Adipocyte Low Density Lipoprotein Receptor-related Protein Gene Expression and Function Is Regulated by Peroxisome Proliferator-activated Receptor γ*

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The α2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) is a large multifunctional receptor that interacts with a variety of molecules. It is implicated in biologically important processes such as lipoprotein metabolism, neurological function, tissue remodeling, protease complex clearance, and cell signal transduction. However, the regulation of LRP gene expression remains largely unknown. In this study, we have analyzed 2 kb of the 5′-flanking region of the LRP gene and identified a predicted peroxisome proliferator response element (PPRE) from −1185 to −1173. Peroxisome proliferator-activated receptor γ (PPARγ) ligands such as fatty acids and rosiglitazone directly stimulate transcription of the LRP gene through activation of the PPRE. Mutagenesis of the predicted PPRE abolished the ability of this construct to respond to rosiglitazone. These data demonstrate that fatty acids and rosiglitazone increased functional cell surface LRP by 1.5–2.0-fold in primary human adipocytes and in the SW872 human liposarcoma cell line as assessed by activated α2-macroglobulin binding and degradation. These agents were found to increase LRP transcription. Gel shift analysis of the putative PPRE demonstrated direct binding of PPARγ/retinoic X receptor α heterodimers to the PPRE in the LRP gene. Furthermore, these heterodimers could no longer interact with a mutated PPRE probe. The isolated promoter was functional in SW872 cells, and its activity was increased by 1.5-fold with the addition of rosiglitazone. Furthermore, the isolated response element was similarly responsive to rosiglitazone when placed upstream of an ideal promoter. Mutagenesis of the predicted PPRE abolished the ability of this construct to respond to rosiglitazone. These data demonstrate that fatty acids and rosiglitazone directly stimulate transcription of the LRP gene through activation of PPARγ and increase functional LRP expression.

The α2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) is a 600-kDa multifunctional endo-

* This work was supported by Heart and Stroke Foundation of Ontario Grant T-4631. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Ontario Graduate Scholarships in Science and Technology and Ontario Graduate Scholarships.

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§ The abbreviations used are: LRP, low density lipoprotein receptor-related protein; PPAR, peroxisome proliferator-activated receptor; RXX, retinoid X receptor; PPRE, peroxisome proliferator response element; CS, fetal calf serum; RT, reverse transcription; α2M, α2-macroglobulin; α2M*, activated α2M; EMSA, electrophoretic mobility shift assay(s); hACOX, human fatty acyl CoA oxidase; PBS, phosphate-buffered saline; RAP, receptor-associated protein; BSA, bovine serum albumin; HBSS, Hank’s balanced salt solution.

Received for publication, December 19, 2002
Published, JBC Papers in Press, January 27, 2003, DOI 10.1074/jbc.M212989200
have shown that LRP gene expression is not regulated by cholesterol (27).

We have studied the regulation of LRP gene expression during human preadipocyte differentiation and in response to free fatty acid availability. LRP mRNA was absent in human preadipocytes, and the appearance of LRP mRNA during differentiation coincided with that of the peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a transcription factor belonging to the nuclear hormone receptor superfamily. The retinoid X receptor α (RXRα) is the obligate partner of PPARγ (31), and together they form a heterodimer that regulates gene transcription following binding to a peroxisome proliferator response element (PPRE) and activation by specific ligands. The PPRE consists of a hexameric nucleotide repeat of the recognition motif (TGACGT) spaced by one nucleotide (DR-1) (32, 33). PPARγ is activated by a number of ligands including long chain fatty acids (34), prostaglandin J2 derivatives (35), and thiazolidinediones (36, 37). The effects of PPARγ ligands on gene expression are direct results of increased transcription of the target gene containing a PPRE. In our own analysis of the LRP promoter, we have identified a novel sequence (TGAAC/TGACAT) in the 5′-flanking sequence at positions −1185 to −1173 with high homology to the PPRE.

We report here that functional cell surface LRP is increased by PPARγ ligands via the activation of PPARγ transcriptional complexes that bind the newly identified PPRE in the LRP promoter. This is the first report demonstrating regulation of LRP gene expression via a discrete promoter element.

EXPERIMENTAL PROCEDURES

Human Preadipocyte Isolation and Culture—Subcutaneous adipose tissue was collected from healthy normolipemic subjects undergoing reduction mammoplasty procedures. Preadipocytes were isolated and cultured from adipose tissue through collagenase digestion, centrifugation, and filtration as previously described (38–41). The preadipocytes were cultured in differentiation media for 10–14 days. The cells were then insulin-starved in the presence or absence of varying concentrations of the PPARγ ligand rosiglitazone for 24 h prior to assays. The control cells were treated with vehicle only (MeSO).

Cell Culture—The human liposarcoma cell line SW872 (American Type Culture Collection, Manassas, VA) was previously characterized and has been shown to be a good cell model for adipocyte gene expression (42–44). The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (3:1) (Invitrogen) supplemented with 5% fetal bovine serum and 1% l-glutamine (Invitrogen), 10 mM NaCO3, and 5% CO2 at 37 °C under humidified conditions and 5% O2.

The cells were transfected with plasmids expressing reporter constructs containing the LRP message by the 488-bp band corresponding to the 18S rRNA. 18S PCR products were amplified to give similar yields so that they could be used for all subsequent PCR reactions. The 18S primer:competitor mix with the following PCR conditions; 1 cycle of denaturation at 94 °C for 1 min, followed by 30 cycles of 15 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR products were subjected to ethidium bromide staining. The band intensities were measured using a CCD camera (MicroMax) and WinView software from Princeton Instrument Corporation.

LightCycler was repeated using relative quantitative RT-PCR as described below. For SW 872 samples, relative quantitative RT-PCR was performed using the Quantum RNA 18 S Internal Standards kit from Ambion. This kit has been previously shown to allow the accurate determination of relative changes in gene expression between samples (45, 46).

Briefly, first strand cDNA was synthesized using 2.5 μg of total RNA and random decamer primers (Ambion, Austin, TX) with 500 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and incubated at 42 °C for 1 h. Consecutive PCR reactions were then performed on the first strand cDNA using the primers shown below and the SYBR green "taq-start" polymerase and the LightCycler Apparatus according to the manufacturer's instructions (Roche Molecular Biochemicals). The data from the LightCycler was repeated using relative quantitative RT-PCR as described below. For SW 872 samples, relative quantitative RT-PCR was performed using the Quantum RNA 18 S Internal Standards kit from Ambion. This kit has been previously shown to allow the accurate determination of relative changes in gene expression between samples (45, 46).

Cell Surface Fluorescent Detection of LRP—The cells were cultured on 0.15-mm cover glass bottom dishes (MatTek, Ashland, MA) as described above and supplemented with 160 μM arachidonic acid, 500 nM rosiglitazone, or control. α2M was activated by incubating purified α2M with 400 μM maleimide for 16 h at room temperature. α2M was fluorescently labeled using Cy3 monofunctional reactive dye (Amersham Biosciences) to a dye-protein ratio of 1.5 according to the manufacturer's instructions. The cells were plated on ice for 3 h in 2M* Dulbecco's modified Eagle's medium/Ham's F-12 medium supplement with 2 mg/ml BSA buffered with 10 mM HEPES. Labeled α2M was diluted in the same medium to a concentration of 1 μg/ml and was added to the cells at 0 °C for 45 min. The cells were rinsed with ice-cold PBS that was also pre-cooled to 0 °C and washed with cold PBS at room temperature for fluorescence microscopy. Binding was competed with 30-fold excess of unlabelled α2M. The cells were viewed with an Olympus IX50 fluorescent microscope, and the images were taken using a cooled CCD camera (MicroMax) and WinView software from Princeton Instrument Corporation.

Determination of LRP binding to cytosolic, nuclear, and membrane fractions was accomplished using affinity chromatography on LRP-coated columns (Qiagen) and the purified DNA was subsequently labeled with 30-fold excess of unlabelled α2M.

The probes were cleaned up with NICK columns (Amersham Biosciences), and the specificity of the probes was determined by use of a scintillation counter. Hybridizations and washes were performed according the NorthernMax-Gly kit instructions (Ambion).

RT-PCR was performed using a two-step approach. First strand cDNA was synthesized using 2.5 μg of total RNA, 1 μM random decamer primers (Ambion), and 500 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and incubated at 42 °C for 1 h. Consecutive PCR reactions were then performed on the first strand cDNA using the primers shown below and the SYBR green "taq-start" polymerase and the LightCycler Apparatus according to the manufacturer's instructions (Roche Molecular Biochemicals). The data from the LightCycler was repeated using relative quantitative RT-PCR as described below. For SW 872 samples, relative quantitative RT-PCR was performed using the Quantum RNA 18 S Internal Standards kit from Ambion. This kit has been previously shown to allow the accurate determination of relative changes in gene expression between samples (45, 46).

Transcription Assay—To determine the transcriptional effect of rosiglitazone, 500 nM of this ligand was added to cells cultured as described above in the presence or absence of 10 μg/ml α-amanitin (Sigma), a protein inhibitor that inhibits DNA polymerase II (47).

RNA Extraction, Northern Blot, and RT-PCR—Total cellular RNA was isolated from both differentiated primary human adipocytes and SW872 cells with Tri-Reagent (Bio/Can, Mississauga, Canada) according to the manufacturer's instructions. RNA samples from differentiated primary adipocytes that were to be used in RT-PCR reactions were treated with amplification grade DNase I to deplete the samples of any DNA contamination according to the manufacturer's instructions (Invitrogen). RNA concentration was determined spectrophotometrically using an A260/280.

Total RNA (5 μg) was separated by agarose gel electrophoresis using the NorthernMax-Gly kit and transferred to BrightStar-Plus nylon membrane according to the manufacturer's instructions (Ambion, Austin, TX). DNA probes were synthesized by RT-PCR; first strand DNA was synthesized as described below, and PCR was performed using the following primers: LRP5′-AGATACAGGTGCTGATACGCTG-3′, 3′-CTCGTCAATCAGGGCACCAGACG-3′, 5′-GGCCCTTCATCTGCACCAGC-3′, and β-actin: 5′-GGGGACAGAGGCTCATT-C3′. The PCR products were gel-purified using the QiAexII kit (Qiagen), and the purified DNA was subsequently labeled using the Rediprime II random prime labeling kit according the manufacturer's instructions (Amersham Biosciences). The probes were cleaned up with NICK columns (Amersham Biosciences), and the specificity of the probes was determined by use of a scintillation counter. Hybridizations and washes were performed according the NorthernMax-Gly kit instructions (Ambion).

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Briefly, first strand cDNA was synthesized using 2.5 μg of total RNA, 1 μM random decamer primers (Ambion), and 500 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and incubated at 42 °C for 1 h. PCR forward primer (5′-AGATACAGGTGCTGATACGCTG-3′) and reverse primer (5′-CTCGTCAATCAGGGCACCAGACG-3′) were designed to amplify a region of the LRP mRNA that is ~400 bp in size, whereas the primers provided in the 18 S Internal Standards kit produced a band that is ~500 bp. A cycle number of 23 was determined to be within the linear range of PCR and was used for all subsequent PCR reactions. The 18 S primer:competitor ratio of 3:7 was experimentally determined so that the LRP and 18 S PCR products were amplified to give similar yields so that they could be compared between samples. PCR was performed on 1 μl of the RT reaction using 20 pmol of each primer, 100 μl of Life Technologies' PCR buffer, 0.2 μM of each primer, and 1 μl of the PCR primer:competitor mix with the following PCR conditions; 1 cycle of 95 °C for 3 min and 23 cycles of 95 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s. Cocktails containing all shared components were used to reduce variation between samples. The PCR products were subjected to electrophoresis through a 1% agarose gel and visualized with ethidium bromide staining. The band intensities were measured using the ChemiDoc apparatus and Quantity One software (Bio-Rad). Relative intensity was calculated by dividing the 400-bp band corresponding to the LRP message by the 488-bp band corresponding to the 18 S message.
Western Blotting of LRP—Total cellular protein (5 μg) from SW872 cells incubated in the presence or absence of various PPARγ ligands was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (49). The LRP was detected (49) using a polyclonal rabbit antisera (from Dr. G. Bu) followed by chemiluminescent detection (Pierce) of a secondary antibody conjugated to horseradish peroxidase. The blot was developed, and the bands were quantified using the ChemiDoc apparatus and Quantity One software (Bio-Rad). Truncate cell samples were processed and were summarized in the graph. Molecular biology techniques were essentially as described by Sambrook et al. (50).

Preparation of Nuclear Protein Extracts—The nuclear proteins were extracted from SW872 cells as previously described (51) or from primary human adipocytes as described (52). Protein concentration was determined using BCA protein reagent (BioLynx, Brockville, Canada) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assays (EMSA)—Double-stranded oligonucleotides (oligomers) corresponding to the PPRE of LRP (5′-GCCGCCTTCCTTTGAAGAAGACTCAGAGACACC-3′) were radioactively end-labeled with [γ-32P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (Invitrogen) and purified from unincorporated nucleotides by gel filtration through G-50 spin columns (Amersham Pharmacia Biotech). The same procedure was used for oligomers corresponding to the PPRE of the human fatty acyl CoA oxidase gene (hACOX) (5′-TCCGGTTCTGGTCCTTGCTGCTGTTTTT-3′) and oligomers corresponding to the mutated form of the LRP PPRE (the mutated half-site is underlined) (5′-CCCCGCTCTTTGAACCTACGGATGACACC-3′). The specific activities of the oligomers were ~250 cpm/μmol. These were diluted to 60 fmol/μl for use in the assay. PPARγ (from Dr. Bruce Spiegelman) was subcloned into the MluI and NofI sites of pSPORT1 (Invitrogen) using PCR-based methods. RXRα was prepared by Dr. Michael Saunders in pSG5. Both constructs are driven by the T7 RNA polymerase promoter for use in the TNT T7 coupled reticulocyte lysate system (Promega) for in vitro transcription/translation. All of the EMSA reactions were carried out on ice in 20 μl of binding buffer (12.5 mM HEPES-KOH, pH 7.6, 6 mM MgCl2, 0.5 mM EDTA, and 50 mM KCl) supplemented with 50 μl dithiothreitol, 0.2 μg of low fat milk, 0.05 μg of poly(dI-dC), and 10% glycerol. For EMSA reactions with TNT-puriﬁed proteins, 2 μl of the TNT reaction was added to the reaction mix along with 1 μl (60 fmol) of labeled oligomers. For EMSA reactions with nuclear protein extracts, 6 μl of nuclear extracts were added to the reaction mix with 1 μl (60 fmol) of labeled oligomers. These reactions were left on ice for 20 min. Following the 20-min incubation, 2 μl of 20% Ficoll was added to the samples. DNA-protein complexes were then resolved by electrophoresis through 6% polyacrylamide gels in 0.25× Tris borate running buffer (20 mM Tris borate, pH 7.2, 0.5 mM EDTA). Supershift assays were performed using the PPARγ NusShift kit following the manufacturer’s protocol (Active Motif). The nonspeciﬁc antibody used was mouse monoclonal anti-PPARγ (Santa-Cruz).

LRP Reporter Gene Constructs—Complementary primers with ﬂanking Nhel and XhoI restriction sites (5′-TACGCTCTTAGACCTACTGAGA-3′) were annealed and subcloned into the luciferase reporter vector, pGL3-Promoter (Promega). This new vector contains a single copy of the putative PPRE upstream of an ideal promoter and is designated pGL3-PPRE. We also prepared a 1.9 kb of the 5′-flanking region of LRP by PCR ampliﬁcation from the LRP-BAC construct prepared by Dr. Jan Boren (53), which contains the entire 92-kb gene of human LRP, using the primers 5′-GAGACGACCTCGGAATAGGGGAG-′3′ and 5′-GACAGCAGATCTTCCGCTGGAAG-′3′. This fragment was subcloned into the XhoI sites of the lucase reporter vector, pGL3-Basic (Promega), and designated pGL3-LRP. Mutagenesis of the PPRE was performed by PCR using PFUTurbo (Stratagene, La Jolla, CA) according to their Quikchange site-directed mutagenesis protocol. The complementary primers (5′-GCCGCCTTCCTTTGAAGAAGACTCAGAGACACC-3′) were designed to mutate a single half-site of the PPRE so that PPRE would no longer bind the response element (mutated nucleotides are underlined). This construct was designated pGL3-LRPmutantPPRE. Both pGL3-LRP and pGL3-LRPmutantPPRE were sequenced to conﬁrm that the promoter sequence was correct (compared with GenBank™ accession number Y18524) and to verify the mutations.

Transient Transfection Assays—Confluent SW872 cells were trypsinized and seeded at a density of 1.25 × 10^5 cells/well in 12-well plates 48 h prior to transfection. The cells were transfected with expression plasmids either alone or with a firefly luciferase reporter vector (either pGL3-basic, pGL3-LRP, pGL3-PPRE, or pGL3-LRPmutantPPRE and 0.25 μg of the Renilla luciferase-bearing reporter vector, pRL-CMV (Promega) using the calcium phosphate-DNA precipitate method (54). The cells were shocked with 15% glycerol for 2 min, 4 h after the transfection, and washed three times with PBS before the addition of medium. The cells were treated 12 h later with Me2SO alone (vehicle control) or varying concentrations of rosiglitazone, and the luciferase activity was then used to standardize for transfection efficiency.

Luciferase activities derived from both ﬁreﬂy (LRP constructs) and Renilla (pRL-CMV) proteins were measured using the dual luciferase reporter assay system (Promega) and recorded using a Monolight 1030 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Renilla luciferase activity was then used to standardize for transfection efficiency.

Statistical Analysis—The results are expressed as the means ± S.E. Where indicated, the statistical signiﬁcance of the differences between groups was determined using Student’s t test or analysis of variance.

RESULTS

Functional Cell Surface LRP Is Increased upon Exposure to PPARγ Ligands—The effects of PPARγ ligands on differentiated primary human adipocytes were examined by an 125I-α2M binding assay (Fig. 1A). The Bmax of cells incubated with 1 μM rosiglitazone (27.0) is ~1.5 times greater than that of the vehicle-treated cells (17.3), indicating that there is an increase in the levels of functional cell surface LRP. The difference in binding was found to be highly signiﬁcant with a two-tailed p value of less than 0.0001. When the cells were treated with RA (30 μM), the amount of binding was reduced to background levels, demonstrating that this process is LRP-speciﬁc. There was no statistically signiﬁcant difference in the Kd between the treated and control cells as illustrated in the Scatchard plot (Fig. 1B), indicating that the binding affinities have not changed. 125I-α2M degradation assays were also performed in the presence or absence of PPARγ ligands for the differentiated primary human adipocytes and SW872 cells. In primary adipocytes, there was a direct relationship between the amount of rosiglitazone added and the amount of α2M degradation over 8 h (Fig. 2A) with very signiﬁcant increases ranging from 1.2- to 1.7-fold over control (p < 0.001). The RAP is an antagonist of all identiﬁed LRP ligands including α2M; therefore we used puriﬁed RAP to block LRP function in our assays. When cells were treated with RAP, the amount of 125I-α2M degraded was diminished to background levels, demonstrating that this process is LRP-speciﬁc. Degradation assays were also performed in the SW872 cells treated with rosiglitazone (Fig. 2B) or arachidonic acid (Fig. 2C). There was a 1.5-fold increase in the amount of 125I-α2M degraded over 8 h in the treated cells versus the control cells for both of the PPARγ ligands.

The increase in functional cell surface LRP was conﬁrmed in SW872 cells by cell surface labeling experiments using ﬂuorescently labeled α2M in the presence of 160 μM arachidonic acid or 500 nM rosiglitazone (Fig. 3A). In addition, total cellular LRP was increased in those cells as determined by Western blot analysis (Fig. 3B). The increases seen in LRP using these methods were ~1.5–2-fold, supporting the binding and degradation data above.

Endogenous LRP mRNA Levels Are Increased upon Exposure to PPARγ Ligands—The effect of PPARγ ligands on levels of LRP mRNA was determined in the adipocytic cell line, SW872, by relative quantitative RT-PCR. The relative intensity was determined by the ratio of the LRP band intensity compared with the 18S band intensity, and these values were normalized to the control samples to give values of fold increase. The fold increases of LRP mRNA in cells upon treatment with oleic acid, arachidonic acid, and rosiglitazone are summarized in Fig. 5 below (A, B, and C, respectively). PPARγ ligands, rosiglitazone
(500 nM), arachidonic acid (160 μM), and oleic acid (0.8 mM) increased LRP mRNA levels by 1.5-, 1.6-, and 1.3-fold, respectively. The maximum effect of the ligands was observed after 24 h of treatment. Increases in LRP mRNA levels were similar to those shown in Fig. 4 (B and C).

The effect of rosiglitazone on LRP mRNA levels was also determined in differentiated primary human adipocytes, as measured by Northern blot and real time PCR, and these results are summarized in Fig. 5. According the Northern blot analysis, LRP mRNA is increased by rosiglitazone treatment by ~1.6-fold. Using real time RT-PCR, there was a dose-dependent increase in LRP mRNA levels (ranging from 1.4- to 1.8-fold) after 24 h of ligand treatment. The levels of LRP mRNA were also verified using relative quantitative RT-PCR, and the fold increases in LRP expression were similar to those shown in Fig. 5.

PPARγ Ligands Act at the Transcriptional Level to Increase LRP mRNA—The increase in LRP mRNA could be due to an mRNA stabilization effect or to increased transcription of the mRNA. To distinguish between these possibilities, a potent inhibitor of RNA polymerase II activity, α-amanitin, was used to inhibit new transcriptional activity. If the level of mRNA were increased by a stabilization effect of rosiglitazone or other PPARγ ligands, then the increase in mRNA levels would still be observed when both α-amanitin and rosiglitazone were added to cells concomitantly. We did not observe an increase in LRP mRNA in cells cultured with both rosiglitazone and α-amanitin (Fig. 4C). Although there was a small decrease in LRP mRNA levels with the addition of α-amanitin, this decrease was identical in both the vehicle-treated and rosiglitazone-treated samples. The results are summarized in Fig. 5.
zone-treated cells, suggesting this is due to normal turnover of the mRNA. These results support a role for these ligands as transcriptional up-regulators of LRP gene expression.

PPARγ-RXRα Heterodimers Selectively Bind the PPRE Identified in the LRP Promoter Region—We identified a PPRE located at −1185 to −1173 of the LRP promoter through sequence scanning of the 5′-flanking region. The sequence homology to the consensus PPRE is 83%; there is a single mismatch per half-site of the DR-1 (Fig. 6A). The TNT in vitro transcription/translation of pSPORT1-PPARγ and pSG5-RXRα was first shown to express PPARγ and RXRα; when [35S]methionine was added to the reaction mix, proteins of the correct molecular mass were synthesized as verified by SDS-PAGE and autoradiography. For the EMSA, these constructs were subjected to the TNT reaction (without [35S]methionine) and incubated with 32P-end-labeled oligomers corresponding to the PPRE of LRP. A clear shift was evident in the presence of these transcription factors (Fig. 6B, lane 2) compared with when the TNT reaction was carried out with the empty pSPORT1 vector (unprogrammed lysate) (lane 1). The interaction between the protein complex and the DNA could be competed by the addition of increasing amounts of excess of unlabeled hACOX oligomer (lanes 3–6). Identical experiments using the hACOX probe yielded very similar results (Fig. 6B, lanes 7–12). The doublet bands that appear have been observed in other studies where rabbit reticulocyte lysate was used to produce PPARγ and RXRα. The interaction between the probes with nuclear protein extracts from primary adipocytes gave a shift of the same size as the TNT proteins (Fig. 6C, lane 2). This shift could also be inhibited by increasing amounts of excess unlabeled hACOX oligomer (lanes 2–5). Nuclear extracts from the SW872 cell line yielded similar results. These results are comparable with those obtained using oligomers corresponding to the hACOX PPRE (lanes 6–10). A probe containing a mutated PPRE half-site, LRFmut, could no longer interact and bind TNT proteins (Fig. 6B, lane 14) or nuclear extracts (Fig. 6C, lane 11).

The shift caused by the nuclear extracts was further analyzed by gel supershift analysis to confirm that PPARγ was a component of this complex (Fig. 6D). Free probe was run in lane 1 as a control reaction, whereas a reaction containing nuclear protein extracts from primary human adipocytes is shown in
The second lane. The nuclear protein extracts were preincubated with anti-PPARγ antibody prior to being added to the reaction containing labeled oligomers, and this was run in the third lane. A band of the same size as that in the second lane was present as well as a larger band that was absent in all other lanes (supershift). The same results were obtained for the oligomer corresponding to the hACOX PPRE. A supershift was not observed when a nonspecific antibody was used in place of the anti-PPARγ antibody. Similar results were also obtained using nuclear extracts from SW872 cells supporting the involvement of PPARγ in the protein-DNA complex.

**LRP PPRE Luciferase Constructs Are Responsive to Rosiglitazone**—Although the basal transcriptional activity of the pGL3-PPRE construct was 2-fold higher than the pGL3-LRP construct because of the context of the SV40 ideal promoter (data not shown), the promoter context of the endogenous LRP promoter, which is present in pGL3-LRP, is the more physiologically relevant of the two constructs. This portion of the promoter drove the basal activity of the reporter gene as determined by the dual luciferase reporter assay system (Fig. 7A). The values were normalized to the cells treated with Me2SO only (vehicle control). The empty pGL3-basic vector had no basal activity above the background measurements of the instrument (data not shown). For pGL3-LRP (Fig. 7A), as well as pGL3-PPRE (data not shown), there was a dose-dependent response of the luciferase activity upon treatment with rosiglitazone that corresponded to the increases in LRP mRNA shown in Fig. 4. The ratio of firefly and Renilla luciferase activities are shown in the figures; it is important to note that the luciferase activity increases with rosiglitazone treatment and that the Renilla does not decrease.
Mutagenesis of the PPRE Results in the Loss of Enhancer Activity—A single half-site of the PPRE, to which PPARγ binds, was mutated using the Quikchange mutagenesis protocol (Fig. 7B) and designated pGL3-LRPmutant_PPRE. This construct was sequenced, and the point mutations within the PPRE were verified. The basal activity of this reporter construct (vehicle control) was similar to that of the pGL3-LRP construct (Fig. 7C). In the presence of 500 nM rosiglitazone there was approximately a 1.5-fold increase in promoter activity of the pGL3-LRP construct. This increase, however, was lost in the pGL3-LRPmutant_PPRE construct, indicating a role for this PPRE as a transcriptional enhancer.

**Discussion**

Despite the importance of LRP in lipoprotein, serum protease, and β-amyloid metabolism, this is the first study to demonstrate that LRP expression is regulated at both mRNA and protein levels via a discrete promoter element. We have demonstrated that functional LRP expression is regulated by PPARγ ligands by a mechanism that involves the ligand-induced up-regulation of transcription via the activation of PPARγ-RXRα heterodimers that bind a newly identified PPRE in the promoter of the LRP gene. Levels of functional cellular LRP were measured by binding and degradation of a well characterized LRP ligand, αM* (58, 59). Furthermore, we have demonstrated that LRP mRNA levels are modulated at the transcriptional level by ligands that activate PPARγ and that this response is dose-dependent. The increase in LRP mRNA levels with rosiglitazone was shown to result from direct binding of PPARγ-RXRα heterodimers to the PPRE identified in the promoter.

As anticipated, there was an inverse correlation between the amount of ligand required and the affinity of the ligand for PPARγ (36, 60). Rosiglitazone, the most potent ligand used, was found to have a maximal effect at ~500 nM in SW872 cells, although there was significant up-regulation of LRP at 50 nM, a concentration much closer to the reported Kd of 40 nM (36). Concentrations of 750 nM or higher did not alter LRP mRNA abundance (data not shown) or promoter activity of pGL3-LRP in SW872 cells. In differentiated human adipocytes, LRP mRNA levels were up-regulated by rosiglitazone in a dose-dependent manner at concentrations up to 1 μM, and the decreased efficacy was observed only at a concentration of 2 μM. It has been suggested that rosiglitazone might act as a partial antagonist at these high concentrations (61). In addition, activation of PPARγ at the AP2 domain enhances its degradation (62), which would explain the reduced efficacy of rosiglitazone at higher concentrations (Figs. 5 and 7A). In both cell types, LRP expression and function were increased by 1.5–2-fold, similar to that reported for other genes containing a PPRE in the promoter region (57, 63, 64). For example, the PPARγ agonist fenofibrate increases apoA-I expression by 1.5–2.0-fold; yet this translates into a clinically important high density lipoprotein raising effect (65).

Previous studies have examined the effect of sterols on LRP transcription and reported that LRP, unlike the low density lipoprotein receptor and other members of this receptor family, was not down-regulated by sterols (27). Further study identified a sequence corresponding to a sterol response element in the 5′-untranslated region of the LRP transcript (28), which appears inactive because LRP does not show any response to sterols. This is in agreement with our data demonstrating that LRP mRNA levels are similar when cells are cultured in CS as compared with lipoprotein-deficient fetal calf serum.

PPARγ is not expressed in preadipocytes and is turned on during differentiation prior to the expression LRP3 and other adipocyte genes (66). In addition to LRP, many adipocyte proteins important in triglyceride accumulation, such as lipoprotein lipase, fatty acid transport protein-1, acyl-CoA synthase, CD36, and apoA2 (67–70), all contain at least one PPRE in their 5′-flanking sequences and are all regulated by PPARγ. Adipocyte LRP functions in chylomicron remnant cholesterol clearance both in vitro and in vivo (24). We have recently demonstrated that LRP also plays a role in the selective uptake of high density lipoprotein–CE by human adipocytes (71). Thus, co-ordinate regulation of LRP and fatty acid transporters may be a mechanism by which adipocytes can regulate cholesterol.

\(^3\) F. Benoist and R. McPherson, unpublished data.
uptake to match TG synthesis during differentiation and maturation of the preadipocytes into fat cells.

LRP is expressed in various tissues (23), and its function in each cell type differs widely. Thus, the regulation of LRP gene expression and function by insulin-sensitizing agents of the glitazone class or by fibrates could have considerable clinical importance. Three PPAR subtypes (α, γ, and δ) have been identified. Within a given species, the DNA-binding domains of the three PPARs are 80% identical (slightly higher between PPARγ and PPARα) (74). However, their ligand-binding domains only share ~65% homology (73). It has been demonstrated that PPARα and PPARγ bind the same core DR-1 PPRE (74). The distinct tissue-specific expression of the different PPARs as well as their specific activation by ligands suggests a mechanism for highly tissue-specific regulation of genes with a PPRE, including LRP. PPARs are predominantly expressed in liver, heart, kidney, intestinal mucosa, and brown adipose tissue (33). These are all sites with high fatty acid catabolism and peroxisomal metabolism. PPARδ is ubiquitously expressed, whereas PPARγ is expressed mainly in adipose tissue, skeletal muscle, heart, brain, vascular smooth muscle cells, and macrophages (31, 33, 75). PPARα is relatively adipose-specific although in animal models of obesity, hepatic expression of PPARα has been documented (76). The transcriptional activity of the PPAR subtypes is enhanced by a multitude of compounds. Prostaglandin J2 is a natural ligand for PPARγ, whereas thiazolidinediones (e.g. BRL49653 or rosiglitazone) are synthetic ligands for PPARγ (36) and do not activate PPARα. PPARα ligands include 8β-hydroxyicosapentaenoic acid, leukotriene B4, and the synthetic fibitres. Long chain fatty acids are less specific ligands recognizing all PPAR subtypes (33, 34). By administration of selective PPAR ligands, it may be possible to regulate the expression of LRP in a tissue-specific manner.

We have demonstrated that adipocyte LRP expression and function is up-regulated by rosiglitazone, a widely used insulin-sensitizing agent. Rosiglitazone has been shown to enhance plasma triglyceride clearance by an unknown mechanism (75), and this effect has been primarily attributed to an increase in lipoprotein clearance by rosiglitazone, a widely used insulin-sensitizing agent, although in animal models of obesity, hepatic expression of LRP is increased (74). The distinct tissue-specific expression of the different PPARs as well as their specific activation by ligands suggests a mechanism for highly tissue-specific regulation of genes with a PPRE, including LRP. PPARs are predominantly expressed in liver, heart, kidney, intestinal mucosa, and brown adipose tissue (33). These are all sites with high fatty acid catabolism and peroxisomal metabolism. PPARδ is ubiquitously expressed, whereas PPARγ is expressed mainly in adipose tissue, skeletal muscle, heart, brain, vascular smooth muscle cells, and macrophages (31, 33, 75). PPARα is relatively adipose-specific although in animal models of obesity, hepatic expression of PPARα has been documented (76). The transcriptional activity of the PPAR subtypes is enhanced by a multitude of compounds. Prostaglandin J2 is a natural ligand for PPARγ, whereas thiazolidinediones (e.g. BRL49653 or rosiglitazone) are synthetic ligands for PPARγ (36) and do not activate PPARα. PPARα ligands include 8β-hydroxyicosapentaenoic acid, leukotriene B4, and the synthetic fibitres. Long chain fatty acids are less specific ligands recognizing all PPAR subtypes (33, 34). By administration of selective PPAR ligands, it may be possible to regulate the expression of LRP in a tissue-specific manner.

We have demonstrated that adipocyte LRP expression and function is up-regulated by rosiglitazone, a widely used insulin-sensitizing agent. Rosiglitazone has been shown to enhance plasma triglyceride clearance by an unknown mechanism (75), which we hypothesize may involve adipocyte LRP. Fibrates substantially decrease plasma triglyceride levels, and this effect has been primarily attributed to an increase in lipoprotein lipase activity and decreased expression of apoCIII (33, 34, 75). We propose that a PPARα-mediated increase in hepatic LRP expression may explain part of the triglyceride-lowering effects of fibric acid derivatives. In ongoing studies, we are investigating PPAR-mediated regulation of LRP expression and function in other cell types, including hepatocytes and neuronal cells. The involvement of LRP in a variety of important metabolic processes including amyloid precursor protein processing, β-amloid clearance, lipoprotein metabolism, cellular remodeling, and protease complex clearance suggest a possible therapeutic role for LRP up-regulation by PPAR ligands in a variety of disease states.

Acknowledgments—We are grateful to Dr. Steven Smith (Smith Kline Beecham) for provision of rosiglitazone, Dr. Michael Saunders (Glaxo Wellcome Inc.) for RXRα, and Dr. Bruce Spiegelman for PPARγ. Thanks to Dr. Xiaohui Zha and members of the Lipoprotein Group for technical advice and critical review of this manuscript.
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