LXR-Agonists Regulate ApoM Expression Differentially in Liver and Intestine

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Abstract: Apolipoprotein M (apoM) has been suggested to play a role in reverse cholesterol transport. Here we studied the influence of liver X-receptor (LXR) agonist on the transcriptional regulation of apoM. Studies were performed in murine liver and intestinal mucosal cells *in vivo* and in human intestinal Caco-2 cells *in vitro*. The expression of apoM was analyzed by quantitative real time PCR, and compared to well-established LXR target genes.

Mice fed with TO901317 for six days showed a downregulation of apoM and apoAI in the liver to 40 % and 60 % respectively and an upregulation of Cyp7A1 to 280 %. In the small intestine, however, apoM and apoAI were upregulated by 30-60 % and ABCA1 by 250-430 %. In Caco-2 cells TO901317 caused a 60 % upregulation and the natural LXR agonist 22-hydroxycholesterol a 40 % upregulation of apoM. Possible causes for the differential effects in liver and intestine are discussed.

Keywords: Intestine, lipid metabolism, lipocalin, expression profiling.

INTRODUCTION

ApolipoproteinM (apoM) has been described first by Xu and Dahlback [1]. It is a glycoprotein of 26 kD that belongs to the lipocalin protein family. ApoM is likely to be of significant physiological importance since it has been preserved among many species ranging from puffer fish, African clawed frog through mammals including humans and mice [2]. Although its exact function and mode of action is unknown, it has been suggested that apoM plays an important role in cellular cholesterol efflux, and thus might be protective against atherosclerosis [3]. ApoM exists in three isoforms whose structural differences are not known yet [4]. Like other apolipoproteins, apoM is mainly expressed in the liver, and upon secretion it binds to high-density lipoproteins (HDL) [1]. Small amounts of apoM are also found in very low-density lipoproteins (VLDL). It is concluded from *in situ* hybridization experiments that apoM is strongly expressed in the adult liver and kidney and more weakly expressed in fetal liver and kidney [5]. Very weak expressions have also been described in various other organs of man and mouse (see http://symatlas.gnf.org/SymAtlas/symcard?oid= 40160578&type= Expression&chartType). In kidney appreciable amounts of apoM are bound to megalin, which prevents its urinary loss [6].

Recently, Zhang *et al.* [15] demonstrated that apoM is a target gene of the LXR agonist TO901317. The synthetic LXR agonist downregulated hepatic apoM expression *in vivo* and *in vitro*. The present report confirms these results and in extension demonstrates that appreciable amounts of apoM are also expressed in the small intestine. In contrast to liver, TO901317 as well as the natural LXR agonist 22-(R)-hydroxy-cholesterol (22(R)-OHC) upregulated the expression of apoM in the intestine.

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MATERIALS AND METHODS

Cell Culture

The colorectal adenocarcinoma cell line, Caco-2, was obtained from American Type Culture collection (ATCC, HTB-37), Manassas, VA. Caco-2 cells were cultured in 6 cm^2 Petri dishes (Szabo-Scandic, Vienna, Austria) with EMEM supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine, 1 % non essential amino acids, 1 % penicillin, and 1 % streptomycin under standard culture conditions (37 °C, 5 % CO_2, 95 % relative humidity). After reaching confluence, cells were incubated for additional 20 days, with changes of the medium every 72h in order to promote differentiation to a more specific phenotype.

Prior to the experiment, cells were washed twice with PBS and incubated with EMEM supplemented alternatively with 1 μM of the non-steroidal LXR agonist TO901317, or with 5 μM of the natural LXR agonist 22-(R)-hydroxy-cholesterol (22(R)-OHC) (all from Sigma, St. Louis, MO, USA). The agonists were solubilized in DMSO and added to the medium at a DMSO concentration of 1 %. For control experiments cells were cultivated under the same conditions with 1 % DMSO alone. Unless stated otherwise, cells were incubated at 37 °C for 24 h.

Animals

All animal experiments were carried out according to the established standards of the Austrian Federal Ministry of

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Education, Science and Culture. Two-month old female wild type C57BL/6 mice were maintained in a temperature controlled room at 21 °C, (14 h light and 10 h dark). During the whole experimental period, mice were kept on a standard rodent chow diet. Six mice were treated with a daily oral dose of 50 mg/kg/d of TO901317 solubilized in sesame oil. Control mice received standard diet plus vehicle. At the end of the experiment intestinal mucosal cells from duodenum, jejunum, and ileum as well as livers were isolated from anesthetized animals and snap-frozen in liquid nitrogen. Biological materials were stored at -70 °C until analysis.

Plasma and Liver Lipids

At the end of the experiment mice were fasted for four hours and blood samples for lipid analysis were drawn into EDTA containing tubes, and centrifuged at 8000 rpm at 4 °C for 8 min. Plasma triglyceride (TG), total cholesterol (TC), and free cholesterol (FC) concentrations were measured enzymatically using commercially available kits from Wako, Osaka, Jp. To determine tissue lipid contents total lipids were extracted from livers [7] and lipid parameters were determined using kits mentioned above.

Isolation of Intestinal Mucosal Cells

Small intestine was resected proximally after pylorus and distally before caecum and immediately put on ice. The gut was then divided into duodenum (5 cm), jejunum (15 cm), and ileum (10 cm). Contents of the intestine were removed by rinsing with ice-cold phosphate-buffered saline (PBS) followed by smoothing over with blunt tweezers. The specimens were several times washed with PBS. After a longitudinal section mucosal cells were scraped off from the submucosa with a glass slide, and subsequently snap-frozen in liquid nitrogen.

Isolation of Total RNA

Caco-2 cells were harvested by trypsinization, and total RNA was extracted using the RNeasy mini Kit (Qiagen) following the instructions of the manufacturer. Tissues from murine liver and intestinal mucosa were removed surgically, weighed, and subsequently frozen in liquid nitrogen. Total RNA was isolated with Trizol® (Invitrogen, Lofer, Austria) following the recommendations of the company. RNA concentration was calculated from the absorbance at 260 nm. Only intact, non-degraded RNA was used as monitored on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Quantitative Real Time PCR

Total RNA was isolated from approx. $2x10^6$ cells as described above. 1 µg of high-quality RNA was reversely transcribed according to the manufacturer's instructions using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Fermentas, Leon-Rot, Germany). For *in vitro* experiments glycerolaldehyde phosphate dehydrogenase (GAPDH) and for *in vivo* experiments peptidyl-prolyl isomerase A (cyclophilin A, PPIA) were used as reference genes. Primer express software was applied to design human and mouse apoM, apoAI, ABCA1, Cyp7a1, GAPDH, and PPIA primers (Table **2**). The primers were purchased from Invitrogen (see Table **2**). Quantitative gene expression was performed on a LightCycler 2.1 (Roche diagnostics, Mannheim, Germany) using DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). PCRs were run in a total volume of 10 µl including 3 µl 1:100 diluted template cDNA. PCR efficiencies were determined using three dilutions (1:10, 1:100, 1:1000) analyzed in triplicates of a pool of all available cDNAs. Melting curve analysis was performed to ensure that a single PCR product was amplified and no primer dimers were generated. The following experimental protocol in LightCycler experiments was used: denaturation (95°C, 10 min), amplification and quantification, 45 cycles (95°C, 5 s; 62°C, 20s for apoM, 60°C, 20 s for ABCA1, apoAI, Cyp7a1, GAPDH and PPIA; 72°C, 10s for apoM and ABCA1, 72°C, 12 s for apoAI and GAPDH, 72°C, 8 s for PPIA), melting curve (57°C- 95°C with heating rate of 0.1°C/s and continuous fluorescence detection) and a final cooling step (40°C, 10 s).

Statistical Analysis

Data were analyzed as described by Pfaffl [8]. Results are expressed as mean ± S.E.M. Two-tailed Student's *t*-test was used for calculating statistical significance among groups using Prism software (Graphpad; * $p < 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) [8].

RESULTS

TO901317 Downregulates apoM Expression in the Liver

In the course of our studies aiming at elucidating LXR regulated genes we noticed that TO901317 downregulated apoM expression in the liver. Six mice were treated with a daily oral dose of 50 mg/kg/d of TO901317 and the expression of several relevant genes was followed. Here we confirmed recent findings from Zhang *et al.* [15] demonstrating that apoM expression was 60 % downregulated by TO901317. Also apoAI expression was reduced by 40 %. For comparison the expression of Cyp7a1 was monitored and showed the well-known upregulation of this gene by a factor of 2.8 Fig. (**1**).

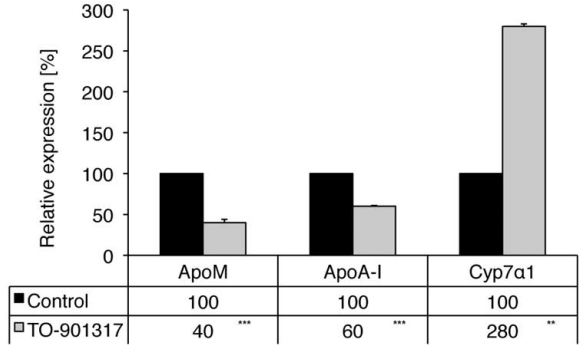


Fig. (1). Effect of TO901317 on the expression of apoM, apoAI and Cyp7a1 mRNA in murine liver.

Six female C57BL/6 mice were orally fed with TO901317, solubilized in sesame oil and six control mice were fed with sesame oil for six days. RNA levels were determined by real time PCR analysis relative to the control group. RNA amounts were normalized to cyclophilin A. Data are displayed as mean ± S.E.M.. * $p < 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

In addition to Liver and Kidney apoM is Expressed in Murine Intestinal Mucosal Cells

Basal apoM expression in mice: It has previously been published that apoM is primarily expressed in liver and to a lower extent in kidney. In our expression profiling studies in Caco-2 cells (see below) we noticed that apoM was an LXR-regulated gene. To study whether these findings were relevant for the situation *in vivo*, we first measured the basal expression levels of apoM in the three segments of small intestine in relation to liver and other organs by quantitative real time PCR. The experiments were carried out in two-month-old C57BL/6 mice on a standard chow in four replicates. Relative to liver we observed an apoM expression of 13 % in the kidney. In lung, testis, and spleen apoM expression was 2 %, 2 %, and 1 %, that of liver respectively. In intestinal mucosal cells apoM expression in duodenum was 1 %, in jejunum 1 %, and in ileum 0.4 % in comparison to liver.

TO901317 Stimulates apoM Expression in Murine Intestinal Cells

The expression of apoM, apoAI, and ABCA1 in C57BL/6 mice after oral administration of TO901317 for six days was monitored by real time PCR. As shown in Fig. (**2A**), TO901317 caused a significant upregulation of apoM expression of 30 % in duodenum and of 40 % in ileum. In jejunum no significant effect could be observed. ApoAI expression was stimulated by TO901317 in all three segments of intestine by 50-60 % Fig. (**2A**). ABCA1 expression showed a 3.3-fold upregulation in duodenum, and a 2.5-fold in jejunum, and a 4.3-fold in ileum Fig. (**2B**).

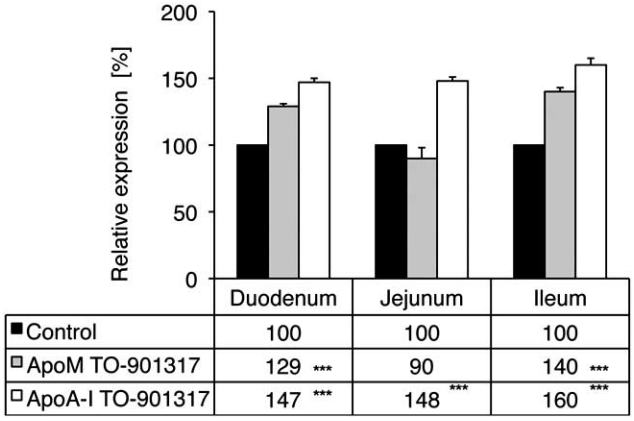
LXR Agonists also Upregulate apoM Expression in Caco-2 Cells

Caco-2 cells were grown for 20 days prior to use and treated with the synthetic (TO901317) or natural (22(R)-OHC) LXR agonists for 24 h and relative expression levels were measured. Both nuclear receptor agonists caused qualitatively comparable modulations of gene expression, yet there were quantitative differences Fig. (**3**). TO901317 had a greater effect and stimulated the expression of apoM, apoAI, and ABCA1 by 60 %, 30 %, and 310 %, respectively. The corresponding values after incubation with 22(R)-OHC were 40 %, 30 %, and 60 %, respectively.

The Impact of TO901317 on Plasma and Liver Lipid Levels in C57BL/6 Mice

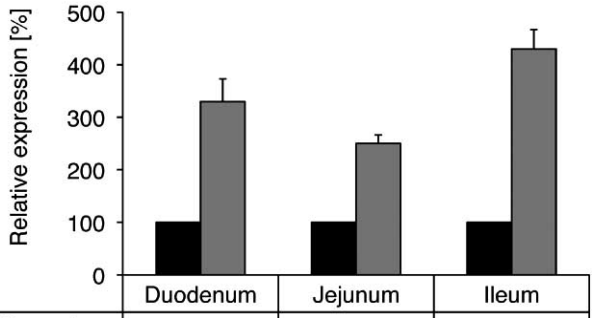
The oral administration of TO901317 to C57BL/6 mice for 6 days resulted in a small but significant body weight increase of 10 % (Table **1**). Plasma lipids increased quite dramatically by TO901317 feeding: total cholesterol increased 2-fold, unesterified cholesterol 1.9-fold, and phospholipids 1.7-fold. These changes were mainly due to elevations in plasma HDL (data not shown). In liver a significant increase of triglycerides was observed which confirms earlier studies indicating that TO901317 causes the development of fatty liver. Liver cholesterol was somewhat reduced by TO901317.

A)



	Duodenum	Jejunum	Ileum
Control	100	100	100
ApoM TO-901317	129 ***	90	140 ***
ApoA-I TO-901317	147 ***	148 ***	160 ***

B)



	Duodenum	Jejunum	Ileum
Control	100	100	100
TO-901317	330 ***	250 ***	430 ***

Fig. (2). Effect of the LXR agonist TO901317 on the expression of apoM, apoAI (A), and ABCA1(B) mRNA in murine small intestinal mucosal cells.

The same experimental onset as in Fig. (**1**), yet the expression of genes was measured in the three segments of small intestine: duodenum, jejunum, and ileum. RNA amounts were normalized to cyclophilin A. Data are displayed as mean ± S.E.M. * $p < 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. Fig.(**2A**): Comparison of the expression of apoM and apoAI; Fig. (**2B**): modulation of the expression of ABCA1.

DISCUSSION

ApoM is a relatively "new" apolipoprotein, mainly expressed in the liver, whose function has not been elucidated in full detail. Recent findings strongly suggest that apoM is an anti-atherogenic protein involved in the conversion of large HDL to pre-ß HDL, and in turn serves as an acceptor of peripherally deposited cholesterol. In fact it has been demonstrated that the overexpression of apoM protects LDL knockout mice against atherosclerosis [3]. ApoM is a lipocalin with a hydrophobic ligand-binding pocket [9]. Its expression has also been demonstrated in the kidney where it binds to megalin [5, 6]. This suggests that it might play a role in binding and possibly in selective tubular reabsorption of hydrophobic substances from kidney. With this respect it is noteworthy that J. Ahnstrom, *et al.* (personal communication) recently reported on the high-affinity binding of retinol and retinoic acid to human and mouse apoM with a Kd value in the order of 1μM. Whether or not, this is a true physiological role remains to be investigated. In view of the possible function mentioned before it is of interest to note that we could

demonstrate apoM expression in duodenum and ileum of small intestine where it might play a similar role.

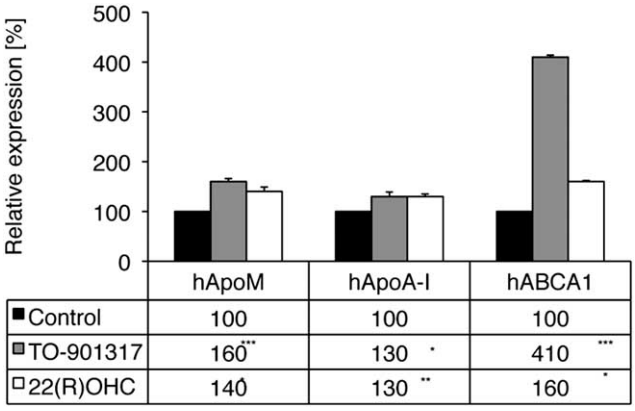


Fig. (3). Effect of two LXR agonists on apoM, apoAI and ABCA1 mRNA levels in the human intestinal Caco-2 cell line. Caco-2 cells were grown as described in *Methods* and treated with 1μM of the synthetic LXR agonists TO901317 or 5μM of the natural agonist 22(R)-OHC for 24h. Cells were harvested after trypsinization and 1μg total RNA was reversely transcribed. cDNA levels were determined by real time PCR analysis relative to the control group. The values were normalized to GAPDH. Each experiment consisted of three replicates and real time PCR was run in triplicate. Data are displayed as means ± S.E.M. * $p < 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

The human apoM gene has a size of approximately 2.3 kB and contains six exons, which encode for a protein with 188 amino acids [1]. The total plasma apoM concentration is estimated to range from 20-150 mg/L. The apoM promoter contains recognition sites for hepatic nuclear factor HNF-1α that are functional in mice and human [10]. HNF-1α knockout mice do not express apoM and in turn, plasma apoM concentrations are virtually zero [11]. Patients suffering from MODY3, which is characterized by mutations in HNF-1α, exhibit alterations in plasma apoM levels [10].

Searching for genes whose expressions were modulated in the intestine by LXR agonists that might imply a role in lipid absorption or metabolism, we noticed that apoM belonged to the group of genes that were stimulated to a high extent in Caco-2 cells. Caco-2 cells plated for 20 days are known to express many genes that are also found in intestinal mucosal cells *in vivo*.

LXR is a master regulator of genes involved in cholesterol and free fatty acid metabolism [reviewed in 12]. Currently much emphasis is put on the identification of synthetic LXR ligands since it has been found that the expression of ABCA1, a key regulator of reverse cholesterol transport, is strongly activated by the synthetic LXR ligand TO901317 in intestine and macrophages. LXR ligands, however, also increase the expression of genes involved in fatty acid metabolism *via* transcriptional SREBP-1c activation [13], a rather unwanted side effect of this class of potential antiatherogenic drugs. Moreover, it emerged from recent investigations that the modulation of a single nuclear receptor might cause an avalanche of effects that may hardly be anticipated. There exists a transcriptional regulatory network of nuclear receptors including LXR, FXR, PPAR's, SREBP's, ZNF2, and HNF's with regulatory feed-back loops which stimulate or suppress each other in a concerted action in an as yet poorly understood manner [13]. It is therefore also hardly possible to predict as to what extent a given ligand of these nuclear receptors may alter the expression of lipogenic genes in cultured cells or *in vivo*. Differential effects in various organs have also been frequently observed.

In a recent report, Zhang *et al.* demonstrated that TO901317 downregulates apoM expression in the liver [15]. They could show that mice treated with 100 mg/kg/d of

Table 1. Body Weight and Plasma and Liver Lipid Parameters of Control Mice and of Mice Fed TO901317 for 6 Days (50mg/kg Body Weight/Day)

	Controls (n = 6)	**TO901317 treated (n= 6)**	**p value**
Body weight (g)	16.2 ± 0.5	17.9 ± 0.2	**
PLASMA LIPIDS			
Total cholesterol (mg/dl)	74.5 ± 7.1	150.5 ± 7.2	***
Free cholesterol (mg/dl)	12.8 ± 0.6	24.0 ± 1.4	***
Phospholipids (mg/dl)	157.2 ± 12.2	272.2 ± 9.4	***
Triglycerides (mg/dl)	66.3 ± 7.8	48.8 ± 1.9	*
Free fatty acids (mM)	0.47 ± 0.07	0.40 ± 0.02	n.s.
LIVER LIPIDS			
Cholesterol (mg/g wet weight)	2,37±0.05	1.96±0.12	*
Triglycerides (mg/g wet weight)	39.49±1.47	93.13±10.26	***

* $p < 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$; n.s: not significantOptional.

Table 2. Primer Sequences Used for Expression Analysis in Caco-2 Cells and in Murine Liver and Intestine

Gene	Spe-cies	Acession-Number	Forward primer Reverse primer
hapo M	Human	NM_019101	5'-GTCTTCAATATGGCTGCTGG -3'
			5'-TGGCCTGTCTCATTCAGCAT -3'
hapoA-I	Human	NM_000039	5'-AAGGACCTGGCCACTGTGT -3'
			5'-TCCTTGCTCATCTCCTGCCT -3'
hABCA1	Human	NM_005502	5'-CTCTTCATGACTCTAGCCTGGA -3'
			5'-ACACAGACAGGAAGACGAACAC -3'
hGAPDH	Human	NM_002046	5'-ACCACAGTCCATGCCATCAC -3'
			5'-TCCACCACCCTGTTGCTGTA -3'
mapoM	Mouse	NM_018816	5'-CCTGGGCCTGTGGTACTTTA -3'
			5'-CCATGTTTCCTTTCCCTTCA -3'
mapoA-I	Mouse	NM_009692	5'-GAACAGATGCGCGAGAGCC -3'
			5'-GCTGGCCTTGTCGATCACAC -

TO901317 had significantly lower apoM plasma levels as compared to control mice. In cultured Hep-G2 cells, TO901317 caused a downregulation of apoM expression, which was in line with the *in vivo* findings. To our surprise, however, we noticed an upregulation of apoM in Caco-2 cells upon TO901317 treatment. Thus we studied the effects of the synthetic and the natural LXR agonist in more detail in comparison to other genes involved in reverse cholesterol transport. In the liver, the findings of Zhang *et al.* [15] could be confirmed. Contrary to liver, TO901317 and 22(R)-OHC increased the expression of apoM, apoAI, and ABCA1 in intestine. It was also a new finding that apoM, with respect to its regulation in intestinal cells by LXR agonists, behaved comparably to the two other main genes involved in reverse cholesterol transport, ABCA1, and apoAI. Whether the effect of LXR ligands on apoM mRNA expression is direct or indirect cannot be answered from our experiments. ApoM expression was shown to be under strict control of the MODY gene HNF-1α since HNF-1α deficient mice completely lacked expression of apoM in kidney and liver [11]. As mentioned above, HNF1-α belongs to a transcriptional regulatory network where its expression is under the control of HNF-4α; HNF-4α by itself is regulated by the LXR-FXR cascade, and the latter being modified by PPAR's. Concerning ABCA1 it has recently been shown that in liver its expression is driven by SREBP-2, whereas in peripheral cells including intestine ABCA1 expression is mediated by LXR which is reportedly due to differential splicing [14]. Whether similar effects might be responsible for the differential regulation of apoM expression in liver and intestine remains to be investigated.

Taken together, this report demonstrates for the first time that apoM, which plays a role in reverse cholesterol transport and which is under the control of the MODY gene, shows a measurable expression in intestinal mucosal cells. LXR agonists significantly modulate the expression of apoM in a similar manner as apoAI and ABCA1. The expressional stimulation of this lipocalin family member in the intestine suggests a possible role in the selective absorption of specific lipophilic substances.

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