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Tissue-specific liver X receptor activation promotes macrophage RCT *in vivo*

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Abstract

Objective—We previously reported that a systemic liver X receptor (LXR) agonist promoted macrophage reverse cholesterol transport (mRCT) *in vivo*. Because LXRs are expressed in multiple tissues involved in RCT (macrophages, liver, intestine), we analyzed the effect of tissue-specific LXR agonism on mRCT.

Methods and Results—In initial studies the systemic LXR agonist GW3965 failed to promote mRCT in a setting where LXR was expressed in macrophages but not in liver or intestine. To evaluate the effect of LXR activation specifically in small intestine on mRCT, wild-type mice were treated with either intestinal-specific LXR agonist (GW6340) or systemic LXR agonist (GW3965). Both GW3965 and GW6340 significantly promoted excretion of [^{3}H]-sterol in feces by 162% and 52%, respectively. To evaluate the requirement for macrophage LXR activation, we assessed the ability of GW3965 to promote mRCT in wild-type mice using primary macrophages deficient in LXRα/β versus wild-type macrophages. While GW3965 treatment promoted fecal excretion compared with vehicle, its overall ability to promote mRCT was significantly attenuated using LXRα/β KO macrophages.

Conclusion—We demonstrate that intestinal-specific LXR agonism promotes macrophage RCT *in vivo*, and macrophage LXR itself plays an important, but not predominant, role in promoting RCT in response to an LXR agonist.

Introduction

Reverse cholesterol transport (RCT) is a physiological process that helps to prevent accumulation of excess cholesterol in peripheral tissues1. Efflux of cholesterol from vessel wall macrophages is thought to be a critical first step by which the RCT pathway protects against atherosclerosis2. Liver X receptors (LXRs) are nuclear receptors activated by endogenous oxysterols that play an important role in the control of cellular and whole body cholesterol homeostasis3 The main target tissues of LXR agonists relevant to RCT are macrophages, liver, and small intestine. LXR agonists increase ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) expression in macrophages resulting in increased cholesterol efflux 4, ABCG5/83, and cholesterol 7 alpha-hydroxylase (CYP7A1)5

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expression in liver5, 6 resulting in increased sterol excretion into bile, and ABCG5/8 expression in small intestine5 resulting in increased cholesterol excretion in feces. We developed an assay that specifically traces RCT from the macrophage to the feces *in vivo*1 and previously reported that a systemic LXR agonist promoted macrophage RCT *in vivo*7. However, the relative roles of LXR agonism in macrophages, liver, and intestine in RCT are not known. Therefore, we tested the effects of tissue-specific LXR agonism on macrophage RCT.

Methods

Materials

Phosphate buffered saline (PBS), RPMI/HEPES were purchased from Invitrogen. [1,2-^{3}H]cholesterol and cholesteryl oleate (cholesteryl-[1,2,6,7-^{3}H]) were purchased from Perkin-Elmer Life Science. Hydroxypropyl methyl cellulose (HMPC K100) was purchased from Shin-etsu. Other reagents without citation in this article were purchased from Fisher Scientific. A systemic LXRα/β agonist (GW3965) and an intestine-specific LXRα/β agonist (GW6340) were obtained from GlaxoSmithKline medicinal chemistry department. In vitro GW3965 is highly specific for LXR receptors8, and GW6340 is the ester form of GW3965. GW6340 is described in a patent (PCT/US01/27622) (GW6340=example 4) which can be accessed through the following link. http://www.wipo.int/pctdb/en/. The structure of the intestinal specific LXR agonist GW6340, a close analog of GW3965, is shown in supplemental Figure I. This compound has an EC50=7 *in vitro* potency in a LXR transactivation assay measuring ABCA1 promoter activity, a value comparable to the potency of GW3965. The dose for GW6340 was chosen in order to achieve a similar intestinal gene induction compared to GW3965 (Figure 2B).

Animals

Wild-type C57BL/6 mice were obtained from the Jackson Laboratory, and LXRα/β double knockout (LXR DKO) mice were obtained from Taconic. Mice were fed a standard chow diet (Lab diet 5010; 4.8 % fat and 267 ppm cholesterol) ad libitum before and during the study. For plasma lipid analyses, animals were fasted for 4 hours and then bled from the retro-orbital plexus. All animals were housed according to guidelines of the Institutional Animal Care and Usage Committee of the University of Pennsylvania. All protocols were considered and approved by the Institutional Animal Care and Usage Committee.

Lipid analysis

Total cholesterol, HDL cholesterol (HDL-C), phospholipid, and triglyceride levels were measured with the use of diagnostic reagents from Thermo Scientific. Pooled plasma from each group (150 μL), was separated by fast protein liquid (FPLC) gel filtration. Total cholesterol in each fraction was measured with the Total Cholesterol E kit from Wako Pure chemicals.

Real time PCR

Total RNA was isolated from mouse tissue using TRIzol Reagent from Invitrogen. cDNA was produced from total RNA with reverse transcription using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Quantification of mRNA expression using Taqman assay systems was performed by ABI 7300 Real Time PCR System from Applied Biosystems. All reagents necessary for running a TaqMan RT-PCR assay including primers and probes were purchased from Applied Biosystems and used according to the manufacturer's instructions9.

Macrophage RCT Study

Experiments were performed in wild-type mice and LXR DKO mice. For each experiment, each group was dosed with 30 mg/kg body weight GW3965, GW6340, or vehicle (HPMC K100 0.5 % and Tween 80 1 % in phosphate buffer 60 mM pH7) once a day for 10 days by oral gavage. On day 10, RCT studies were performed as previously described10, 11. GW3965, GW6340, or vehicle were continued to be gavaged during the 48-hour experiment. J774 macrophages were obtained from American Type Culture Collection, and were grown in suspension in RPMI 1640 medium supplemented with 10 % FBS, followed by being labeled with [^{3}H]-cholesterol and loaded with acLDL. Bone marrow macrophages (BMMs) were isolated from femurs and tibias and cultured in Dulbecco modification of Eagle media (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 30 % L929 conditioned medium for 7 days12. BMMs were grown in 100-mm Petri dishes, labeled with 5 μCi/ml [^{3}H]-cholesterol, and loaded with 25 μg/ml acetylated LDL (acLDL). Twenty-four hours later, cells were washed and equilibrated overnight in DMEM-0.2 % BSA with 10 μM GW3965. Before the injection, cells were collected in PBS-10 mM EDTA, washed, and suspended in RPMI/HEPES.

In vivo HDL turn over studies

Wild-type C57BL/6 were fed for 2 weeks a control or LXR agonist GW3965 (10 mg/kg/day) supplemented chow diet ad libitum. GW3965 was grinded and added to the chow diet at 100 mg/kg diet (Research diets, NJ). HDL was prepared from pooled human plasma by sequential ultracentrifugation (density 1.063<d<1.21 g/mL). After extensive dialysis against dialysis buffer (0.15 M NaCl, EDTA 1 mM, ph 7.4), HDL was exchange labeled with cholesteryl oleate as previously described13. After labeling, the HDL was re-isolated by ultracentrifugation (density 1.063<d<1.21 g/mL), extensively dialyzed, filter sterilized and stored at 4°C until injection. [^{3}H]-cholesteryl oleate-labeled HDL (1 to 2 million cpm per animal) was injected intravenously via tail veins into mice (n=5-6 per group). Blood samples were drawn by retro-orbital bleeding at 2min, 1h, 3h, 6h, 9h, 24h and 48h (≈ 25μL at each time point). Feces were collected continuously from 0 to 48 hours and stored at 4°C before extraction of cholesterol and bile acids. At study termination (48 hours after injection), mice were exsanguinated, perfused with ice cold PBS and liver samples were collected for analysis. Plasma decay curves for both tracers were normalized to radioactivity at the initial 2-min time point after tracer injection. To measure [^{3}H]-tracer recovery in liver, lipid extraction was performed according to the procedure of Bligh and Dyer7. Briefly, a 50-mg piece of tissue was homogenized in water, and then lipids were extracted with a mixture of chloroform/methanol 2:1 (vol/vol). The lipid layer was collected, evaporated, resuspended in toluene, and counted in a liquid scintillation counter. Results were expressed as a percentage of the injected dose. The injected dose was calculated by multiplying the initial plasma counts (2-min time point) by the estimated plasma volume (3.5% of the body weight).

Statistical Analyses

All Values are presented as mean ± SD. Comparisons between control and agonist-treated mice were made with the use of the Student *t* test (2 tailed) and ANOVA with the use of GraphPad Prism Software.

Results

LXR agonism in macrophages alone is insufficient to substantially promote RCT in the absence of hepatic and intestinal LXR expression

In order to test whether activation of LXR in macrophages is by itself sufficient to promote macrophage RCT in the absence of LXR in all other tissues, wild-type and LXR DKO mice

were gavaged for 10 days with GW3965 or vehicle before injection of cholesterol-labeled J774 macrophages, and continued to be gavaged during the 48-hour experiment. At day 5, plasma HDL-C levels were increased in GW3965-treated wild-type mice (111 +/− 12 vs 73 +/− 10 mg/dl), but didn't change in GW3965-treated LXR DKO mice (Supplemental Table I). [^{3}H]-cholesterol counts in plasma of GW3965-treated mice showed a trend toward an increase in wild-type mice (Figure 1A), which was not observed in LXR DKO mice (Figure 1B). There was no difference in [^{3}H]-cholesterol in the liver at the end of 48-hour time period among 4 groups (Figure 1C). GW3965 treatment promoted significantly greater fecal excretion of total tracer and cholesterol tracer but not bile acid tracer (Figure 1D) in wild-type mice but not in LXR DKO mice. These data suggest that LXR activation in macrophages alone is insufficient to promote RCT and that LXR activation in the liver and/or the intestine is also required for promoting macrophage RCT.

An intestinal specific LXR agonist increased macrophage RCT in wild-type mice

In order to determine the role of intestinal LXR activation in promoting RCT, we used an intestinal specific LXR agonist, GW6340. Wild-type mice were gavaged with GW6340, the systemic LXR agonist GW3965, or vehicle for 10 days before injection of cholesterol-labeled J774 macrophages, and continued to be gavaged during the 48-hour experiment. To confirm the intestinal-specific effect of GW6340, gene expression in liver and small intestine of vehicle, GW3965- and GW6340-treated wild-type mice were assessed by quantitative PCR (Figures 2A and B). GW3965-treated mice had significantly increased LXR target gene expression both in liver and small intestine, whereas GW6340-treated mice had increased LXR target gene expression exclusively in small intestine. In addition, treatment with GW3965 significantly increased hepatic triglyceride content whereas treatment with GW6340 did not (Supplementary Table II). These observations confirm that, *in vivo*, GW6340 is an intestinal-specific LXR agonist and unlike the systemic LXR agonist GW3965 does not modulate hepatic gene expression or triglyceride content.

At day 5, plasma HDL-C levels were significantly higher in GW3965-treated mice compared with vehicle-treated mice (100 ± 16 vs 57 ± 7 mg/dl) (Supplemental Table III). While there was a trend toward an increased HDL-C level in GW6340--treated mice, it was not significant (Supplemental Table III). In the FPLC analysis, GW3965 substantially increased cholesterol in the usual HDL fraction as well as a large HDL fraction, whereas GW6340 modestly increased cholesterol in the usual HDL fraction (Figure 3A). The [^{3}H]-cholesterol counts in plasma after J774 macrophage injection, expressed as a percentage of total [^{3}H]-counts injected, were significantly increased in GW3965-treated mice compared with control mice, while they trended higher but were not significantly different in the GW6340-treated mice compared with the vehicle-treated mice (Figure 3C). The [^{3}H]-cholesterol in plasma tracked closely with the cholesterol mass in plasma lipoprotein distribution and were consistent with HDL [^{3}H]-counts highest in GW3965-treated mice, next highest in GW6340-treated mice, and lowest in vehicle-treated mice (Figure 3B). There were no differences in [^{3}H]-cholesterol in the liver at the end of 48-hour time period among the three groups (Figure 3D). As expected, GW3965-treated mice excreted a significantly 162 % higher amount of macrophage-derived tracer in the feces compared with the control mice (Figure 3E). Interestingly, GW6340-treated mice also excreted significantly more (52 %) amount of macrophage-derived tracer in the feces compared with the control mice (Figure 3E). Thus intestine-specific LXR activation promotes macrophage RCT, though it may be less effective than systemic LXR activation.

Macrophage LXR contributes to the ability of a systemic LXR agonist to promote macrophage RCT

We tested whether macrophage LXR is critical for the ability of a systemic LXR agonist to promote RCT. As expected, in BMMs from wild-type mice, both GW3965 and acLDL treatment increased cholesterol efflux, whereas in BMMs from LXR DKO mice, GW3965 and acLDL treatment had no effect on efflux *ex vivo* (Supplemental Figure II). Next, we evaluated RCT *in vivo* using primary BMMs from LXR DKO and wild-type mice. Wild-type mice were gavaged for 10 days with GW3965 or vehicle before injection of BMMs from either wild-type or LXR DKO mice, and continued to be gavaged during the 48-hour experiment. At day 5, plasma HDL-C levels were increased in GW3965-treated mice (Supplemental Table IV). The [^{3}H]-cholesterol counts in plasma were significantly increased by GW3965-treatment in mice injected with wild-type BMMs (Figure 4A). In contrast, there was no effect of GW3965-treatment on plasma [^{3}H]-cholesterol counts in mice injected with LXR DKO BMMs (Figure 4B). There was no difference in [^{3}H]-cholesterol in the liver at the end of 48-hour time period among 4 groups (Figure 4C). GW3965 treatment significantly increased fecal excretion of total tracer in mice injected with wild-type BMMs but not in mice injected with LXR DKO BMMs (Figure 4D). Excretion of cholesterol tracer was markedly increased by GW3965 in wild-type BMMs, and was increased, modestly but significantly, by GW3965 in LXR DKO BMMs (Figure 4D). In contrast, GW3965 had no effect on fecal excretion of bile acid tracer in either group (Figure 4D). These data confirm that macrophage LXR is an important determinant of the ability of a systemic LXR agonist to promote macrophage RCT, but that LXR agonist promotes the fecal excretion of macrophage-derived cholesterol even in the absence of macrophage LXR.

In order to confirm that activation of LXR in the liver and intestine promotes the fecal excretion of HDL-derived cholesterol, we performed an [^{3}H]-HDL-cholesteryl-ester kinetics study with GW3965-treated mice. Compared to the control group treated with vehicle, GW3965 treatment did not alter the rate of plasma turnover of [^{3}H]-HDL-cholesteryl-ester (Figure 5A) or the amount of [^{3}H]-cholesterol in the liver (Figure 5B), but significantly increased the fecal cholesterol excretion of the HDL-derived cholesterol (Figure 5C). These data confirm the macrophage RCT study results and suggest that LXR agonism promotes RCT at least in part by accelerating the rate by which HDL-cholesterol is targeted for biliary and fecal excretion.

Discussion

In these studies, we provide evidence that LXRs in all three major LXR-expressing tissues, macrophages, liver, and intestine, play a role in promoting macrophage RCT in response to LXR activation. We show that that ability of an LXR agonist to promote macrophage RCT requires LXR expression in tissues other than just the macrophage. We provide the first evidence that an intestine-specific LXR agonist promotes macrophage RCT *in vivo*. Finally, we show that macrophage LXR is an important, but not the sole component of the ability of a systemic LXR agonist to promote RCT and that an LXR agonist promotes the fecal excretion of HDL-derived cholesterol. These observations are potentially relevant to therapeutic targeting of LXR for reduction of atherosclerotic vascular disease.

The small intestine plays a critical role in cholesterol homeostasis. It regulates cholesterol absorption and excretion and also produces apoA-I and nascent HDL. Indeed, intestine-specific ABCA1 deficiency resulted in a 30 % reduction of plasma HDL levels14, whereas a systemic LXR agonist significantly increased plasma HDL levels even in liver-specific ABCA1 deficient mice15. These data indicate that intestinal ABCA1 is a major determinant of plasma HDL-C levels. In this study, we used an intestine-specific LXR agonist GW6340

to address the question of how intestine-specific LXR agonist influences macrophage RCT and showed that it significantly promoted macrophage RCT. GW6340 significantly upregulated ABCA1, ABCG5, and ABCG8 expression in the small intestine but not in the liver. FPLC analysis suggested that it modestly increased cholesterol mass and macrophage-derived cholesterol tracer in the HDL fraction, providing one possible mechanism by which it promoted macrophage RCT In addition, ABCG5/8 promotes cholesterol excretion in the small intestine16. Recently, Veen et al17 reported that direct intestinal secretion of plasma cholesterol significantly contributes to fecal neutral sterol loss in mice. This trans-intestinal cholesterol excretion was upregulated by a LXR agonist and impaired in ABCG5 deficient mice. Thus, the intestine-specific LXR agonist might stimulate trans-intestinal excretion of plasma cholesterol partially via activating expression of the ABCG5/ABCG8 heterodimer. Therefore, the intestine-specific LXR agonist may promote RCT through at least two independent pathways: increasing intestinal HDL production as well as promoting intestinal excretion of HDL-derived cholesterol.

We previously reported that ABCA1 and ABCG1 deficient macrophages showed a significant reduction of macrophage-derived fecal cholesterol excretion11. LXR agonist treatment is known to upregulate ABCA1 and ABCG1 and cholesterol efflux from macrophages18. These data suggest that macrophage LXR is crucial for the ability of an LXR agonist to promote RCT. We show here that macrophage-specific LXR deficiency resulted in a significant blunting, but not abolishment, of the ability of the systemic LXR agonist GW3965 to promote RCT. However, even in mice injected with labeled macrophages lacking LXR, GW3965-treated mice still increased fecal cholesterol excretion compared with vehicle group. Once cholesterol efflux occurred from macrophages to plasma through an LXR-independent pathway, activated LXR in liver and small intestine likely promoted cholesterol excretion from plasma to feces. We confirmed using HDL labeled with cholesteryl ester that GW3965 promoted the fecal excretion of HDL-derived cholesterol in a macrophage-independent manner.

Some systemic LXR agonists activate SREBP-1c in the liver, leading to elevated plasma TG and fatty liver19. This has slowed the clinical development of LXR agonists as a potential treatment for atherosclerosis. Based on our studies, we suggest that intestinal specific LXR agonism, which avoids undesired effects of activating hepatic LXRs, might be expected to promote macrophage RCT and thus potentially be anti-atherogenic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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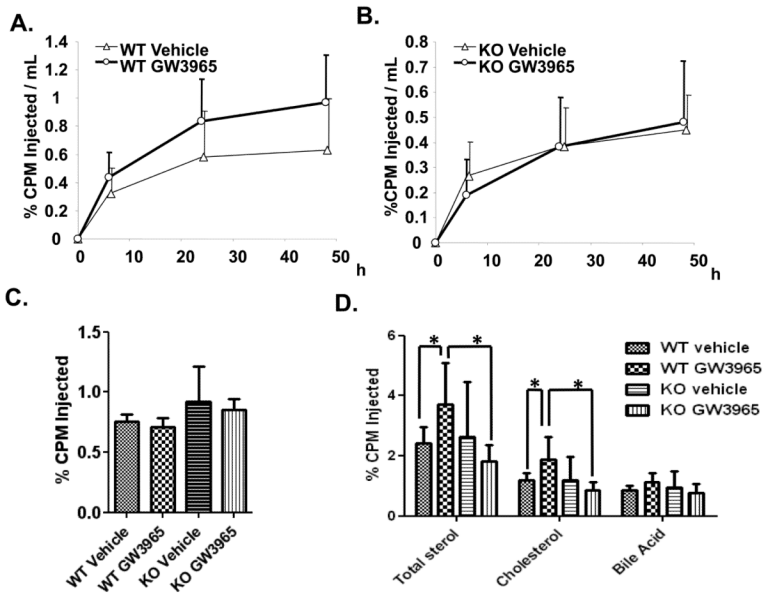

Figure 1.

Wild-type (WT) and LXR DKO(KO) mice were treated with GW3965 or vehicle for 10 days and RCT study was performed with J774 macrophages as described in Methods (n=6/each group). (A) [^{3}H]-cholesterol in plasma after macrophages injection in wild-type mice. (B) [^{3}H]-cholesterol in plasma after macrophages injection in LXR DKO mice. (C) [^{3}H]-cholesterol in liver. (D) [^{3}H]-tracer distribution in feces. *$p<0.05$. Data are expressed as mean ± SD.

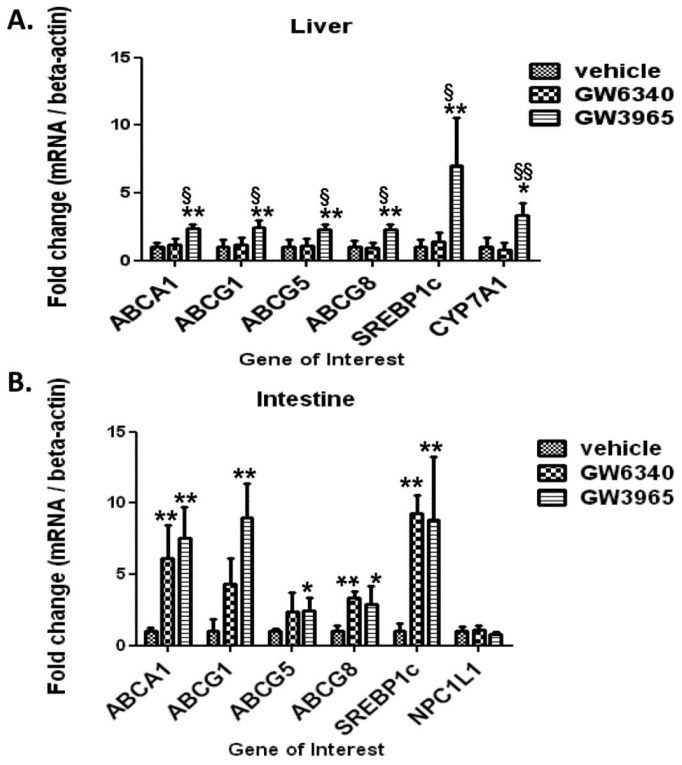

Figure 2.

Gene expression analysis in Liver (**A**) and intestine (**B**) of wild-type mice determined by real-time PCR analysis. Animals were treated with vehicle, GW3965, or GW6340 by oral gavage for 12 days. Mice were sacrificed after 4 h gavage and fasting (n=5/each group). Data are expressed as fold change ± SD vs vehicle treated mice and normalized to beta-actin mRNA. *p<0.05,**p<0.01 vs vehicle group. §p<0.05, §§p<0.01 vs GW6340 group.

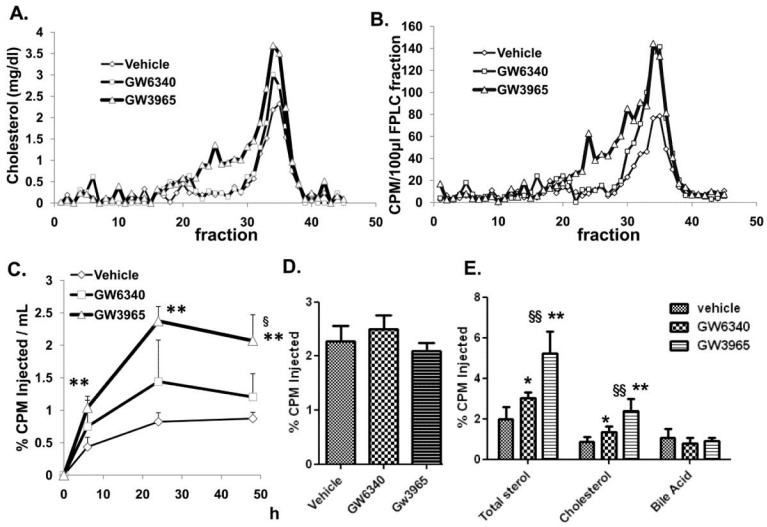

Figure 3.

Intestine-specific LXR agonist promotes RCT *in vivo*. Wild-type mice (n=5/each group) were treated with indicated drug for 10 days and RCT study was performed with J774 macrophages as described in Methods. (A) FPLC analysis of cholesterol mass profile of pooled 48-hours plasma from wild-type mice treated with indicated drug. (B) FPLC analysis of [^{3}H]-cholesterol profile of pooled 48-hours plasma from wild-type mice treated with indicated drug. (C) [^{3}H]-cholesterol in plasma after macrophages injection. (D) [^{3}H]-cholesterol in liver. (E) [^{3}H]-tracer distribution in feces. *p<0.05, **p<0.01 vs vehicle group. §p<0.05, §§p<0.01 vs GW6340 group. Data are expressed as mean ± SD.

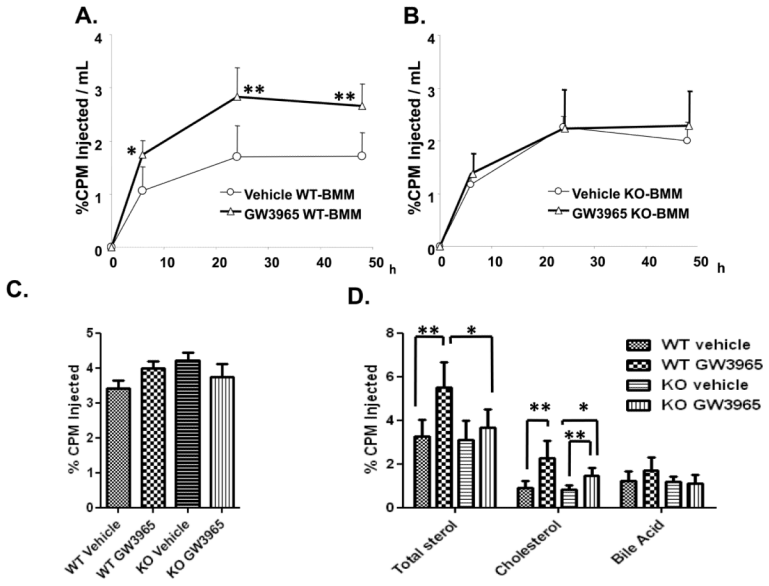

Figure 4.

Wild-type mice were treated with either GW3965 or vehicle for 10 days and RCT study was performed with BMMs from either wild-type mice (WT-BMM) or LXR DKO mice (KO-BMM) as described in Methods (n=6/each group). (A) [^{3}H]-cholesterol in plasma after macrophages injection in wild-type mice. *p<0.05, **p<0.01 vs vehicle group. (B) [^{3}H]-cholesterol in plasma after macrophages injection in LXR DKO mice. (C) [^{3}H]-cholesterol in liver. (D) [^{3}H]-tracer distribution in feces. *p<0.05, **p<0.01. Data are expressed as mean ± SD.

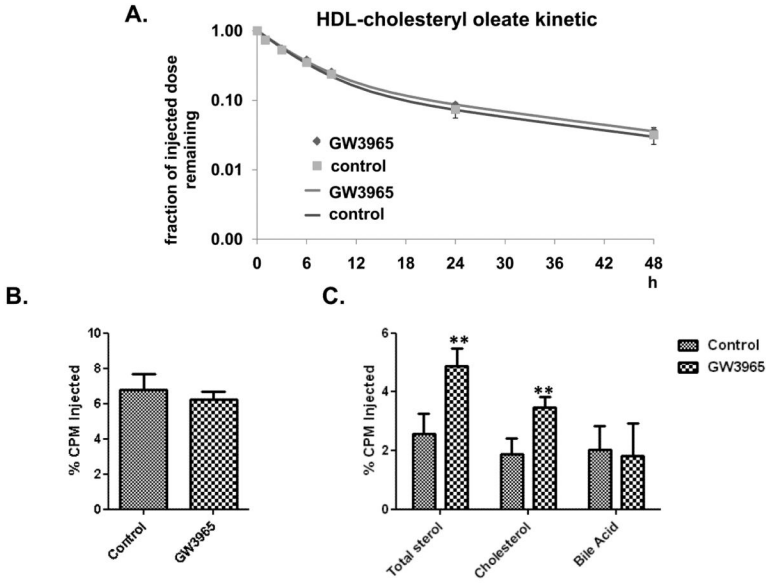

Figure 5.

Wild-type mice were fed the experimental diets for 2 weeks and HDL turnover studies were performed as described in Methods (n=6/each group). (A) The change of [^{3}H]-HDL-cholesteryl-ester in plasma. (B) [^{3}H]-cholesterol in liver. (C) [^{3}H]-tracer distribution in feces. **P<0.01 vs. control group. Data are expressed as mean ± SD.