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Sterol O-Acyltransferase 2-Driven Cholesterol Esterification Opposes Liver X Receptor-Stimulated Fecal Neutral Sterol Loss

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Abstract

Statin drugs have proven a successful and relatively safe therapy for the treatment of atherosclerotic cardiovascular disease (CVD). However, even with the substantial low-density lipoprotein (LDL) cholesterol lowering achieved with statin treatment, CVD remains the top cause of death in developed countries. Selective inhibitors of the cholesterol esterifying enzyme sterol-O acyltransferase 2 (SOAT2) hold great promise as effective CVD therapeutics. In mouse models, previous work has demonstrated that either antisense oligonucleotide (ASO) or small molecule inhibitors of SOAT2 can effectively reduce CVD progression, and even promote regression of established CVD. Although it is well known that SOAT2-driven cholesterol esterification can alter both the packaging and retention of atherogenic apoB-containing lipoproteins, here we set out to determine whether SOAT2-driven cholesterol esterification can also impact basal and liver X receptor (LXR)-stimulated fecal neutral sterol loss. These studies demonstrate that SOAT2 is a negative regulator of LXR-stimulated fecal neutral sterol loss in mice.

Keywords

Cholesterol; Intestine; Atherosclerosis; Esterification

Introduction

Although statins have been used to modestly decrease cardiovascular disease (CVD) mortality, the fact remains that CVD still accounts for roughly one-third of all deaths in the United States and many other developed countries [1, 2]. Clearly, there is an unmet need for additional therapeutic strategies to prevent or treat CVD. Inhibition of the cholesterol esterifying enzyme sterol O-acyltransferase 2 (SOAT2), also known as acyl-coA:cholesterol o-acyl transferase 2 (ACAT2), has long been considered an attractive strategy [3–5]. We have previously demonstrated that genetic deficiency [6–12], antisense oligonucleotide (ASO)-mediated knockdown [13–16], or selective small molecule-mediated inhibition [17,

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Compliance with Ethical Standards

Conflict of interest All authors have no conflicts of interest to declare.

18] of SOAT2 can effectively prevent the progression of atherosclerosis in mice. Also, SOAT2 inhibition can also stabilize highly advanced plaques when given in the late phases of atherosclerosis progression [16]. The ability of SOAT2 inhibitors to protect against atherosclerosis can be in part attributed to decreased intestinal cholesterol absorption, reduced hepatic very low density lipoprotein (VLDL), and blunted retention of low density lipoprotein (LDL) in the artery wall [6–18]. However, there is also developing evidence that SOAT2 is a negative regulator of removal of cholesterol from the body via fecal excretion.

The first evidence that SOAT2-driven cholesterol esterification may interplay with fecal cholesterol disposal and high density lipoprotein (HDL) metabolism was elucidated in the small intestine of SOAT2 knockout mice. In this case it was demonstrated that SOAT2 deficient mice have elevated expression of the cholesterol efflux pump ATP-binding cassette transporter A1 (ABCA1) in the small intestine, and that this compensatory ABCA1-mediated cholesterol efflux can maintain moderate levels of cholesterol absorption despite the lack of chylomicron CE packaging [19–22]. This strongly suggested a potential cross talk between SOAT2-driven cholesterol esterification and HDL biogenesis in the intestine. Although less dramatic, there is also a compensatory upregulation of ABCA1 in the liver of mice treated with SOAT2 ASOs [14, 23], which has the potential to alter hepatic high density lipoprotein biogenesis. More recently, we have demonstrated that either chronic or acute inhibition of SOAT2 promotes a non-biliary pathway of reverse cholesterol transport (RCT) called transintestinal cholesterol excretion (TICE) [14, 15, 24–26]. Although these results suggest a potential role for SOAT2 as a regulator of RCT, the ability of either genetic deficiency or pharmacological inhibition of SOAT2 to alter circulating HDL levels of markers of RCT have been inconsistently seen [6–16]. Given that the non-biliary TICE pathway for RCT can be stimulated by drugs that activate the liver X receptor (LXR) [27–30], and the fact that acute inhibition of SOAT2 can likewise stimulate the TICE pathway, we hypothesized that SOAT2 may be a negative regulator of LXR-stimulated fecal cholesterol disposal. To address this, we have studied basal and LXR-stimulated fecal cholesterol disposal in mice with whole body or tissue-selective deletion of SOAT2. Our results suggest that SOAT2-driven cholesterol esterification opposes LXR-stimulated fecal cholesterol disposal, and that the combination of SOAT2 inhibition with LXR agonist treatment results in a marked negative cholesterol balance.

Materials and Methods

Animals

Generation of whole body SOAT2 knockout and tissue specific knockouts using Cre/LoxP technology have been previously described [6–12]. Mice transgenically overexpressing Niemann-Pick C1-Like 1 (NPC1L1) in hepatocytes have also been previously described [24, 26, 31], and we used the high expressing line in this work (line 112) [31]. For all studies, male mice ranging from 14 to 20 weeks of age were maintained on standard rodent diet for all studies. For LXR agonist studies, mice were gavaged with either vehicle or 25 mg/kg T0901317 once daily for a period of 7 days as previously described [24, 26]. All experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) at Wake Forest University School of Medicine.

Cholesterol Absorption, Biliary and Fecal Sterol Excretion Measurements

Intestinal cholesterol absorption was measured using the dual fecal isotope assay, and fecal neutral sterol loss and gall bladder bile cholesterol levels were measured by gas chromatography as previously described [14, 15, 19, 24, 26].

Statistical Analyses

Data are expressed as the mean ± standard error of the mean (SEM) and were analyzed using two-way analysis of variance (ANOVA) followed by Student's *t* tests. All analyses were performed using JMP version 5.0.12 (SAS Institute; Cary, NC) software.

Results and Discussion

Global Deficiency of SOAT2 Enhances LXR-Stimulated Fecal Neutral Sterol Loss

In order to understand the role of SOAT2-driven cholesterol esterification in LXR-stimulated fecal neutral sterol loss, we treated wild type or SOAT2 global knockout mice (lacking SOAT2 in all tissues) with a vehicle or a pharmacological agonist of LXR (T0901317) (Fig. 1). As previously reported [27–30], LXR treatment of WT animals increased fecal neutral sterol loss (Fig. 1a), and reciprocally decreased fractional cholesterol absorption (Fig. 1b). However in SOAT2$^{-/-}$ mice, LXR-stimulated increases in fecal neutral sterol loss (Fig. 1a) and reductions in cholesterol absorption (Fig. 1b) were enhanced when compared to their wild type counterparts. Collectively, SOAT2's key role in promoting intestinal cholesterol absorption [19, 22] and suppressing the non-biliary TICE pathway [14, 15, 24] are both likely contributing mechanisms underlying SOAT2's ability to oppose LXR-stimulated fecal cholesterol disposal.

Intestine or Liver Specific Deletion of SOAT2 is Not Sufficient to Enhance LXR-Stimulated Fecal Neutral Sterol Loss

SOAT2 is primarily expressed in enterocytes in the intestine and hepatocytes in the liver [32, 33], where it plays a major role in the cholesterol ester cycle. However, modest expression has also been seen in other cell types in culture [34–37]. To understand whether hepatic or intestinal SOAT2 is responsible for suppressing LXR-stimulated fecal neutral sterol loss, we subjected control (fl/fl), liver specific (L−/L−) or intestine specific (SI−/SI−) SOAT2 knockout animals to LXR agonist treatment. As expected, T0901317 treatment increased fecal sterol levels and reduced cholesterol absorption in control floxed mice (Fig. 2). However, to our surprise, LXR-stimulated alterations in cholesterol absorption and fecal sterol excretion were similarly altered in either intestine- or liver-specific SOAT2 knockout mice (Fig. 2). These results suggest that SOAT2 deficiency in either intestine or liver alone is not sufficient to augment LXR-stimulated fecal neutral sterol loss. It remains possible that SOAT2 activity in other cell types [33–36] is important here, but more likely these results indicate that both intestine and liver deletion are needed to see robust augmentation of LXR-stimulated fecal neutral sterol loss. It is tempting to speculate that SOAT2's role in both promoting intestinal cholesterol absorption [11, 19] and suppression of the hepatic organization of TICE [14, 15, 24] is involved in LXR responses *in vivo*. However, in full disclosure, another potential explanation of the lack of difference in LXR responsiveness in

the tissue-specific SOAT2 knockout mice may reflect the fact that the floxed lines (Fig. 2) had not been completely backcrossed into a pure C57BL/6 background as had the global SOAT2 knockout mice (Fig. 1).

The Ability of SOAT2 to Suppress LXR-Stimulated Fecal Neutral Sterol Loss Relies In Part on LXR-Stimulated Biliary Cholesterol Secretion

Although LXR agonists primarily stimulate the non-biliary TICE pathway of RCT [26, 29, 30], they also can modestly increase the classic hepatobiliary route [27, 28]. To understand the relative contribution of biliary versus non-biliary pathways in SOAT2's ability to suppress LXR-stimulated fecal neutral sterol loss we crossed global SOAT2 knockout mice to mice transgenically overexpressing Niemann-Pick C1-Like 1 (NPC1L1) in hepatocytes. We have previously shown that NPC1L1-liver-transgenic mice have >90 % reduced levels of cholesterol in gall bladder bile, and preferentially use the non-biliary TICE pathway for RCT [26, 31]. As expected, transgenic overexpression of NPC1L1 reduced biliary cholesterol levels both in vehicle and T0901317-treated mice (Fig. 3c). Interestingly, LXR-stimulated alterations in cholesterol absorption and fecal sterol excretion seen in global SOAT2$^{-/-}$ mice were normalized when biliary cholesterol was suppressed by transgenic NPC1L1 overexpression (Fig. 3a, b). These results strongly suggest that the ability of SOAT2-driven cholesterol esterification to oppose LXR-stimulated fecal cholesterol loss involves the classic hepatobiliary route of excretion.

SOAT2 Only Modestly Alters LXR-Driven Reorganization of Cholesterol-Sensitive Gene Expression in the Liver and Small Intestine

Cellular cholesterol levels are carefully sensed and regulated by at least two major transcriptional mechanisms involving LXR and the sterol regulatory element-binding proteins (SREBPs) [38, 39]. Interestingly, when we examined LXR- and SREBP2-driven alterations in gene expression, we found that SOAT2 deletion only altered a subset of LXR and SREBP2 target genes (Fig. 4). In the liver, SOAT2$^{-/-}$ mice exhibited normal LXR-stimulated upregulation of ABCA1, but had blunted LXR-stimulated upregulation of ABCG5 (Fig. 4a, b). While SOAT2$^{-/-}$ mice showed normal LXR-stimulated upregulation in the liver (Fig. 4c, d), basal and LXR-stimulated regulation of ABCA1, ABCG5, HMGCS, and LDLr in the small intestine was unchanged in SOAT2$^{-/-}$ mice (Fig. 4e–h). Collectively, although there are modest changes in LXR-stimulated gene expression in the liver of SOAT2$^{-/-}$ mice, these changes likely do not fully explain the dramatic reorganization of intestinal cholesterol absorption and fecal cholesterol loss seen in global SOAT2$^{-/-}$ mice (Fig. 1).

Conclusions

The key finding of the current study is that SOAT2-driven cholesterol esterification negatively regulates LXR-stimulated fecal neutral sterol loss. Given that small molecule LXR agonists [40, 41] and SOAT2-selective inhibitors [42–47] have recently been identified as potential antiatherogenic drugs, it remains possible that dual therapy could provide unexpected benefit. In particular, our results suggest that a dual therapy of LXR agonists in combination with SOAT2-selective inhibitors can impressively stimulate cholesterol removal

from the body by dampening intestinal cholesterol absorption and stimulating fecal cholesterol disposal. Logically such a dual therapy has promise for the cholesterol-driven atherosclerotic CVD, but this strategy may also hold promise for other diseases driven by pathological storage of cholesterol. Along these lines, it has recently been shown that selective SOAT2 inhibitors can relieve some aspects of lysosomal acid lipase (LAL) deficiency [48, 49]. An important consideration in developing such therapies is the species-specific differences in the expression of NPC1L1, where humans have abundant expression and mice lack endogenous expression of NPC1L1 in the liver [50]. Furthermore, NPC1L1 itself can be transcriptionally regulated by LXR agonists in the intestine [51]. Hence, further studies are warranted in non-human primates or humans to determine whether similar effects of dual LXR agonists and SOAT2 inhibitors can potently stimulate fecal cholesterol disposal in species where endogenous hepatic NPC1L1 plays a key role in hepatobiliary cholesterol disposal [31]. Collectively, our results provide support that such a combination therapy (SOAT2 inhibitor + LXR agonist) holds promise for those suffering from diseases of excessive cholesterol burden.

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Abbreviations

ASO	Antisense oligonucleotide
CE	Cholesteryl ester
FC	Free cholesterol
HDL	High density lipoprotein
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LXR	Liver X receptor
NPC1L1	Niemann-Pick C1-Like 1
RCT	Reverse cholesterol transport
Soat2	Sterol O-acyltransferase 2
TG	Triacylglycerol
VLDL	Very low density lipoprotein

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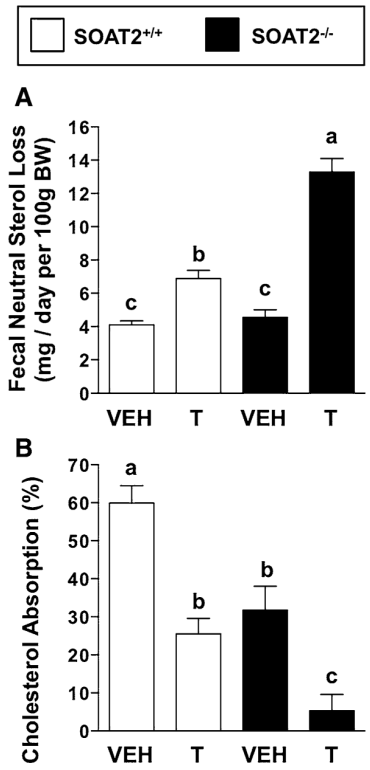

Fig. 1.

Global deficiency of SOAT2 enhances LXR-stimulated fecal neutral sterol loss. Male wild type ($SOAT2^{+/+}$) and SOAT2 total body knockout mice ($SOAT2^{-/-}$) were fed a standard chow diet and orally gavaged either with vehicle (VEH) or the LXR agonist T0901317 (T) for 7 days. **a** Mass fecal neutral sterol loss was determined by gas–liquid chromatography. **b** Fractional cholesterol absorption was measured using the fecal dual-isotope method. Data represent mean ± S.E from 12 to 14 mice per group and values not sharing a common superscript differ significantly ($p \leq 0.05$)

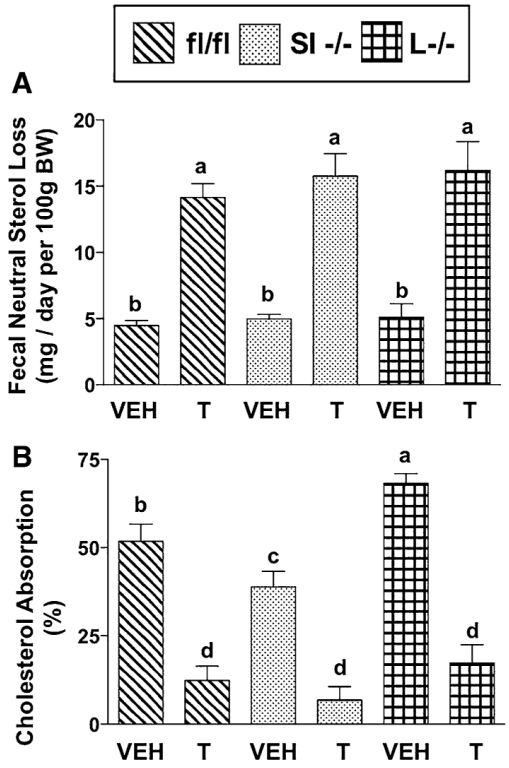

Fig. 2.

Intestine or liver specific deletion of SOAT2 is not sufficient to enhance LXR-stimulated fecal neutral sterol loss. Male control mice with two SOAT2 floxed alleles (fl/fl), enterocyte-specific SOAT2 knockout mice ($SI^{-}/^{-}$), or hepatocyte-specific SOAT2 knockout mice ($L^{-}/^{-}$) were fed a standard chow diet, and were either treated with vehicle (VEH) or the LXR agonist T0901317 (T) for 7 days. **a** Mass fecal neutral sterol loss was determined by gas–liquid chromatography. **b** Fractional cholesterol absorption was measured using the fecal dual-isotope method. Data represent mean ± S.E from 9 to 11 mice per group and values not sharing a common superscript differ significantly ($p \leq 0.05$)

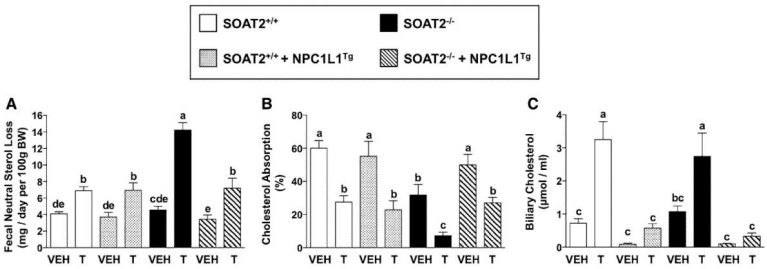

Fig. 3.

The ability of SOAT2 to suppress LXR-stimulated fecal neutral sterol loss relies in part on LXR-stimulated biliary cholesterol secretion. Male wild type ($SOAT2^{+/+}$) or global SOAT2 knockout mice ($SOAT2^{-/-}$), were studies in the presence of absence of hepatocyte-specific overexpression of NPC1L1 ($NPC1L1^{Tg}$) to blunt biliary cholesterol loss. These four genotypes of mice were maintained on a standard chow diet, and were either treated with vehicle (VEH) or the LXR agonist T0901317 (T) for 7 days. **a** Mass fecal neutral sterol loss was determined by gas–liquid chromatography. **b** Fractional cholesterol absorption was measured using the fecal dual-isotope method. **c** Gall bladder bile was collected and analyzed for biliary cholesterol by gas–liquid chromatography. Data represent mean ± S.E from 5 to 14 mice per group and values not sharing a common superscript differ significantly ($p \leq 0.05$)

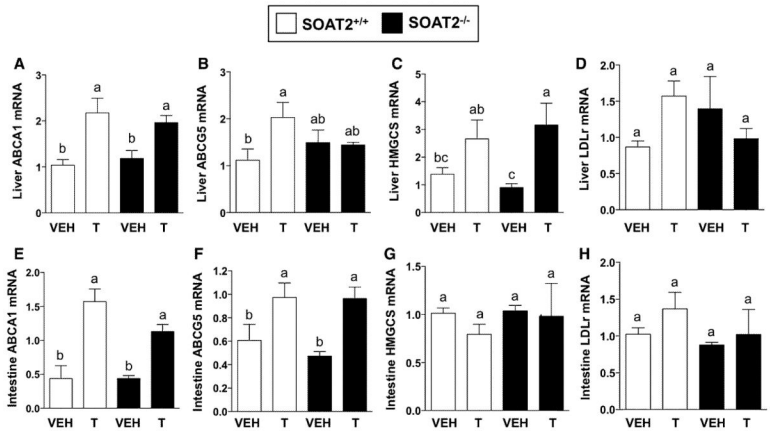

Fig. 4.

Expression of cholesterol-regulated gene expression in the liver and small intestine (jejunum). Male wild type ($SOAT2^{+/+}$) and SOAT2 total body knockout mice ($SOAT2^{-/-}$) were fed a standard chow diet and orally gavaged either with vehicle (VEH) or the LXR agonist T0901317 (T) for 7 days. The relative levels of mRNA for ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G5 (ABCG5), 3-hydroxy-3methylgltaryl-CoA synthase (HMGCS), and LDL receptor (LDLr) were quantified by quantitative real time PCR in the liver (panels **a**–**d**) and proximal small intestine (panels **e**–**h**). Data represent mean ± S.E from four mice per group and values not sharing a common superscript differ significantly ($p \leq 0.05$)