Cell Metabolism

Supplemental data

Short article

Biliary sterol secretion is not required for macrophage reverse cholesterol transport

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Supplemental Experimental Procedures

Mice, Diets, and Treatments

Male C57BL/6N mice (8-12 weeks old) were purchased from Harlan (Indianopolis, IN, USA). Creation of NPC1L1-^{LiverTg} mice has been described previously (Temel et al., 2007). For purposes of this paper the moderate overexpressing line corresponds to L1-Tg20, and the high overexpressing line corresponds to L1-Tg112 (Temel et al., 2007). For data shown in Figure 1, both transgenic lines were maintained on a B6D2F1 background; cholesterol levels in gall bladder bile for NPC1L1-^{LiverTg} mice have been previously reported (Temel et al., 2007) and are included here (Figure 1A) only for comparison purposes. For all other experiments, the high expressing line (L1-Tg112) backcrossed onto the C57BL/6N background for four generations was used. All mice were maintained on standard rodent chow. For LXR agonist studies, T0901317 was suspended in a vehicle containing 1.0% carboxymethylcellulose (CMC) and 0.1% Tween 80. Mice were gavaged with either vehicle or 25 mg/kg T0901317 once daily for a period of seven days. All mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved pathogen-free animal facility, and all experimental

protocols were approved by the institutional animal care and use committee at the Wake Forest University School of Medicine.

J774 Cell Culture and [^{3}H]-Cholesterol Loading

J774 mouse macrophages were a generous gift from Dr. George Rothblat (The Children's Hospital of Philadelphia). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. [^{3}H]-Cholesterol loading was initiated by incubating cells for 48 hours with 5 μCi/ml [^{3}H]-cholesterol and 100 μg/ml acetylated LDL in 10% FBS containing RPMI-1640. The resulting foam cells were washed twice with phosphate buffered saline and equilibrated for an additional 12-hour period in serum free RPMI-1640 supplemented with 0.2% bovine serum albumin (BSA). Cells were then harvested and resuspended in serum-free RPMI-1640 immediately before injection. An aliquot of cells was extracted using the method of Bligh and Dyer (1959), and lipids were separated by thin layer chromatography (TLC) using a 70:30:1 (hexane:diethyl ether:acetic acid) solvent system. Following TLC separation J774 foam cells were found to have ~62% [^{3}H]-cholesteryl ester and ~38% [^{3}H]-free cholesterol. On average, the cell suspension contained ~10x10^6 cells/ml at ~3x10^6 dpm/ml. All cell suspensions were analyzed microscopically in order to count and to ensure viability before injection, and all mice were injected within 5 minutes of resuspension of freshly isolated foam cells.

In Vivo Macrophage RCT Studies in NPC1L1-^{LiverTg} Mice

In vivo measurement of macrophage RCT was conducted essentially as described by Rader and colleagues (Zhang et al., 2003; Naik et al. 2006), with minor modifications. Briefly, mice were gavaged with either vehicle or 25 mg/kg T0901317 daily for seven consecutive days. On the 5th day of treatment, mice were injected intraperitoneally with ~500 μl [^{3}H]-cholesterol labeled foam cell suspension containing ~10x10^6 cells/ml at ~3x10^6 dpm/ml. To allow quantitative fecal collection, mice were housed individually on wire bottom cages for 48 hours with *ad libitum* access to food and water. At 6, 24, and 48 hours post injection, blood was collected via the submandibular vein, and 20 μl of isolated plasma was used to determine [^{3}H]-cholesterol recovery. The [^{3}H]-cholesterol distribution across plasma lipoproteins was determined for the 48-hour time point following size exclusion chromatography of plasma as previously described (Brown et al., 2008, Temel et al., 2007).

At 48 hours post injection, mice were anesthetized with isofluane (4% induction, 2% maintenance), and common bile duct cannulations were performed to collect newly secreted bile for a 20-minute period (Brown et al., 2008; Temel et al. 2007). 20 μl of newly secreted bile was extracted by adding 1 ml diH_20 and 3 ml 2:1 chloroform:methanol and then vortexing. The phases were split by adding 2 ml chloroform, vortexing, and centrifuging at 1000 x g for 10 minutes. The top phase containing the [^{3}H]-bile acids was subsequently extracted with 3 additional volumes (5 ml) of chloroform to completely remove [^{3}H]-cholesterol. All chloroform phases were pooled. The phases containing the [^{3}H]-cholesterol and [^{3}H]-bile acids were dried under N_2 and resuspended in scintillation cocktail to determine [^{3}H] recovery. Biliary lipid

mass was analyzed using enzymatic methods as previously described (Brown et al., 2007, Temel et a. 2007).

To determine [^{3}H] recovery and sterol mass in feces, feces were quantitatively collected for 48 hours and then dried in a vacuum oven at 70^{o}C overnight. For the first set of fecal [^{3}H] recovery experiments, fecal extraction was conducted exactly as described by Naik and colleagues (2006), with one minor modification. Following saponification and acidification of the [^{3}H] bile acid-containing aqueous phase, addition of ethyl acetate did not cause adequate phase separation. As an alternative, the acidified aqueous phase (pH <1) was extracted 4 times with 6 ml hexane to quantitatively recover [^{3}H]-bile acids. All extracts were pooled, dried under N_2, and resuspended in scintillation cocktail for recovery determination. For the second set of fecal [^{3}H] recovery experiments, feces were ground into a fine powder, and a measured amount of powdered feces (~100 mg) was extracted as follows. Samples were saponified by adding 2 ml 95% ethanol and 200 μl 50% KOH (w/v in diH_2O) and then incubating for 3 hours at 70^{o}C with periodic vortexing. Thereafter, the samples were extracted by adding 2 ml hexane and 2 ml diH_2O with vortexing after each addition. The samples were then centrifuged at 2700 rpm at room temperature for 10 minutes to split the phases. The upper hexane phase (containing [^{3}H]-cholesterol) was removed, and the remaining lower phase was re-extracted 3 times with 2 ml hexane. All [^{3}H]-cholesterol hexane phases were pooled. The remaining bottom phase (containing [^{3}H]-bile acids) was acidified by adding 200 μl concentrated HCL and then vortexed. After confirming pH was < 1, the [^{3}H]-bile acids were extracted four times with 2 ml hexane and the [^{3}H]-bile acid hexane phases were pooled. After drying under N_2, samples were resuspended in scintillation cocktail for [^{3}H] recovery determination.

Both fecal extraction protocols yielded similar results, and therefore the data have been pooled. Mass fecal neutral sterol analysis was performed by gas liquid chromatography (GLC) as previously described (Brown et al., 2008; Temel et al., 2007).

For tissue (ie liver & small intestine wall) [^{3}H]-cholesterol recovery, a piece of the liver (~100 mg) or entire intestinal segments were extracted for a minimum of 24h at room temperature in 9 ml 2:1 chloroform:methanol with frequent vortexing. Following extraction, a measured volume of solvent was dried down under N_2 and resuspended in scintillation cocktail for count recovery determination. Total tissue recovery is calculated per total organ weight.

***In Vivo* Macrophage RCT Studies With Acute Surgical Biliary Diversion**

C57BL/6N mice maintained on standard rodent chow were gavaged with either vehicle or 25 mg/kg T0901317 daily for seven consecutive days prior to surgery. On day 7, mice were administered ketoprofen (5mg/kg) for analgesia just prior to surgery. Thereafter, mice were anesthetized with isoflurane (4% for induction, 2% for maintenance) and were maintained on a 37^{o}C heating pad to control body temperature. A minimal midline laparotomy was performed and the common bile duct was visualized. Just above the sphincter of oddi, a tie (4-0 silk) was put in place to completely obstruct the common duct. Thereafter, another tie was loosely placed mid way between the liver and the sphincter of oddi, to be tied later when the cannulation was complete. A small hole was made in the common duct near the sphincter of oddi using a $30G_{1/2}$ needle, and a cannula (PFTE, Braintree # SUBL-120; O.D.= 0.012 x I.D. = 0.006) pre-loaded with a Teflon-coated guide wire (Braintree # ST003-7) was inserted. Following successful cannulation, the

proximal 4-0 silk was gently tightened to secure the cannula taking care not to obstruct bile flow. The chest was gently massaged while the guide wire was slowly removed to promote bile flow into the cannula. Once bile flow was confirmed, the cannula was tunneled through the abdominal wall and exteriorized through a hole in the right flank. Once externalized, the bile cannula was attached to larger tubing (Micro-Renathane; O.D.= 0.025 x I.D. = 0.012) to promote bile flow into a tube for collection. Another hole was made just beside the bile cannula, and a second cannula (Micro-Renathane; O.D.= 0.025 x I.D. = 0.012) was gently inserted to provide an injection port for macrophage dose. This was important because it ensured that there was no opportunity to puncture the intestines or other organs when administering the dose, which can happen easily with IP administration. The midline incision was closed using 4-0 absorbable suture with interrupted stitches and sealed using surgical glue. Surgical glue was applied to the holes where the cannulas were externalized. Because > 1ml of bile was lost during eight hours of collection, hydration was maintained by implanting a subcutaneous cannula into the back of the mouse and infusing saline at the physiologic flow rate for murine biliary secretion (2.5 µl/minute or 150 µl per hour). Once the surgery was complete, the mice were allowed to recover from anesthesia (~5 minutes), and the trunk of the mouse was wrapped with surgical tape. To restrain the mice, a circular piece of plastic was inserted through a loop of the tape and bent to form a grip, with the externalized cannulas strategically placed out of reach of the mouse (Figure 3A). Once restrained on the wheel, mice were injected intraperitoneally via the externalized port with ~500 µl [^{3}H]-cholesterol labeled foam cell suspension containing ~10x10^6 cells/ml at ~3x10^6 dpm/ml. Following dose administration, the mouse was then allowed to rest or walk on an exercise

wheel with only partial restraint of movement for the subsequent 8 hours. This orientation of the mouse allowed for some movement and enhanced bile flow. After 8 hours the animal was terminally anesthetized with ketamine/xylazine (100-160mg/kg ketamine-20-32mg/kg xylazine), and a midline laparotomy was performed. A whole body flush was conducted by puncturing the inferior vena cava and slowly delivering 10 ml of saline into the heart to clear plasma lipoprotein-associated [^{3}H]-cholesterol from tissues. Thereafter, the liver and small intestine (SI) were removed. The intestine was divided into four equal segments that were classified proximal to distal as SI-1, SI-2, SI-3, and SI-4. All SI segments were gently flushed twice with 2 ml saline to collect intestinal lumenal contents, and the remaining intestine was blotted dry. To extract lumenal contents for separation of [^{3}H]-cholesterol and [^{3}H]-bile acids, 9 ml 2:1 chloroform:methanol was added to the lumenal contents (in ~4ml of saline), and samples were extracted with frequent vortexing for a minimum of 24h at room temperature. Following centrifugation at 2700 rpm for 10 minutes, the lower organic phase (containing [^{3}H]-cholesterol) was removed, and the remaining upper [^{3}H]-bile acid-containing aqueous phase was re-extracted 3 times with 4 ml of chloroform. The pooled chloroform phases and the aqueous phase was dried to completion under N_2. Samples were resuspended in scintillation cocktail to determine count recovery. For tissue (ie liver & small intestine wall) [^{3}H]-cholesterol recovery, a small piece of the liver (~100 mg) or intestinal segments were extracted in 9 ml 2:1 chloroform:methanol with frequent vortexing for a minimum of 24h at room temperature. A measured volume of the extract was dried down under N_2 and resuspended in scintillation cocktail for count recovery determination. Total

tissue recovery is calculated per total organ weight, and all data are expressed as the percent of the total dose injected into each recipient mouse (tissue dpm/dose dpm × 100).

Plasma Lipoprotein Separation

Detailed descriptions have been previously reported for plasma lipid analysis and lipoprotein separation by size exclusion chromatography (Brown et al. 2008a; Temel et al., 2007; Brown et al., 2008b).

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). All data were analyzed using two-way analysis of variance (ANOVA) followed by Student's t tests for post hoc analysis. Differences were considered significant at $p < 0.05$. All analyses were performed using JMP version 5.0.12 (SAS Institute; Cary, NC) software.

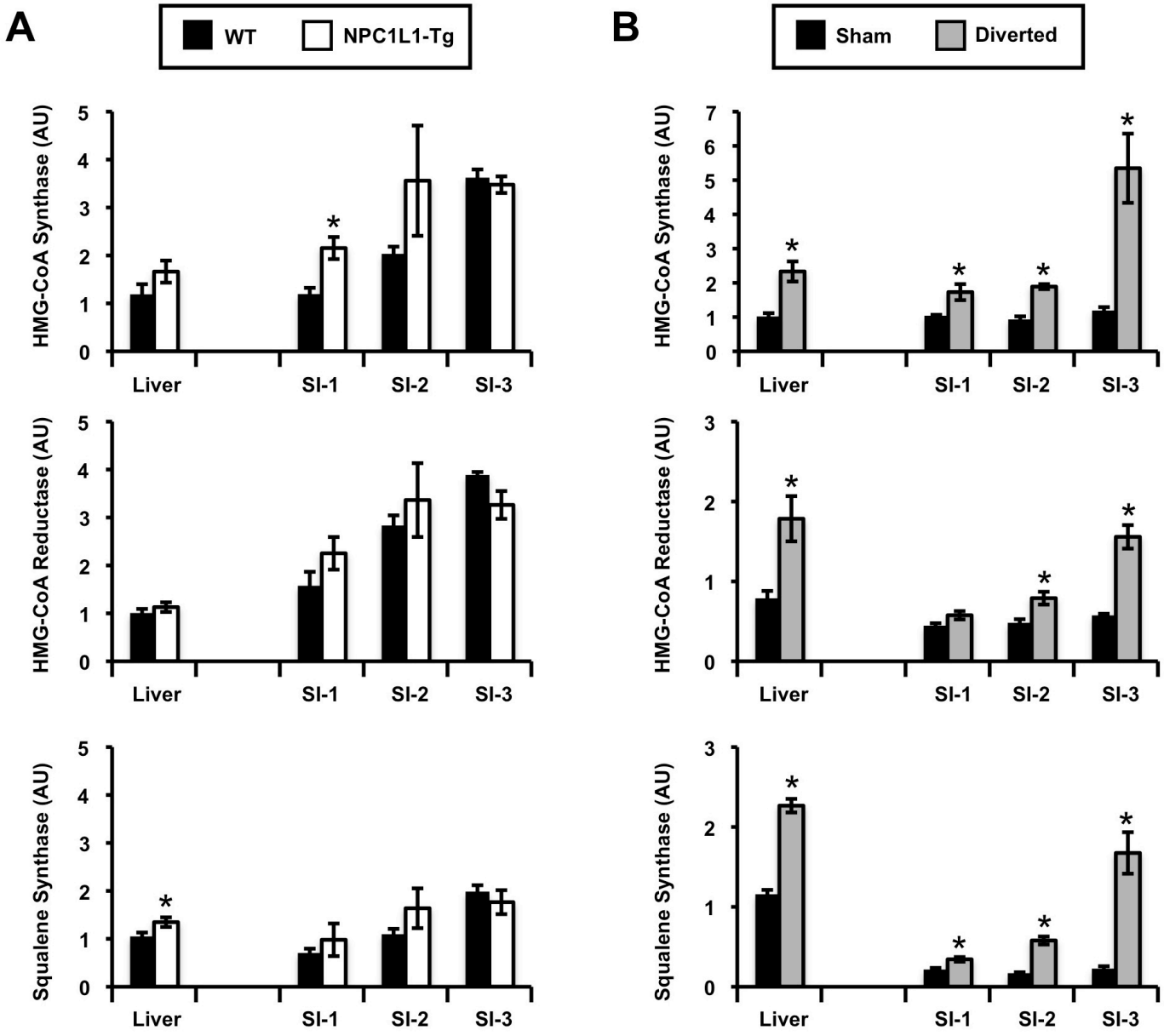

Figure S1. Expression Levels of Cholesterol Synthetic Genes in the Liver and Small Intestine. **(A)** Male wild type mice (WT) or mice overexpressing NPC1L1 in the liver (NPC1L1-Tg) were maintained on a standard chow diet for a period 1-4 months. **(B)** Male wild type C57BL/6N mice were maintained on a standard chow diet, and at eight weeks of age underwent either a sham (Sham) surgery or complete bile diversion (Diverted) as described in materials and methods. RNA was extracted from the liver or the small intestine (SI), which was segmented into 3 equal parts represented proximal to distal as SI-1, SI-2, SI-3. The relative expression of 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA Synthase), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), squalene synthase, and cyclophilin were determined by real-time PCR using the ΔΔ-CT method, and are expressed as arbitrary units (AU). Data represent the means ± SEM from 3-4 mice per group, * = significantly different than matching tissue from control group (WT group in panel A or Sham group in panel B), $P < 0.05$.