Mol Cell Biochem (2007) 302:203–213
DOI 10.1007/s11010-007-9442-5

Adipocyte fatty acid-binding protein (aP2), a newly identified LXR target gene, is induced by LXR agonists in human THP-1 cells

Qiang-Yuan Liu · Elaine Quinet · Ponnal Nambi

Received: 31 October 2006 / Accepted: 02 March 2007 / Published online: 30 March 2007

Abstract The liver X receptors (LXRα and LXRβ), ligand-activated transcription factors, belong to the superfamily of nuclear hormone receptors and have been shown to play a major role in atherosclerosis by modulating cholesterol and triglyceride metabolism. In this report, we describe a novel LXR target, the adipocyte fatty acid binding protein (aP2), which plays an important role in fatty acid metabolism, adipocyte differentiation and atherosclerosis. While LXR agonists induce aP2 mRNA expression in human monocytes (THP-1 cells) and macrophages in a time- and concentration-dependent manner, they have no effect on aP2 expression in human adipocytes. The increase in aP2 mRNA level was additive when THP-1 cells were treated with LXR and PPARγ agonists. Also, an RXR agonist induced aP2 expression in these cells. While no additive effect was observed with LXR and RXR agonists, additive effects were observed with RXR and PPARγ agonists. GW9662, a potent PPARγ antagonist, inhibited PPARγ-induced aP2 expression without affecting LXR-mediated aP2 expression indicating the induction is mediated directly through LXR activation. Analysis of human aP2 promoter revealed a potential LXR response element (LXRE). Gel shift data showed that the LXRα/RXRα heterodimer bound to the LXRE motif in aP2 promoter in vitro in a sequence-specific manner. Deletion and mutation analyses of the proximal aP2 promoter confirm that this is a functional LXRE. These data indicate for the first time that human macrophage aP2 promoter is a direct target for the regulation by LXR/RXR heterodimers.

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Keywords Liver X receptors · Adipocyte fatty acid binding proteins · Target gene · Atherosclerosis

Introduction

The cytoplasmic fatty acid binding proteins (FABPs) are small proteins that are expressed in a tissue-specific manner and bind fatty acids as ligands [1, 2]. Adipocyte fatty acid binding protein (aP2) is a member of the FABPs family. The aP2 was first detected in adipose tissue, where it is an abundant cytoplasmic protein. Expression of aP2 is highly regulated during differentiation of adipocytes [3, 4], and its messenger RNA is transcriptionally controlled by fatty acids [5, 6]. Studies conducted using aP2-deficient mice have shown that aP2 plays an important role in obesity-induced insulin resistance and hyperglycemia [7]. Since both insulin resistance and abnormal lipid metabolism are risk factors for cardiovascular disease, aP2 may have an important role in the development of atherosclerosis by modulating these factors. Recent studies have shown an important role of aP2 expression in atherosclerosis [8, 9]. ApoE–/– mice null for aP2 have reduced atherosclerosis. Furthermore, macrophage aP2 deficiency also protects ApoE–/– mice from atherosclerosis independently of effects on insulin sensitivity or lipid metabolism [8, 9]. aP2-Deficient macrophages show reduced cholesterol ester accumulation and production of inflammatory cytokines in vitro [8, 9].

LXRα and LXRβ are members of the nuclear hormone receptor superfamily that form obligatory heterodimers with RXRs and can be activated by both RXR and LXR ligands [10]. RXR/LXR heterodimers activate their target genes by binding to specific response elements (LXREs) that contain a hexameric nucleotide direct repeat spaced

by four bases (DR4). LXRs have been shown to regulate the expression of a number of genes involved in cholesterol metabolism [11–13]. The expression of the human LXRα gene was recently shown to be autoregulated by activated LXRα through a process that is dependent on the LXRE in the proximal promoter of the LXRα gene [14, 15]. Recent studies have shown that the cholesterol-induced increase in fatty acid synthesis is due to the direct activation by LXRs of the gene encoding SREBP-1c, which is the primary transcription factor responsible for regulating fatty acid synthesis in the liver and peripheral tissues [16–18]. These data suggest that, in addition to sterol metabolism, LXRs also regulate fatty acid metabolism.

Previous studies have shown that aP2 mRNA was increased in mouse adipocytes and fat tissues by LXR agonists [19]. No data are available to demonstrate aP2 is a direct target gene of LXR. The present investigation was initiated to test whether aP2 were regulated by LXR agonists in human macrophages and adipocytes. We demonstrate here that the expression of aP2 is highly induced in human THP1 monocytes/macrophages cells in response to LXR agonists in a concentration- and time-dependent manner, but the regulation of aP2 expression was not observed in human adipocytes. Moreover, we also demonstrate that LXR and PPARγ agonists display additive effects on aP2 expression and 9-cis-RA (RXR agonist) also induces aP2 expression in THP-1 cells. Analysis of the aP2 promoter revealed a functional LXR response element (LXRE). These data define the aP2 promoter as the direct target of LXRs and further expand the role of the LXRs as key regulators of lipid metabolism.

Materials and methods

Reagents

All cell culture reagents were obtained from Gibco-BRL (Grand Island, NY). Phorbol 12, 13-dibutyrate was obtained from Sigma. LXR agonists T0901317 [N-(2,2,2,-trifluoro-ethyl)-N-[4-(2,2,2,-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide] [13, 17] and GW3965 [3-(3-(2-chloro-3-trifluoromethylbenzyl-2,2-diphenylethylamino)propoxy)phenylacetic acid] [17, 18] were prepared following standard chemical syntheses from published literature. 9-cis-RA was from Sigma. PPARγ agonist Ciglitazone was from BIOMOL Research Labs. Inc. PPARγ antagonist, GW9662, was obtained from Cayman Chemical (Ann Arbor, MI). Lipoprotein-deficient serum (LPDS) was obtained from Intracel Corp. (Rockville, MD).

Plasmid constructions

Human aP2 promoter was amplified by PCR using the published genomic structure and sequence. Two primers (aP2pFor: 5′-CGGGTACCCACGCCTGTAATCCCAGCGC and aP2pRev: 5′-CGCTCGAGGACCCTCTTGAGTCCA GATAAC, restriction sites are underlined) were used to amplify a fragment spanning from –1852 to +1 (relative to the transcription start site from exon 1) of the aP2 promoter. This fragment was subcloned into Kpn I/Xho I sites of pGL3 basic plasmid to create pGL3-haP2-Luc. A nested forward primer (aP2pdel: 5′-CGGGTACCTTATCCAGG-CATGTGGTGGG, restriction site is underlined) and the aP2pRev primer were used to amplify a –1729/+1 fragment (without the putative LXRE sequence). The PCR product was subcloned into pGL3 basic plasmid to generate pGL3-haP2del-Luc construct. Site-directed mutagenesis of pGL3-haP2-Luc plasmid was performed with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the following mutagenic primer (only sense primer is shown), aP2pmut: 5′-GAGACTGAGGCGAGTCCCAA ACCTGCCCACAGGAGTTCAAGACC (the mutated LXRE sequences are underlined) to create the pGL3-haP2-mut-Luc construct. The entire coding regions of human LXRα, LXRβ and RXRα were amplified by RT-PCR according to the sequences in GeneBank and subcloned into Sal I/Not I sites of a pCMV expression vector (Invitrogen, Carlsbad, CA), and KpnI/XhoI sites of pcDNA 3.1(+) (Invitrogen, Carlsbad, CA). All constructs were confirmed by sequencing.

Cell culture and cotransfections

THP-1 cells were obtained from ATCC (cat # TIB-202, Manassas, VA) and cultured in RPMI medium containing 10% fetal bovine serum (FBS). For gene expression analysis in differentiated THP-1 cells, the cells were incubated in RPMI medium supplemented with 10% LPDS and treated with 100 nM phorbol ester for 3 days followed by treatment in RPMI medium (no phorbol ester added) supplemented with 10% LPDS plus LXR, RXR and PPARγ agonist and antagonist as indicated. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Human preadipocytes and adipocytes were obtained from Zen-Bio, Inc (Research Triangle Park, NC) and were grown in Preadipocyte Medium (cat # PM-1, Zen-Bio) and Adipocytes Medium (cat # AM-1, Zen-Bio) respectively. The preadipocytes were differentiated into adipocytes using Differentiation Medium (cat # DM-2, Zen-Bio) and Adipocyte Medium according to the manufacturer's instructions. Transfections were performed in triplicate in 24 well plates using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Each well was transfected with

500 ng of reporter plasmid, 100 ng of receptor expression vector, and 200 ng of pCMV-βgal reference plasmid containing a bacterial β-galactosidase gene. Additions to each well were adjusted to contain constant amounts of DNA and pCMV expression vector. After 4–6 h, (following transfection), the cells were washed once with phosphate-buffered saline, and incubated with fresh medium containing 10% LPDS (Intracel Corp, Rockville, MD) and the indicated LXR and RXR agonists, or vehicle control for 24 h. The cells were lysed and the extracts were assayed for luciferase and β-galactosidase activity in a microplate luminometer/photometer reader (Lucy-1; Anthos, Salzborg, Austria). Luciferase activity was normalized to β-galactosidase activity.

Electrophoretic mobility shift assays

Human LXRα and RXRα proteins were synthesized from pcDNA3.1(+)-hLXRα and pcDNA3.1(+)-hRXRα expression plasmids using the TNT T7 Quick Coupled Transcription/Translation Systems (Promega, Madison, WI) according to the manufacturer's instructions. Electrophoretic mobility shift assay (EMSA) was performed using DIG Gel Shift Kit, 2nd Generation (Roche Applied Science, Penzberg, Germany). Briefly, oligonucleotide probes were labeled at 37°C for 15 min in a total volume of 20 µl consisting of 200 mM potassium cacodylate, 25 mM Tris–HCl, 0.25 mg/ml bovine serum albumin, pH 6.6, 5 mM $CoCl_2$, 0.05 mM DIG-ddUTP, and 20 U/µl Terminal transferase (Roche Applied Sciences). Binding reactions were performed in a total volume of 20 µl consisting of 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 50 mM KCL, 0.2% Tween (w/v), 50 ng/µl poly[d(I-C)], 20 ng/µl poly L-lysine, 30 fmol of DIG labeled aP2 WT probes, and 2 µl of synthesized receptors. For competition binding analysis, unlabeled oligonucleotides (100-fold molar excess) were added to the reaction mixture just prior to the addition of the DIG labeled probe. Samples were separated on nondenaturing 10% polyacrylamide gel and transferred onto the Nylon membrane and signal was detected by using anti-Digoxigenin-AP conjugate (Rocke Applied Science). The DNA sequence of double-stranded oligonucleotides for the probes were as follows (only one strand is shown): aP2-WT, 5′-GCGAGTGGGTCACCTGAGGTCAGGAGTT-3′; aP2-MUT, 5′-GCGAGTCCCAAACCTGCCCACAGGAGTT-3′ (mutant nucleotides are underlined).

RNA analysis

Total RNA was isolated using QIAGEN RNA miniPrep kit (Qiagen, Valencia, CA). Real-time quantitative RT-PCR assays were performed using an Applied Biosystems 7700 sequence detector. Briefly, each amplification mixture (50 µl) contained 50 ng of total RNA, 400 nM forward primer, 400 nM reverse primer, 200 nM dual-labeled fluorogenic probe (Applied Biosystems), 5.5 mM MgCl2, 1 U Rnase inhibitor, 1.25 U Gold Taq (Applied Biosystems) and 10 U Subscript II reverse transcriptase. The RT-PCR thermocycling parameters were 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. together with the samples and no-RT controls, a serially diluted RNA standard was analyzed in parallel. All samples were analyzed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in parallel in the same run using probe and primers from predeveloped assays for GAPDH (Applied Biosystems, Foster City, CA). All of the target gene expression was normalized to the expression of GAPDH. Quantitative analysis was performed using the threshold procedure (Perkin-Elmer protocol), and relative amounts were calculated from the standard curve. The primers and probe used in these studies were as follows: human aP2 (Genbank accession no. BC003672) aP2-forward (5′-GGGCTTTGCCACCAGGA-3′), aP2-reverse (5′-TCATCACATGGGGATTCACACT-3′), aP2 TaqMan probe (FAM-TGGCTGGCATGGCCA AACCTAACA-TAMRA).

Statistical analysis

Statistical significance of differences between two groups (vehicle treatment and drug treatment) was determined by conducting Student's t test. $P = <0.05$ are considered to be significant.

Results

LXR agonists induce aP2 mRNA expression in THP-1 macrophages

To investigate whether LXR compounds were involved in the regulation of aP2 expression, differentiated THP-1 (dTHP-1) cells were treated with various LXR ligands including synthetic agonists and oxysterol. As shown in Fig. 1, aP2 mRNA was increased in dTHP-1 macrophages treated with the oxysterol LXR ligand 22(R)-hydroxycholesterol (5 µM) or with two synthetic LXR agonists T0901317 (5 µM) or GW3965 (5 µM) for 24 h. In the same experiment, 22 (S)-hydroxycholesterol (10 µM), which binds but neither activate LXRs [20] nor alter aP2 expression (Fig. 1A).

We then treated dTHP-1 cells with varying concentrations of synthetic LXR agonists. As shown in Fig. 1B, the

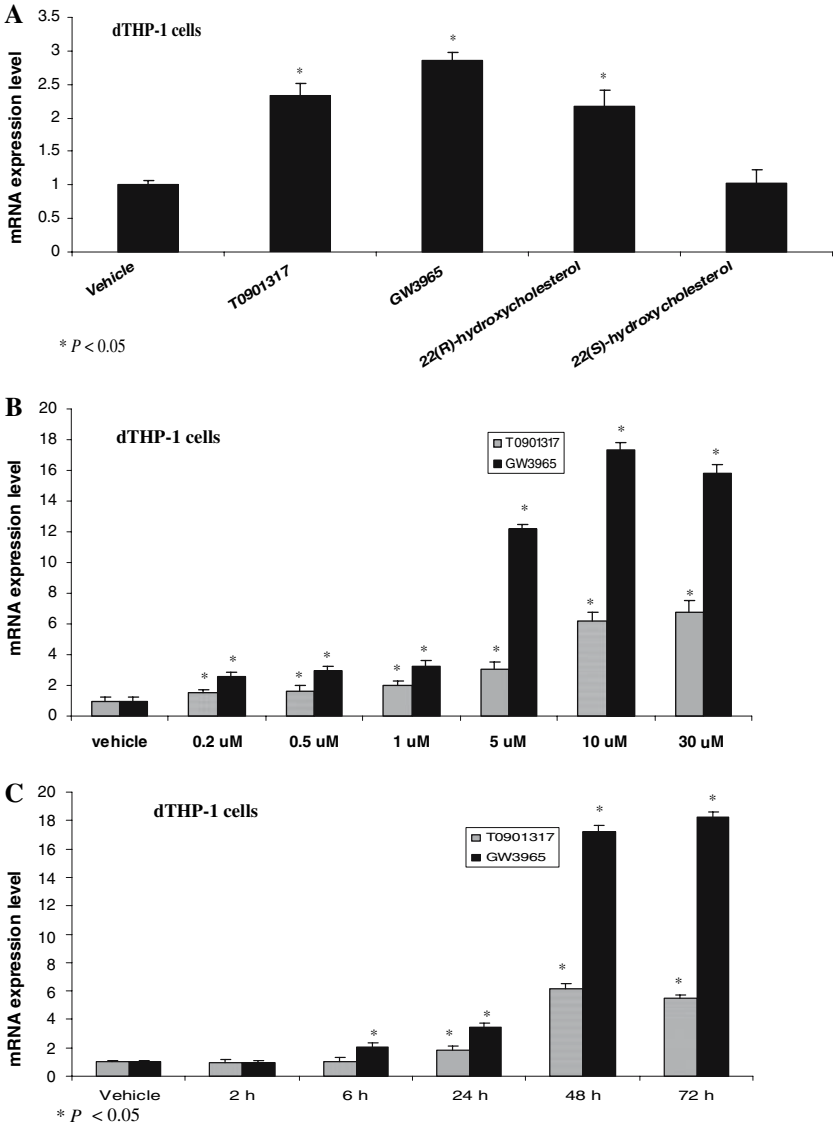

Fig. 1 (**A**) LXR agonists induce aP2 expression in differentiated THP-1(dTHP-1) cells. THP-1 cells were incubated in RPMI medium supplemented with 10%LPDS and were differentiated for 72 h with phorbol ester. Synthetic LXR ligands (5 μM of T0901317, 5 μM of GW3965), Oxysterol 22(R) HC (5 μM) or 22(S)HC (10 μM) were added to the medium and were incubated for 24 h. The total RNA of the cells was isolated and analyzed using real-time PCR as described under Materials and Methods. Data are expressed as the fold increase above vehicle treatment (<0.1% DMSO or <5% ethanol) and are expressed as the mean + SEM of triplicate samples. Each experiment was repeated three times. (**B**) & (**C**) The LXR agonists, T0901317 and GW3965 increase aP2 mRNA levels in a dose-and time-dependent manner. (**B**) dTHP-1 cells were treated with increasing concentrations of the agonists for 48 h. aP2 mRNA levels were assessed with real-time PCR analysis. (**C**) dTHP-1 cells were treated with 10 μM of T0901317 or 10 μM of GW3965 for various times. At the indicated time points, total RNA of the cells were isolated and analyzed using real-time PCR

treatment of dTHP-1 macrophages with two synthetic LXR agonists, T0901317 or GW3965, led to a prominent concentration-dependent induction of aP2 mRNA expression, with the maximal increase occurring at 10 μM for both compounds. Further increase of aP2 mRNA was not observed with the compound concentrations higher than 10 μM (Fig. 1B). GW3965 was more efficacious (16-fold of maximal increase in aP2 expression) compared with T0901317 (4-fold).

Next, the time-course of induction of aP2 mRNA was studied by treating dTHP-1 cells with T0901317 (10 μM) or GW3965 (10 μM) for various times ranging from 2 h to 72 h. As shown in Fig. 1C, aP2 mRNA expression was increased by 2-fold after 6 h treatment and 17-fold after 48 h treatment with GW3965. Interestingly, T0901317 did not induce aP2 expression until 24 h after treatment, with a maximal level of only 6-fold over control levels after 48 h treatment.

Additive effects of LXR and PPARγ agonists on aP2 expression

Previous work has shown that aP2 expression is induced by PPARγ agonists in human monocytes [21]. We, therefore, examined the effects of simultaneous activation of both the LXR and PPARγ pathways on macrophage aP2 gene expression. In agreement with this finding [21], we found an increase in aP2 mRNA expression in d THP-1 cells after treating the cells with synthetic PPARγ ligand (10 μM Ciglitazone) alone (Fig. 2A). Combination of the LXR ligands (T0901317 and GW3965) and PPARγ ligand (Ciglitazone) had an additive (GW3965 + Ciglitazone) or

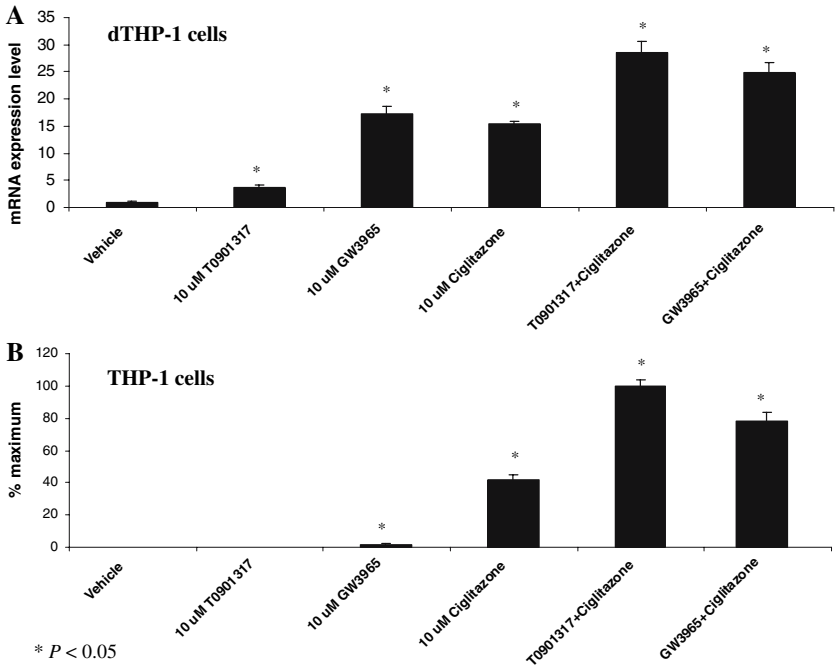

Fig. 2 LXR and PPARγ agonists induce aP2 expression in an additive manner in dTHP-1 cells. (**A**) dTHP-1 cells were incubated in RPMI medium containing 10%LPDS plus synthetic LXR agonists (T0901317 or GW3965) and PPARγ agonist (Ciglitazone) at indicated concentrations for 48 h. The level of mRNA expression relative to control (fold induction) is indicated. (**B**) undifferentiated THP-1 cells were also treated under identical conditions as differentiated THP-1 cells and aP2 expression was quantitated. Since there was no aP2 expression in the vehicle-treated undifferentiated THP-1 cells, the data are represented as percent maximum. In this particular series of experiments, combination of T0901317 and Ciglitazone gave the maximum response and this was kept as 100. All other responses were calculated as percent of this response

synergistic (T0901317 + Ciglitazone) effect on aP2 expression.

It has been shown that aP2 was not expressed in resting monocytes and a significant increase in aP2 mRNA was observed when the cells were differentiated with phorbol ester [9, 21]. We further addressed the ability of LXR ligands and PPARγ ligand to regulate aP2 expression in undifferentiated THP-1 cells. As shown in Fig. 2B, there was no detectable aP2 mRNA in undifferentiated THP-1 cells, although the average yield of total RNA was very similar to the number of differentiated and undifferentiated THP-1 cells. Treatment of these cells with LXR ligand T0901317 had no effect on aP2 mRNA expression. In contrast, PPARγ agonist (Ciglitazone) as well as another synthetic LXR agonist GW3965 induced aP2 expression in undifferentiated THP-1 cells, although the induction by GW3965 was much lower compared to Ciglitazone (Fig. 2B). Unlike differentiated THP-1cells, a synergistic effect on aP2 expression was found when the undifferentiated THP-1 cells were treated with both PPARγ ligand (Ciglitazone) and GW3965. Although, T0901317 did not have any stimulatory effect on its own, it potentiated the effect of PPARγ agonist. Since control (vehicle-treated) cells did not have any measurable aP2 expression, the data are presented as percent maximum response (maximum being the response mediated by T0901317 + PPARγ agonist and this was kept as 100). Since PPARγ has been shown to upregulate LXR in macrophage, it was interesting to determine whether the increased aP2 expression by LXR agonists was through PPARγ pathway. This was addressed using a potent PPARγ antagonist, GW9662. So shown in Fig. 3, while PPARγ induced increase of aP2 expression was completely blocked by GW9662, treatment of differentiated THP-1 cells with GW9662 did not inhibit the upregulation of aP2 by LXR agonists, indicating that the upregulation of aP2 by LXR agonists was independent of PPARγ activation and directly mediated through LXR activation.

Effect of 9-cis-retinoic acid (RA) on aP2 expression in THP-1 cells

Previous studies performed in preadipocytes have shown that 9-cis-RA (retinoid X receptor (RXR) agonist) had minimal effect on increasing aP2 expression (2-fold), and had no additive effects when 9-cis-RA and PPARγ agonist were added simultaneously [22]. We, therefore, addressed whether 9-cis-RA has an effect on aP2 expression in

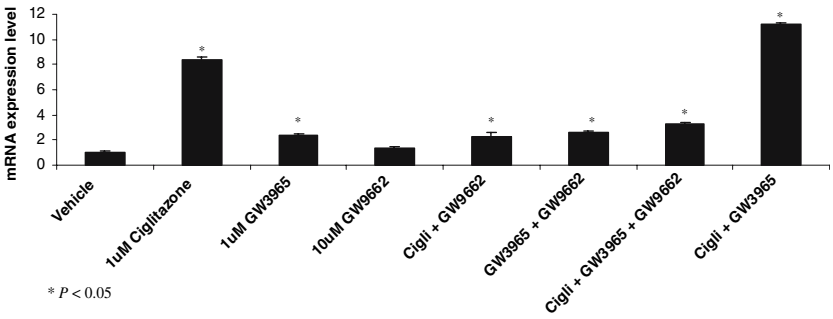

Fig. 3 Effects of PPARγ antagonist GW9662 on LXR induction of aP2 expression. DTHP-1 cells were pretreated with 10 uM GW9662 for 2 h and followed by treatment by LXR or PPARγ agonists for another 24 h. Total RNA of the cells was analyzed using real-time PCR as described under Materials and Methods

differentiated as well as undifferentiated THP-1 cells. As shown in Fig. 4A, treatment of dTHP-1 cells with 9-cis-RA resulted in a significant increase in aP2 expression (15-fold increase compared to vehicle treatment). While no additive effects were observed when cells were treated with 9-cis-RA and LXR agonists (T0901317 or GW3965) simultaneously, an additive effect was observed when the cells were treated with 9-cis-RA and PPARγ ligand (Ciglitazone) (Fig. 4A). Interestingly, treatment of undifferentiated THP-1 cells with 9-cis-RA alone induced aP2 expression (Fig. 4B), and had no additive effects when combined with LXR ligands (T0901317 or GW3965) (Fig. 4B). In contrast, when undifferentiated THP-1 cells were treated with both 9-cis-RA and PPARγ ligand (Ciglitazone) simultaneously, a significant synergistic effect was observed (Fig. 4B).

Cell selective regulation of aP2 by LXR

aP2 has been shown to be upregulated in mouse adipocytes and fat tissues in response to LXR agonist treatment [19]. We therefore examined whether LXR agonists have an effect on aP2 expression in human adipocytes. Preadipocytes were differentiated with Preadipocyte Medium (Zen-Bio), on day 7 and day 14, 10 μM of LXR or PPARγ compounds were added to medium and the cells were incubated for another 48 h. Total RNA was isolated and the aP2 mRNA expression levels were evaluated by using real-time RT-PCR analysis. In contrast to the results obtained with human macrophages, treatment of human adipocytes with LXR agonists did not alter aP2 expression (Fig. 5). While PPARγ agonist (Ciglitazone) significantly increased aP2 expression in this cell lines (Fig. 5).

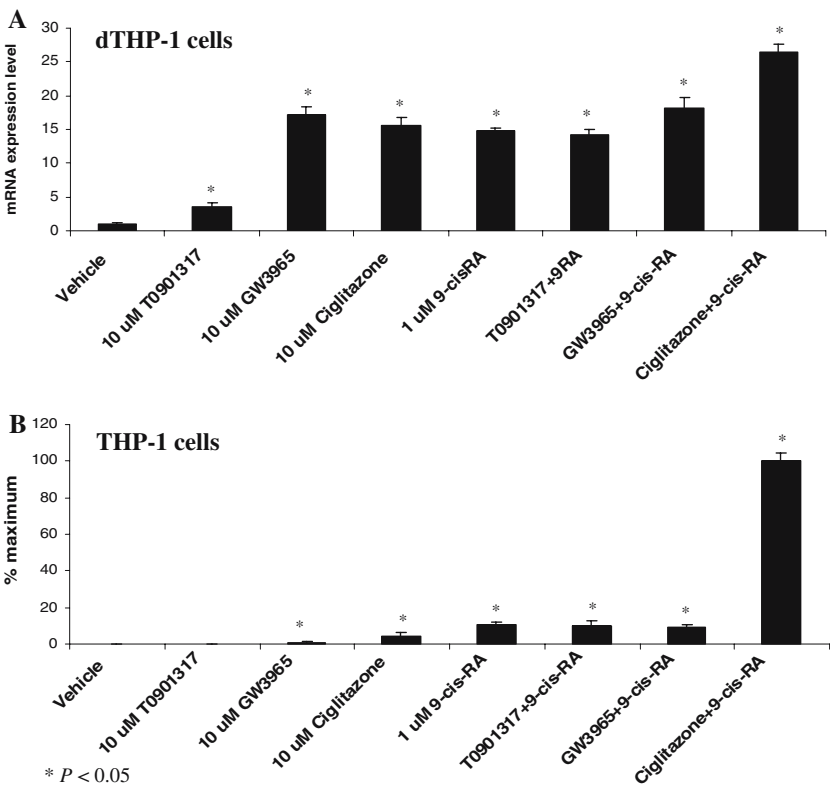

Fig. 4 Effects of RXR agonist 9-cis-RA on aP2 expression in THP-1 and dTHP-1 cells. (**A**) dTHP-1 cells were incubated in RPMI medium containing 10%LPDS plus synthetic LXR agonists (T0901317, GW3965), PPARγ agonist (Ciglitazone) and 1 μM of 9-cis-RA at indicated concentrations for 48 h. The level of mRNA expression relative to control (fold induction) is indicated. (**B**) Undifferentiated THP-1 cells were also treated under identical conditions and aP2 mRNA was quantitated. Since there was no aP2 expression in vehicle-treated THP-1 cells, the data are represented as percent of maximum and in this series of experiments, the maximum response mediated by 9-cis-RA + Ciglitazone was kept as 100%

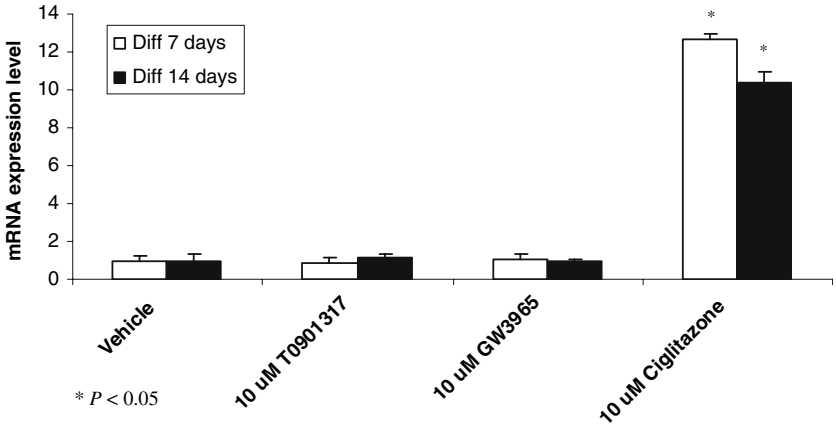

Fig. 5 Regulation of aP2 expression in human adipocytes. Differentiated human adipocytes (7 and 14 days) were treated with 10 μM T0901317, 10 μM GW3965 or 10 μM Ciglitazone for 48 h. The total RNA of the cells were isolated and analyzed using real-time PCR as described under Materials and Methods

Identification of RXR/LXR binding sites (DR4) in aP2 promoter

To determine whether the aP2 promoter has LXR response elements, human aP2 gene promoter was obtained from GenBank. Using a computer search algorithm, we identified a potential LXR response element in human aP2 promoter (Fig. 6). This response element (5′-GGGTCAcctgAGGTCA-3′) is in the –1797 bp of sequence upstream from the transcription initiation site of aP2 exon 1 and its sequence was 100% identical to the published LXR response element (LXRE) sequence [23].

The ability of LXR/RXR to specifically bind to the identified DR4 sequence was tested by EMSA (Fig. 7). The wild-type LXRE (aP2-WT, Fig. 7A) was DIG (Digoxigenin) labeled and incubated with LXRα and/or RXRα, then separated from free aP2-WT probe by gel electrophoresis. Only in the presence of both receptors a specific DNA-protein complex of retarded mobility was observed (Fig. 7B, lane 4). The specificity of binding was shown by the ability of the unlabeled aP2-WT oligonucleotide added in 100 molar excess to compete for binding with the DIG labeled probe, thereby completely abolishing the detectable shifted complex (Fig. 7B, lane 5). A mutated form of LXRE (aP2-MUT, Fig. 7A) was ineffective in competing for binding with the DIG labeled aP2-WT probe (Fig. 7B, lane 6).

-1852

cacgcctgtaatcccagcactttgggagactgaggcgagt**gggtca**cctg**aggtca**ggag
ttcaagaccagcctggccaacatggcgaaaccctgtctctactaaaaatatgagaaaaaa
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aaaaggaagttatctggactcaagagggtcAcagcaccctcctgaaaactgcagcttcct
+1
tctcaccttgaagaataatcctagaaaactcacaaa ATG TGT GAT GCT
Met Cys Asp Ala

Fig. 6 The human aP2 5′-flanking region. The sequence of the human aP2 5′-flanking region (1850 bp) is shown with the LXRE sequence denoted by bold and underlined. The transcription start site is capitalized and labeled as +1

The DR4 in aP2 promoter is a functional LXRE

To demonstrate that the DR4 sequence identified above functions as an LXRE in the context of aP2 gene promoter,

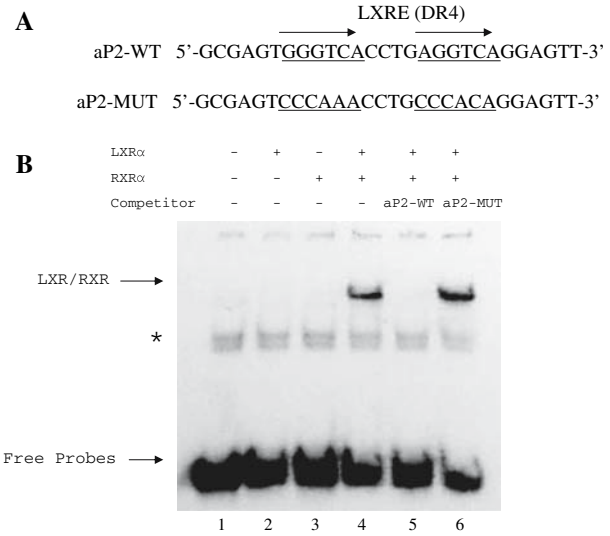

Fig. 7 Direct binding of LXRα/RXRα heterodimer to the LXRE sequence in aP2 promoter. (**A**) Sequences of LXRE derived form wild-type human aP2 promoter region (aP2-WT), and mutant human aP2 promoter region (aP2-MUT). (**B**) Electrophoretic mobility shift assay was performed as described in Materials and Methods. The DIG labeled aP2-WT LXRE probe was incubated with in vitro synthesized human LXRα and RXRα proteins as indicated. 100 molar excess of unlabeled aP2-WT (lane 5) or aP2-MUT (lane 6) were added to the reaction mixture just prior to the addition of the DIG labeled aP2-WT probe. LXR/RXR complex, free probe are denoted by arrows and nonspecific bands are indicted by asterisks

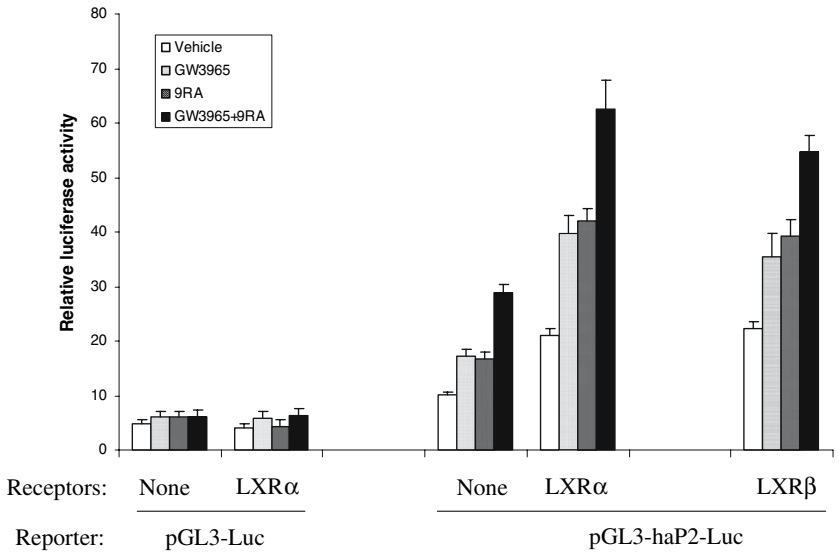

Fig. 8 LXRα/RXRα and LXRβ/RXRα heterodimers activate the –1850 bp aP2 proximal promoter. 293 cells were cotransfected with either control pGL3-Luc or pGL3-aP2-Luc reporters with or without pCMV-hLXRα/pCMV-hRXRα or pCMV-hLXRβ/pCMV-hRXRα and β-galactosidase. After transfection, cells were incubated in DMEM supplemented with 10% LPDS and 10 μM GW3965, 5 μM 9-cis-RA, combination of GW3965 and 9-cis-RA or DMSO for 24 h. Lucifierase activity was normalized to the transfection efficiency using β-galactosidase activity. Error bars represent the mean ± SD from three separate transfection experiments

the human aP2 promoter (from –1852 to +1) was cloned into the luciferase vector (pGL3-basic) directly upstream of the luciferase gene to generate reporter construct pGL3-haP2-luc. Reporter construct activity was examined in transient transfection assays in HEK 293 cells. The cells were transfected with each reporter construct either without exogenous expression of receptors or in the presence of hRXRα and either hLXRα or hLXRβ expression vectors. Treatments included vehicle or the synthetic LXR agonists GW3965 as well as T0901317 (data not shown). As shown in Fig. 8, while there was no effect on the control pGL3-luc reporter, the LXRα/RXRα and LXRβ/RXRα heterodimers activated the aP2 promoter in a ligand dependent manner. Since HEK-293 cells express endogenous LXRs, a background level of ligand-dependent induction of the reporter gene is seen in the absence of transfected receptors. Interestingly, as shown in Fig. 8, when expression vectors for LXRα and RXRα were cotransfected with the pGL3-haP2-luc reporter construct, an additive effect on the activation of aP2 promoter was observed with the treatment of both GW3965 (LXR agonist) and 9-cis retinoic acid (RXR agonist).

To determine whether the potential LXRE in the human aP2 promoter contributes to the LXR-mediated reporter activation, deletion and mutation of the LXRE were performed. The data presented in Fig. 9 showed that deletion of nucleotide –1852 to –1729 (containing LXRE sequence) on human aP2 promoter resulted in complete loss of LXR ligand-dependent activation. Furthermore, mutation of this LXRE abolished aP2 promoter activation by LXRs (Fig. 9). These data indicate that LXRE in human aP2 promoter represents the functional LXR response element. Taken together, these results demonstrate that the human aP2 promoter is a direct target for regulation by LXR/RXR heterodimers.

Discussion

Cytoplasmic FABPs are a family of 14–15 kDa proteins that are expressed in tissue-specific manner. They are proposed to play critical roles in the shuttling of fatty acids to specific enzymes and compartments, intracellular lipid metabolism, and gene expression. The adipocyte fatty acid binding protein (aP2), originally identified in adipocytes, is highly regulated during adipocyte differentiation. It has been proposed to be a marker of differentiation. Its expression is transcriptionally regulated by fatty acids [5, 6]. Interestingly, aP2 expression was recently revealed in human monocytes following stimulation with PPAR agonists [21], and oxidized low-density lipoprotein has been reported to induce expression of aP2 in human THP-1 macrophages [24]. AP2 deficiency is protective in the setting of advanced atherosclerosis induced by a Western diet in ApoE–/– mice and does not result in significant changes in insulin resistance or lipid metabolism in this model [9, 25]. Clearly, aP2 plays a role in atherogenesis in its early and late stages, making it a promising therapeutic target in the prevention and treatment of atherosclerosis and obesity-induced insulin resistance [25].

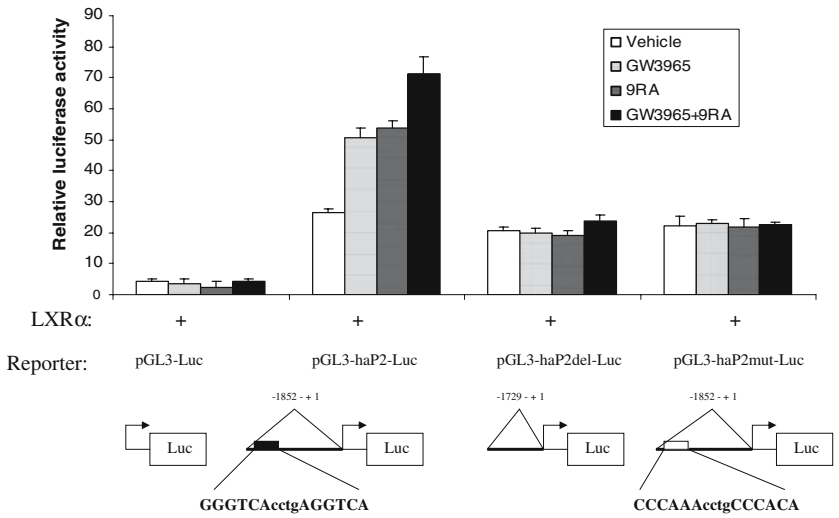

Fig. 9 Deletion and mutation analysis of LXRE in aP2 promoter. 293 cells were transfected with either a empty pGL3 vector, a construct containing the putative LXRE sequence (black box) in aP2 promoter (–1830/+1), a construct in which putative LXRE was deleted (1729/+1), or a construct containing a mutation of LXRE (open box, the sequence is shown at the bottom). The cells were cotransfected with pCMV-hLXRα/pCMV-hRXRα and incubated with appropriate compounds as indicated for 24 h. Luciferase activity was normalized to β-galatosidase activity

The present investigation was initiated to evaluate the role of LXR in aP2 regulation in human macrophages and adipocytes since LXRs have been shown to play critical roles in cholesterol/lipid metabolism as well as atherosclerosis. Recent studies have revealed that LXRs directly controls the expression of SREBP-1c [16, 26], which regulates lipogenic enzymes in liver [18]. Treatment of wild-type mice with LXR agonists led to a marked increase in hepatic triglyceride content [17, 27], which was not observed in LXRα/β double knockout mice [17]. These reports implicate a broad role for LXRs in both sterol and fatty acid metabolism. In the present study, we have shown that aP2 gene is induced in human THP-1 macrophages in response to the endogenous and synthetic LXR agonists in a time- and concentration-dependent manner. Also, 9-cis-retinoic acid (RXR-ligand) itself induced aP2 expression in these cells. To our knowledge, there is no information on the involvement of 9-cis-retinoic acid in macrophage aP2 expression and atherosclerosis. Nuclear hormone receptors such as LXRs, PPARs, and FXR have been shown to form heterodimers with RXR and mediate appropriate target gene expression. Soe et al. have previously reported that aP2 was increased by LXR agonist in mouse adipocytes and fat tissues [19]. In contrast to the results obtained with human macrophages, and with the previous studies [19], we found that treatment of human adipocytes with LXR agonists did not significantly alter aP2 expression. Our data suggest that regulation of aP2 expression is species as well as tissue-specific. Nuclear hormone receptors have been shown to regulate their target genes in tissue-specific manner. Fan et al. [28] have shown the PPARγ activation results in an increase of lipoprotein lipase in muscle tissues and a decrease in adipose tissues with no change in serum levels. Furthermore, we have shown that this induction is likely to be mediated by the direct binding of LXR/RXR heterodimers to the human aP2 promoter. Analysis of human aP2 promoter revealed a potential LXR response element. The data presented here show that the LXRα/RXRα heterodimer bound to the LXRE motif in aP2 promoter in vitro in a sequence-specific manner. Deletion and mutation analysis of the proximal aP2 promoter firmly demonstrates that this is a functional LXRE indicating that human aP2 is a direct target gene of LXR. Even though aP2 expression has been shown to be induced by LXR agonists in mouse adipocytes, there are no data available to demonstrate if aP2 is a direct target gene of LXR. To our knowledge, this is the first demonstration showing aP2 as the direct target gene of LXR. Our findings define a new mechanism for regulation of aP2 expression in macrophages and further expand the role of the LXRs as key regulators of lipid metabolism.

LXRα expression was recently shown to be regulated by PPARγ in macrophages [28] and in human adipocytes and in obese Zucker rats [29]. LXRα promoter is a direct target of PPARγ and PPARγ plays a role in macrophage cholesterol efflux through a transcriptional cascade involving LXRα and ABCA1 [30]. Previous work demonstrated that mouse embryonic stem cells that lack both copies of the PPARγ gene lack expression of aP2 gene and are not able to develop into fat cells [31]. Recent studies have shown that Troglitazone, a synthetic agonist of PPARγ, is able to induce aP2 gene expression in human THP-1 macrophages

and skeletal muscle cells [21, 32]. However, the molecular mechanism for the upregulation of aP2 by PPARγ has not been addressed and functional PPRE has not been identified in the promoter of human aP2 gene. In agreement with the previous reports, we found that aP2 expression was increased in differentiated THP-1 cells following the treatment with PPARγ compound and an additive increase of aP2 expression observed in the presence of LXR and PPARγ agonists. But sequence analysis showed that there was no PPRE-like motif in human aP2 promoter. In addition, we have provided evidence here that human aP2 is the direct target gene of LXR. Together with the previous findings [29, 30], our data indicate that one of the mechanisms by which aP2 was upregulated by PPARγ agonist in human THP-1 cells might be through the upregulation of LXR.

Activation of LXR has been shown to regulate a number of genes in cholesterol homeostasis [13, 16] and fatty acid biosynthesis [17, 32]. While the activation of genes such as ABCA1, ABCG1, ApoE, and CETP are critical for reverse cholesterol transport (a process by which excess cholesterol is transported from the peripheral tissues to the liver for elimination), activation of genes such as FAS and SREBP1c involved in triglyceride synthesis is an undesirable side effect of the LXR activation. Major efforts are being focused on the identification of molecules that will selectively regulate genes involved in reverse cholesterol transport without affecting genes such as SREBP1c and FAS. Synthetic LXR agonist such as T0901317 has been shown to increase liver SREBP1c expression as well as plasma triglyceride levels in animal models of atherosclerosis. The identification of aP2 that is involved in fatty acid transport as a LXR target gene adds another level of complexity to its involvement in triglyceride metabolism. Studies conducted with ApoE and aP2 double knock out mice indicate that these mice developed less atherosclerosis compared to ApoE knockout mice suggesting that aP2 inhibition may lead to beneficial effect. Thus, compounds that act on LXR to selectively upregulate ABCA1 without affecting SREBP1c or aP2 may be better candidates for the treatment of atherosclerosis.

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