LUND UNIVERSITY

ABCA1 upregulating apolipoproein M expression mediates via the RXR/LXR pathway in HepG2 cells

Di, Dongmei; Wang, Zongchun; Liu, Yang; Luo, Guanghua; Shi, Yuanping; Berggren Söderlund, Maria; Nilsson-Ehle, Peter; Zhang, Xiaoying; Xu, Ning

Link to publication

ABCA1 upregulating apolipoproein M expression mediates via the RXR/LXR pathway in HepG2 cells

Dongmei Di[1], Zongchun Wang[1], Yang Liu[1], Guanghua Luo[2], Yuanping Shi[2], Maria Berggren-Söderlund[3], Peter Nilsson-Ehle[3], Xiaoying Zhang[1]*, and Ning Xu[3]**

[1]Department of Cardiothoracic Surgery and [2]Comprehensive Laboratory, the Third Affiliated Hospital of Soochow University, Changzhou 213003, P.R. China; [3]Division of Clinical Chemistry and Pharmacology, Department of Laboratory Medicine, Lunds University, S-221 85 Lund, Sweden

*Corresponding author

Department of cardiothoracic surgery, the Third affiliated hospital of Soochow university, Changzhou 213003, P.R. China. Tel: +86 519 68871278; Fax: + 86 519 86621235; E-mail: zhangxy6689996@163.com

**Corresponding author

Division of clinical chemistry and pharmacology, Department of laboratory medicine, Lunds University, Lund S-221 85, Sweden. Tel: +46 46 173487; Fax: + 46 46 130064; E-mail: ning.xu@med.lu.se

Emails: Dongmei Di: ddm502@sohu.com

Zongchun Wang: wzongchun@yahoo.com.cn

Yang Liu: eddiffier@126.com

Guanghua Luo: shineroar@163.com

Yuanping Shi: luhao64@163.com

Maria Berggren-Söderlund: maria.berggren_soderlund@med.lu.se

Peter Nilsson-Ehle: peter.nilsson-ehle@med.lu.se

Xiaoying Zhang: zhangxy6689996@163.com

Ning Xu: ning.xu@med.lu.se.

Abstract

We have previously reported that liver X receptor (LXR) agonist, TO901317, could significantly inhibit hepatic apolipoprotein M (apoM) expression. It has been reported that TO901317 could activate the ATP-binding cassette transporter A1 (ABCA1) that mediates cholesterol efflux to the lipid-poor apoAI, which is an essential step for the high-density lipoprotein (HDL) formation. It is unknown if ABCA1 may regulate hepatic apoM expression. In the present study, HepG2 cells were cultured with the synthetic LXR agonists, TO901317 or GW3965 in the presence or absence of ABCA1 antagonist, disodium 4,4′-diisothiocyanatostilbene- 2,2′-disulfonate (DIDS). The mRNA levels of ABCA1, apoM and liver receptor homologue-1 (LRH-1) determined by the real-time RT-PCR. It demonstrated that both TO901317 and GW3965 could significantly enhance ABCA1 expression, and simultaneously, inhibit LRH1 expression. However, TO901317 alone could significantly inhibit apoM expression, while GW3965 alone did not influence apoM expression. ABCA1 antagonist, DIDS, have no effects on GW3965 induced upregulation of ABCA1 and downregulation of LRH1. However, apoM mRNA level was significantly decreased when the cells cultured with GW3965 together with DIDS. The present study demonstrated that apoM expression could be elevated by ABCA1 via the RXR/LXR pathway and LRH1 does not involve in the regulation of apoM by the activation of ABCA1, although the direct regulative pathway(s) between ABCA1 and apoM gene is still unknown yet. The detailed mechanism needs further investigation.

Apolipoprotein M (apoM), one of the most recently discovered plasma apolipoproteins, is mainly associated with high density lipoproteins (HDL), with only a small proportion located in low density lipoprotein (LDL) and very low density lipoprotein (VLDL) particles [1]. Human apoM is exclusively expressed in the hepatocytes in liver and tubular epithelial cell in kidney, and small amounts were also found in fetal liver and fetal kidney [2]. Luo, et al., [3] demonstrated that apoM could also be abundantly expressed in human colorectal tissues, although the pathophysiological importance of this expression and if it could influence plasma apoM pool are unknown. Wolfrum, et al., [4] demonstrated that apoM, by influencing preβ-HDL formation, being an important regulator of HDL metabolism *in vivo*, which could influence cholesterol efflux and the susceptibility of atherosclerosis. They elucidated the molecular mechanism by which apoM affects HDL particle size and to exploit a possible new pathway for therapeutic strategies aiming to reduce the development or progression of atherosclerosis [4]. ApoM mRNA levels could be regulated by many intracellular and extracellular factors, including platelet activating factor (PAF), insulin, leptin, transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), hepatic growth factor (HGF), etc., although the function of apoM is unknown yet [5,6,7,8,9,10,11]. Further investigating the regulation of hepatic apoM expression may explore the pathophysiological functions of apoM *in vivo*.

Liver X receptor (LXR) was initially identified as orphan member of the nuclear receptor superfamily that forms obligate heterodimers with retinoid X receptor (RXR) [12]. LXR-RXR can be activated by the endogenous oxysterols and by synthetic agonists such as T0901317 and GW3965 [13,14]. LXR has been established to regulate intracellular cholesterol levels by transactivating the expression of ATP-binding cassette transporter A1 (ABCA1), which modulates cholesterol efflux and mediates reverse cholesterol transport from peripheral tissues [15]. It is still unknown if ABCA1 regulates hepatic apoM expression. In the present study, we examined effects of LXR agonists on ABCA1 activation in relation to the regulation of apoM expression HepG2 cells.

Materials and methods

Materials. The HepG2 cell line obtained from the American Type Culture Collection (Manassas, VA, USA). Six-well cell culture clusters and 75-cm^2 vented cell culture flasks purchased from Nunc (Roskilde, Denmark). GW3965 and disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) purchased from Sigma-Aldrich (St. Louis, USA). TO901317 purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fetal bovine serum (FBS), RPMI 1640 and bovine serum albumin (BSA) purchased from Invitrogen (CA, USA). Total RNA purification kits purchased from the Shenergy Biocolor BioScience and Technology Company (Shanghai, China). First strand cDNA synthesis kits obtained from Fermantas (Vilnius, Lithuania). The LightCycler real-time RT-PCR System was from Roche Applied Science (Mannheim,

Germany).

Cell cultures. HepG2 cells were cultured in the RPMI 1640 supplemented with 10% FBS in presence of 100 U/ml penicillin, 100 μg/ml streptomycin and 1% Glutamax at 37 °C under 5% CO_2 atmosphere. Cells were plated in 6-well cell culture clusters at a density of 1×10^5 cells/dish with RPMI 1640 containing 10% FBS. Cell monolayer of approximately 50-70% confluence was grown for 24 hrs in the above media, then washed and incubated in serum-free medium with different concentrations of TO901317 and GW3965 for 24 hrs. In another series experiments, cells were cultured with TO901317 or GW3965 with or without ABCA1 antagonist DIDS. TO901317 and GW3965 were dissolved in the DMSO (the final concentration of DMSO in medium containing TO901317 and GW3965 was at 0.067% and 0.033%, respectively). DIDS was dissolved in RPMI 1640 containing 0.5% BSA.

Total RNA extraction and real time RT-PCR. Total RNA of HepG2 cells was extracted by using the total RNA purification kit according to the manufacturer's instructions. Primer Express software (Applied Biosystems) was used to design human ABCA1, LRH1 and apoM primers and probes for the TaqMan based RT-PCR assay (see Table 1). The quality of the RNA samples was determined by the absorbance measurements at 260/280 nm. According to the manufacturer's instructions of the first strand cDNA synthetic kit, 2μg total RNA was reverse transcribed to cDNA. The real-time PCR reaction for each gene was performed in a 25μL volume, in a glass capillary, containing 0.1μL 100mM each primer and probe, 2μL cDNA, 2.5μL 10 $\times$ buffer, 1.5μL $MgCl_2$ (25mM), 0.5μL dNTP (10mmol/L),

and Taq DNA polymerase 0.25μL. Finally, 17.95μl H_2O were added to the reaction mixture. Thermal cycling conditions included following steps: initial denaturation at 95 ºC for 2 min, followed by 40 cycles at 95 ºC for 5 sec and 60 ºC for 15 sec (for LRH1 and apoAI, 62 ºC, 12 sec and 20 sec, respectively). All PCRs were performed on the LightCycler real-time PCR system. Samples were amplified simultaneously in triplicates in one-assay run. The prospective amplicon of each gene was amplified and purified, then ligated into the pMD19-T vector. The ligated product was transformed into the E.Coli JM109 competent cells. In brief, a serial dilution of extracted plasmid DNA was used to generate a standard curve by plotting the cycle threshold versus the log initial copy number of input plasmid DNA. Standard curves of ABCA1, LRH1, apoM and GAPDH achieved a very high correlation coefficient (r=1.00). The ratio between the target gene and GAPDH was calculated as the relative gene expression.

Statistics. Data are expressed as means±SE. Statistical analyses were performed with the software Prism (version 5.0). Multiple comparisons were performed with one-way ANOVA/dunnett-t, and comparisons between two groups were statistically evaluated by Mann Whitney test. Significance was established at a *P* value less than 0.05.

Results

As shown in figure 1, GW3965 could significantly increase ABCA1 mRNA levels and, at meanwhile, decrease LRH1 mRNA levels, which was dose dependent (Fig. 1). When the cells cultured with GW3965 at 10μM the ABCA1 mRNA level increased

by 15-times and LRH1 mRNA level decreased by more than 50%. However, apoM mRNA level was not significantly influenced in the cells after cultured with GW3965 (Fig. 1). DIDS, an ABCA1 antagonist, have no statistical significant influence on GW3965 induced upregulation of ABCA1 and downregulation of LRH1 (Fig. 2). Interestingly, apoM mRNA level was significantly decreased when the cells cultured with GW3965 together with DIDS (Fig. 2). TO901317 could also enhance ABCA1 expression and suppress LRH1 expression in HepG2 cells (Fig. 3). Moreover, it could also significantly inhibit apoM expression (Fig. 3). When cells cultured with TO901317 together with DIDS, ABCA1 mRNA levels were even higher than cells cultured with TO901317 alone, but TO901317 induced inhibition of LRH1 was disappeared (Fig. 3). ApoM mRNA level was decreased even more when the cells cultured with TO901317 together with DIDS (Fig. 3).

Discussion

ABCA1, an integral membrane protein consisting of 12 transmembrane domains and two-ATP-binding domains, functions as a key role for the intracellular cholesterol efflux [16]. Mutations in the ABCA1 gene result in the Tangier disease (TD) [17,18]. ABCA1 promotes efflux of phospholipids and cholesterol to the lipid-poor apoAI in a process that involves the direct binding of apoAI to the transporter [19]. Many factors, including cAMP, sterols, *cis*-retinoic acid, peroxisome proliferator-activated receptor (PPAR) agonists, interferon-γ (IFN-γ) and LXR agonist could regulate ABCA1 expression [20], further influences cholesterol efflux to apoAI. ApoM is one of the

last discovered apolipoproteins that is also involved in the HDL formation. It is still unknown if moderation of ABCA1 may influence hepatic apoM expression. Lacking apoM expression in the Tcflα−/− (hepatic nuclear factor 1α) mice or in apoM siRNA-injected mice leads to the formation of unusual large-sized HDL1 particles and the disappearance of prebeta HDL in plasma, suggesting that apoM may also play an important role in the HDL metabolism, particularly with regard to the formation and/or catabolism of prebeta HDL [4]. The formation of prebeta HDL has been known to be important for the reverse cholesterol transport. Overexpression of human apoM in mice increases HDL cholesterol concentrations, while apoM deficiency is associated with lower HDL cholesterol concentrations [21]. Ventenclef, et al., [9] reported that LRH-1 could directly regulate human and mouse apoM transcription by binding to an LRH-1 response element（5′CAAGG3′）located in the proximal apoM promoter region. Our previous studies demonstrated that the synthetic LXR agonist, TO901317, could downregulate hepatic apoM expression *in vivo* and *in vitro* [11], which was confirmed by Calayir, et al. [22]. A hormone response element (HRE) that presents in the proximal apoM promoter could mediate apoM gene transcription by ligands of hormone nuclear receptor superfamily, such as HNF-4α, heterodimers of RXRα with retinoic acid receptor (RAR), thyroid hormone receptor β (TRβ) and liver X receptor (LXR) [22]. Moreover, it has been reported that TO901317 is a dual FXR/LXR agonist that activates the FXR more efficiently than its natural ligand, the bile acids CDCA chenodeoycholic acid (CDCA) [13,23], and have been demonstrated that the suppression of apoM by bile acids and the synthetic FXR agonist is most

likely mediated by FXR-SHP-LRH1 cascade [9]. Mitro, et al., [23] demonstrate that GW3965 does not activate FXR but LXR. Venkateswaran, et al., [24] suggest a model in which activation of LXRs by oxysterols in response to cellular sterol loading leads to induction of the ABCA1 transporter and the stimulation of lipid efflux to extracellular acceptors. Repa, et al., [25] identify the heterodimer of LXR/RXR on the upregulation of ABCA1 expression. The responsible control element was mapped to an imperfect direct repeat of the nuclear receptor half-site TGACCT separated by four bases (DR-4) that binds LXR/RXR heterodimers [26]. Those studies suggest that ABCA1 is one of the targets of LXR.

Our results show that LXR agonist GW3965 could significantly upregulate ABCA1 and downregulate LRH1 expression, however, apoM expression was not significantly influenced. Importantly, DIDS, an ABCA1 antagonist, could significantly decrease apoM expression, but not affect on GW3965 induced supression of LRH1 (figure 2), which suggests that the activation of ABCA1 could upregulate apoM expression, but not via the LRH1 pathway. Furthermore, as shown in figure 3, it demonstrated that DIDS could even enhance TO901317 induced inhibition of apoM expression and it could abolish TO901317 induced inhibition of LRH1, which further confirms that LRH1 does not involve in the regulation of apoM expression. In addition, TO901317, but not GW3965 could directly influence apoM expression. We suppose that ABCA1 upregulated by RXR/LXR could increase apoM expression. Thus, as shown in figure 4, TO901317 could inhibit LRH1 expression, further decrease apoM expression via the RXR/LXR and RXR/FXR pathway, but

GW3965 only through RXR/LXR pathway, while both TO901317 and GW3965 increase apoM expression via two different pathways, i.e., RXR/LXR and RXR/LXR-ABCA1 pathway. The different effects of TO901317 and GW3965 on apoM expression are due to that TO901317 may have additional suppressive pathway on apoM comparing to GW3965.

The present study indicates that apoM expression could be elevated through ABCA1 upregulation via the RXR/LXR pathway and LRH1 does not involve in the regulation of apoM expression by ABCA1. It is possible that certain response elements may locate in the proximal apoM promoter region for binding with ABCA1 or target protein of ABCA1 to regulate apoM expression. The detailed mechanism needs further investigation.

References

[1] N. Xu, B. Dahlback, A novel human apolipoprotein (apoM), J Biol Chem 274 (1999) 31286-31290.

[2] X.Y. Zhang, X. Dong, L. Zheng, G.H. Luo, Y.H. Liu, U. Ekstrom, P. Nilsson-Ehle, Q. Ye, N. Xu, Specific tissue expression and cellular localization of human apolipoprotein M as determined by in situ hybridization, Acta Histochem 105 (2003) 67-72.

[3] G. Luo, X. Zhang, Q. Mu, L. Chen, L. Zheng, J. Wei, M. Berggren-Soderlund, P. Nilsson-Ehle, N. Xu, Expression and localization of apolipoprotein M in human colorectal tissues, Lipids Health Dis 9 (2010) 102.

[4] C. Wolfrum, M.N. Poy, M. Stoffel, Apolipoprotein M is required for prebeta-HDL formation and cholesterol efflux to HDL and protects against atherosclerosis, Nat Med 11 (2005) 418-422.

[5] N. Xu, X.Y. Zhang, X. Dong, U. Ekstrom, Q. Ye, P. Nilsson-Ehle, Effects of platelet-activating factor, tumor necrosis factor, and interleukin-1alpha on the expression of apolipoprotein M in HepG2 cells, Biochem Biophys Res Commun 292 (2002) 944-950.

[6] G. Luo, M. Hurtig, X. Zhang, P. Nilsson-Ehle, N. Xu, Leptin inhibits apolipoprotein M transcription and secretion in human hepatoma cell line, HepG2 cells, Biochim Biophys Acta 1734 (2005) 198-202.

[7] N. Xu, P. Nilsson-Ehle, M. Hurtig, B. Ahren, Both leptin and leptin-receptor are essential for apolipoprotein M expression in vivo, Biochem Biophys Res Commun 321 (2004) 916-921.

[8] N. Xu, B. Ahren, J. Jiang, P. Nilsson-Ehle, Down-regulation of apolipoprotein M expression is mediated by phosphatidylinositol 3-kinase in HepG2 cells, Biochim Biophys Acta 1761 (2006) 256-260.

[9] N. Venteclef, A. Haroniti, J.J. Tousaint, I. Talianidis, P. Delerive, Regulation of anti-atherogenic apolipoprotein M gene expression by the orphan nuclear receptor LRH-1, J Biol Chem 283 (2008) 3694-3701.

[10] C. Wolfrum, J.J. Howell, E. Ndungo, M. Stoffel, Foxa2 activity increases plasma high density lipoprotein levels by regulating apolipoprotein M, J Biol Chem 283 (2008) 16940-16949.

[11] X. Zhang, Z. Zhu, G. Luo, L. Zheng, P. Nilsson-Ehle, N. Xu, Liver X receptor agonist downregulates hepatic apoM expression in vivo and in vitro, Biochem Biophys Res Commun 371 (2008) 114-117.

[12] S.M. Ulven, K.T. Dalen, J.A. Gustafsson, H.I. Nebb, LXR is crucial in lipid metabolism, Prostaglandins Leukot Essent Fatty Acids 73 (2005) 59-63.

[13] K.A. Houck, K.M. Borchert, C.D. Hepler, J.S. Thomas, K.S. Bramlett, L.F. Michael, T.P. Burris, T0901317 is a dual LXR/FXR agonist, Mol Genet Metab 83 (2004) 184-187.

[14] L.R. Brunham, J.K. Kruit, T.D. Pape, J.S. Parks, F. Kuipers, M.R. Hayden, Tissue-specific induction of intestinal ABCA1 expression with a liver X receptor agonist raises plasma HDL cholesterol levels, Circ Res 99 (2006) 672-674.

[15] N. Zelcer, P. Tontonoz, Liver X receptors as integrators of metabolic and inflammatory signaling, J Clin Invest 116 (2006) 607-614.

[16] M.F. Luciani, F. Denizot, S. Savary, M.G. Mattei, G. Chimini, Cloning of two novel ABC transporters mapping on human chromosome 9, Genomics 21 (1994) 150-159.

[17] J.R. Nofer, A.T. Remaley, Tangier disease: still more questions than answers, Cell Mol Life Sci 62 (2005) 2150-2160.

[18] S. Rust, M. Rosier, H. Funke, J. Real, Z. Amoura, J.C. Piette, J.F. Deleuze, H.B. Brewer, N. Duverger, P. Denefle, G. Assmann, Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1, Nat Genet 22 (1999) 352-355.

[19] J.F. Oram, R.M. Lawn, M.R. Garvin, D.P. Wade, ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages, J Biol Chem 275 (2000) 34508-34511.

[20] S. Santamarina-Fojo, A.T. Remaley, E.B. Neufeld, H.B. Brewer, Jr., Regulation and intracellular trafficking of the ABCA1 transporter, J Lipid Res 42 (2001) 1339-1345.

[21] C. Christoffersen, M. Jauhiainen, M. Moser, B. Porse, C. Ehnholm, M. Boesl, B. Dahlback, L.B. Nielsen, Effect of apolipoprotein M on high density lipoprotein metabolism and

atherosclerosis in low density lipoprotein receptor knock-out mice, J Biol Chem 283 (2008) 1839-1847.

[22] E. Calayir, T.M. Becker, A. Kratzer, B. Ebner, U. Panzenbock, J. Stefujl, G.M. Kostner, LXR-agonists regulate ApoM expression differentially in liver and intestine, Curr Pharm Biotechnol 9 (2008) 516-521.

[23] N. Mitro, L. Vargas, R. Romeo, A. Koder, E. Saez, T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR, FEBS Lett 581 (2007) 1721-1726.

[24] A. Venkateswaran, B.A. Laffitte, S.B. Joseph, P.A. Mak, D.C. Wilpitz, P.A. Edwards, P. Tontonoz, Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha, Proc Natl Acad Sci U S A 97 (2000) 12097-12102.

[25] J.J. Repa, S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R.A. Heyman, J.M. Dietschy, D.J. Mangelsdorf, Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers, Science 289 (2000) 1524-1529.

[26] K. Schwartz, R.M. Lawn, D.P. Wade, ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR, Biochem Biophys Res Commun 274 (2000) 794-802.

Legend to figures

Fig. 1 *Effects of GW3965 on mRNA levels of ABCA1, LRH1and apoM.*

HepG2 cells were cultured with GW3965 at different concentrations for 24 hrs. The mRNA levels of ABCA1, LRH1 and apoM were determined by RT-PCR as described in the materials and methods. Data are means±SE (n=6) and controls were represented as 100%. * $P<0.05$；**$P<0.01$ ； *** $P<0.001$ vs. controls.

Fig. 2 *Combination of GW3965 and DIDS on mRNA levels of ABCA1, LRH1 and apoM.*

HepG2 cells were cultured with GW3965 (10 μM) alone or together with DIDS (400 μM) for 24 hrs. The mRNA levels of ABCA1, LRH1 and apoM were determined by RT-PCR as described in the materials and methods. Data are means±SE (n=6). The cells were cultured without GW3965 nor DIDS were as controls and were represented as 100%. * $P<0.05$ and **$P<0.01$ vs. controls.

Fig. 3 *Effects of TO901317 and DIDS on mRNA levels of ABCA1, LRH and, apoM.*

HepG2 cells were cultured with TO901317 (10µM) alone or together with DIDS (400 µM) for 24 hrs. The cells were cultured without TO901317 nor DIDS were as controls and were represented as 100%. The mRNA levels of ABCA1, LRH1 and apoM were determined by RT-PCR as described in the materials and methods. Data are means±SE (n=6). *$P<0.05$; ** $P<0.01$.

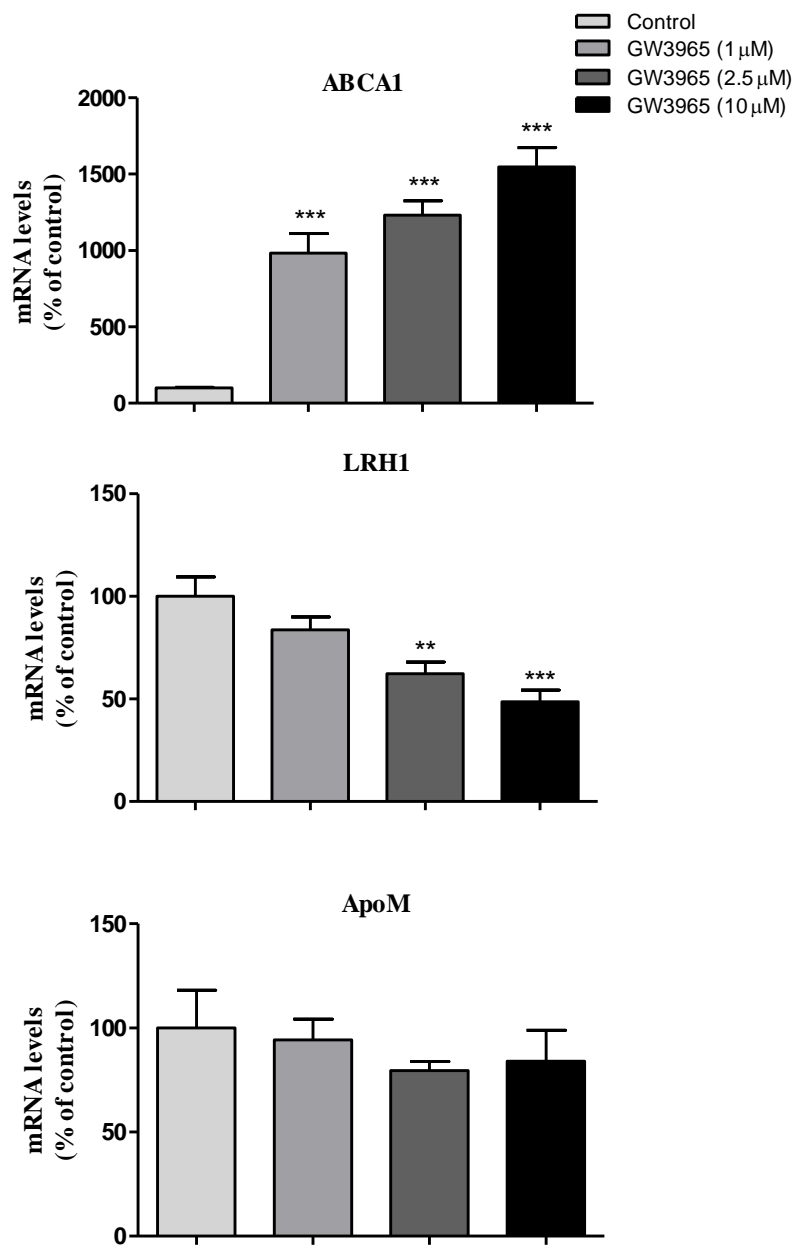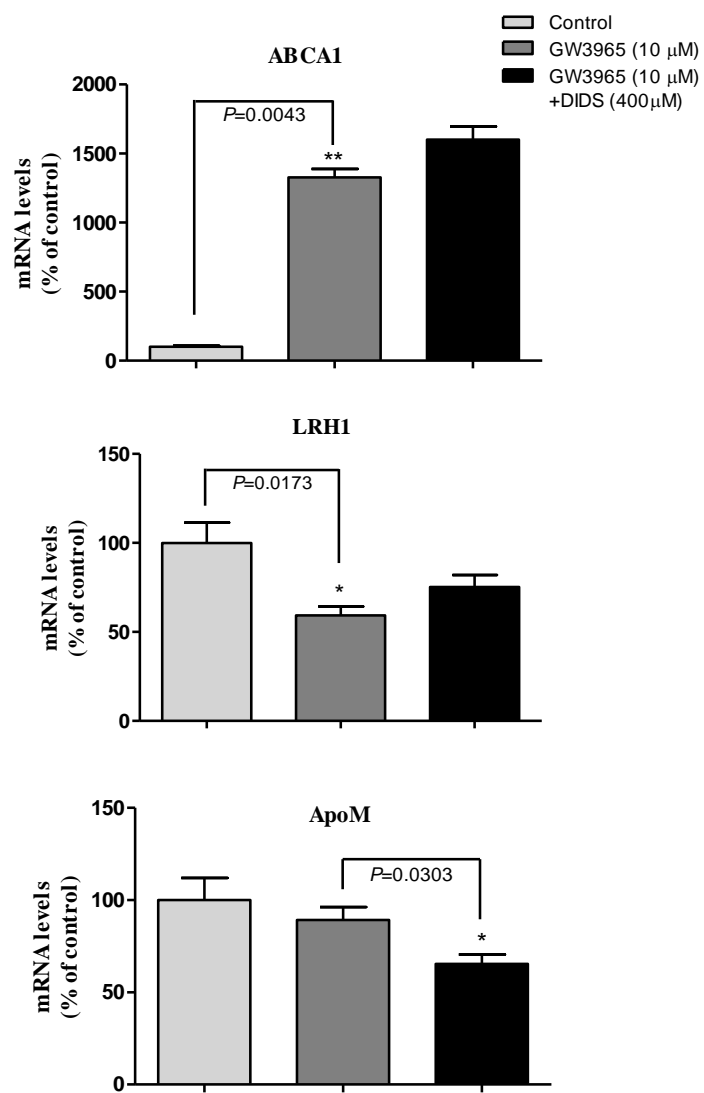**Fig. 4** *Possible mechanism of different effects of GW3965 and TO901317 on mRNA levels of apoM.*
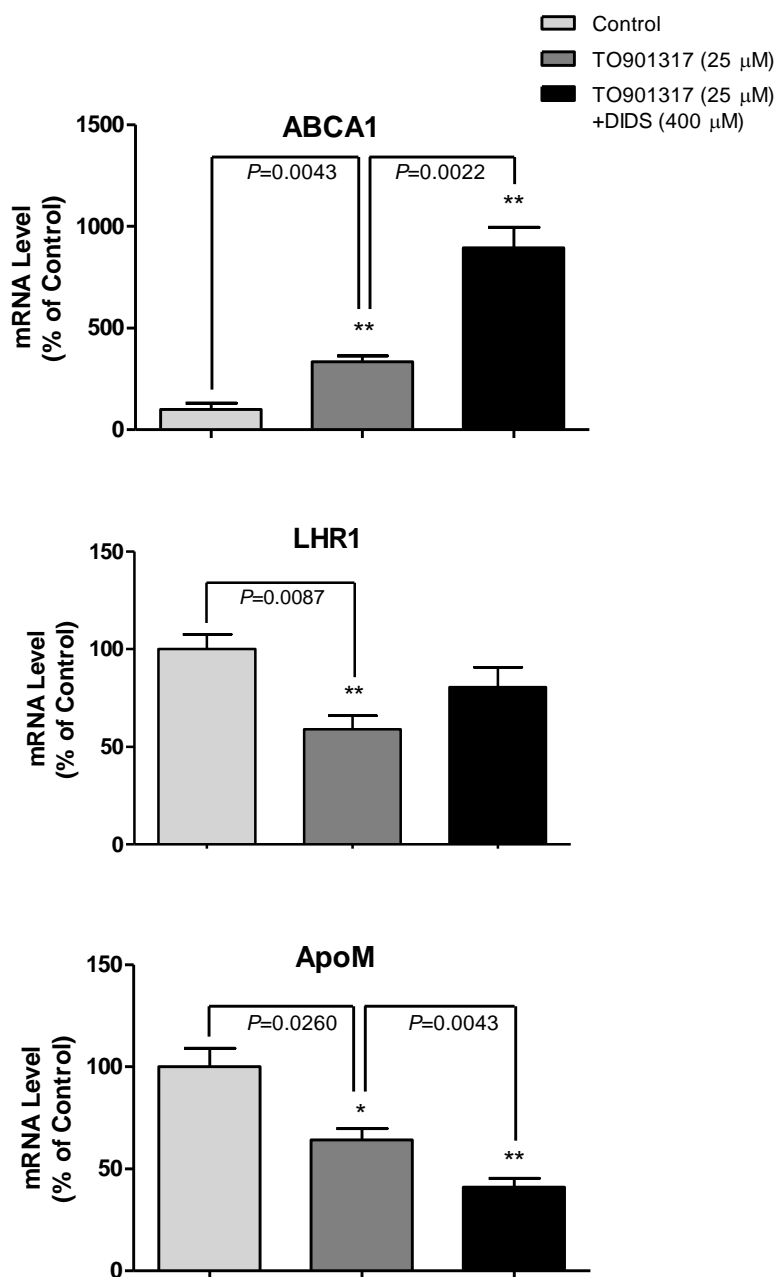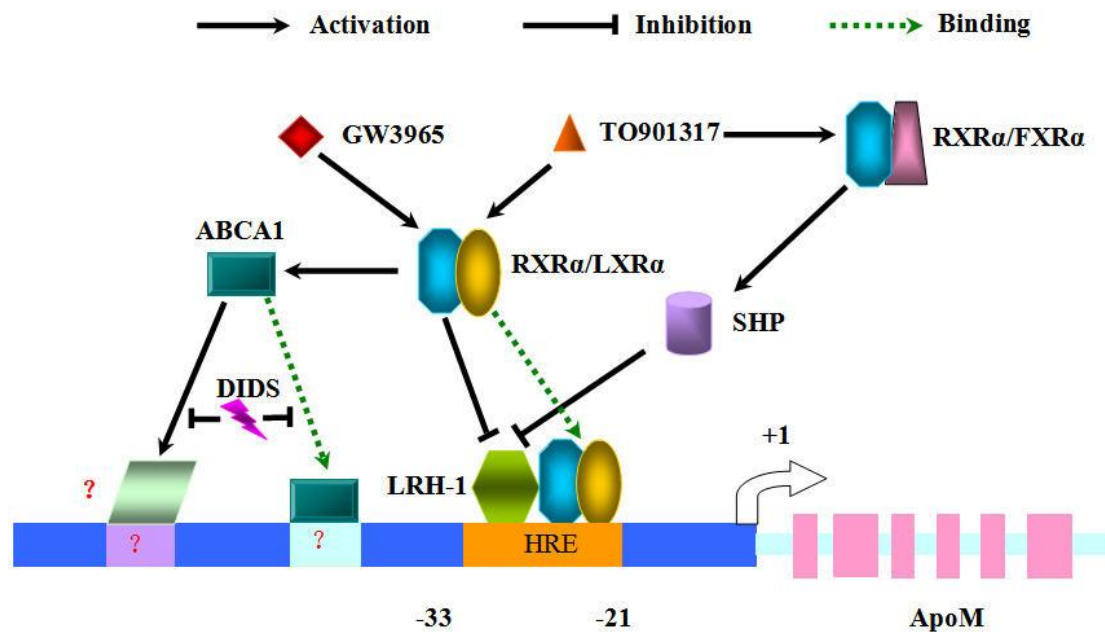
Fig 1

Fig 2

Fig 3

Fig 4

Table 1 Primers and fluorescent probes for real-time RT-PCR

gene	Forward primer	Reverse primer	Probe
Human apoM	TGCCCCGGAAATGGATCTA	CAGGGCGGCCTTCAGTT	FAM-CACCTGACTGAAGGGAGCACAGATCTCA -TAMRA
Human GAPDH	GGAAGGTGAAGGTCGGAGTC	CGTTCTCAGCCTTGACGGT	FAM-TTTGGTCGTATTGGGCGCCTG-TAMRA
Human ABCA1	CAAGGGGTAGGAGAAAGAGACGC	CTCAGCCAGCACCCCCAG	FAM-CCAGCCACGGCGTCCCTGCTGT-TAMRA
Human LRH1	TTAGTGGCAAAACTTCGTTCTCTCC	AGGGCGGCATTGACTTGTTC	FAM-AGTTCGTATGTCTGAAATTCTTGGTGCTCT-TAMRA