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Induction of Liver abcg5/abcg8 Expression Is an Important Determinant of the Macrophage-To-Feces Reverse Cholesterol Transport Response to Treatment with Ezetimibe

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INDUCTION OF LIVER ABCG5/ABCG8 EXPRESSION IS AN IMPORTANT DETERMINANT OF THE MACROPHAGE-TO-FECES REVERSE CHOLESTEROL TRANSPORT RESPONSE TO TREATMENT WITH EZETIMIBE

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ABSTRACT

Reverse cholesterol transport (RCT) consists of the transfer of cholesterol from peripheral tissues for excretion in the feces. The RCT from macrophages in atherosclerotic lesions is an important determinant of arterial wall atherosclerotic lesion formation. Previous studies by our group have shown that treatment with ezetimibe (EZ), a potent inhibitor of cholesterol absorption from the intestine, results in a 2-6 fold increase in RCT. To determine whether EZ may increase RCT by mechanisms that are independent of its well established cholesterol absorption inhibitory effects, we examined the expression of genes involved in the RCT pathway in the jejunum and liver tissues of C57BL/6J mice fed a chow diet or chow supplemented with 0.005% EZ. These studies revealed that treatment with EZ specifically stimulates the expression of Abcg5/Abcg8 in the liver, but not in the intestine. Further experiments clearly demonstrated that stimulation of liver Abcg5/Abcg8 expression was due to the inhibition of cholesterol absorption from the intestine and not a direct effect of EZ in the liver. This conclusion was further supported by the absence of an ABCG5/ABCG8 expression response to treatment of primary human hepatocytes with a glucuronated form of EZ. Finally, we found that the induction of liver Abcg5/Abcg8 accounts for nearly 50% of the EZ-dependent stimulation of RCT. To our knowledge, our studies are the first to demonstrate increased liver Abcg5/Abcg8 expression in response to EZ treatment which, in conjunction with suppression of intestinal cholesterol absorption, synergistically stimulate the macrophage-to-feces RCT.

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CHAPTER I

INTRODUCTION

Atherosclerosis is a complex chronic inflammatory disease and the underlying cause of most myocardial and cerebral infarctions (Ross, 1993). The atherosclerotic lesion is characterized by the accumulation of lipids and inflammatory cells within the arterial wall, especially by cholesterol laden macrophages. Cholesterol in macrophages is removed through a process called reverse cholesterol transport (RCT). This process consists of cholesterol efflux from macrophages to high density lipoproteins (HDL) which then delivers cholesterol to the liver (Goedeke and Fernandez-Hernando, 2012). Once in the liver, cholesterol is excreted into bile either as neutral cholesterol or, alternatively, in the form of bile acids. Biliary sterols that are transported through the gastrointestinal tract are partly reabsorbed by enterocytes and in part excreted into feces. The process that transfers cholesterol from macrophages in the arterial wall to the feces is designated macrophages-to-feces RCT.

Niemann-Pick C1 like 1 (Npc1l1) is localized to the brush border membrane of enterocytes, and serves as a permease that mediates the uptake of cholesterol from the intestinal lumen (Xie et al., 2012). Ezetimibe (EZ) is a potent drug that selectively binds

Npc1l1 and blocks the uptake of cholesterol by enterocytes (Xie et al., 2012). Animal studies have shown a dramatic 86% decrease in intestinal cholesterol absorption in C57BL/6J mice treated with 0.005% EZ (Sehayek and Hazen, 2008). Our group was the first to show that treatment with EZ also increases macrophage-to-feces RCT by 626% (Sehayek and Hazen, 2008). These observations led us, as well as others, to hypothesize that EZ increases macrophage-to-feces RCT through decreased intestinal absorption of macrophage-derived cholesterol that reaches the gastrointestinal tract through biliary secretion. It should be noted however that this hypothesis has not been carefully examined. Therefore, we set out to determine whether EZ may increase RCT by mechanisms that are independent of its well established cholesterol absorption inhibitory effects. To examine our hypothesis we first investigated the expression of genes involved in the RCT pathway in the jejunum and liver tissues of chow fed C57BL/6J mice treated with 0.005% EZ and chow fed controls. These preliminary experiments revealed that EZ treatment resulted in the induction of the liver neutral sterol hemitransporters Abcg5 and Abcg8, which are major determinants of liver cholesterol secretion into bile. These findings were of particular interest, because in mice, Npc1l1, the molecular target of EZ, is not expressed in liver tissue (Altmann et al., 2004) and raised major questions regarding the mechanisms whereby EZ stimulates the expression of these hemitransporters. The main goal of this project was to elucidate the mechanisms whereby EZ induced liver Abcg5/Abcg8 expression and to determine the role of these hemitransporters on EZ-dependent stimulation of RCT. To address these questions, we utilized animals that are targeted for Npc1l1 or Abcg8. From these sets of experiments we concluded that EZ-dependent stimulation of liver Abcg5/Abcg8 expression is due to

inhibition of cholesterol absorption from the intestine and that stimulation of these hemitransporters accounts for nearly 50% of the EZ-dependent increase in RCT.

CHAPTER II

MATERIALS AND METHODS

Mice Husbandry

Mice were kept at the Cleveland Clinic's Biological Resource Unit which has a 14/10 light/dark cycle. Animals were bred in-house and pups were weaned at 21 days of age. Adults were housed in groups of up to 5 mice per cage with ad libitum access to food and water. Mice were fed either a standard Teklad 2918 chow diet (Harlan Laboratories) or chow supplemented with 0.005% w/w EZ (Merck Sharp & Dohme Corp.). All experiments were performed on mice at 14-16 weeks of age. All breeding and experiments were approved by the Institutional Animal Care and Use Committee. Npc1l1-KO mice on a C57BL/6J background were obtained courtesy of Yiannis A. Ioannou at the Mount Sinai School of Medicine in New York (Davies et al., 2005). Abcg8-KO mice on a C57BL/6J background were obtained courtesy of Shailendra B. Patel at the Medical College of Wisconsin (Klett et al., 2004).

Mouse Genotyping and PCR

Ear tissue was harvested from pups at 17 days of age and digested overnight in 500 µl lysis buffer (4 M Urea, 0.5% Sarkosyl, 0.1 M Tris-HCl pH 8, 0.2 M NaCl, 0.01 M EDTA

pH 8) and 10 mg/ml Fungal Proteinase K (Invitrogen Life Technologies). Digests were centrifuged at 15,000 rpm for 5 minutes and 450 µl supernatant was mixed with 1 ml 100% ethanol to precipitate the DNA. DNA was spooled, dissolved in 0.1% TE buffer, and diluted in ddH_2O (Gibco Life Technologies) to approximately 30 ng/µl. PCR reactions were performed using JumpStart Taq DNA Polymerase (Sigma-Aldrich) on C1000 Thermocycler (Bio-Rad Laboratories). Wild-type, knockout, or heterozygous genotypes were determined by gel migration of the corresponding wild type and targeted allele PCR amplicons.

Bone Marrow Macrophage Harvest and Labeling

Twelve days before injection, 5 wild-type mice were euthanized by isoflurane overdose followed by cervical dislocation. The hind fur was wiped with 100% ethanol and cut open to expose the hind legs. The muscles around the femurs and the knee joints were severed, the femoral joints exposed and the femurs removed. Femurs were wiped clean and kept in ice cold PBS supplemented with 0.5 unit/ml penicillin and 0.5 µg/ml streptomycin (pen/strep). Femur epicondyles were removed and femurs were placed into a 0.7 ml centrifuge tube with 100 µl pen/strep supplemented PBS. Femurs were spun for 5 seconds (~6000 rpm), and bone marrow pellets were resuspended and pooled in 10 ml of pen/strep supplemented PBS. Marrow was centrifuged at 2000 rpm for 5 minutes and pellet was washed once with an additional 10 ml pen/strep supplemented PBS. Marrow pellet was resuspended in RPMI medium supplemented with 0.5 unit/ml penicillin, 0.5 µg/ml streptomycin, 0.2 mM glutamine, 50 µM β-mercaptoethanol, 10% heat-inactivated FBS, and 10% L-Cell cultured media (as a source of macrophage stimulating factor). Cells were grown in 162 cm^2 polystyrene flasks at 37°C. Two days before the

experiment, cells were labeled with RPMI media supplemented with 20 μCi ^{14}C-cholesterol (PerkinElmer Inc.), 100 μg/ml of human acetylated LDL, 0.5 unit/ml penicillin, 0.5 μg/ml streptomycin, and 0.2 mM glutamine. On the day of experiment, the media was removed, cells were scraped in ice cold PBS and pelleted at 2000 rpm for 5 minutes. Cell pellets were washed twice with ice cold PBS and resuspended in 1.8 ml DMEM/flask. A triplicate of 25 μl resuspended cell aliquots were counted (on a Packard 1900CA Liquid Scintilation beta counter) for ^{14}C-label incorporation. Cell suspension was kept on ice until injected into mice.

Macrophage-to-Feces Reverse Cholesterol Transport

Fourteen days before injection, mice were fed a chow diet or chow supplemented with 0.005% w/w EZ (Merck Sharp & Dohme Corp.). Two days before injection, mice were transferred and housed individually in conventionally vented cages with wire rack floors. Mice were weighed, subjected to isoflurane anesthesia, and subcutaneously injected with 300 μl of ^{14}C-cholesterol labeled bone marrow macrophage suspension. Two days after injection, feces were collected and dried overnight at 65°C. Dried feces were weighed and pulverized by mortar and pestle. Sterols in one gram of feces were extracted in 8 ml of folch organic solvent (2:1 v/v chloroform/methanol) and centrifuged once at 3500 rpm for 15 minutes at 4°C. A 2 ml sample of the supernatant was mixed with 0.7 ml 0.88% KCl in a clean glass tube and centrifuged at room temperature for 5 minutes at 3500 rpm. Next, 1 ml of the organic phase was transferred and evaporated in a scintillation vial at 65°C. After complete evaporation, 4 ml of Ecolite(+) scintillation fluid (MP Biologicals) was added and ^{14}C-label counted in a Packard 1900CA Liquid Scintilation beta counter.

Percent total fecal ^{14}C-label excrete was calculated and expressed as percent of total DPM in subcutaneously injected bone marrow macrophages.

For neutral sterol extraction, 2 ml of the fecal chloroform/methanol extract was transferred into a fresh glass tube and evaporated to dryness at 95°C. Sterols were resuspended in 75% ethanolic solution supplemented with 200 µl 1 N NaOH and ethanolic hydrolysis was achieved by incubation at 95°C. Neutral sterols were extracted three times with 2 ml of hexane and centrifuged at 3500 rpm for 5 minutes at 4°C. The organic phases were pooled and evaporated in a scintillation vial, resuspended in 4 ml of Ecolite(+) scintillation fluid (MP Biologicals), labeled neutral sterols were counted and percent neutral fecal ^{14}C-sterol excretion calculated and expressed as percent of total fecal ^{14}C-sterol excretion.

For acidic sterol extraction, remaining aqueous phase was evaporated down to approximately 2 ml (by incubation at 95°C), acidified (by adding 4 ml ddH_2O and 800 µl 2 N HCl), acidic sterols extracted three times in 2 ml of ethyl acetate and centrifuged at 3500 rpm for 5 minutes at 4°C. The organic phases were pooled and evaporated in a scintillation vial, resuspended in 4 ml of Ecolite(+) scintillation fluid (MP Biologicals), labeled acidic sterols were counted and percent acidic fecal ^{14}C-sterol excretion calculated and expressed as percent of total fecal ^{14}C-sterol excretion.

Animal Sacrifice and Tissue Harvest

Animals were fasted for 6 hours and anesthetized with intramuscular injection of ketamine-xylazine. The peritoneal cavity was exposed and animals were exsanguinated via a heart puncture. Next the jejunum was cut distal to the Treitz ligament, flushed with ice cold PBS and stripped of mesentery and fat deposits. A 4 cm segment of the jejunum

was removed and stored in 1 ml RNAlater solution (Ambion Life Technologies). The ileum was cut open proximal to the cecum, flashed with ice cold PBS, a 4 centimeter piece proximal to the ileocecal sphincter was resected and stored in RNAlater solution (Ambion Life Technologies). Next, the left lobe of the liver was harvested and stored in RNAlater solution (Ambion Life Technologies). Finally, the right lobe of the liver was harvested and snapped frozen in liquid nitrogen. All samples were stored at -80°C.

RNA Isolation, Reverse Transcription and qPCR Analysis

Tissue samples were homogenized in 1 ml Trizol reagent (Invitrogen Life Technologies) by using TissueLyser II (Qiagen), cooled on ice, incubated at room temperature for 5 minutes and supplemented with 200 µl of chloroform. Samples were spun at 12000 rpm for 15 minutes at 4°C and the aqueous phase transferred into isopropanol containing tube, and centrifuged again at 12000 rpm for 10 minutes at 4°C. RNA pellet was washed in 75% ethanol, centrifuged at 9000 rpm for 5 minutes at 4°C and resuspended in DEPC treated ddH_2O. RNA quality was determined by gel electrophoresis and 5 µg treated with DNAse (Invitrogen Life Technologies). DNAse treated RNA was subjected to reverse transcription by incubation with Superscript II (Invitrogen Life Technologies) and qPCR reactions were performed in triplicate using SYBR Advantage qPCR premix (Clonetech Labratories Inc.) on a CFX384 Real-Time PCR platform (Bio-Rad Laboratories). Expression levels were determined by using gene specific standard curves after normalization to house keeping genes and expression level of controls. Values were expressed as mean ± SEM. A list of gene specific primers used can be found in Appendix Table AI.

Liver Cholesterol Analysis by GC-MS

Approximately 10 mg snap-frozen liver tissue was homogenized in 2 ml ddH_2O using TissueLyser II (Qiagen) and a 15 µl aliquot was transferred into a coprostanol (internal standard) spiked glass tube, volume completed to 1 ml with ddH_2O and mixed with 1.8 ml of solution A (80:20:2 isopropanol:hexane:2 M acetic acid). Lipids were extracted three times in hexane and centrifuged at 3000 rpm for 10 minutes. The organic phase was split into two glass tubes and dried under nitrogen (one for total cholesterol and the other one for free cholesterol determination). For determination of liver total cholesterol content, one dried tube was resuspended in 100 µl 0.5 M KOH, hydrolyzed at 37°C, acidified by 1 M HCl and mixed with 900 µl of solution A. Hydrolyzed sterols were extracted in hexane and organic phase dried under nitrogen. Dried samples were resuspended in 40 µl Sylon HTP (3:1:9 HMDS:TMCS:Pyridine) (Sigma-Aldrich), incubated at 90°C for 1 hour, and transferred into airtight GC vials. Finally, 1 µl was injected into a GC-MS (Agilent Technologies) by following a previously established protocol (Robinet et al., 2010). GC-MS chromatograms were integrated at m/z = 329 and 370 for cholesterol and coprostanol, respectively, peak areas integrated and liver content of total and free cholesterol content were expressed as µg cholesterol/ mg liver tissue. Cholesteryl ester content was determined as the difference between total and free cholesterol levels.

Primary Human Hepatocytes

Confluent plates of primary human hepatocytes (form a normal 16-year old female donor) were purchased from Yecuris Corporation (OR). Upon receiving, cells were incubated with HMM Maintenance Media (Lonza) supplemented with 50 µg/ml gentamicin sulfate and 50 ng/ml amphotericin-B (Lonza). After 24 hours, media was

replaced and cells were treated with 0.001% DMSO or 0.001% DMSO plus either 50 μM chenodeoxycholic acid (CDCA), 30 μM SCH60663 or 120 μM SCH60663 (a glucuronated metabolite of EZ) (Merck Sharp & Dohme Corp.). After 24 hour incubation, cells were scraped in Trizol reagent (Invitrogen Life Technologies) and RNA was processed for cDNA synthesis as described above.

Statistical Analysis

Unless otherwise indicated, group differences were analyzed by using two-tailed unpaired Students t-test. To pool multiple experiments, raw data were converted into z-scores (distance from the mean value within an individual experiment in SD). Throughout this thesis results were expressed as means ± SD unless otherwise indicated.

CHAPTER III

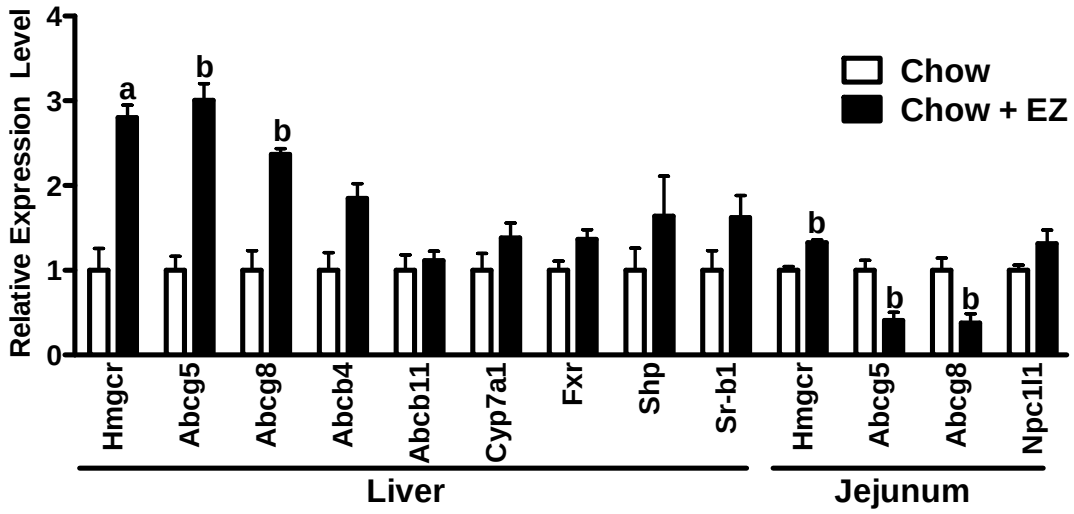
RESULTS

Ezetimibe upregulates liver Abcg5/Abcg8 expression in C57BL/6J mice

As previously reported, treating C57BL/6J mice with 0.005% ezetimibe (EZ) results in a dramatic 86% decrease in intestinal cholesterol absorption and a 626% increase in macrophage-to-feces reverse cholesterol transport (RCT) (Sehayek and Hazen, 2008). To determine whether EZ may increase RCT by mechanisms that are independent of its well established cholesterol absorption inhibitory effects, we examined the expression of genes involved in sterol metabolism (Table I) in the jejunum and liver of chow fed C57BL/6J mice treated with 0.005% EZ and in chow fed controls. We expected that treatment with EZ would decrease the delivery of dietary cholesterol from the intestine to the liver, thereby increasing the expression of Hmgcr, the rate limiting enzyme in the de-novo cellular cholesterol biosynthesis pathway in this organ (Goedeke and Fernandez-Hernando, 2012). Indeed, as shown in Figure 1, EZ treated animals displayed a 180% increase in liver Hmgcr expression levels when compared to chow fed controls. Moreover, we expected that treatment with EZ will deplete both enterocytes and hepatocytes of their cholesterol content, thus decreasing the expression of Abcg5 and

Gene	Full Name	Expression	Function
Hmgcr	3-hydroxy-3-methylglutaryl-coenzyme A reductase	Liver and Jejunum	The rate-limiting enzyme in cholesterol synthesis
Abcg5	ATP-binding cassette sub-family G member 5	Liver and Jejunum	Transports neutral sterols from hepatocytes into bile or enterocytes into the lumen
Abcg8	ATP-binding cassette sub-family G member 8	Liver and Jejunum	Transports neutral sterols from hepatocytes into bile or enterocytes into the lumen
Abcb4	ATP-binding cassette sub-family B member 4	Liver	Transports phospholipids from hepatocytes into bile
Abcb11	ATP-binding cassette sub-family B member 11	Liver	Transports bile acids from hepatocytes into bile
Cyp7a1	Cholesterol 7 alpha-hydroxylase	Liver	The rate-limiting enzyme in cholesterol to bile acid synthesis
Fxr	Farnesoid X receptor	Liver	Transcription factor that regulates Shp expression
Shp	Small heterodimer partner	Liver	Suppressor of Cyp7a1 expression
Sr-b1	Scavenger receptor class B	Liver	Receptor for HDL
Npc1l1	Niemann-Pick C1-Like 1	Jejunum	Transports cholesterol into enterocytes

Table I: Genes involved in sterol metabolism



[a]P<0.02, [b]P<0.006

Figure 1: EZ specifically stimulates liver Abcg5/Abcg8 expression. Gene expression in liver and jejunum tissues in chow or chow supplemented with 0.005% EZ fed C57BL/6J mice, N=5 per group.

Abcg8, two neutral sterol hemitransporters responsible for the efflux of excess cellular cholesterol into the intestinal lumen or bile, respectively. Indeed, when compared to chow fed controls, treatment with EZ resulted in 60% decreased expression of these hemitransporters in the jejunum (Figure 1). Interestingly however, and contrary to our expectations, EZ treatment resulted in a 240-300% induction in liver Abcg5/Abcg8 expression (Figure 1). Increased liver cholesterol content has been shown to induce the liver expression of Abcg5/Abcg8 (Escola-Gil et al., 2011). Considering the induction of liver Hmgcr, the rate limiting enzyme in de-novo cellular cholesterol biosynthesis, we examined the liver cholesterol content of EZ treated C57BL/6J mice and controls to determine if this was the cause. However, we did not find a difference in liver total cholesterol, free cholesterol, or cholesteryl ester content (Figure 2). Therefore, the induction of liver Abcg5/Abcg8 raised major questions regarding the mechanisms whereby EZ stimulates the expression of these hemitransporters, particularly since Npc1l1, the molecular target of EZ, is not expressed in the liver tissue of mice (Altmann

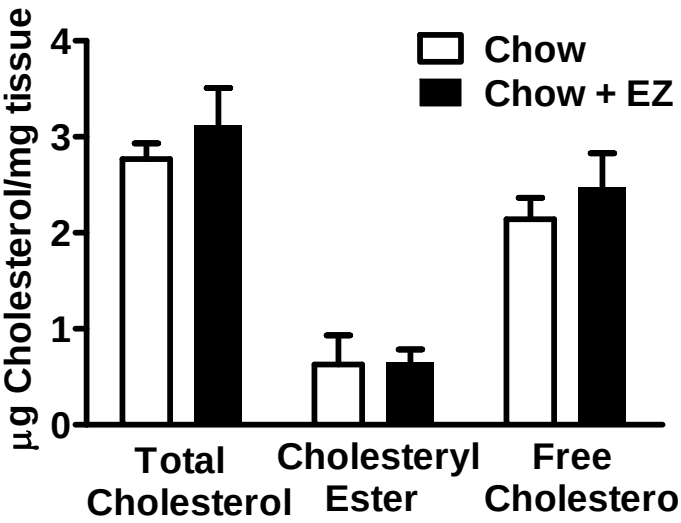


Figure 2: EZ treatment has no effect on liver cholesterol content. Liver total cholesterol, cholesteryl ester, and free cholesterol content in chow or chow supplemented with 0.005% EZ fed C57BL/6J mice, N=5 per group.

et al., 2004). To determine whether EZ-dependent induction of liver Abcg5/Abcg8 expression is due to direct stimulation of these genes in the liver or, alternatively, is downstream to this drug's effect in the intestine, we turned to study the effect of EZ in animals that were targeted for the Npc1l1 gene.

Induction of RCT and liver Abcg5/Abcg8 expression is an indirect effect of ezetimibe

Ezetimibe prevents intestinal cholesterol absorption by selectively blocking endocytosis of the Npc1l1-cholesterol complex (Xie et al., 2012). Therefore, Npc1l1-KO mice, which lack this critical permease, are characterized by a 70% decrease in cholesterol absorption rates (Altmann et al., 2004). For that reason, we utilized Npc1l1-KO mice to determine whether increased liver expression of Abcg5/Abcg8 is due to decreased cholesterol absorption and/or is a direct effect of EZ on Abcg5/Abcg8 expression in the liver.

First, we investigated the effect of decreased cholesterol absorption on RCT. If induction of RCT was a direct result of decreased cholesterol absorption, we anticipated that Npc1l1-KO mice will display an increase in RCT like the EZ treated C57BL/6J mice. Indeed, as shown in Figure 3A, when compared to wild-type (WT) controls, chow fed Npc1l1-KO mice displayed a 230% increase in RCT. Furthermore, this increase was entirely attributable to increased fecal neutral radiolabeled sterol excretion (266%) with no effect on fecal acidic radiolabeled sterol secretion (Figure 3C). These results were confirmed in a second set of mice and data was pooled via Z-scores to show a highly significant induction of RCT (Figures 3B and 3D). Our findings indicated that, as expected, Npc1l1- KO mice displayed an increase in RCT that was similar to what has

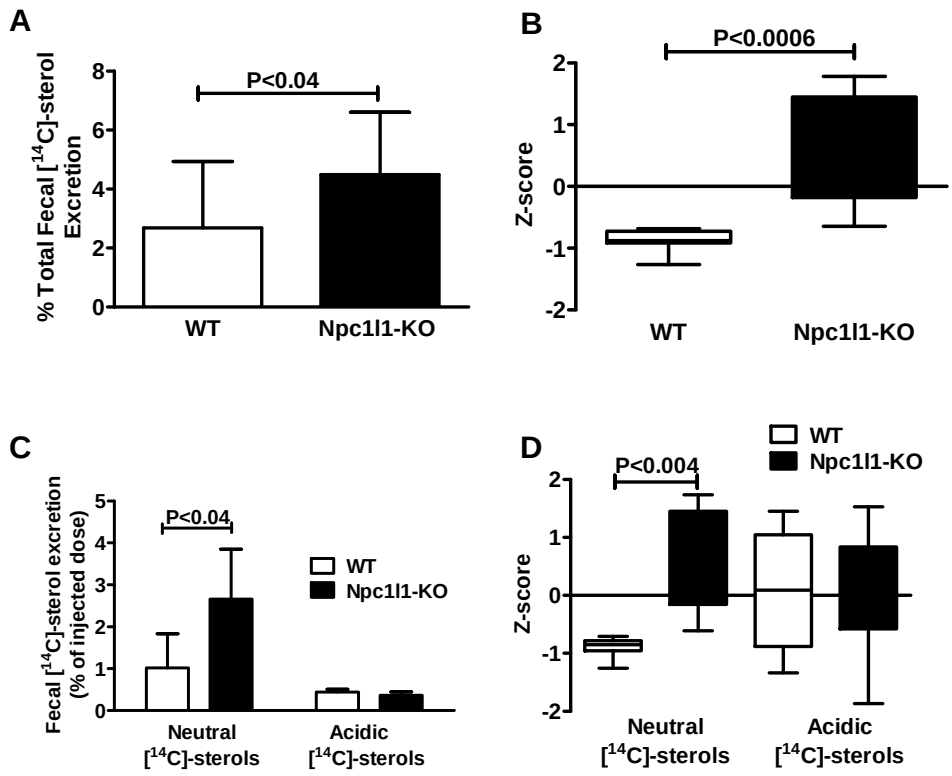


Figure 3: Npc1l1-KO mice were characterized by increased RCT due to increased fecal neutral sterol excretion. A) Total RCT, N=5 per group, **B)** Z-score of total RCT of two pooled experiments, N=7-10 per group, **C)** Fecal neutral and acidic ^{14}C-sterols, N=5 per group, **D)** Z-score of fecal neutral and acidic ^{14}C-sterols of two pooled experiments, N=7-10 per group.

been observed in EZ treated C57BL/6J mice. Next, we turned to examine the effect of targeting Npc1l1 on Abcg5/Abcg8 expression in the liver. If EZ increases Abcg5/Abcg8 expression through blocking the absorption of cholesterol, then chow fed Npc1l1-KO mice should also show increased liver expression of these genes. Indeed, as shown in Figure 4, Npc1l1-KO mice displayed a gene expression profile that was nearly identical to EZ treated C57BL/6J mice. Specifically, although stimulation of liver Hmgcr failed to reach statistical significance, jejunal Hmgcr was significantly induced by 160% (Figure 4). Moreover, jejunal Abcg5/Abcg8 was suppressed by 40-50% and liver Abcg5 and Abcg8 were induced by 70% and 29%, respectively (Figure 4). Together, these

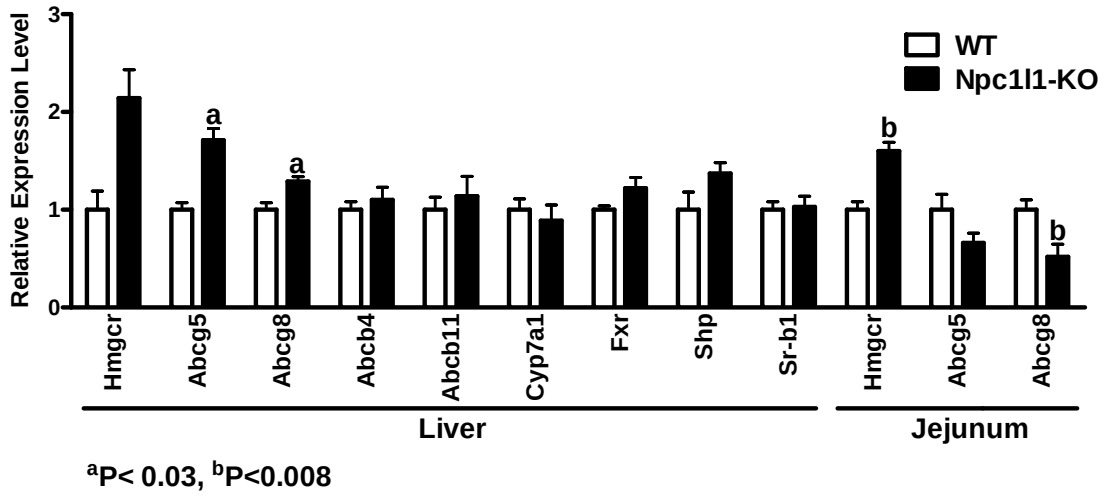


Figure 4: Npc1l1-KO mice are characterized by increased liver Abcg5/Abcg8 expression. Gene expression in liver and jejunum tissues of wild-type (WT) and Npc1l1-KO mice, N=5 per group.

experiments indicated that, similar to EZ treated C57BL/6J mice, chow fed Npc1l1-KO mice were characterized by elevated liver expression of Abcg5/Abcg8.

It should be noted however, that these experiments fell short in excluding a direct effect of EZ on liver Abcg5/Abcg8 expression. To address this possibility we treated Npc1l1-KO mice with EZ and examined the add-on effect of this drug to RCT and liver Abcg5/Abcg8 expression. As shown in Figure 5, EZ treated Npc1l1-KO mice displayed RCT, fecal neutral and acidic radiolabeled sterol secretions that were comparable to the values seen in controls. These findings were validated in a second experiment and data was pooled via Z-scores (Figure 5). Most importantly, with the exception of an increase in jejunal Abcg5, EZ treated Npc1l1-KO mice displayed a gene expression profile that was indistinguishable from EZ treated wild-type mice (Figure 6). Specifically, EZ treated Npc1l1-KO mice exhibited liver Hmgcr, Abcg5, and Abcg8 expression levels that were similar to those seen in their control group (Figure 6). Collectively, these results unequivocally showed that in Npc1l1-KO mice, EZ treatment had no effect on RCT or

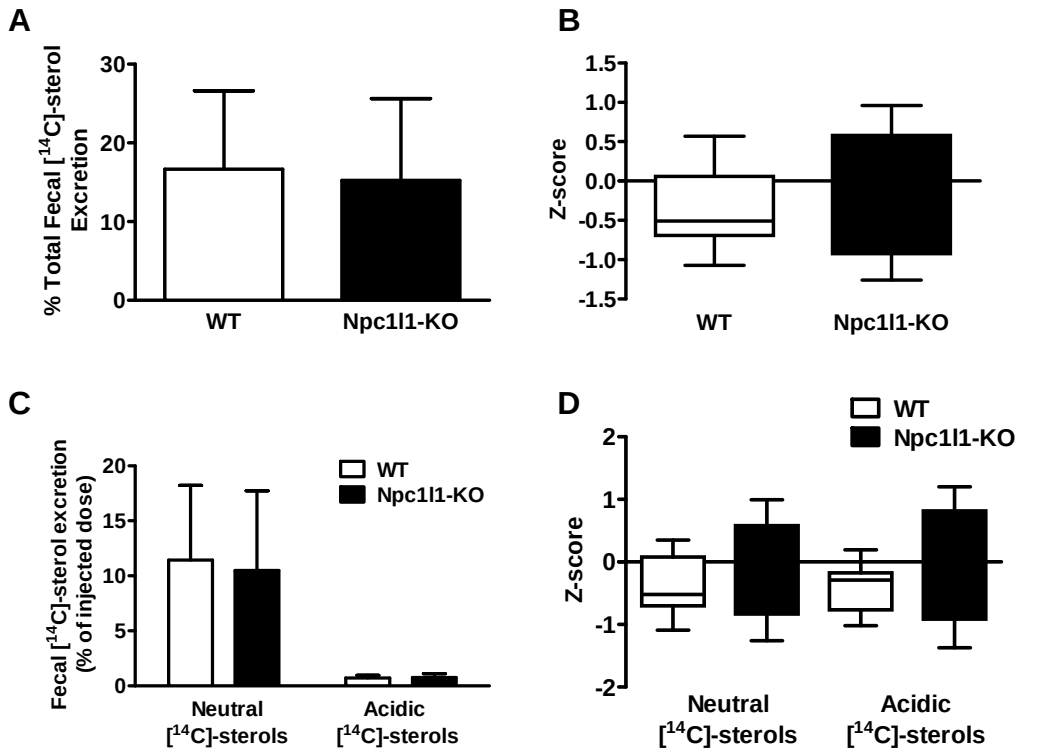


Figure 5: EZ treatment has no effect on RCT in Npc1l1-KO mice. A) Total RCT, N=5 per group, **B)** Z-score of total RCT of two pooled experiments, N=9 per group, **C)** Fecal neutral and acidic ^{14}C-sterols, N=5 per group, **D)** Z-score of fecal neutral and acidic ^{14}C-sterols of two pooled experiments, N=9 per group. WT = wild-type mice.

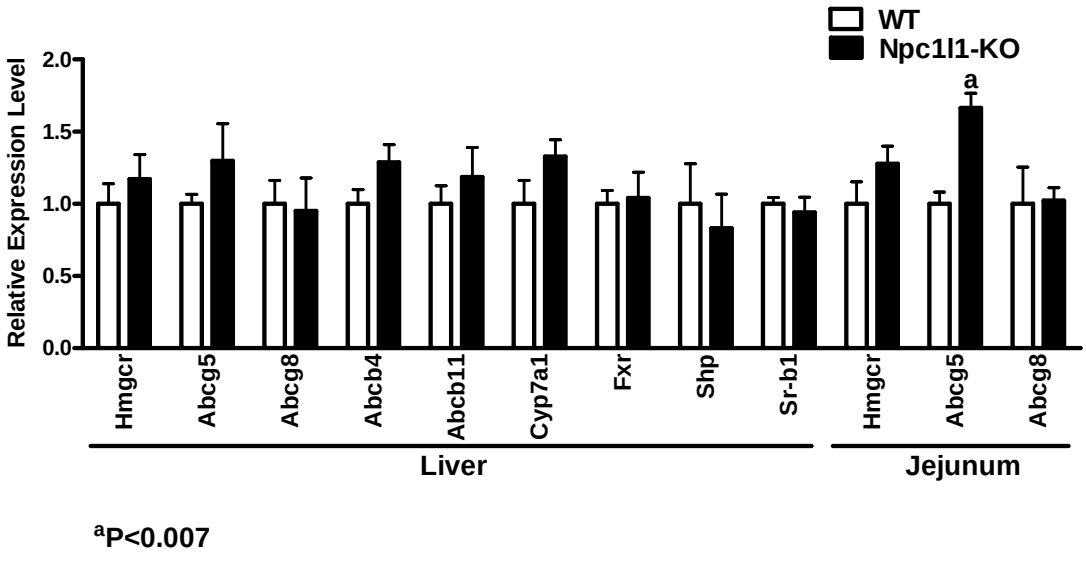


Figure 6: EZ treatment has no effect on liver Abcg5/Abcg8 expression in Npc1l1-KO mice. Gene expression in liver and jejunum tissues of EZ treated wild-type (WT) and Npc1l1-KO mice, N=5 per group.

liver expression of Abcg5/Abcg8.

This set of experiments strongly suggested that EZ-dependent stimulation of RCT and liver expression of Abcg5/Abcg8 is due to inhibition of cholesterol absorption from the intestine. It should be mentioned however, that the above-described experiments were done in-vivo and may mask potential direct EZ effects on liver Abcg5/Abcg8 expression. To ascertain the absence of a direct EZ effect on liver Abcg5/Abcg8 expression we turned to examine the effect of this drug on ABCG5/ABCG8 in human primary hepatocytes.

Ezetimibe metabolite has no effect on Abcg5/Abcg8 expression in primary human hepatocytes

Primary human hepatocytes (PHH) express NPC1L1, ABCG5, and ABCG8, making them an ideal in-vitro model to test the indirect effect of EZ. Ezetimibe is largely glucuronated in the small intestine and its glucuronated metabolites reach the liver through the portal system (Phan et al., 2012). Therefore, to examine the effect of EZ on gene expression in PHH we used a glucuronated form of EZ, SCH60663. In these experiments, chenodeoxycholic acid (CDCA), a major bile acid in human bile, served as a positive control. CDCA is a well established stimulator of small heterodimer partner (SHP), a liver transcription inhibitor that regulates key enzymes and transporters in bile acid metabolism. Indeed, and as shown in Figure 7, when compared to DMSO treated cells, PHH treated with 50 μM CDCA displayed a 395% increase in SHP expression. These findings clearly indicated that our primary human hepatocytes were an appropriate model for in-vitro sterol metabolic studies. Our findings in Npc1l1-KO mice strongly suggested that EZ indirectly effected Abcg5/Abcg8 expression through the inhibition of

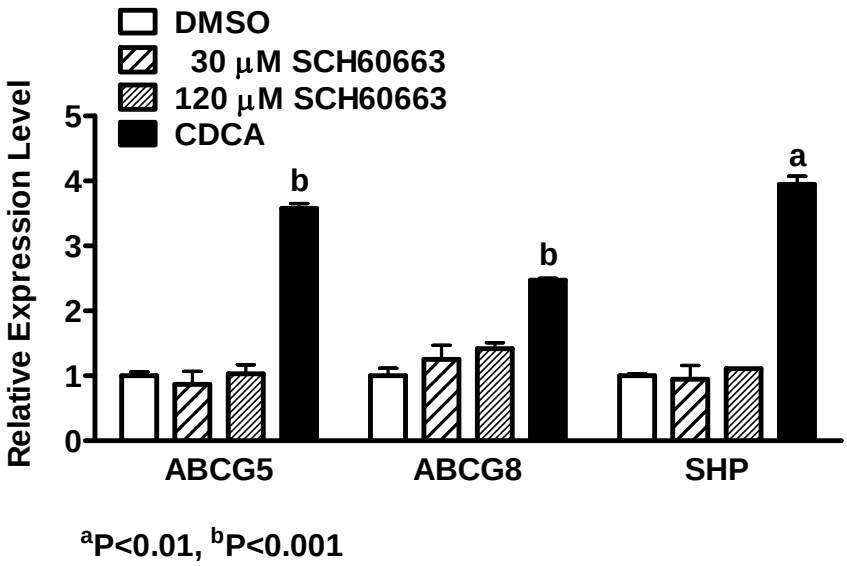


Figure 7: SCH60663 treatment of primary human hepatocytes has no effect on ABCG5/ABCG8 expression. Gene expression in primary human hepatocytes treated with 0.001 % DMSO supplemented with 30 μM and 120 μM SCH60663, or 50 μM CDCA, N=3 per group.

cholesterol absorption from the intestine. Based on these findings we expected that incubating PHH with SCH60663 will have no effect on ABCG5/ABCG8 expression. Indeed, as shown in Figure 7, treating PHH with incremental doses of SCH60663 (which correspond to concentrations measured in EZ treated patients) displayed no effect on ABCG5/ABCG8 expression. Interestingly, CDCA treatment induced ABCG5/ABCG8 expression by 247-358% (Figure 7). However, further investigation into CDCA-induced increase of ABCG5/ABCG8 was outside the scope of this study. This set of experiments clearly excluded a direct effect for this drug on liver expression of these genes and indicated that in C57BL/6J mice, the stimulation of Abcg5/Abcg8 is downstream to the effect of EZ on cholesterol absorption.

Liver Abcg5/Abcg8 expression accounts for 50% of the ezetimibe induced RCT effect

To determine the role of liver Abcg5/Abcg8 on EZ-dependent stimulation of RCT, we examined the effect of EZ in mice targeted for the Abcg8 gene. Liver Abcg5/Abcg8 hemitransporters are responsible for the efflux of cholesterol from hepatocytes into bile and Abcg8-KO mice are characterized by largely decreased biliary cholesterol secretion (Klett et al., 2004). Therefore, we utilized Abcg8-KO mice to determine whether the EZ-dependent stimulation of RCT is, at least in part, attributable to increased biliary cholesterol secretion into bile.

First, we examined whether targeting of Abcg8 has an effect on RCT. As shown in Figure 8A, chow fed Abcg8-KO mice displayed RCT levels that were similar to those in controls. Interestingly, however, Abcg8-KO mice were characterized by a shifted balance in fecal sterol secretion, as reflected by a 56% decrease and 50% increase in fecal neutral and fecal acidic radiolabeled sterol secretion respectively (Figure 8B). We also examined the gene expression profile in chow fed Abcg8-KO mice. Consistent with previous reports (Klett et al., 2004) and as seen in Figure 9, chow fed Abcg8-KO mice had a 45% decrease in liver Hmgcr expression and liver Abcg5 expression was below our qPCR detection limit. In addition, when compared to their controls, Abcg8-KO mice displayed

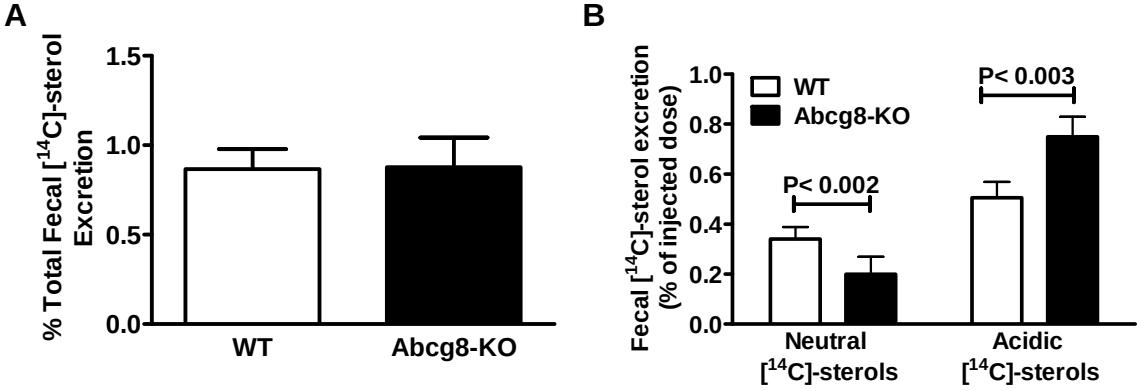


Figure 8: Abcg8-KO mice display RCT that was indistinguishable from controls, but opposing neutral and acidic fecal ^{14}C-sterol excretion. A) Total RCT, N=5 per group, **B)** Fecal neutral and acidic ^{14}C-sterols, N=5 per group. WT= wild-type mice.

a 38% decrease in liver Farnesoid X receptor (Fxr) expression, a bile acid receptor that regulates the transcription of critical genes in bile acid metabolism (Figure 9). However, studies into mechanisms that modify Fxr suppression in Abcg8-KO mice are beyond the scope of this project. From this series of studies we concluded that targeting of Abcg8 had no overall effect on RCT.

Next, we addressed the effect of EZ in Abcg8-KO mice. We expected that, in Abcg8-KO mice, EZ treatment would not be able to induce RCT to the same extent as in wild type animals. Indeed, as shown in Figure 10A, when compared to their controls, EZ treated Abcg8-KO mice displayed a 50% decrease in RCT. Furthermore, this decrease was largely attributable to a 38% decrease in fecal neutral radiolabeled sterol secretion (Figure 10C). This observation was confirmed in a second set of mice and pooling of data after conversion into Z-scores showed a highly significant reduction in RCT (Figures 10B

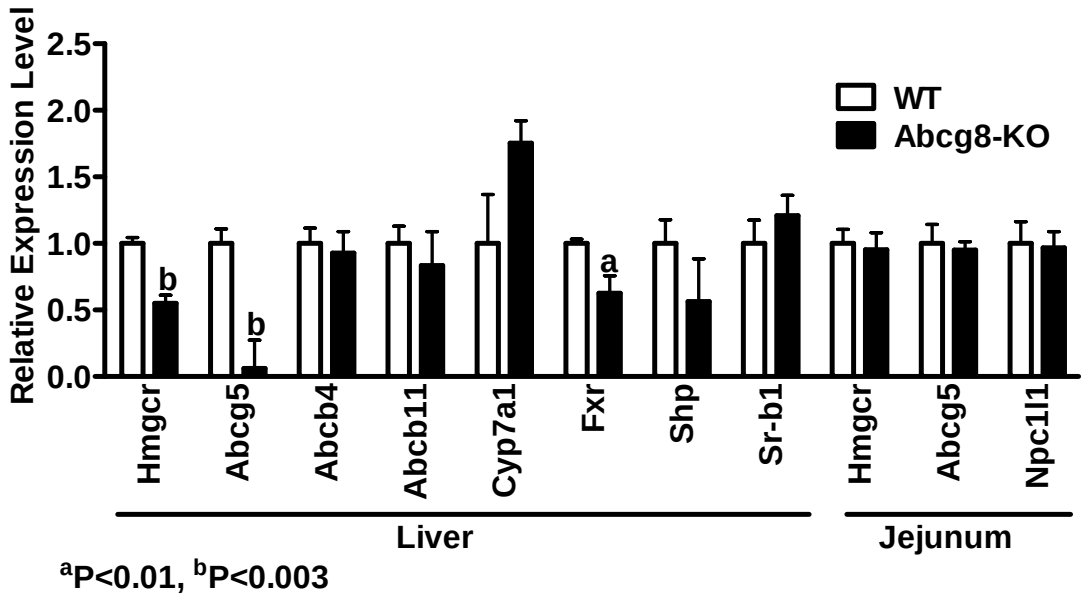


Figure 9: Abcg8-KO mice display decreased expression of liver Hmgcr, Abcg5, and Fxr. Gene expression in liver and jejunum tissues of wild-type (WT) and Abcg8-KO mice, N=5 per group.

and 10D). Finally, we examined the gene expression profile in EZ treated Abcg8-KO mice. Unexpectedly, EZ treatment in Abcg8-KO mice induced the expression of liver Abcb4, the biliary phospholipid transporter, by 34% and the expression of Shp by 233% (Figure 11). Further studies are needed to clarify the mechanisms whereby EZ induces the expression of Abcb4 and Shp in Abcg8-KO mice. However, these studies are beyond the scope of this project. Taken together, our experiments showed that increased Abcg5/Abcg8 expression accounts for nearly 50% of EZ-dependent increase in RCT.

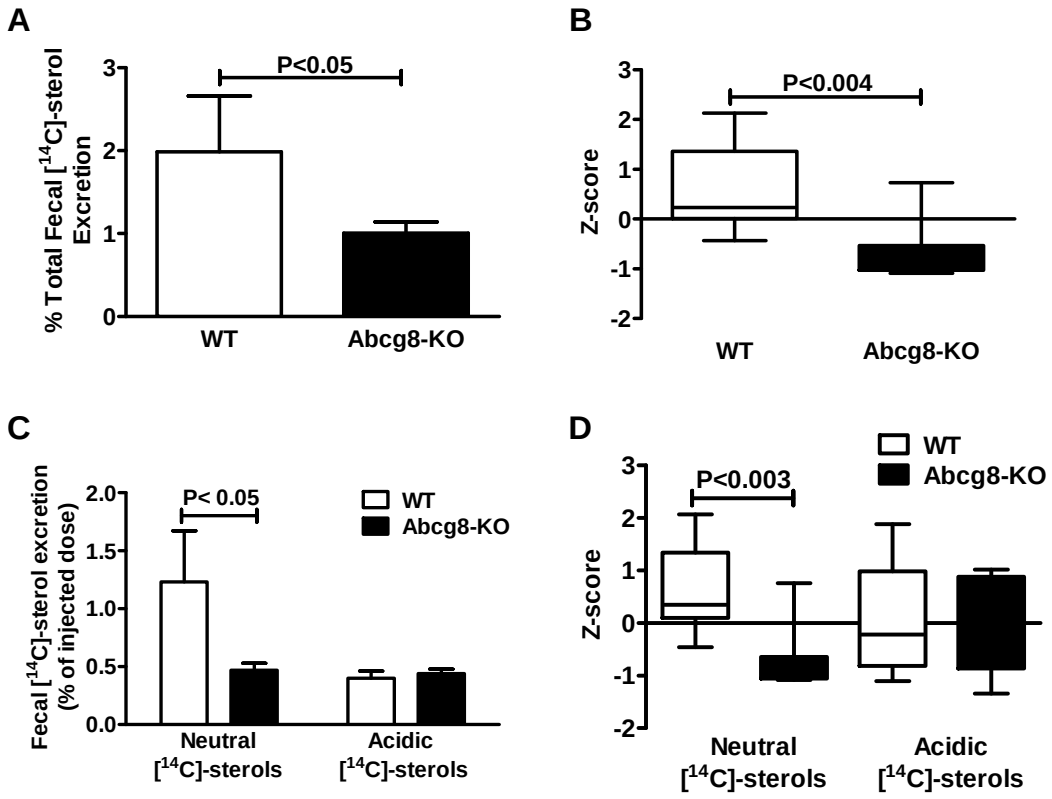


Figure 10: EZ treated Abcg8-KO mice are characterized by decreased RCT due to suppressed fecal neutral sterol excretion. A) Total RCT, N=5 per group, **B)** Z-score of total RCT of two pooled experiments, N=8-9 per group, **C)** Fecal excretion of neutral and acidic ^{14}C-sterols, N=5 per group, **D)** Z-score of fecal neutral and acidic ^{14}C-sterols of two pooled experiments, N=8-9 per group. WT = wild-type mice.

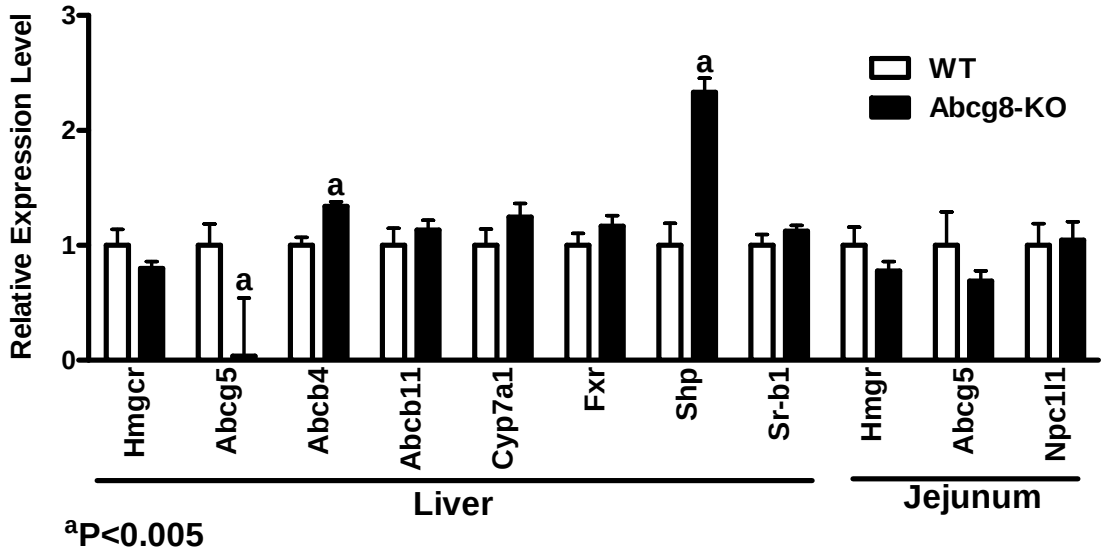


Figure 11: EZ treatment stimulates liver Abcb4 and Shp expression in Abcg8-KO mice. Gene expression in liver and jejunum tissues of EZ treated wild-type (WT) and Abcg8-KO mice, N=5 per group.

CHAPTER IV

DISCUSSION

Our studies showed that treatment with EZ specifically stimulates the expression of Abcg5/Abcg8 in the liver, but not in the intestine (Figure 1). Our experiments in Npc1l1-KO mice clearly demonstrated that stimulation of liver Abcg5/Abcg8 expression is due to the inhibition of cholesterol absorption from the intestine and not a direct effect of EZ in the liver (Figures 4 and 6). This conclusion is supported by the absence of an ABCG5/ABCG8 response to the glucuronated form of EZ in primary human hepatocytes (Figure 7). Finally, we found the up-regulation of Abcg5/Abcg8 accounts for nearly 50% of the EZ-dependent stimulation of RCT in response to EZ treatment (Figure 10).

Our studies showed for the first time that, in the mouse, decreased intestinal cholesterol absorption due to either EZ treatment or deletion of its molecular target, Npc1l1, stimulates the expression of Abcg5/Abcg8 in the liver, but not in the intestine (Figures 1 and 4). The expression of Abcg5/Abcg8 in the mouse is regulated by three distinct transcriptional factors: Liver X receptor alpha (Lxrα), Forkhead box O1 (Foxo1), and Hepatocyte nuclear factor 4 alpha (Hnf-4α) (Brown and Yu, 2010). Probably the best-studied regulator of Abcg5/Abcg8 expression is Lxrα, a sterol-sensing transcription factor that is activated by hydroxylated forms of cholesterol, designated oxysterols. Since

liver cholesterol is the precursor of oxysterols, increased liver sterol content in response to high cholesterol feeding results in Lxrα activation and increased Abcg5/Abcg8 expression (Repa et al., 2002). Interestingly, we found that treatment with EZ in animals fed a chow diet, which is a poor source of cholesterol (0.02% w/w), stimulated the expression of Abcg5/Abcg8 in the liver. Moreover, our studies found no change in liver cholesterol content in chow fed controls and EZ treated C57BL/6J mice (Figure 2). It is therefore conceivable that treatment of chow fed animals with EZ results in a small, but critical, depletion in the liver regulatory cholesterol pool which controls Abcg5/Abcg8 expression, a depletion that cannot be detected by our GC-MS method. An unambiguous indication for such depletion is provided by the stimulation of Hmgcr expression, the rate limiting enzyme in de-novo cellular cholesterol biosynthesis (Figure 1). Therefore, in addition to conditions where liver cholesterol content is increased, our findings indicate, for the first time, that expression of liver Abcg5/Abcg8 is inducible under conditions where the regulatory cholesterol pool is depleted. These findings raise an intriguing question: what is the mechanism whereby depletion of liver cholesterol content increases the expression of Abcg5/Abcg8? Brown and Goldstein have shown that endoplasmic reticulum-bound sterol regulatory element-binding protein 2 (Srebp-2) is responsible for orchestrating the transcriptional regulation of genes under conditions where the cellular cholesterol regulatory pool is depleted (Sheng et al., 1995). It is therefore conceivable that Srebp-2 is responsible for activation of Abcg5/Abcg8 in EZ treated animals. Indeed, studies in animals targeted for Srebp cleavage-activating protein (Scap), a cholesterol sensing protein which resides and tethers the inactive Srebp-2 to the endoplasmic reticulum, indicated that Srebp-2 is responsible for stimulation of Abcg5 expression in

the liver (Horton et al., 2003). It is of note that in our studies, stimulation of Abcg5 was consistently higher than the corresponding stimulation of its Abcg8 partner. Interestingly, other studies have demonstrated that Srebp-2 can positively interact with Hnf-4α (Misawa et al., 2003), a known transcription factor for Abcg5/Abcg8 expression. A definitive link between Abcg5/Abcg8, Hnf-4α, and Srebp-2 needs further experimentation that is beyond the scope of this thesis.

Reverse cholesterol transport consists of the transfer of cholesterol from peripheral tissues for ultimate excretion in the feces. However, the pathways involved are not fully understood. The classical view of the RCT process holds that cholesterol from peripheral tissues is transferred to the liver, excreted into bile, reaches the intestine and, ultimately is eliminated in the feces. This view has been recently challenged by observations that suggested a bypass of the biliary tract and direct excretion of cholesterol into the intestine. This process has been designated transintestinal cholesterol excretion or in short TICE (Temel et al., 2010, Temel and Brown, 2012). Our studies strongly support the importance of biliary excretion in determining the RCT process. Our experiments in Abcg8-KO mice strongly indicate that absence of liver Abcg5/Abcg8 induction in response to EZ treatment results in a 50% decrease in RCT (Figure 10). While our macrophage-to-feces RCT studies cannot exclude TICE as a potential mechanism, the induction of liver, but not intestinal Abcg5/Abcg8 strongly suggests that, in EZ treated animals, increased fecal secretion of neutral radiolabeled sterols is biliary-dependent. Therefore, our studies indicate that biliary cholesterol secretion is an important determinant of RCT. It should be noted, however, that our studies couldn't absolutely

exclude a role for TICE. A definitive answer awaits elucidation of the molecular basis of the TICE pathway.

To our knowledge, our studies are the first to demonstrate increased liver Abcg5/Abcg8 expression in response to EZ treatment and, in conjunction with suppression of intestinal cholesterol absorption, synergistically stimulate macrophage-to-feces RCT. Our findings highlight the role of liver Abcg5/Abcg8 in determining the RCT process. The exact mechanisms whereby depletion of liver cholesterol content increase the expression of Abcg5/Abcg8 and clarification of the balance between biliary cholesterol secretion and TICE in determining the RCT response to EZ needs further experimentation.

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APPENDIX

Primer	Forward Sequence	Reverse Sequence
hABCG5	CGTTCTGCGGGTCCGAAGCA	AGCTCGCAGCACGGGAAACAG
hABCG8_RT	TTCCTGCTGGTGTGGCTGGTGGTC	CCCCCGGCGAGGTAGAAGGAGTTG
hBACTIN	CCCCCATGCCATCCTGCGTCTG	CTGGGCCGTGGTGGTGAAGC
hNPC1L1	GCCTCCAGGTTCATGGCCTATCACA	GGCACTTTCCGCAGGTCAGCA
hSHP	CTCACGGCCTCCACCCTCAAGTCC	GTCCCCAAGAAGGCCAGCGATGTC
mAbcb11	CAGGGAGGCCAAAGGTGAGC	ATGGTGGCAGGGAATGAAAAGTAG
mAbcb4	CCGCTATGGCCGTGGGAATGTAA	ACTCAGCTGCGCCCCTCTATCACC
mAbcg5	CTGCTGAGGCGAGTAACAAGAAAC	GACGCGGAGAAGGTAGAAAATGAG
mAbcg8	GTACGTGGGGTGTCCGGGGGTGAG	GCGAGGCTGGTGGAGGGAGATGAG
mBactin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC
mCyp7a1	TGCCTTCTGCTACCGAGTGATGTT	CGGGCTTTATGTGCGGTCTTGA
mFxr	TTGTGCCGGAAGGGATGAGTGTG	TCCGGTTGTTGGGGGTAGAAGC
mHmgcr	GAGGCCCAGTGGTGCGTCTTCC	GGTTGCGTCCTGCCATCGTCAC
mNpc1l1	AGTGCGGTGTTTGCTGGAGTGG	AGGAGGTTGAGGCGGAAGAAGAAA
mPabpc1	AGCCATGCACCCTTCTCTTG	AGTTGGAACAACAGTGGCAC
mShp	AGCGCTGCCTGGAGTCTTTCTG	ACGGAGGCCTGGCACATCTG
mSr-b1	ATGGGCCAGCGTGCTTTTATGAAC	ACGCCCGTGAAGACAGTGAAGACC

Table AI: Primers used for gene expression studies