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Enolase is regulated by Liver X Receptors

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ABSTRACT

Enolase is a glycolytic enzyme known to inhibit cholesteryl ester hydrolases (CEHs). Cholesteryl ester loading of macrophages, as occurs during atherosclerosis, is accompanied by increased Enolase protein and activity. Here, we describe that J774 macrophages treated with LXR agonists exhibit reduced Enolase transcript and protein abundance. Moreover, we show that this reduction is further potentiated by activation of the LXR/RXR heterodimer with the RXR ligand 9-*cis* retinoic acid. Enolase levels are also reduced *in vivo* following activation of LXRs in the intestine, but not in the liver. This effect is lost in *Lxrαβ−/−* mice. In aggregate, our study identified Enolase as a new target of LXRs *in vivo*, which may promote cholesterol mobilization for subsequent efflux.

1. Introduction

The enzyme Enolase (ENO1, EC.4.2.1.11) acts in glycolysis to convert 2-phosphoglycerate to phosphoenolpyruvate. Twenty years ago, Shand and West had proposed that next to its glycolytic function Enolase can also inhibit cholesteryl ester hydrolases (CEHs) [1,2]. The transition of macrophages to "foam cells" in the atherosclerotic plaque is accompanied by storage of cholesterol esters in lipid droplets [3,4]. This process, which can be mimicked experimentally *in vitro* by loading macrophages with acetyl-LDL or oxidized-LDL, requires enhanced cholesterol esterification activity. Accordingly, cholesterol loading of macrophages results in a marked increase in ENO1 protein [5,6], which can potentially inhibit CEHs on the surface of lipid droplets [7,8].

The Liver X Receptor-α and -β (LXRs, NR1H3 and NR1H2, respectively) are members of the nuclear receptor superfamily that play a central role in controlling cholesterol homeostasis [9,10]. In macrophages, LXRs can decrease the cellular sterol burden by inducing expression of the cholesterol efflux transporters *Abca1* and *Abcg1* [11,12], and by limiting uptake of LDL-derived cholesterol due to induction of *Idol*, an E3 ubiquitin ligase that promotes lysosomal degradation of the LDLR [13]. Despite the crucial role of LXRs in cholesterol homeostasis, their effect on cellular cholesterol storage is not well understood. Through transcriptional profiling, we have identified that Enolase is subject to LXR-dependent regulation [14]. Here, we show that Enolase transcript and protein abundance are reduced by LXRs in macrophages and intestine and discuss the impact this may have on mobilization of cholesterol towards efflux pathways.

2. Materials and methods

2.1. Cell culture and treatments

J774-A1 murine macrophage cell line was cultured in DMEM medium supplemented with L-glutamine (2 mM), penicillin streptomycin (100 μg/ml) and 10% of Fetal Bovine Serum. Twenty-four hours after seeding, cells were treated with synthetic LXR agonists, GW3965 (Sigma–Aldrich, L'Isle d'Abeau, France) or T0901317 (Cayman Chemical, Montigny-le-Bretonneux, France) and/or RXR ligand 9-*cis* RA (Sigma–Aldrich) diluted in DMSO as indicated in the figure legends in a 1% lipoprotein-deficient serum (LPDS) medium.

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2.2. Animals

Mice lacking *Lrrα* and *Lrrβ* (LXR double knockout mice) and their wild-type controls were maintained and housed as previously described [14]. Male mice were orally gavaged daily with methylcellulose or 25 mg/kg T0901317 for 3 consecutive days. Animals were sacrificed at day 4, and organs collected and stored at −80 °C prior to RNA or protein extraction, or paraformaldehyde (PFA)-fixed and embedded in paraffin for immunofluorescence experiments. All experiments were approved by the local Regional Ethics Committee.

2.3. RNA and real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Saint Aubin, France) according to manufacturer instructions. RNA was reverse transcribed to cDNA with 200 U of Moloney murine leukemia virus-reverse transcriptase (Promega, Charbonnières, France), 5 pmol of random primers (C1181, Promega), 40 U RNAsin (Promega), and 2.5 mM deoxynucleotide triphosphate mix. Quantitative PCR was performed on a Mastercycler epRealplex (Eppendorf, LePecq, France) using MESA GREEN quantitative PCR masterMix Plus for SYBR (Eurogentec, Angers, France). Primer sequences are: m*Abca1* Fw: 5′-GGAGCTGGGAAGTCAACAAC-3′, m*Abca1* Rev: 5′-ACATGCTCTCTTCCCGTCAG-3′; m*Abcg1* Fw: 5′-GCTGTGCGTTTTGTG CTGTT-3′, m*Abcg1* Rev: 5′-TGCAGCTCCAATCAGTAGTCCTAA-3′; mEno1 Fw: 5′-TGATCCTGCCTGTGGGGGCA-3′; mEno1 Rev: 5′-GCCG GCCTTTGCGATTGCAG-3′; m*36b4* Fw: 5′-GTCACTGTGCCAGCTCA-GAA-3′, m*36b4* Rev: 5′-TCAATGGTGCCTCTGGAGAT-3′.

2.4. Western blotting

Proteins from J774-A1 cells or mice organs were extracted, transferred and detected as described previously [15]. Primary antibodies used are the following: ABCA1 (NB400-105, Novus Biologicals, Littleton, CO), ENO1 (#3810, Cell Signaling, Danvers, MA), α-Tubulin (T6074, Sigma Aldrich), ABCG1 (NB400-132, Novus Biologicals) and GAPDH (NB300221, Novus Biologicals). Detection was performed using HRP-conjugated secondary antibodies (P.A.R.I.S., Compiègne, France) and Western Lightning System kit (Perkin Elmer, Villebon s/ Yvette, France) on a MF-ChemiBIS imager (DNR bio-imaging systems, Jerusalem, Israel).

2.5. Immunofluorescence

Following necropsy, jejunum was collected, paraformaldehyde (PFA)-fixed, embedded in paraffin, and 5-μm-thick sections were prepared for immunofluorescence analysis as described previously [14]. Antibody used was ENO1 (#3810, Cell Signaling). Stained slides were visualized with a Carl Zeiss Axiocam digital camera on a Zeiss Axioplan 2 microscope.

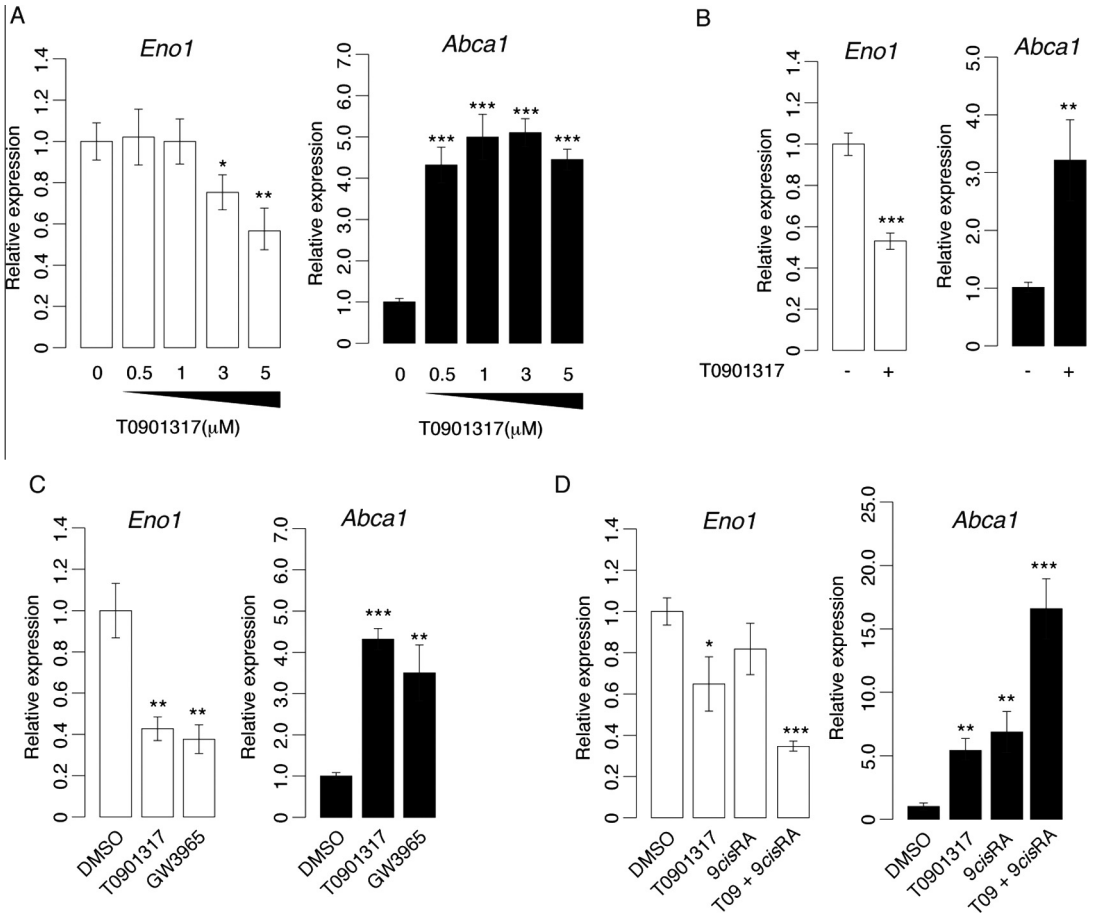

Fig. 1. LXRs agonists decrease *Eno1* expression in J774 macrophages. (A) *Eno1* and *Abca1* expression levels were quantified by RTqPCR on cells treated with 0, 0.5, 1, 3 and 5 μM of T0901317 for 8 h. (B) Expression levels of *Eno1* and *Abca1* were monitored on cells incubated with 5 μM of T0901317 for 48 h. (C) Expression levels of *Eno1* and *Abca1* were analyzed on cells incubated with either 3 μM of T0901317 or 3 μM of GW3965 for 24 h. (D) *Eno1* and *Abca1* expression were quantified on cells incubated for 24 h with 3 μM of T0901317 and/or 3 μM of 9-*cis* retinoic acid. RTqPCR were normalized using *36b4* gene expression. Analyzes results from three independent experiments of each realized in triplicates Data are expressed as the means ± SEM. Statistical analysis: $^{*}p < 0.05$ and $^{**}p < 0.01$; $^{***}p < 0.001$.

2.6. Statistical analysis

Values are expressed as means ± SEM. Statistical comparisons were performed using a two-tailed Student's t test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of Eno1 expression by LXR agonists

To investigate the potential role of LXRs in regulation of *Eno1* expression, we tested the effect of T0901317, a LXR synthetic ligand [16], treatment in J774 cells, which represent an established murine macrophage cell line. In these cells, we observed dose-dependent repression of *Eno1* by LXR activation, upon 8 h, that was mirrored by a concomitant upregulation of the canonical LXR target gene *Abca1* (Fig. 1A). The repression of *Eno1* expression by LXRs was stable and maintained over time, as it was still observed after 48 h of treatment with T0901317 (Fig. 1B). To further establish involvement of LXRs we challenged J774 macrophages with a second LXR synthetic agonist, GW3965 [17]. Inhibition of *Eno1* expression in response to GW3965 treatment was similar to that observed with T0901317 (Fig. 1C). There is ample evidence demonstrating that LXRs bind their obligate heterodimer partner RXR to form a permissive nuclear receptor complex that can be activated by ligands of any of the two interacting receptors, often with additive/synergistic transcriptional activity [18]. We tested this idea by treating the cells with either synthetic ligands of LXR, RXR, or a combination of both. As depicted in Fig. 1D, concomitant stimulation of LXR and RXR using

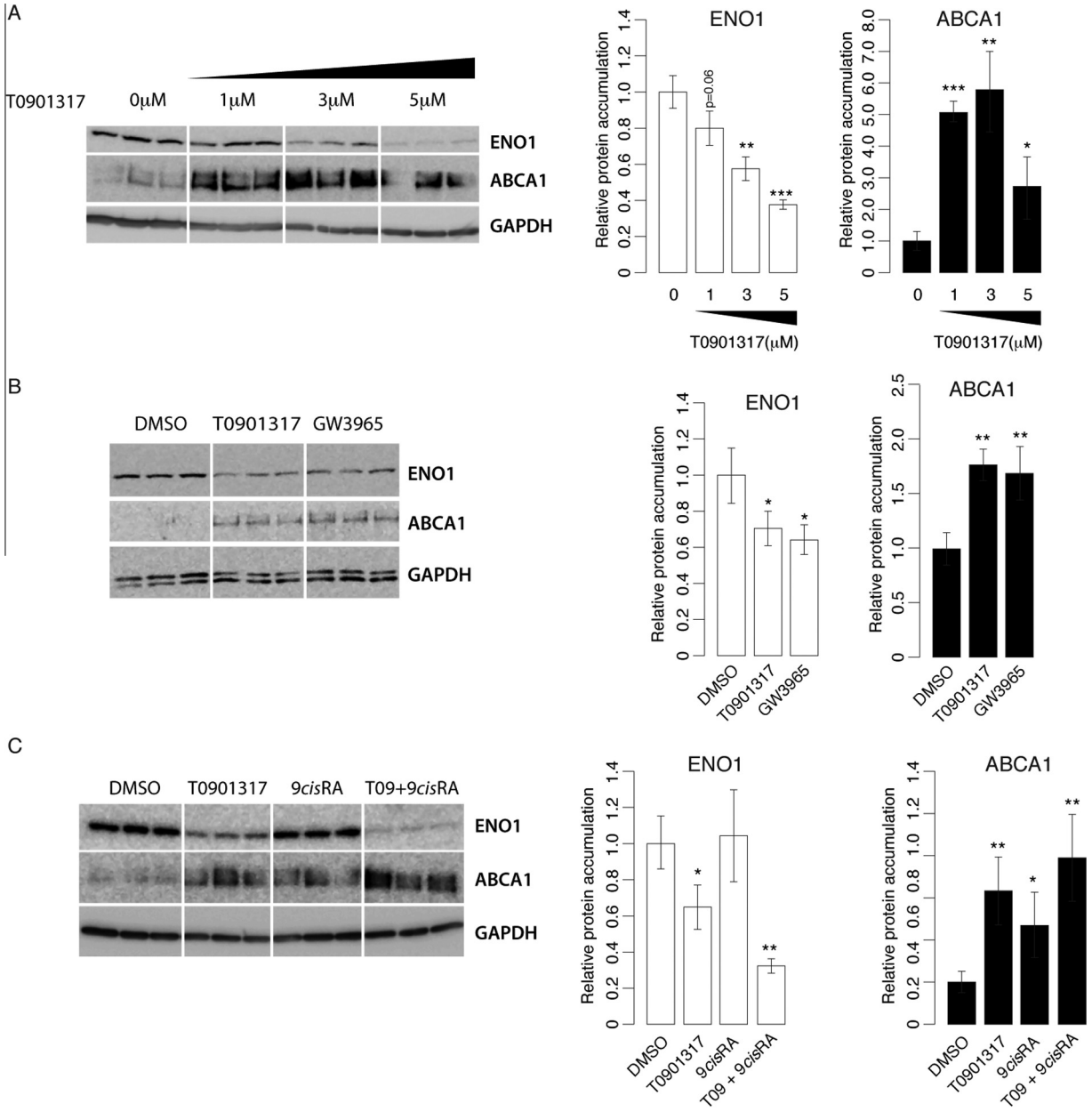

Fig. 2. ENO1 protein accumulation is decreased by LXRs agonists in J774 macrophages. (A) ENO1 and ABCA1 accumulations were quantified by western blot on cells treated with 0, 1, 3 and 5 μM of T0901317 for 48 h. (B) ENO1 and ABCA1 accumulation levels were analyzed on cells incubated with 48 h of T0901317 (3 μM) or GW3965 (1 μM) treatments. (C) ENO1 and ABCA1 accumulation were quantified on cells incubated for 48 h with 3 μM of T0901317 and/or 3 μM of 9-*cis* retinoic acid. Signals are normalized using GAPDH signal quantification. Pictures are representative and results from at least three independent experiments. Data are expressed as the means ± SEM. Statistical analysis: $^{*}p < 0.05$ and $^{**}p < 0.01$; $^{***}p < 0.001$.

T09013147 and 9-*cis* retinoic acid, respectively, results in an additive decrease in *Eno1* transcript levels, suggesting that *Eno1* repression depends on RXR/LXR heterodimer activity. LXR and/or RXR stimulation induces a parallel induction of *Abca1*, as expected.

3.2. LXR stimulation decreases ENO1 protein abundance

To address whether the reduction in *Eno1* transcript is paralleled by a decrease in abundance of Enolase protein ENO1 in murine macrophages, we monitored its level by western blot. After T0901317 treatment, the level of ENO1 decreased in a dose dependent manner (Fig. 2A), whereas ABCA1 protein was conversely increased. Furthermore, treatment of J774 macrophages using GW3965 resulted in a comparable reduction in ENO1 abundance to that observed with T0901317 (Fig. 2B). In line with their additive effect on *Eno1* transcript, treatment of macrophages with both T0901317 and 9-*cis* retinoic acid dramatically decreased the level of ENO1 (Fig. 2C). Collectively, these results indicate that the LXR/RXR heterodimer represses *Eno1* expression and as a consequence reduces the level of Enolase protein in murine macrophages.

3.3. LXRs regulate Enolase in vivo

To further establish LXRs as regulators of *Eno1*, we measured the levels of Enolase in mice that have been pharmacologically dosed with methylcellulose or T0901317. We found that treating mice with the LXR ligand resulted in a marked reduction in ENO1 in intestine, but not in liver (Fig. 3A, upper panel). Supporting the role of LXR in regulating Enolase levels *in vivo* we found that the protein level of Enolase remains insensitive to T0901317 in the intestine of *Lrxαβ−/−* mice. We also determined the level of ABCA1 and ABCG1 in the intestine and liver, respectively, and the increase in their protein abundance serves as a positive controls for LXR activation in wild-type mice. Interestingly, although T0901317 did not change ENO1 abundance in the liver of wild type or in *Lrxαβ−/−* mice, mice lacking LXRs have a lower level of detectable Enolase protein in both organs (Fig. 3A). These findings suggested that although ENO1 abundance is repressed by LXR stimulation, their expressions are required for a normal basal expression. Conversely to *Abcg1* expression, Enolase regulation seems to occur at the post-transcriptional level given that *Eno1* expression remains unchanged in liver whatever the genotype and treatment (Fig. 3B). We also determined the level of *Eno1* and *Abca1* expression in the intestine. These were largely in agreement with the western blotting results showing a decrease in *Eno1* and increase in *Abca1* following ligand treatment. (Fig. 3B). In *Lrxαβ−/−* mice the ability of the ligand to increase *Abca1* was abolished, as expected. Unexpectedly, we found that loss of LXRs reduces basal levels of *Eno1*, which likely explains the reduced Enolase protein (Fig. 3A). Finally, we investigated ENO1 representation *in situ* using intestine slides and confirmed a decreased signal in villi of mice challenged with T0901317 (Fig. 4). Altogether, these findings therefore suggest that the influence of LXRs on *Eno1 in vivo* is complex and likely involved direct and indirect transcriptional processes.

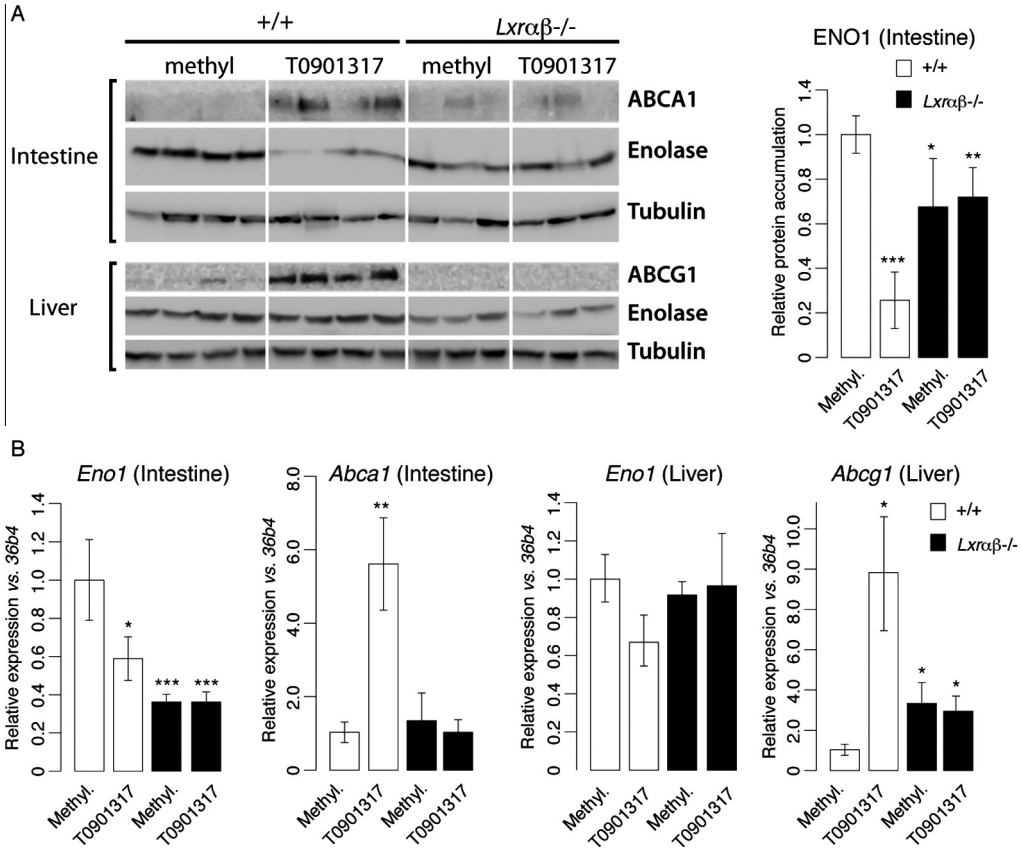

Fig. 3. Enolase is regulated by LXRs *in vivo*. (A) ENO1, ABCA1 and ABCG1 accumulations were quantified by western blot on intestine and/or liver samples from wild type (white squares) and *Lrxαβ−/−* mice (black squares) gavaged with methylcellulose (vehicle) or T0901317 (25 mg/kg) (n = 6/8 animals per group). (B) *Eno1*, *Abca1* and *Abcg1* expression levels were quantified by RTqPCR on intestinal and/or liver samples from wild type (white squares) and *Lrxαβ−/−* (black squares) as (A). RTqPCR were normalized using *36b4* gene expression. Data are expressed as the means ± SEM. Statistical analysis: $^{*}p < 0.05$ and $^{**}p < 0.01$; $^{***}p < 0.001$.

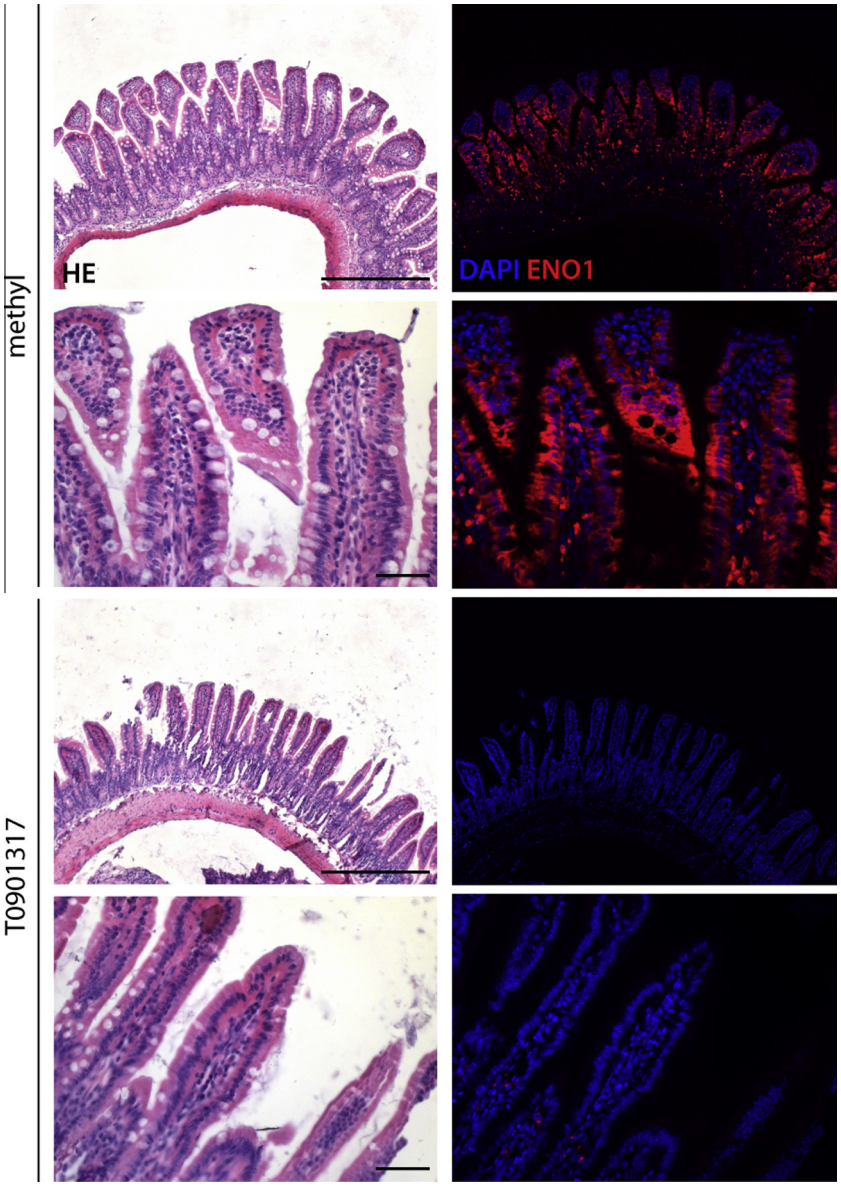

Fig. 4. Enolase accumulation is decreased in intestine villi. Immunofluorescence detection of ENO1 on intestine slides from wild type mice dosed with methylcellulose (methyl) as a vehicle or T0901317 (25 mg/kg). Scale bar represent 100 μm.

4. Discussion

We report herein the regulation of Enolase by LXRs. The major finding of this study is that activated LXRs decrease *Eno1* expression and corresponding protein levels in murine macrophages and *in vivo* in a tissue-specific and LXR-dependent manner.

Despite being an important metabolic enzyme the regulation of *Eno1* expression is poorly understood. Recently, Cai et al. reported that Estrogen-Related Receptors (ERRs) α, β, and γ (NR3B1, 2 and 3, respectively) can bind and drive transcriptional activity of the *Eno1* promoter in cooperation with hypoxia-inducible factors under hypoxic conditions [19]. Expression of *ENO1* is also highly responsive to proinflammatory signals such as IL- 1β, IL-6, PGE2, or TNF-α in peripheral blood mononuclear cells [20]. These cytokines, largely acting through the NF-κB pathway, increase expression of ENO1 as part of the inflammatory program. Our finding that LXRs are potent repressors of *Eno1* expression further illustrates the complex regulation of this enzyme. An important question that emerges from our study relates to the mechanism underlying repression of *Eno1* expression by LXRs. LXR binding has been observed by ChIP-seq analysis in the vicinity of the *ENO1* gene in human macrophages [21]. However, careful *in silico* analyses of both human and mouse promoters failed to reveal potential bindings sites (not shown). Alternatively, it is well established that LXR are potent anti-inflammatory factors in macrophages, largely due to their ability to *trans*-repress inflammatory gene signaling [22,23]. Accordingly, ligand activated LXRs inhibit expression of NF-κB-responsive genes such as *COX2*, *iNOS* and *MMP-9* during the inflammatory response [24]. Given that *ENO1* gene expression is also enhanced by NF-κB signaling we postulate that repression of *Eno1* by LXRs may follow a similar mechanism, an hypothesis that warrants future studies. The question is still open in the context of human macrophages. Preliminary data lead us to confirm that ENO1 regulation by LXRs is present in THP1 human cell line (data not shown) but occurs only at the protein level. This observation suggest that molecular mechanism underlying *Eno1* regulation by LXRs is complex and probably organism specific.

In the context of cellular cholesterol homeostasis regulation of *Eno1* is of particular interest. There is ample evidence pin pointing changes in ENO1 abundance as a key determinant in the

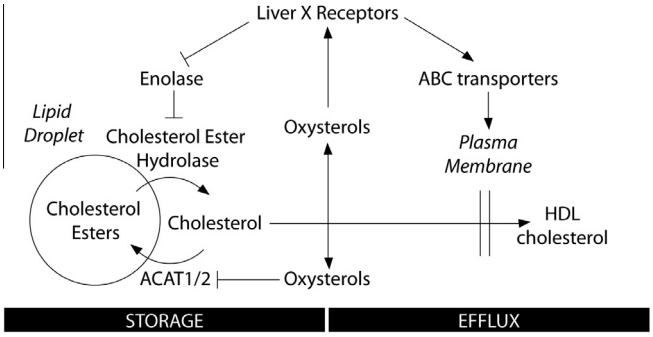

Fig. 5. Schematic diagram of cholesterol storage control mediated by LXRs through Enolase regulation (for more details, see text).

transformation of macrophages to "foam cells" in response to cholesterol loading [5,6]. Shand and West [2] demonstrated that ENO1 inhibits activity of CEHs. Together with increased *ENO1* expression this activity may contribute to enhanced-cholesteryl ester accumulation in macrophages loaded with lipoprotein-derived cholesterol. Whereas the roles of LXRs in cholesterol efflux and uptake are well established, their effect on cholesterol-ester storage is less-well studied. In this context, it is interesting to point out some oxysterols, which are endogenous LXR ligands, are reported to modulate cholesterol esterification by ACAT1 and 2 independent of LXRs activation [25–28]. Nevertheless, mobilization of cholesterol from intracellular pools to the plasma membrane is an essential step in cholesterol efflux [29]. Indeed, NPC1 and NPC2 are required for efficient cholesterol efflux supported by cholesterol transporters such as ABCA1 and ABCG1 [30]. Furthermore, overexpression of CEH have been shown to enhances cholesterol elimination and reverse cholesterol transport *in vivo* [31]. Repression of Enolase expression by activated LXRs could therefore relieves their inhibition of CEH activity and enhances cholesteryl ester hydrolysis in macrophages. This in turn could provide a source of free cholesterol that is accessible to cholesterol efflux transporters (Fig. 5). In conclusion, Enolase repression by LXRs could represent a novel regulation node of cholesterol homeostasis network within the cell.

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