Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]

Variable	Dietary Treatment				P value
	HCHS (0.15 ppm)	HCHS + Se (0.85 ppm)	HCHS + Se (1.7 ppm)	HCHS + Se (3.4 ppm)	
Se (µmol/g wet wt)	0.51±0.03[a]	0.62± 0.03[b]	0.81± 0.03[c]	1.03± 0.03[d]	P <0.005
TC[2] (µmol/g wet wt)	101±5.7	[3]98± 6.2	[3]96± 6.2	[4]109± 6.5	NS
CE[2] (µmol/g wet wt)	[4]98±5.7	[3]88± 5.4	[3]88± 5.4	98± 5.2	NS
FC (µmol/g wet wt)	10±0.8	11± 0.8	9± 0.8	9± 0.8	NS
TG[2] (µmol/g wet wt)	160±23[a]	171± 29[a]	228± 24[ab]	262± 23[b]	P <0.05

[1]Values are mean ± SEM (n=10) except where noted, [3] n= 9, [4] n=8. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: Se, Se; TC, Total cholesterol; CE, cholesterol Ester; FC, Free cholesterol; TG, Triglycerides.

[2] Block significant (TC, $P < 0.05$; CE, $P < 0.005$; TG, $P < 0.05$) in statistical model.

Table 4.6 ANOVA Of The Effects Of Dietary Se Supplementation On Liver Oxysterols Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]

Variable	Dietary Treatment				P value
	HCHS (0.15 ppm)	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
7-Keto[2] (pmol/g wet wt)	666±72	[3]584±75	[3]634±72	[3]587±75	NS
7-OHC (pmol/g wet wt)	134±30[ab]	201±45 [ab]	[3]119±27 [a]	[4]263±65 [b]	*P*=0.08[5]
24(S)-OHC[2] (pmol/g wet wt)	298±47	353±45	399±55	430±50	NS
25-OHC (pmol/g wet wt)	228±30	221±30	226±30	246±32	NS
27-OHC (pmol/g wet wt)	204±65 [a]	243±65[ab]	402±70[b]	387±70[ab]	*P*=0.09[5]

[1]Values are mean ± SEM (n=10) except where noted, [3] n= 9, [4] n=8. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: 7-Keto, 7-Ketocholesterol; 7-OHC, 7-hydroxycholesterol; 24(S)-OHC, 24-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol. [2] Block significant (7-Keto, $P < 0.05$; 24(S)-OHC, $P < 0.05$) in statistical model.

[5] ANOVA was marginally significant for 7-OHC ($P = 0.08$), and for 27-OHC ($P = 0.09$).

Table 4.7 ANOVA Of The Effects Of Dietary Se Supplementation On Liver mRNA Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]					
Variable	**Dietary Treatment**				**P value**
	HCHS (0.15 ppm)	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
cyp27a1[2]	151 ± 17	179± 16	196± 15	174± 16	NS
cyp7a1[3,4]	62± 5	74± 7	79± 16	73± 12	NS
abcg5[3]	106± 8	118± 8	117± 8	122± 8	NS
abcg8[3]	283± 18[a]	[5]383± 38[b]	353±15[b]	388±33[b]	$P < 0.05$
hmgcr[4]	84± 5	87±4	96±6	96± 3	NS

ldlr[4]	325±30[a]	400±30[b]	450± 30[bc]	475±30[c]	$P < 0.05$

[1]Values are mean ± SEM (n=9) except where noted, [5] n= 8. The average of duplicate samples was taken. Quantities are relative to each other. Means within rows with no common superscript roman letter differ significantly ($P < 0.05$). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: *cyp27a1*, sterol 27-hydroxylase; *cyp7a1*, 7α-hydroxylase; *abcg5,* ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP-binding cassette (ABC) transporter g8; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase*; ldlr*, low density lipoprotein cholesterol receptor. [2] Intake significant ($P < 0.005$) in statistical model. [3] Block significant (*cyp7a1*, $P < 0.05$; *abcg5*, $P < 0.005$; *abcg8*, $P < 0.05$) in statistical model. [4] Plate significant (*cyp7a1*, $P < 0.05$; *hmgcr,* $P < 0.005$; *ldlr*, $P < 0.005$) in statistical model.

Table 4.8 ANOVA Of The Effects Of Dietary Se Supplementation On Jejunal mRNA Of Adult Male Syrian Hamster Fed BF Diets For 4 Wk[1]					
Variable	**Dietary Treatment**				**P value**
	HCHS (0.15 ppm)	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
npc1l1[2]	300± 20[a]	325± 35[a]	425± 50[a]	[7]675±75[b]	P < 0.05
abcg5	120± 20	111± 20	121± 20	123± 30	NS
abcg8[3,4,5]	117± 6[a]	112± 6[a]	122± 6[a]	[6]142± 6[b]	P < 0.05
Hmgcr	248± 13	258±23	255±18	255± 25	NS
ldlr[5]	300± 43[a]	425± 43[b]	500± 43[b,c]	[6]600± 43[c]	P < 0.05

[1]Values are mean ± SEM (n=9) except where noted, [6]n= 8, [7] n=6. The average of duplicate samples was taken. Quantities are relative to each other. Means within rows with no common superscript roman letter differ significantly ($P<0.05$). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP-binding cassette (ABC) transporter g8; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase *; ldlr*, low density lipoprotein receptor. [2]Initial weight used as covariate in the statistical model.

[3]Intake used as covariate in the statistical model.

[4]Plate used as covariate in the statistical model.

[5]Final weight used as covariate in the statistical model.

Table 4.9 ANOVA Of The Effects Of Dietary Se Supplementation On Biliary Bile Acids And TC Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]					
Variable	**Dietary Treatment**				**P value**
Bile bile acid (µmol/mL bile)	**HCHS (0.15 ppm)**	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
CA[2]	49±3	60±8	63±9	47±7	NS
CDCA	7.4±1.2	5.3±1.1	7.9±1.1	6.9±1.2	NS
DCA	5.7±0.53	6.1±0.52	5.9±0.49	5.8±0.53	NS
LCA	1.5±0.25	1.9±0.18	2.1±0.18	2.2±0.19	NS
UDCA	[7]0.32±.08	[5]0.22±.03	[4]0.27±.04	[7]0.23±.04	NS
HDCA	[7]0.24±.04[a]	[5]0.11±.01[b]	[4]0.16±.03[ab]	[7]0.13±.01[b]	P<0.05
UCA	[4]0.06±.01	[4]0.04±.01	[4]0.04±.01	[7]0.04±.01	NS
TC	13±1.5	11±1.3	12±1.4	10±1.4	NS
Total bile acids[3]	58±5.8	71±8.7	80±5.5	71±8.8	NS

Total bile acids/TC ratio	5.1±0.98	7.8±0.85	5.8±0.79	6.8±0.85	NS

[1]Values are mean ± SEM (n=10) except where noted, [4] n= 9, [5] n=8, [6]n=7, [7]n=5. Means within rows with no common superscript roman letter differ significantly (P<0.05). Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; UCA, ursocholanic acid.

[2] Final weight significant (P < 0.005) in statistical model.

[3] Intake significant (P < 0.005) in statistical model.

Table 4.10 ANOVA Of The Effects Of Dietary Se Supplementation On Fecal Bile Acids And Cholesterol Of Adult Male Syrian Hamster Fed BF Diets For 4 Wk[1]					
Variable	**Dietary Treatment**				**P value**
umol/g feces	**HCHS (0.15 ppm)**	**HCHS + Se (0.85 ppm)**	**HCHS + Se (1.7 ppm)**	**HCHS + Se (3.4 ppm)**	
LCA	[4]0.54±0.08	0.50±0.09	[4]0.64±0.08	[4]0.54±0.08	NS
DCA[2]	0.41±.04	0.29±.06	0.32±.04	0.37±.05	NS
Ursocholic	0.21±0.03	0.23±0.03	0.21±0.03	0.24±0.03	NS
CA	0.05±.01	0.08±.03	0.08±.04	0.08±.01	NS
CDCA	0.01±.01	0.02±.01	0.03±.01	0.01±.01	NS
HDCA	ND	ND	ND	ND	
UDCA	ND	ND	ND	ND	
Cholesterol	[3]5.8±1.1	[3]7.0±2.61	[3]8.3±2.5	[3]9.2±1.9	NS
Total bile acids	2.0±0.51	2.0±0.52	1.6±0.47	1.4±0.51	NS

Total bile acids/cholesterol ratio	0.3±0.04	0.3±0.05	0.4±0.05	0.3±0.05	NS

[1]Values are mean ± SEM (n=10) except where noted, [3] n= 9, [4] n=8. Diets and abbreviations are as indicated in Table 4.1. Other abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; UCA, ursocholanic acid; ND, not detected.

[2] Intake used as covariate in the statistical model.

Table 4.11 List Of Oligonucleotide Primers Used To Amplify mRNA Of Various Genes By Real-Time PCR[1]

Gene	Accession Number		Forward primer 5'→ 3'	Reverse primer 5'→ 3'
	Forward	Reverse		
gapdh	AF106860	U10983	TCA AGA AGG TGG TGA AGC AGG C	GCA TCA AAG GTG GAA GAG TGG G
hmgcr	M12705	X00494	AGC AAG TGG TCC CAC GAA TGA AGA	GCT CCT TGA ACA CCT AGC ATC TGC
cyp27a1	NM_178847	AY178622	GGA TCC AAC ACC CAT TTG GCT CTG	TGT ATC AGC CTT GAC AGC AGG AGT
cyp7a1	L04690.1	NM_012942.1	GCA TTT GGA CAC AGA AGC ATT GAC CC	GTG ACC CAG GCA TCA CTC TTT GAT
ldlr	NM_175762	M94387	TTG GAC AAC AAT GGT GGC TGT TCC	TTG CAG ACC CTG GTG TGA GG
abcg5	NM_031884.1	NM_053754.2	AGG ACT GGA CTG CAT GAC TGC AAA	CAG AAC ACC AAC TCT CCG TAA GTC AG

abcg8	NM_026180.2	NM_130414.2	ACC TAC AGT GGT CAG TCC AAC ACT	TTT CAT CTT GCC ACC GTG GTC TCT
npc1l1	AY437866	AY437865	ACA GGC CAG CTA CAG GAT ATG ACT	ACT GTT GAC ACA GCA ATC GGA GAG

[1]**ABBREVIATIONS**: *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase ; *cyp27a1*, sterol 27-hydroxylase; *cyp7a1*, cholesterol 7α-hydroxylase; *ldlr*, low density lipoprotein receptor ; *abcg5*, ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP –binding cassette (ABC) transporter g8; *npc1l1*, niemann-pick C1-like 1 protein.

Table 4.12 ANOVA Of The Effects Of Dietary Se Supplementation On Average Daily Cholesterol Intake, Average Daily Cholesterol Intake/100 g Body Wt, Total Fecal Steroids, Total Fecal Steroids/ 100 g Body Wt, Cholesterol Balance mg/d and Cholesterol Balance mg/100 g Body wt /d Of Adult Male Syrian Hamster Fed HCHS Diets For 4 Wk[1]

Variable	Dietary Treatment				P value
	HCHS (0.15 ppm)	HCHS + Se (0.85 ppm)	HCHS + Se (1.7 ppm)	HCHS + Se (3.4 ppm)	
Average daily cholesterol intake(µmol/d)	19±0.3	[2]17±0.4	[2]18±0.7	[2]19±0.4	P=0.11
Average daily cholesterol intake (µmol /100 g Body Wt)	16±0.3[a]	[2]16±0.4 [a]	[2]16±0.4 [a]	[2]17±0.3 [b]	P<0.05
Total fecal steroids (µmol/g feces)	8±0.7	10±3	11±3	11±2	NS
Total fecal steroids (µmol/g feces/ 100 g Body Wt)	7±0.7	9±2	9±2	10±2	NS

[3]**Cholesterol balance (Intake minus excretion) (µmol /d)**	9±0.7	6±3	7±2	7±2	NS
[3]**Cholesterol balance (Intake minus excretion) (µmol /100 g Body wt/d)**	10±0.9	7±3	7±3	8±2	NS

[1]Values are mean ± SEM (n=9) except where noted, [2]n=10. Means within rows with no common superscript roman letter differ significantly ($P<0.05$). Diets and abbreviations are as indicated in Table 4.1.

[3]Cholesterol balance or absorbed cholesterol was determined by subtracting the average fecal steroid output from average cholesterol intake.

CHAPTER 5 - CONCLUDING REMARKS

5.1 Final Conclusion and Overall Summary

The work described in the present thesis has examined hepatic and jejunal gene expression levels of key cholesterol modulators that are responsive to LXR as a potential mechanism of the blood cholesterol lowering effects of Se supplementation in Golden Syrian hamsters fed hypercholesterolemic diets. To further study the LXR mechanism of hypocholesterolemic action of Se, the present thesis also studied the possible role of Se-mediated hepatic induction of oxysterol ligands of LXR. In particular, the relationship of oxysterols with the LXR responsive genes *abcg5* and *abcg8* in hepatic and jejunal tissues and *npc1l1* in the jejunum was investigated. To examine the possible oxysterol synthetic pathways that might be involved in relation to the Se-mediated induction of oxysterols, the activities of CYP7A1 and CYP27A1 as well as the expression levels of *cyp7a1* and *cyp27a1* mRNA were also studied. To obtain a comprehensive overview of the impact of Se on cholesterol metabolism, *ldlr* and *hmgcr* genes were examined to relate in hepatic cholesterol and plasma cholesterol modulation in response to Se supplementation. Finally, cholesterol and bile acid content in both bile and feces was studied to relate possible Se-mediated enhancement of *abcg5* and *abcg8* activities in the secretion of cholesterol into bile and the intestinal lumen as a possible hypocholesterolemic mechanism of action. In these animal studies, hepatic Se content was assessed to ensure that the Se-enriched diets were efficacious in increasing tissue Se concentrations.

The Syrian hamster was chosen since this animal is considered to be a better model for human lipid metabolism as compared to murine models that have been the primary animal models used for the study of LXR responsive genes (Zelcer and Tontonoz, 2006). Importantly, both hamsters and humans possess a CETP gene (Tsutsumi et al., 2001; Luo and Tall, 2000) which is a known LXR-responsive gene containing a LXRE (Michael et al., 2005). The up-regulation of CETP in humans could result in a more atherogenic profile as CETP

exchanges TG in apolipoprotein B containing lipoproteins for HDL-C which reduces HDL-C (Wang, 2003). Also, *cyp7a1* is not a LXR target gene in either the human or the hamster as opposed to mice and rats (Repa and Mangelsdorf, 2000). Therefore, the hamster is expected to be a much more representative model in regards to the human response to LXR agonists. The rat model has been typically used to study the hypocholesterolemic effects of Se; however, hamsters are sensitive to dietary cholesterol to a similar degree as humans (Spady, 1983a) whereas rats and mice are resistant to diet-induced hypercholesterolemia due to the stimulation of CYP7A1 activity, which makes them highly efficient at converting cholesterol into bile acids (Wilson, 1964; Horton et al., 1995; Peet et al., 1998). Therefore, the hamster is a more representative model in regards to the human response to the supplementation of Se in conjunction with a HCHS diet on plasma lipids.

The choice of highest level of Se (3.4 ppm) for the studies conducted in chapters 3 and 4 is based on the results of a former study which showed an effect of Se at this dose on plasma lipids (Poirier et al., 2002). In chapter 3, the range of doses of Se used in the cell culture study were chosen based on the usual plasma levels of Se which range from (1.3 – 1.9 µmol Se/L; 20-30 µg Se/L) in Se-poor countries to (6.3 – 12.6 µmol Se/L; 100-200 µg Se/L) in Se-rich countries (Thorling et al., 1986).

The form of Se in the study in chapter 3 was selenate. As inorganic forms of Se do not appear to demonstrate a differential effect on plasma lipids in previous animal studies, Se in the form of selenite was tested in cell culture using a dose response study design. Because Se in the form of selenite was shown to have an effect on concentrations of 27-OHC in a dose response manner Se in the form of selenite was used in the in vivo study in chapter 4.

In chapter 3, hamsters consuming the Se diet showed a lower tendency to increase weight than hamsters consuming the other dietary groups in the same study. Also, a significant decrease in intake was noted in hamsters consuming the Se diet as compared to animals ingesting the control diet which might partially explain the reason for the lower tendency to increase weight shown in

this dietary group. The reason for the effect of Se on intake in chapter 3 is not clear. In chapter 4 the lowest dose of Se was shown to be associated with a significant decrease in intake as compared to control group. The addition of Se to the diet in the studies conducted in both chapters may have made the diets less palatable however in chapter 4 the consumption of diets containing the highest Se doses (1.7 and 3.4 ppm) was not different from control hamster consumption which also showed a decrease in weight. Hamsters in chapter 4 were fed for one week longer than hamsters fed in chapter 3 which may partially explain the weight loss shown in hamsters ingesting Se diets in chapter 4 as compared to a tendency for weight gain in animals ingesting the Se diet in chapter 3.

In chapter 4, the final weight, liver weight and liver weight/final weight ratios of hamsters consuming the diets containing Se were not different from control which suggests that the loss of weight shown in chapter 4 had minimal impact on measured parameters. Feed intake in chapter 4 was included in the model for the analysis of change in weight and *cyp27a1* mRNA analysis. Also in chapter 4, final weight was found to be significant as a covariate in the statistical model in the analysis of liver weight, bile CA, jejunal *ldlr* and jejunal *abcg8*. Taken together, these findings indicate that final weight and intake were important influences on the above-mentioned measured parameters however did not influence other parameters measured in chapter 4.

Novel primers were needed to be developed for all eight genes described in the present thesis as the primers for hamster were not available, or the primers tested in the literature with hamsters (Field et al., 2004) did not result in satisfactory results. To date, one study only used the hamster using an LXR agonist to study hepatic *abcg5* and *abcg8* (Groot et al., 2005) whereas another studied intestinal *abcg5, abcg8, npc1l1* (Field et al., 2004). Both studies indicated the use of quantitative real time PCR analysis in order to quantitate mRNA of hamster genes tested, similar to the method used in the present thesis. However, in the present thesis, Ribogreen quantitative reagent was used in order to quantitate RNA. As opposed to conventional methods used to quantitate RNA, the use of Ribogreen circumvents contributions from interfering substances by

exhibiting an emission max at 530 nm when bound specifically to intact RNA which minimizes bias or incorrect results. The accuracy of gene expression evaluation is known to be influenced by the quantity and quality of starting RNA. Ribogreen which is used for ultra sensitive quantitation of RNA binds to only intact RNA in DNA free solution. Working with low-quality RNA may strongly compromise the experimental results. Using intact RNA is a key element for successful application of quantitative real time PCR. Thus the method developed in the present thesis is more sensitive and specific. Although 8 primer sets were used for the hepatic and jejunal data presented in this thesis, a total of 18 primer pairs were designed and 16 tested. No hamster sequences were available for *cyp27a1* and thus synthesis of the primer was based on sequence-alignment in region of high homology between rat and human. The *cyp27a1* mRNA has never been previously studied in the hamster and thus the findings of the present study show that it is present in hamster liver at levels which allows for its detection by real time quantitive PCR. These novel primers developed in the present thesis are therefore now available in the literature for use in future hamster studies by other researchers. Also, an extensive perusal of the literature was needed in order to identify a housekeeping gene which was abundant and constantly expressed in samples and was not affected by Se treatment. Both *β-actin* and *gapdh* gene primers were tested for their stability with the samples. Due to the fact that *β-actin* was shown to be significantly increased by Se treatment and *gapdh* was not, *gapdh* was chosen as the housekeeping gene.

To date, it has been presumed that naturally occurring oxysterols such as 24(S)-OHC, 25-OHC and 27-OHC regulate LXR activating functions based on: (a) in vitro studies; and (b) from evidence that cholesterol feeding naturally increases hepatic oxysterol concentrations in the animal (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2004) **(Figure 5.1)**. For the most part, studies which investigated the association between oxysterols and LXR-responsive genes have measured the effect of exogenous oxysterols concentrations within cell culture systems (i.e., macrophages, human embryonic kidney cells, Chinese hamster ovary cells) **(Table 2.9)** on LXR-responsive genes

(Sparrow et al., 2000; Venkateswaran et al., 2000; Wong et al., 2007; Rowe et al., 2003). Of the cell culture studies, only one study examined the effect of cholesterol loading on endogenously produced oxysterols in the macrophage (Fu et al., 2001) **(Table 2.8)**. The loading of cholesterol is important as it is believed that the oxysterols derived from cholesterol are involved in the activation of LXR. In animal studies, although cholesterol feeding has been shown to increase levels of hepatic oxysterols (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2004), the association of endogenously produced oxysterols with LXR-resonsive genes has not been extensively studied. One study only measured tissue levels of hepatic oxysterol concentrations on LXR-responsive genes with cholesterol feeding in rats (Xu et al., 2004). A unique aspect of the thesis work is that oxysterol ligands of LXR were measured in vivo in response to dietary intervention on LXR responsive genes in relation to cholesterol metabolism. Previous in vivo studies have either: (a) fed cholesterol and assumed oxysterols were produced; (b) fed oxysterols; or (b) used non-sterol LXR agonists to study their effects on LXR responsive gene expression levels. Very recently, the 24-, 25- and 27-OHC were indicated indirectly to act as LXR ligands via studies involving transgenic 24-, 25- and 27-hydroxylase knockout mice (Chen et al., 2007). Thus, the work from this thesis is the first to demonstrate that these enzymatically generated oxysterols present at physiological levels in vivo are related to changes in lipid metabolism. To our knowledge, no previous study has related in vivo tissue concentrations of oxysterol LXR ligands to dietary or pharmacological interventions on tissue lipids or mRNA abundance. The present study shows that tissue oxysterol LXR ligands can be affected through dietary manipulation. We showed that apart from the effect of dietary cholesterol on in vivo oxysterol concentrations, dietary Se treatment can affect enzymatically formed oxysterols. Thus, these findings suggest the need for future studies to determine if such oxysterols are induced by other dietary cholesterol-modulating factors as a mechanism of their blood cholesterol lowering effects.

Importantly, the present thesis also describes a comprehensive examination of the relationship of the expression levels of liver *abcg5* and *abcg8* and jejunum *abcg5*, *abcg8* and *npc1l1* with respect to hepatic lipid (TG, TC, FC, CE) concentrations, *hmgcr*, *ldlr* expression levels and plasma lipid (LDL-C, HDL-C, TG) concentrations. The study of such a large number of parameters concurrently provides a more comprehensive view regarding the mechanisms of Se-mediated cholesterol modulation, which is not typically carried out with respect to in vivo assessments of the dynamics of cholesterol metabolism.

Our primary objective in Chapter 3 was to compare the separate and combined antioxidant effects of supplemental Se and tocopherol on hepatic oxysterol concentrations in relation to their plasma lipid lowering effects in the Syrian hamster. A semi-purified diet which was well tolerated by the hamsters was designed by the PhD candidate based on National Research Council guidelines specific to Syrian hamster nutritional needs and took into consideration the potential toxicity of Se. Our results in Chapter 3 showed that the enhanced hepatic levels of either Se alone or both tocopherol and Se were associated with increases in hepatic concentrations of the enzymatically generated oxysterol, 27-OHC, thereby indicating that oxysterol generation was associated with Se but not tocopherol supplementation. Hep G2 cell culture studies confirmed that Se treatment at physiological concentrations could specifically induce hepatic enzymatically generated oxysterol concentrations.

To our knowledge, this latter result is the first direct demonstration that tissue levels of an enzymatically generated oxysterol are related to dietary modulation of cholesterol metabolism. In addition, higher enzymatically-generated oxysterol content was positively associated with increased liver concentrations of the supplemented antioxidants of tocopherol and Se, which suggests antioxidant protection of enzymatically generated oxysterols. This result is supported by an in vitro study that showed that antioxidant treatment in the form of Se treatment inhibited cellular depletion of 27-OHC by oxidized LDL (Zhu et al., 2005). Apart from some in vivo studies which have investigated the effect of antioxidants on free radically formed oxysterols, to our knowledge this is the

first study to examine the effect of antioxidants on naturally occurring enzymatically formed hepatic oxysterols. Since Se treatment was associated with increased concentrations of hepatic oxysterols, the effect of Se supplementation on LXR responsive genes at various doses with a HCHS diet was further investigated in the Golden Syrian hamster since 27-OHC is a putative LXR agonist.

The results from Chapter 4 indicated that the supplementation of Se at the two highest doses was associated with an increase in hepatic levels of 27-OHC and *ldlr* and *abcg8* mRNA abundance and a decrease in plasma LDL-C concentrations. The oxysterol, 27-OHC, showed a significant positive correlation with *ldlr* and *abcg8* mRNA suggesting that these genes share a common source of upregulation involving this oxysterol. This latter finding is the first to show a relationship between an enzymatically generated oxysterol measured in vivo and changes in cholesterol metabolism.

The hepatic concentrations of 27-OHC were not related to hepatic biosynthetic pathways in terms of CYP7A1 and CYP27A1 activities and expression levels of *cyp7a1* and *cyp27a1*, which suggests that this oxysterol was generated extrahepatically. Although other studies have suggested that 27-OHC is synthesized primarily in extrahepatic tissues and transported via lipoproteins to the liver (Diczfalusy et al., 1996), the present thesis data is the first study to directly show that hepatic in vivo 27-OHC content is not related to hepatic biosynthetic pathways. In support of this contention, positive correlations were observed between hepatic concentrations of 27-OHC and plasma TC.

A significant positive correlation was observed between hepatic 27-OHC and TG content, which is explainable by the 27-OHC-mediated induction of SREBP-1c, which is involved in hepatic lipogenesis (Groot et al., 2005) but which can also be explained by the effect of Se on PPARγ expression (Mueller and Pallauf, 2006). Hepatic 27-OHC content was negatively correlated with *cyp7a1* expression levels, which is consistent with the known feedback inhibition of 27-OHC on this rate limiting enzyme involved in bile acid synthesis (Souidi et al., 2003). Importantly, the lack of Se-mediated hepatic upregulation of CYP7A1

activity and *cyp7a1* mRNA abundance and the unchanged bile acid and cholesterol content in bile through increased hepatic abundance of *abcg5* and *abcg8* indicates that increased cholesterol secretion in bile is not a key blood cholesterol lowering mechanism of Se supplementation in the hamster.

The increased abundance of hepatic *ldlr* mRNA in response to Se supplementation was not associated with a significant decrease in hepatic levels of cholesterol, which suggested that Se worked through a sterol independent mechanism. This is supported by previous work demonstrating sterol-independent gene regulation of hepatic enzymes responsible for cholesterol homeostasis (Kojima et al., 2004). Although induction of SREBP-2 is linked with increased expression levels of *ldlr* (Goldstein and Brown, 1990), this mechanism is unlikely as activation of SREBP-2 is normally associated with depressed hepatic levels of cholesterol (Goldstein and Brown, 1990) and which was not observed with Se treatment in our study. The significant increase in hepatic *ldlr* abundance and a strong positive correlation between *ldlr* and *hmgcr* is consistent with a plasma cholesterol lowering action of Se mediated via an increased hepatic uptake of LDL. A negative correlation between CYP7A1 activity and *hmgcr* abundance was observed, which is consistent with previous studies showing such an inverse relationship in lead nitrate-treated male rats (Kojima et al., 2004). The positive correlation between *cyp7a1* and *hmgcr* mRNA abundance is in concert with the concept that increased expression levels of *cyp7a1* enhances the abundance of LDL receptors (Spady et al., 1995; Pandak et al., 2001).

As Se influenced only the hepatic *abcg8* transporter but not abundance of the *abcg5* transporter, we suggest that LXR is not the mechanism by which Se exerts hypocholesterolemic effects as previous studies indicate that both transporters are normally concurrently induced by LXR agonists in murine models at the level of the liver (Repa et al., 2002; Dieter et al., 2004; Berge et al., 2000). Also, as both *abcg5* and *abcg8* did not respond to two LXR agonists in a previous study (Groot et al., 2005), the response of liver *abcg8* in the present study suggests that *abcg8* was increased independently of LXR activation.

Hence, further work was carried out to determine if Se was capable of modulating both *abcg5* and *abcg8* expression levels in the hamster jejunum. This latter investigation was important to clarify further whether Se treatment involved LXR since the *abcg5* transporter in the Syrian hamster jejunum is LXR responsive as demonstrated by cholesterol feeding (Field et al., 2004), which activates LXR and intestinal expression levels of *abcg5* and *abcg8,* and as both *abcg5* and *abcg8* are also increased by LXR agonists in the hamster intestine (Groot et al., 2005).

Thus, the specific upregulation of jejunal *abcg8* and *npc1l1* genes by Se treatment is described. Similar to the findings in the liver, *abcg5* was not up-regulated as opposed to *abcg8* by Se treatment further supporting the concept Se upregulates *abcg8* through a mechanism independent of the LXR. The unexpected upregulation of *npc1l1* further supports the findings in the liver that Se exerted effects on lipid metabolism independently of LXR, since LXR agonists decrease intestinal *npc1l1* mRNA (Duval et al., 2006; Davis et al., 2004). The very strong correlation between liver Se and *npc1l1* mRNA ($r^2 = 0.9$, $P < 0.0005$) and with *ldlr* mRNA ($r^2 = 0.7$, $P < 0.0005$) is indicative that Se status played an important modulatory role on jejunal *npc1l1* and *ldlr* expression levels, since liver Se stores are reflective of whole body Se status (Hac et al., 2003). Interestingly, as loss of NPC1L1 expression was shown to protect against diet-induced fatty liver in a previous study (Davies et al., 2005) the positive correlation of *npc1l1* expression and liver TG supports a more recent suggestion that indicates *npc1l1* transport is involved with fatty acid uptake (Mathur et al., 2007) and our finding thus provides support for this novel direction for future studies examining the role of *npc1l1* in lipid absorption (van Heek et al., 2001). Hepatic cholesterol content is a sensitive index of whole body cholesterol absorption (Schwarz et al., 2001). Hence, the lack of correlation was observed between *npc1l1* and hepatic cholesterol content appears to signify that the upregulation of *npc1l1* by Se treatment did not lead to a major increase in cholesterol absorption. This latter observation lends further support to the concept that the the Se-mediated upregulation of *npc1l1* was a response to a possible lowering of enterocyte

cholesterol content mediated by the increased expression levels of jejunal *abcg8* via Se supplementation.

It is conceivable that the upregulation of *npc1l1* expression levels by Se supplementation was a response to reduced cellular cholesterol content, which activates receptors that are involved in cholesterol absorption. Expression levels of NPC1L1 are downregulated by elevated cellular cholesterol (Davis et al., 2004). Thus, upregulation of *npc1l1* abundance might be a response to increased expression of *abcg8* levels as suggested in previous work using the aging mouse model (Duan et al., 2005). In further support of this concept, we observed: (a) a significant increase in hepatic *ldlr* expression levels observed with Se supplementation; and (b) a positive correlation between *ldlr* and *hmgcr* mRNA abundance, which are expected responses to maintain cholesterol homeostasis to the increased abundance of *abcg8*. A negative relationship was observed between *npc1l1* and *hmgcr* mRNA abundance, which can be also related to a feedback inhibition of cholesterol synthesis of HMG-CoA reductase via the increased NPC1L1-mediated cholesterol uptake.

As opposed to the higher 3.4 ppm dietary Se dose, enhanced jejunal abundance of *abcg8* was not observed at the lower dietary dose of Se of 1.7 ppm despite a significant plasma LDL lowering effect, which signifies induction of *abcg8* is not an important mechanism for hypocholesterolemic effects of Se. An analysis of cholesterol balance indicated that Se appears to modulate cholesterol absorption and thus, it appears that the intestine is an important site of the cholesterol-lowering effects of Se supplementation through an unmeasured mechanism.

On the basis of the present thesis data, we strongly conclude that Se is not acting via LXR pathways to induce hypocholesterolemic effects in the Syrian hamster based on: (a) only jejunal *abcg8* mRNA abundance increased with Se treatment in our study whereas both intestinal *abcg8* and *abcg5* mRNA are upregulated when LXR agonists were administered to Syrian hamsters (Groot et al., 2005); (b) similarly only *abcg8* but not *abcg5* mRNA were increased in the liver with Se supplementation in the present thesis whereas hepatic *abcg8* and

abcg5 mRNA were unresponsive when Syrian hamsters were treated with LXR agonists (Groot et al., 2005); (c) *ldlr* mRNA was increased in response to Se supplementation whereas previous Syrian hamster studies have shown that LXR agonists do not increase *ldlr* mRNA (Groot et al., 2005); (d) the upregulation of *npc1l1* by Se treatment in the present work contradicts the concept of LXR activation as LXR agonists normally decrease intestinal *npc1l1* mRNA (Duval et al., 2006; Davis et al., 2004); (e) as opposed to the plasma LDL lowering effect of Se supplementation in the present thesis, LXR agonists were shown to induce blood cholesterol raising effects in the Syrian hamster (Groot et al., 2005); and (f) both cholesterol feeding and LXR agonists have been shown in murine models to upregulate both *abcg8* and *abcg5* mRNA rather than selectively only one of these transporters (Repa et al., 2002; Dieter et al., 2004; Berge et al., 2000). As Se can activate hepatic and jejunal *abcg8* abundance selectively, we propose that other nuclear receptors such as PPAR may be involved with Se supplementation as the *abcg5* and *abcg8* transporters can be selectively up-regulated by PPAR agonists (van der Veen et al., 2005). Moreover, the present thesis data does indicate that the liver is not a primary site of the cholesterol-lowering effects of Se supplementation as: (a) catabolism of cholesterol to bile acids as mediated by the rate limiting CYP7A1 enzyme was not significantly increased; and (b) cholesterol content of bile was not significantly increased.

Although intestinal secretion or absorption of cholesterol as mediated by *abcg5*, *abcg8* and *npc1l1* were not significantly related to alterations in blood cholesterol concentrations, a cholesterol balance study did show that Se decreased absorption of cholesterol at the level of the gut, most favourably in the lower doses (0.85 and 1.7 ppm). This effect of Se through an unmeasured mechanism may have contributed to the significant elevations in hepatic *ldlr* mRNA abundance shown with Se treatment at the level of the liver and which provide indications that this is a key pathway of blood cholesterol lowering as has been demonstrated for a variety of hypocholesterolemic dietary and pharmacological agents (Spady, 1992). Future studies should examine alternative non-sterol mediated pathways as mechanisms of Se-mediated blood

cholesterol lowering, particularly PPAR since activation of PPAR increases oxysterols (Guan et al., 2003). In particular, the human CYP27A1 gene was shown to be under the regulation of PPARγ and induction resulted in an increase in 27-OHC (Szanto et al., 2004). As selenate increases expression of PPARγ (Mueller and Pallauf, 2006) it thus may also influence other forms of PPAR associated with modulation of the expression of *abcg5*, *abcg8* and *npc1l1* mRNA (Mathur et al., 2007; Duval et al., 2006; van der Veen et al., 2005).

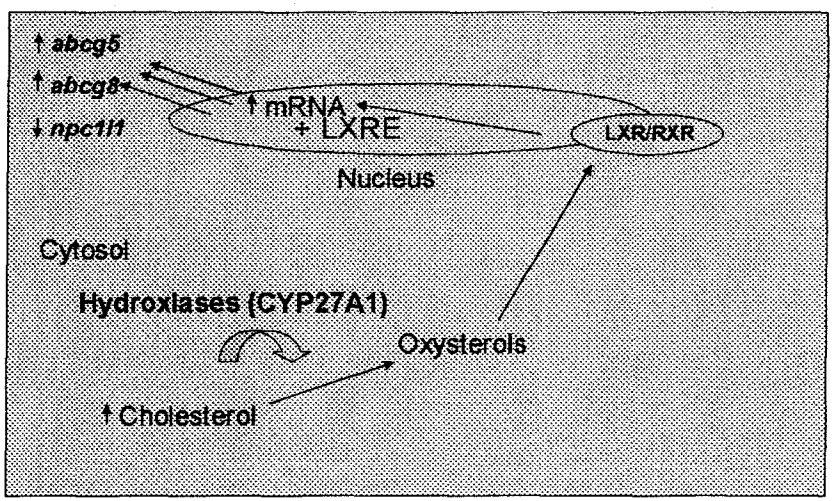
5.2 Limitations of the Thesis and Future Work

The present thesis indicated that Se is unlikely to have acted via the LXR pathway as both hepatic and jejunal *abcg5* and *abcg8* did not respond similarly to Se supplementation **(Figures 5.2 and 5.3)**. Although plasma lipids were decreased, liver cholesterol and bile acids were unaffected, indicating that Se exerts its plasma cholesterol lowering effects through non-sterol mechanisms at the hepatic level. Since *abcg5*, *abcg8* and *npc1l1* genes may be selectively modulated through activation of other nuclear receptors such as FXR, PXR, PPAR, the measurement of these receptors as well as LXR itself would have more clearly confirmed whether Se increased the putative LXR responsive genes via activation of non-LXR mediated pathways. Specifically, studies examining the association between PPAR and Se on *abcg5*, *abcg8* and *npc1l1* could be conducted as previous work has shown the involvement of Se with PPARγ which has been shown to modulate these genes. Since the LXRE has not been yet been found in the promoter of *abcg5*, *abcg8* or *npc1l1*, future studies can focus upon a gene that is known to contain an LXRE and also affects lipid metabolism such *abca1* and *cetp*. The measurement of other transporters such as *abca1*, which is involved in reverse cholesterol metabolism would have been useful as its upregulation leads to lower cellular cholesterol levels. It is conceivable that Se also increased levels of *abca1* similar to the Se-mediated induction of *abcg8*. As *hmgcr* and *ldlr* are modulated by changes in tissue sterol concentrations via

SREBP-2, the inclusion of the measurement of SREBP-2 would have more conclusively determined if increased levels of SREBP-2 were not involved with supplementation of Se to increase levels of *ldlr* mRNA. As mRNA abundance can be affected transcriptionally or post-transcriptionally, protein levels of each transporter would clarify if the transporters *npc1l1* and *abcg5*, *abcg8* were active; however, to our knowledge, these protein measurements have not been developed to date.

Although the mechanism(s) whereby the supplementation of Se influences blood cholesterol levels remain unknown, the present thesis has demonstrated that it is likely that Se supplements lower plasma cholesterol via pathways that do not involve LXR, cholesterol absorption or bile acid secretion although more studies are needed in order to confirm this concept. In particular, the thesis results indicate that futher research should focus upon a better understanding on the association between Se-mediated changes in oxysterols, *ldlr* mRNA and PPAR as the alternate nuclear receptor pathway to LXR.

Modulation of LXR-Response Genes Measured and Oxysterol Synthesis



Cell (Hepatocyte and Enterocyte)

FIGURE 5.1

Figure 5.1: Proposed mechanism of conversion of dietary cholesterol into oxysterols which are naturally occurring ligands of the LXR as studied in rodents (Lund et al., 1992; Saucier et al., 1989; Zhang et al., 2001; Xu et al., 2004). It has been proposed that increased dietary cholesterol causes an increase in cellular cholesterol which is transformed into oxysterols through action of hydroxylases. These naturally occurring oxysterols bind to the LXR/RXR heterodimer which enters the nucleus and binds LXRE in promoter region of LXR-responsive genes. It is suggested that binding to LXRE causes the up-regulation of mRNA for *abcg5* and *abcg8* while causing a down-regulation of *npc1l1* as previous studies have shown *abcg5*, *abcg8* and *npc1l1* to be modulated by dietary cholesterol as studied in rodents (Peet et al., 1998; Berge et al., 2000; Repa et al., 2002; Yu et al., 2002; Duan et al., 2004).

ABBREVIATIONS: *abcg5*, ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP-binding cassette transporter (ABC) g8; *npc1l1*, Neiman-Pick C-Like 1 Protein; *cyp27a1*, sterol 27-hydroxylase; mRNA, messenger ribonucleic acid; LXRE, liver X Receptor binding element; LXR/RXR, liver X receptor/ retinoic acid X receptor heterodimer. ↑ indicates an increase; ↓ indicates a decrease.

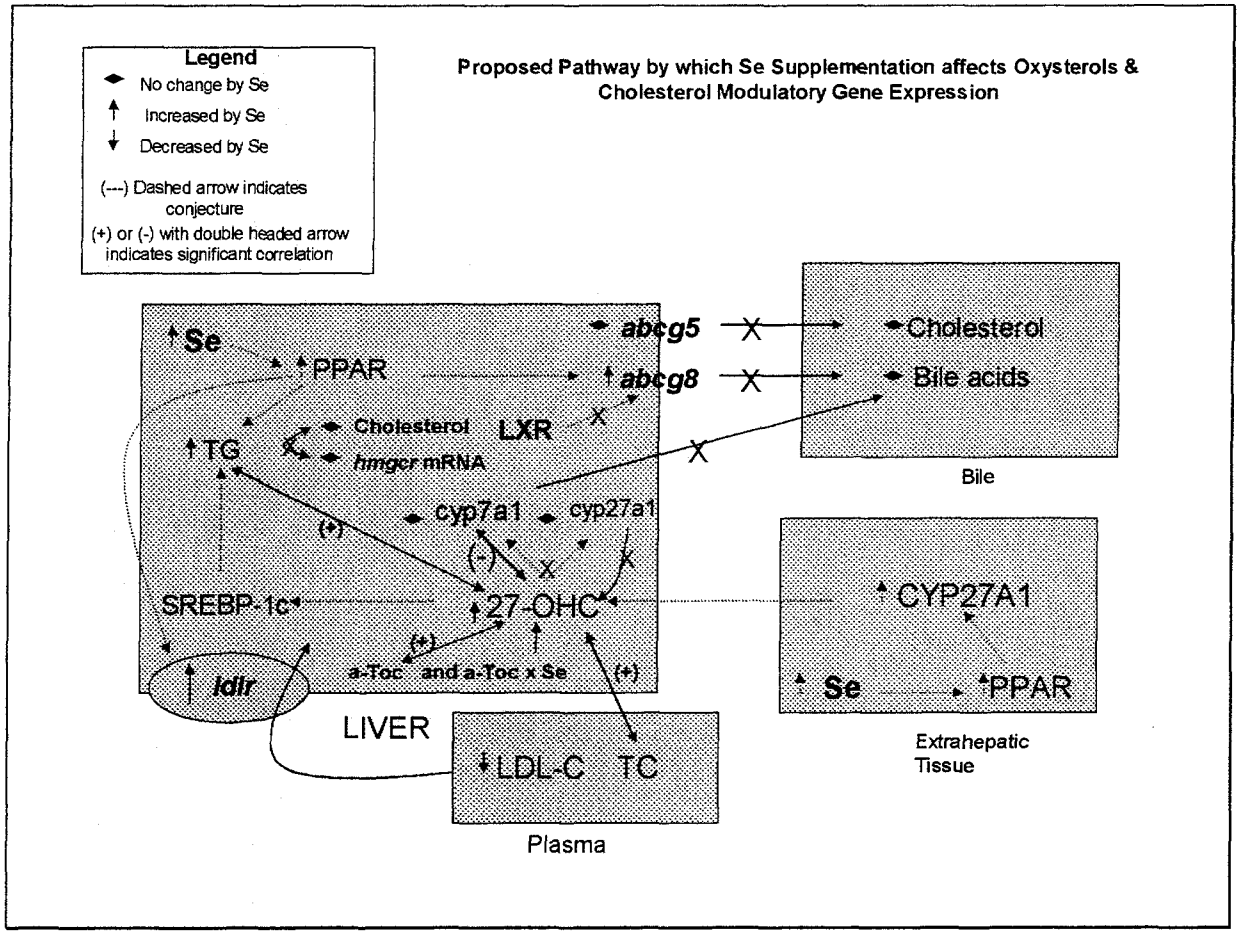

Legend
No change by Se
Increased by Se
Decreased by Se
(---) Dashed arrow indicates conjecture
(+) or (-) with double headed arrow indicates significant correlation
Proposed Pathway by which Se Supplementation affects Oxysterols & Cholesterol Modulatory Gene Expression
Se
PPAR
abcg5
abcg8
Cholesterol
Bile acids
Bile
TG
Cholesterol
hmgcr mRNA
LXR
cyp7a1
cyp27a1
(+)
(-)
SREBP-1c
27-OHC
CYP27A1
Se
PPAR
Extrahepatic Tissue
(+)
a-Toc and a-Toc x Se
(+)
ldlr
LIVER
LDL-C
TC
Plasma

FIGURE 5.2

Figure 5.2: Suggested mechanism for the in vivo regulation of cholesterol modulatory gene expression levels by endogenously produced 27-OHC in the hamster. (↔) Double-headed arrow indicates a correlation between measured parameters in the present thesis, either (+) positive, or (-) negative. The dotted lines represent conjectured mechanisms to explain significant effect of Se on indicated parameters. For example, the supplementation of Se was shown to decrease plasma LDL-C and TC concentrations and increase hepatic levels of Se, *abcg8* mRNA , TG, 27-OHC and *ldlr* mRNA. On the other hand, the supplementation of Se showed no effect on hepatic levels of cholesterol, *abcg5*, *hmgcr*, *cyp7a1*, and *cyp27a1* mRNA. Also, Se was without effect on CYP7A1 and CYP27A1 activity. Finally, Se was without effect on biliary concentrations of cholesterol and bile acids.

Plasma LDL-C lowering effect of Se is not through activation of LXR pathway as both transporters are not increased. It is known that PPAR selectively increases *abcg8* without effect on *abcg5,* this may have prevented increased secretion of hepatic cholesterol into bile, thus effect of Se on decreasing plasma LDL-C is sterol independent as hepatic levels of cholesterol are not changed by Se which is possibly due to absence of effect of Se on *hmgcr* mRNA abundance.

The suggested mechanism of regulation of 27-OHC levels includes proposed Se-induced activation of PPAR in extra-hepatic and hepatic tissues (Mueller and Pallauf, 2006). Thus, dietary Se is incorporated into tissues which increases known activation of PPAR and CYP27A1 (Szanto et al., 2004) in extrahepatic tissues forming 27-OHC. Extrahepatic formation of 27-OHC exits peripheral cells due to increased solubility and can not down-regulate CYP27A1 activity in extrahepatic tissue. 27-OHC is transported with plasma lipids (TC) to liver where its levels increase due to protection from oxidative stress by α-Tocopherol and Se and also due to increased activation of PPAR by Se. Accumulation of 27-OHC down-regulates activity and/or mRNA of *cyp7a1* and *cyp27a1*.

Down-regulation of *cyp7a1* leads to no change in biliary bile acid concentration. PPAR also causes: a) 27-OHC induced increase in hepatic TG concentrations possibly by activation of SREBP-1c; and b) increase in ldlr which leads to increased uptake of plasma levels of LDL-C.

ABBREVIATIONS: Se, selenium; PPAR, peroxisome proliferators-activated receptor ; *abcg5*, ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP-binding cassette (ABC) transporter g8; TG, triglyceride; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase; mRNA, messenger ribonucleic acid; *cyp7a1*, cholesterol 7-hydroxylase; *cyp27a1*, sterol 27-hydroxylase; SREBP-1c, sterol responsive element binding protein 1c ;27-OHC, sterol 27-hydroxycholesterol; *ldlr*, low density lipoprotein receptor; a-TOC, α-Tocopherol; α-Toc x Se, α-Tocopherol x selenium; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol.

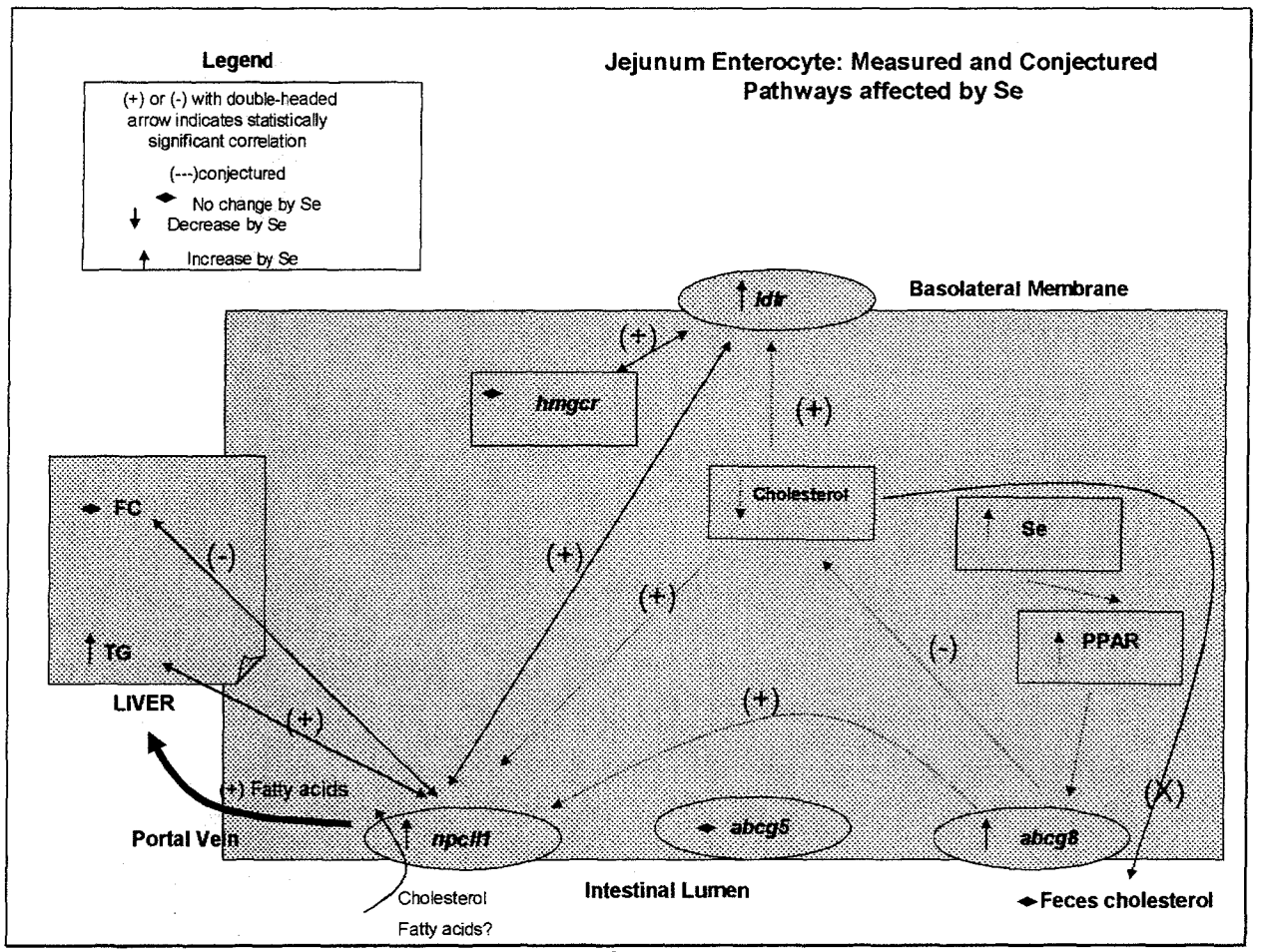

Legend
(+) or (-) with double-headed arrow indicates statistically significant correlation
(---)conjectured
No change by Se
Decrease by Se
Increase by Se
Jejunum Enterocyte: Measured and Conjectured Pathways affected by Se
Basolateral Membrane
ldlr
hmgcr
(+)
(+)
Cholesterol
Se
FC
(-)
(+)
(+)
TG
LIVER
(-)
PPAR
(+)
(+) Fatty acids
Portal Vein
npc1l1
abcg5
abcg8
(X)
Cholesterol
Fatty acids?
Intestinal Lumen
Feces cholesterol

FIGURE 5.3

Figure 5.3: Measured and conjectured pathways affected by Se in the hamster jejunum. (↔) Double-headed arrow indicates a correlation between measured parameters in the present thesis, either (+) positive, or (-) negative. The dotted lines represent conjectured mechanisms to explain significant effect of Se on indicated parameters. For example, the supplementation of Se was shown to increase *ldlr*, *npc1l1* and *abcg8* mRNA without showing an effect on *hmgcr* and *abcg5* mRNA. Also, the supplementation of Se showed no effect on fecal concentrations of cholesterol or bile acids. The increase in *abcg8* mRNA may have decreased enterocyte concentrations of cholesterol as supported by a significant and positive correlation shown between *ldlr* and *hmgcr* mRNA abundance. The increased expression levels of *abcg8* by decreasing cholesterol concentrations of enterocyte may have increased expression levels of *npc1l1* mRNA which is shown to be increased by cholesterol depletion (Alrefai et al., 2007). This is supported by the significant and positive correlation noted between *ldlr* and *npc1l1* mRNA abundance. Increased expression levels of *npc1l1* did not lead to increased hepatic cholesterol concentrations suggesting the the increased expression levels of *npc1l1* were not increasing cholesterol absorption which was supported by a significant and negative correlation shown between *npc1l1* and hepatic FC concentrations. Also, a significant and positive correlation between *npc1l1* abundance and liver TG suggests *npc1l1* abundance might have a role in fatty acid absorption in the hamster jejunum. Activation of LXR with Se was not supported by a differential effect of Se shown on *abcg5* abundance which was not upregulated by Se whereas *abcg8* expression levels were. Increase Se concentrations in jejunum might have increased PPAR expression which is known to differentially upregulate *abcg5* and *abcg8*.

ABBREVIATIONS: Se, selenium; PPAR, peroxisome proliferator-activated receptor ; *abcg5*, ATP-binding cassette (ABC) transporter g5; *abcg8*, ATP-binding cassette (ABC) transporter g8; TG, triglyceride; *hmgcr*, 3β-hydroxy-3β-methyl glutaryl coenzyme A reductase; mRNA, mRNA, messenger ribonucleic acid; *ldlr*, low density lipoprotein receptor mRNA, messenger ribonucleic acid; LDL-C, low density lipoprotein cholesterol; FC, free cholesterol.

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