Published in final edited form as:

Atherosclerosis. 2009 August ; 205(2): 413–419. doi:10.1016/j.atherosclerosis.2009.01.008.

On the mechanism for PPAR agonists to enhance ABCA1 gene expression

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Abstract

Expression of ATP binding cassette transporter A1 (ABCA1), a major regulator of high density lipoprotein (HDL) biogenesis, is known to be up-regulated by the transcription factor liver X receptor (LXR) α , and expression is further enhanced by activation of the peroxisome proliferator activated receptors (PPARs). We investigated this complex regulatory network using specific PPAR agonists: four fibrates (fenofibrate, bezafibrate, gemfibrozil and LY518674), a PPAR δ agonist (GW501516) and a PPAR γ agonist (pioglitazone). All of these compounds increased the expression of LXRs, PPARs and ABCA1 mRNAs, and associated apoA-I-mediated lipid release in THP-1 macrophage, WI38 fibroblast and mouse fibroblast. When mouse fibroblasts lacking expression of PPAR α were examined, the effects of fenofibrate and LY518674 were markedly diminished while induction by other ligands were retained. The PPAR α promoter was activated by all of these compounds in an LXR α -dependent manner, and partially in a PPAR α -dependent manner, in mouse fibroblast. The LXR responsive element (LXRE)-luciferase activity was enhanced by all the compounds in an LXR α -dependent manner in mouse fibroblast. This activation was exclusively PPAR α -dependent by fenofibrate and LY518674, but nonexclusively by the others. We conclude that PPARs and LXRs are involved in the regulation of ABCA1 expression and HDL biogenesis in a cooperative signal transduction pathway.

Keywords

ABCA1; PPAR; LXR; ABCA1; HDL; Cholesterol

1. Introduction

High density lipoprotein (HDL) plays a central role in transporting cholesterol from extrahepatic tissues to the liver for its catabolism to bile acids, and thus it is thought to contribute to removing cholesterol from peripheral tissues and possibly lowering its deposits in atherosclerotic lesions. This idea is based on epidemiological evidence that plasma HDL concentrations are inversely related to cardiovascular risk, and on experimental results showing that HDL may remove cholesterol from vascular cells in culture. HDL removes cholesterol from cells via two independent mechanisms [1]. One is by non-specific exchange of cholesterol, in which the driving forces for its net release may be cholesterol esterification on HDL and the presence of ATP binding cassette transporter (ABC) G1 in the cell membrane. The other mechanism is through HDL biogenesis by helical apolipoproteins and cellular lipids in the presence of ABCA1 [1]. The latter is an almost exclusive source of HDL biogenesis and one of the important rate limiting factors for plasma HDL concentration [2]. Many drug reagents are known to influence this reaction by modulating ABCA1 activity. ABCA1 is therefore an important target for development of drugs that impact atherogenesis.

Peroxisome proliferator activated receptor (PPAR) agonists are known to increase expression of ABCA1 and enhance biogenesis of HDL in vitro and in vivo. Fibrates act mainly as PPAR α agonists [3] to increase ABCA1 gene transcription [4,5]. Fenofibrate and LY518674 exclusively activate PPAR α while bezafibrate and gemfibrozil activate PPAR δ and PPAR γ as well [6–8]. More specific activation of PPAR δ and PPAR γ also results in increased ABCA1 transcription [4,9]. All of these events seem to involve the liver X receptors (LXRs), especially LXR α [4,5,8], one of the main regulators of ABCA1 gene transcription by sensing oxysterol [10], although specific pathways for these cascades are yet to be clarified.

Fibrates are drugs widely used to decrease plasma triglyceride (TG). This effect is expected to reduce atherosclerotic disease through a decrease in TG-rich atherogenic lipoproteins as well as by reducing other risk factors secondarily caused by the increase of TG-rich lipoprotein, such as low HDL and “small and dense” LDL. Fibrates also increase HDL independently of TG reduction by direct up-regulation of the genes related to HDL biogenesis as indicated above [4,5]. Eventually, fibrates were shown to decrease secondary or primary coronary heart disease events in several large-scale prevention trials [11]. Statistical analysis of these data suggested independent contribution of HDL increase to the risk reduction [12].

Chronic inflammation is also thought to be involved in atherogenesis, such that accumulation of cholesterol-rich lipoproteins results in the recruitment of circulating monocytes, their adhesion to the endothelium and differentiation into macrophages. Lipid-loaded macrophages may produce chemokines, cytokines, and reactive oxygen species as early atherogenic process. Activation of PPARs was shown to suppress such processes.

PPARs act as ligand-activated transcription factors mainly to regulate target genes related to energy metabolism. The PPAR family consists of PPAR α , δ and γ , which display distinct expression patterns, different ligand specificities and different biological functions with some degree of overlap between PPAR α and PPAR δ . PPAR α is mainly expressed in liver, kidney, heart, and muscle, tissues with a high rate of fatty acid catabolism. PPAR α up-regulates the expression of genes involved in fatty acids oxidation, lipolysis, HDL metabolism, down-regulates very low density lipoprotein synthesis and cholesterol esterification [13], and inhibits inflammatory mediators [14]. PPAR γ is mainly expressed in adipose tissue, skeletal and cardiac muscle, and also in human monocytes [15]. It plays a role in adipocyte differentiation and fat storage; up-regulation of PPAR γ increases insulin sensitivity [16]. PPAR γ also up-regulates the expression of genes involved in HDL metabolism, down-regulates cholesterol esterification, and inhibits inflammatory mediators [4–17]. PPAR pathways are thus integrated

in atherogenesis and the use of the fibrates (PPAR α agonists) and thiazolidinediones (PPAR γ agonists) may extend beyond the treatment of hyperlipidemia or insulin resistance [18]. PPAR δ is ubiquitously expressed and its role in atherogenesis is controversial. Disruption of the PPAR δ gene suggested its important roles in skin biology, lipid metabolism, and energy homeostasis [19]. PPAR δ agonist (GW501516), however, reportedly enhanced ABCA1 expression and increased apoA1-mediated lipid release to maintain macrophage cholesterol homeostasis [20] and suppressed inflammation to reduce atherosclerosis [21]. On the other hand, a different PPAR δ agonist promoted lipid accumulation in macrophages [22].

As various PPAR agonists are clinically used, it is important to provide information about their detailed reaction mechanism with respect to signaling and cross-talk among the transcriptional factors relating the energy metabolism induced by these drugs. We therefore investigated profiles of the network of transcriptional factors by which PPAR agonists modulate ABCA1 gene expression. The results suggest that PPARs and LXRs are involved in the regulation of ABCA1 expression and HDL biogenesis in a complicated signal transduction network.

2. Methods

2.1. Cell culture

THP-1 (human monocytic leukemia) cells (4.0×10^6 cells per well) in six well plates were differentiated with 3.2×10^{-7} M phorbol 12-myristate 13-acetate (PMA) (Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 h at 37 °C in a humidified atmosphere of 5% CO₂ (THP-1 macrophages). WI38 human fibroblasts (RIKEN Cell Bank) were incubated in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO₂. Fibroblasts were prepared from C57BL6 mice and PPAR-null/C57BL6 (PPAR(-/-)) mice [23] and bred in the Nagoya City University Animal Experiment Facility. Briefly, 13–14th day fetuses were harvested and suspended in Hank's EGTA solution containing 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (PCSM). Associated membranes and placentas were dissected and rinsed thoroughly. The fetal trunks were transferred to fresh solution and finely minced. The suspension was centrifuged at 1000 rpm for 3 min and the cells were re-suspended into MEM medium containing 10% FCS and PCSM. The cells were cultured in 37 °C with 5% CO₂ incubator over five passages and used for the experiments. The experimental protocol was pre-approved by the institutional Animal Welfare Committee. Cells were seeded into a 100-mm dish at a density of 1.5×10^6 cells/ml. When the WI38 cells and mouse primary fibroblasts were grown to 80% of a confluent stage, cells were washed with phosphate-buffered saline (PBS) twice and cultured an additional 20 h in the presence of apoA-I (10 μ g/ml). PPAR α activators, fenofibric acid (Tyger Scientific, referred as fenofibrate hereafter), bezafibrate (Sigma), gemfibrozil (Sigma), and LY518674 [24] (synthesized in house), PPAR δ activator GW501516 [20] (kindly provided by Aska Pharmaceutical Co. Ltd.), PPAR γ activator pioglitazone (kindly provided Takeda Pharmaceutical Co. Ltd.), and an LXR agonist TO901317 (Sigma) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.02% bovine serum albumin (BSA) (Sigma). The experimental procedure had been approved by the animal welfare committee of the institution.

2.2. Cellular lipid release

WI38 cells and mouse primary fibroblasts were grown to 80% confluence, and twice washed with PBS. The cells were cultured for additional 20 h in the presence of apoA-I (10 μ g/ml) and PPAR activators described above. THP-1 macrophages were also treated with PPAR activators similarly for apoA-I-mediated lipid release in 0.02% BSA-RPMI 1640 medium (serum-free). Cholesterol and choline-phospholipid released into the medium by apoA-I were determined

enzymatically and the apoA-I-dependent release was evaluated by subtracting the background with BSA, as described in detail previously [5].

2.3. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Cells incubated with and without apoA-I and PPAR activators for 20 h were harvested in cold PBS and collected by centrifugation. Membrane fractions were prepared for detection of ABCA1. The cell pellet was suspended in 5 mmol/l Tris-HCl, pH 7.5, containing 0.3% protease inhibitor cocktails (Sigma) and 1 mM phenylmethane sulfonyl fluoride and 1 mM benzamidine for 30 min in ice with vortexing at every 10 min. The cell debris and nuclei were removed by centrifugation at $800 \times g$ for 5 min at 4 °C, and the supernatant was centrifuged at $99,000 \times g$ for 60 min to prepare the membrane fraction as a pellet. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethane sulfonyl fluoride, 10 mM benzamidine, 1% Triton X-100, and 1% protease inhibitor cocktails. Membrane fraction protein (20–60 µg) was dissolved in 9 M urea, 2% triton X-100, 1% dithiothreitol and analyzed by 6% polyacrylamide electrophoresis for immunoblotting by using specific antibodies against ABCA1 and BIP/GRP78.

2.4. RNA extraction and real time quantitative polymerase chain reaction (PCR)

Cellular RNA was extracted by using RNA extraction reagent (Isogen, Nippon Gene). Single strand cDNA was synthesized by High capacity cDNA archive kit (applied biosystem) from 5 µg of the total RNA. PCR was carried out for the cDNA by using primers (sense and antisense) of human ABCA1 (5'-GAA CTG GCT GTG TTC CAT GAT-3' and 5'-GAT GAG CCA GAC TTC TGT TGC-3'), human LXRα (5'-TCT GGA GAC ATC TCG GAG GTA-3' and 5'-GGC TCA CCA GTT TCA TTA GCA-3'), human LXRβ (5'-GCG AAG TTA CTT TTG AGG GTA-3' and 5'-CTC CTT TAC AGT GGG TGA AGA-3'), human PPARα (5'-TCG GTG ACT TAT CCT GTG GTC-3' and 5'-TTC TCA GAT CTT GGC ATT CGT-3'), human PPARδ (5'-TCT CTC TTC CCT TCT CCC TTG-3' and 5'-GGC TCA AGT CTT TTG CTC TGA-3'), human PPARγ (5'-TCA CAG AGT ATG CCA AAA GCA-3' and 5'-AAA CTC AAA CTT GGG CTC CAT-3'), mouse ABCA1 (5'-CTC AGA GGT GGC TCT GAT GAC-3' and 5'-CCC ATA CAG CAA GAG CAG AAG-3'), mouse LXRα (5'-TAG GGA TAG GGT TGG AGT CAG-3' and 5'-AGT TTC TTC AAG CGG ATC TGT-3'), mouse PPARα (5'-CTG TCC TCT CTC CCC ACT GGA-3' and 5'-TGA CTG AGG AAG GGC TGG AAG-3'), mouse PPARδ (5'-GGG AAG AGG AGA AAG AGG AA-3' and 5'-AGG AAG GGC AGG AAT TCT G-3'), mouse PPARγ (5'-ATA AAG CAT CAG GCT TCC ACT-3' and 5'-GCA CTT CTG AAA CCG ACA GTA-3'), human and mouse β-actin (5'-CTG ACC CTG AAG TAC CCC ATT-3' and 5'-TCT GCG CAA GTT AGG TTT TGT-3' (synthesized by Hokkaido System Science, Japan). Quantification of mRNA for these primers products were accomplished by using SYBR green PCR master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystem Japan). Results were normalized to β-actin mRNA.

2.5. RNA interference

Human LXRα-specific small interfering RNA (siRNA) and scrambled control RNA oligonucleotides were purchased from Invitrogen. The transfection of siRNA was performed using Nucleofector kit (Amaxa) reagent according to the manufacturer's instructions. Scrambled control RNA oligonucleotide or human LXRα siRNA were added to WI38 cells and transfected by electroporation. The cells were incubated for 20 h, and washed with PBS, then incubated in MEM containing 0.02% BSA and indicated dose of fenofibrate, bezafibrate, gemfibrozil, LY518674, GW501516, pioglitazone were added and incubated for further 20 h. Then the cells were harvested and mRNA levels determined by RT-PCR. The oligonucleotide sequences used to construct siRNA for LXRα in this study were: siLXRα (5'-UUC UCG AUC

AUG CCC AGU UGU UCC G-3') and scrambled control (5'-UUC UUC UUA GUA CCC GGA CGU UCC G-3').

2.6. Construction of luciferase reporter genes

The construct of human PPAR α promoter luciferase reporter gene (containing -1664/+83 of the PPAR α gene, relative to the transcription start site) was prepared as described previously [25]. The 5'-flanking region of this PPAR α gene (corresponding to -1664/+83) was inserted into pGL4 vector (Promega) to generate PPAR α promoter-luciferase reporter construct (pPPAR α -Luc). The reporter plasmid with a mutated and inactivated PPAR α responsive element (PPRE) (mutant PPRE) was generated by using QuickChange Site-Directed Mutagenesis Kit (Stratagene). A mutation was introduced at the PPRE (-1493/-1481 GGGGCAAGTTCA to GcaGCAAGTTCA) which is identical to that reported previously (pPPAR α -mut-Luc) [25]. The luciferase reporter plasmid was prepared to contain four copies of LXRE upstream of thymidine kinase promoter (pLXRE-tk-Luc) [26]. The sequence of LXRE of the LXRE-tk-Luc vector was (ACAG **TGACCG** CCAG **TAACCC** CAG...GGA **CGCCCG** CTAG **TAACCC** CGG) $\times$ 2 (LXREa and LXREb from sterol responsive element binding protein-1c).

2.7. Transient transfections and reporter gene assay

WI38 cells and mouse fibroblasts were co-transfected with pPPAR α -Luc or pPPAR α -mut-Luc vectors (4 μ g), and Renilla phRL-tk vector (Promega) (0.2 μ g) by electroporation using Nucleofector kit reagent (Amaxa) according to instructions supplied by the manufacturer. The activity of luciferase reporter of LXRE, pLXRE-tk-Luc, was examined in human fibroblasts and mouse fibroblasts of wild type and of PPAR α (-/-). After 20 h transfection, the cells were washed with PBS and cultured in the presence of fenofibrate, bezafibrate, gemfibrozil, LY518674, GW 501516, pioglitazone for 20 h. Cellular luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega). Results were standardized by the Renilla luciferase activity derived from phRL-tk vector.

2.8. Other methods

Protein content of each sample was determined with bicinchoninic acid assay reagent (Pierce) using BSA as a standard. Statistical significance was evaluated using two-tailed Student's *t* test and analysis of variance Scheffe's test.

3. Results

3.1. HDL biogenesis in THP-1 macrophages and WI38 fibroblasts

The effects of PPAR agonists on HDL biogenesis were determined by measuring the expression of ABCA1 and cellular lipid release by human apoA-I (Figs. 1 and 2). Fenofibrate, bezafibrate, gemfibrozil, LY518674, GW501516 and pioglitazone increased ABCA1 mRNA and protein levels and consequently cellular lipid release. In addition, all compounds increased mRNAs coding LXR α , LXR β , PPAR α , PPAR δ and PPAR γ . The results were same with WI38 fibroblasts (Fig. 2). Therefore, activation of PPARs by their agonists results in transcriptional enhancement, not only of LXR α and ABCA1 but also LXR β and PPARs themselves in these cell lines.

3.2. HDL biogenesis in PPAR α null mouse fibroblasts

In order to differentiate a role of PPAR α and other PPARs, a similar analysis was done using fibroblasts prepared from wild-type and PPAR α (-/-) mice (Fig. 3). The increase in cellular lipid release was reduced in PPAR α (-/-) cells by fenofibrate and LY518674. In contrast, PPAR α deficiency only marginally influenced lipid release induced by bezafibrate, GW501516

and pioglitazone as expected since these compounds preferably activate PPAR δ or PPAR γ . These results also indicate that their effects are entirely or partially dependent on the presence of PPAR α . Expression of ABCA1, as estimated by mRNA and protein expression was roughly parallel to the results of the lipid release. Increase of LXR α mRNA was also reduced in PPAR α ($-/-$) cells in parallel with the ABCA1-related reactions. Increase of PPAR δ and PPAR γ mRNA was diminished when PPAR α was knocked-out for all the ligands, suggesting that cis or trans activation of PPARs may require the presence of PPAR α for its full activity.

3.3. PPAR α promoter assay

In order to investigate the mechanism of PPAR α induction, the PPAR α promoter was investigated by transfected studies in WI38 cells. As shown in Fig. 4A, PPAR α promoter activity was increased by all of the PPARs ligands, indicating trans activation of the PPAR α gene by all PPAR subtypes. When the PPRE was mutated in the promoter, all of these effects were abolished. The PPAR α promoter was also activated by TO901317, an LXR ligand indicating that activation depends on LXR. To confirm this finding, LXR α was down-regulated by a specific siRNA (by $80 \pm 2\%$ at 100 nM, based on standardization for β -actin). The activation by the PPAR ligands was eliminated under these conditions while transfection of scrambled siRNA had no effect (data not shown in Fig. 4A). The results therefore demonstrated that activation of PPAR α by PPARs ligands requires both the PPRE-mediated activation by LXR α . PPAR α promoter activity was also observed in mouse fibroblasts prepared from wild-type and in PPAR α ($-/-$) mice. As shown in Fig. 4B, the activity was increased by all the ligands and the effects were reduced by introducing mutations into the PPRE. The increases in PPAR α promoter activity by the ligands were all diminished in the PPAR α ($-/-$) cells. Interestingly, fenofibrate retained the positive effect on activity, although somewhat reduced while this effect was lost with LY518674. These results thus show that PPAR α is activated by other PPAR subtypes being dependent on LXR.

3.4. LXRE transcriptional assay

Finally, the activity of the luciferase reporter of LXRE was examined in fibroblasts. Fig. 5A shows that the LXR–LXRE-dependent transcription was activated by all the PPAR ligands, and this effect was canceled by down-regulation of the LXR α gene by a specific siRNA in WI38 cells. When this activity was examined in fibroblasts from wild-type and PPAR α ($-/-$) mice, the activation was diminished with all of the ligands tested in the absence of PPAR α . The effects of fenofibrate and LY518674 were almost completely reduced (Fig. 5B).

4. Discussion

We investigated the mechanism for increased transcription of the ABCA1 gene upon pretreatment with PPAR ligands including four fibrates, fenofibrate, bezafibrate, gemfibrozil and LY518674 that preferentially activate PPAR α , PPAR δ -specific GW501516, and PPAR γ -selective pioglitazone. All ligands induced increased ABCA1 mRNA and protein along with an elevation in HDL biogenesis by apoA-I in fibroblasts and macrophages, consistent with previous findings. In addition, they increased mRNAs encoding LXR α and LXR β , and all three PPARs, PPAR α , PPAR δ and PPAR γ . In the absence of PPAR α , the effects of fenofibrate and LY518674 on the ABCA1/HDL biogenesis pathway were decreased including activation by the LXR α ligand, suggesting predominant dependency of these fibrates on PPAR α . The increase of PPAR δ and PPAR γ mRNAs was reduced in the absence of PPAR α for all of the compounds tested, suggesting their apparent dependence on PPAR α . Transcriptional activation of PPAR α is, at least, induced by all the compounds in an LXR α -dependent manner. We conclude that activation of PPARs induces transcriptional increase of the genes encoding PPARs and LXR α , and that this induction requires LXR α and PPAR α . Fenofibrate and LY518674 have rather narrow specificity to PPAR α but the other compounds, notably

bezafibrate has a broad specificity among PPAR subtypes, in agreement with previous reports. The present results also suggest parallel effects of PPAR δ with other PPAR subtypes in atherogenesis at least with respect to cholesterol transport.

It was previously shown that the increase in ABCA1 expression and HDL biogenesis by Wy1463 and fenofibrate is LXR α -dependent [4,5]. LXR α is activated by oxysterols and its primary function is to maintain cellular cholesterol homeostasis. It is therefore quite relevant that activation of LXR α leads to induction of the genes required for release of cholesterol, such as those encoding ABCA1 and ABCG1 through LXR response element (LXRE) in their proximal promoters [10]. It was also reported that PPAR γ directly induces LXR α expression and leads to coupled induction of ABCA1 through a transcriptional cascade and regulates a pathway of cholesterol removal from macrophages [27]. Moreover, a PPAR δ agonist, GW501516, reportedly increased the expression of ABCA1 and induced apoA1-mediated cell cholesterol release in macrophages, fibroblasts, and intestinal cells [20]. The present data demonstrated that all the PPAR agonists require LXR α for increased transcription of the ABCA1 gene. However, another PPAR δ agonist reportedly promoted lipid accumulation in human macrophages by increasing expression of the class A and class B scavenger receptors (SR-A and CD36) and adipophilin [22].

Interestingly, a requirement of LXR α was demonstrated not only for enhanced ABCA1 gene transcription but also induction of PPARs in this study. Fenofibrate, bezafibrate, gemfibrozil, LY518674, GW501516, and pioglitazone all increased ABCA1 expression and HDL biogenesis in human THP1 macrophages, human fibroblast WI38 cells and primary mouse fibroblasts. All of these compounds also increased LXR α -mRNA and enhanced expression of PPAR isoforms in an LXR α -dependent manner. We therefore examined the PPAR α promoter and LXR-LXRE dependent transcriptional activity in human and mouse fibroblasts. The PPAR α promoter was activated by all compounds in an LXR α -dependent manner and partially in a PPAR α -dependent manner in mouse fibroblast. The former finding is consistent with a recent report by Inoue et al. [28]. LY518674 is more dependent on PPAR α in PPAR α promoter activation than the other PPARs agonists used in this study. We also demonstrated that LXRE-luciferase activity was enhanced by all compounds in an LXR α -dependent manner and partially in a PPAR α -dependent manner in mouse fibroblast, and fenofibrate and LY518674 are more dependent on PPAR α in LXRE-luciferase activity. What is puzzling is that the PPAR α promoter activity was enhanced by fenofibrate in the PPAR α (-/-) cells in the presence of LXR α .

The effects of the PPAR agonists on transcription of the genes encoding ABCA1, LXRs and PPARs were not strictly distinguishable each other. Disruption of PPAR α markedly diminished the effects of fenofibrate and LY518674 indicating the narrow specificity of these agonists toward PPAR α . Interestingly, the effects of GW501516 and pioglitazone were also substantially diminished by loss of PPAR α expression. In addition, the PPAR α promoter was activated by these compounds. These findings indicate two possibilities: (1) GW501516 and pioglitazone may activate PPAR α in addition to their specific respective targets PPAR δ and PPAR γ , respectively; (2) PPAR δ and PPAR γ increase transcription of PPAR α via LXR α to exhibit PPAR α -dependency as part of their activity. However, we cannot differentiate among these possibilities based on the data presently available.

PPAR and LXR form obligate heterodimers with retinoid X receptor (RXR) (PPAR/RXR, LXR/RXR) and regulate gene transcription. PPAR γ but not PPAR α or PPAR δ activates LXR directly because of the presence of a response element upstream of the LXR promoter region [27]. However, PPAR α and LXR negatively regulate their transcription by inhibiting dimerization with RXRs, and by direct interaction with PPAR and LXR [29,30]. In contrast to these reports, all the PPAR ligands increased mRNA encoding LXRs and PPARs and increased

LXRE-luciferase activity and PPAR α promoter activity in the present study. Therefore, PPARs and LXR are necessary for their activation, either partially or completely.

In this study, we employed the experimental conditions that mostly depend on endogenous PPARs and ABCA1 rather than relying on the use of transfected and overexpressed genes employed in previous studies [4,5,9,30]. Thus the data are hopefully of more physiological relevance. Based on these observations, we conclude that PPARs and LXR α consist of complex networks including activation among PPAR subtypes for regulation of target genes. The effects of PPAR ligands exhibit their effect as a result of these complex signal transduction networks. This information should be beneficial for future development of anti-atherogenic compounds targeting PPARs [18]. The results and discussion above are summarized in Fig. 6.

Some questions remain unresolved in this study. Activation of PPARs may induce production of the endogenous ligands for LXRs, and this would contribute to enhancement of transcription of the PPARs and ABCA1 genes in addition to induction of the LXRs gene transcription. Differential effects on the cholesterol and phospholipid release and on cellular ABCA1 protein were observed among the drugs and cell lines (Figs. 1–3A). These findings may be related to unknown cellular factors to regulate cholesterol content in the HDL generated in the ABCA1/apoA-I-dependent HDL biogenesis including subcellular distribution of ABCA1 [31]. Finally, clinical relevance remains to be determined with respect to contribution of transcriptional enhancement of ABCA1 by the mechanism found here to anti-atherogenic effects of PPAR agonists. These questions are to be answered by further investigation of this topic.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Culture and Sports and Technology of Japan and from Japan Health Science Foundation, and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

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Fig. 1. Effects of PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μM , PPAR δ agonist GW501516 (W) at 0.5 μM , and PPAR γ agonist pioglitazone (P) at 5 μM , in comparison to control (C) in THP1 macrophages. The cells were exposed to PPAR agonists for 20 h. (A) ApoA-I-mediated release of free cholesterol and choline-phospholipid were measured in the conditioned medium as described in the text. ABCA1 protein was determined by immunoblotting after incubation with PPAR agonists for 20 h. The numbers in the ABCA1 immunoblotting photo indicate the results of digital densitometric scans of each band standardized for the loading standard and expressed as the relative value to the control. (B) Messenger RNA levels of ABCA1, LXR α , LXR β , PPAR α , PPAR δ , PPAR γ were

determined by quantitative RT-PCR after incubation with PPAR agonists for 20 h and standardized with those of β -actin mRNA levels. Data represent mean $\pm$ S.E. of three independent measurements. All the data are statistically significant for the increase from the control as $P < 0.01$.

Fig. 2.

Effects of PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C) in WI38 cells. The cells were exposed to PPAR agonists for 20 h. The numbers in the ABCA1 immunoblotting photo indicate the results of digital densitometric scan of each band standardized for the loading standard and expressed as the relative value to the control. (A) ApoA-I-mediated release of free cholesterol and choline-phospholipid were measured in the conditioned medium as described in the text. ABCA1 protein was determined by immunoblotting after incubation with PPAR agonists for 20 h. (B) Messenger RNA levels of ABCA1, LXR α , LXR β , PPAR α , PPAR δ , PPAR γ were measured

by quantitative RT-PCR after incubation with PPAR agonists for 20 h and standardized for β -actin mRNA. Data represent mean $\pm$ S.E. of three independent measurements. All the data are statistically significant for the increase from the control as $P < 0.01$.

Fig. 3.

Effects of PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C) in mouse fibroblasts, wild type (WT) and PPAR α (-/-). The cells were exposed to PPAR agonists for 20 h. (A) ApoAI-mediated release of free cholesterol and choline-phospholipid were measured in the conditioned medium as described in the text. ABCA1 protein and its mRNA standardized for those of β -actin were determined after incubation with PPAR agonists for 20 h. The numbers in the ABCA1 immunoblotting photo indicate the results of digital densitometric scan of each band standardized for the loading standard and expressed as the relative value to the control. (B) Messenger RNA levels of

LXR α , LXR β , PPAR α , PPAR δ , PPAR γ were detected after incubation with PPAR agonists for 20 h and standardized for those of β -actin. Data represent mean $\pm$ S.E. of three independent measurements. All the data in the wild type fibroblasts are statistically significant for the increase from the control as $P < 0.01$. For those in PPAR α ($-/-$) fibroblasts, no significant increase from the control is indicated as NS.

Fig. 4.

Human PPAR α promoter activity influenced by PPAR agonists. (A) Reporter gene construct of pPPAR α -Luc and its mutant in PPRE (pPPAR α -mut-Luc) were transfected for 20 h to WI38 cells or WI38 cells in which expression the LXR α gene was down-regulated by 80% by a specific siRNA (siLXR α), and the cells were incubated for further 20 h with an LXR agonist TO901317 (TO), PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C). The cells were lysed and firefly luciferase activity measured by the Dual-Luciferase Reporter Assay System (Promega). (B) Reporter gene construct of pPPAR α -Luc and pPPAR α -mut-Luc were transfected for 20 h to

mouse fibroblasts of wild type (WT) and PPAR α (-/-). After transfection the cells were incubated for further 20 h with PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C), and luciferase activity was determined. Results were standardized for Renilla luciferase activity derived from the phRL-tk vector. The values represent the average $\pm$ S.D. of three independent experiments performed in septuplicate. Significance of the increase from the control is indicated as * P < 0.05 and ** P < 0.01.

Fig. 5.

Activity of the LXR α response element (LXRE)-containing reporter gene (pLXRE-tk-Luc) transcription influenced by PPAR agonists. (A) The luciferase reporter plasmid for LXRE (pLXRE-tk-Luc) was transfected with and without siRNA specific to LXR α (siLXR α) or its scramble control (siScrambl) to WI38 cells for 20 h. The cells were incubated for further 20 h with PPAR α agonists fenofibrate (F), bezafibrate (B), gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C), luciferase activity was determined. B: The reporter gene pLXRE-tk-Luc was transfected to mouse fibroblasts, wild type (WT) and PPAR α (-/-), for 20 h. The cells were incubated for 20 h with PPAR α agonists fenofibrate (F), bezafibrate (B),

gemfibrozil (G) and LY518674 (L) at 30 μ M, PPAR δ agonist GW501516 (W) at 0.5 μ M, and PPAR γ agonist pioglitazone (P) at 5 μ M, in comparison to control (C), luciferase activity was determined. Results were standardized by the Renilla luciferase activity derived from phRL-tk vector. The values represent the average $\pm$ S.D. of three independent experiments performed in septuplicate. Significance of the increase from the controls is indicated as * $P < 0.05$ and ** $P < 0.01$.

Fig. 6.

Summary of the results and discussion. Thick and solid arrows indicate the pathways for transcriptional activation demonstrated in this study. Thin and broken arrows with question marks indicate potential but unproven pathways. Relative selectivity of the fibrates is illustrated as fenofibrate and LY 518674 for PPAR α , GW501516 for PPAR β/δ , pioglitazone for PPAR γ and bezafibrate and gemfibrozil for relatively broad spectrum.