Biochemical Pharmacology 82 (2011) 1950–1962

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Very low density lipoprotein receptor promotes adipocyte differentiation and mediates the proadipogenic effect of peroxisome proliferator-activated receptor gamma agonists

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ARTICLE INFO

Article history:
Received 6 July 2011
Accepted 2 September 2011
Available online 9 September 2011

Keywords:
Very low density lipoprotein receptor
Adipocyte
Adipogenesis
15-Deoxy-delta^{12,14}-prostaglandin J_2
Peroxisome proliferator-activated receptor gamma

ABSTRACT

Very low density lipoprotein receptor (VLDLR) is a member of the low density receptor family, expressed mostly in adipose tissue, heart, and skeletal muscles. VLDLR binds apolipoprotein-E-triglyceride-rich lipoproteins and plays a key role in lipid metabolism. In adipocytes, VLDLR expression increases with differentiation but it is not known whether it plays a role in the adipogenesis. Here we report that VLDLR expression in 3T3-L1 adipocytes is upregulated by PPARγ agonist 15-deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2) in dose- and time-dependant manners. Knockdown of peroxisome proliferator-activated receptor-γ (PPARγ) with siRNA abolished pioglitazone- and 15d-PGJ_2-induced VLDLR expression and simultaneously reduced VLDL uptake in adipocytes. In addition, PPARγ-agonist treatment of control mouse adipocytes (*vldlr*$^{+/+}$) enhanced adipogenesis and VLDL uptake concurrently with the induction of VLDLR expression. However, *vldlr* deficiency (*vldlr*$^{-/-}$) significantly blunted the proadipogenic effects of PPARγ agonists. Sequence analysis revealed the presence of a putative PPARγ responsive sequence (PPRE) within the *vldlr* promoter, which is responsive to natural (15d-PGJ_2) and synthetic (pioglitazone) PPARγ agonists. Reporter gene assays using serial deletion of the 5′-flanking region showed that this putative PPRE site induced promoter transactivation, while a site-targeted mutation abolished transactivation. Moreover, electrophoresis mobility shift assay (EMSA) and chromatic immunoprecipitation (ChIP) assays showed the specific binding of PPARγ to the PPRE sequence.

Together, these results support a crucial function for VLDLR in adipocyte differentiation and mediation of the proadipogenic effect of PPARγ.

1. Introduction

Very low density lipoprotein receptor (VLDLR) is a member of the low density lipoprotein receptor (LDLR) family [1]. In contrast to LDLR, VLDLR is highly expressed in adipose tissue, heart, and skeletal muscle [2]. It binds apoprotein-E-containing triacylglycerol-rich (TG-rich) lipoproteins including very low density-lipoprotein (VLDL), beta-migrating VLDL and intermediate-density lipoprotein (IDL) [1,3]. Previous investigations [4–6] reported an increase in plasma triglycerides in *vldlr* null (*vldlr*$^{-/-}$) mice postprandial and after long fasting, and suggested a link between VLDLR expression and hyperlipidemia. Moreover, there is increasing evidence to indicate that VLDLR controls TG-rich lipoprotein uptake and TG deposition in adipocytes [6]. For example, expression of VLDLR is virtually absent in preadipocytes, the non-differentiated progenitor cell of adipocytes [6], and increases dramatically in mature adipocytes [6]. These results are consistent with *in vivo* findings showing a delayed clearance of plasma TG-rich lipoproteins [6] and lower adiposity in *vldlr*$^{-/-}$ mice [5,7].

Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated nuclear receptor with a key role in the initiation of adipocyte differentiation, lipogenesis, and maintenance of mature adipocyte phenotype [8,10]. Agonists of PPARγ include the pharmacological agents thiazolidinediones (TZDs) and natural molecules, especially polyunsaturated fatty acids (PUFAs) and their metabolite 15-deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2) [11,12]. After ligand-dependent activation, PPARγ heterodimerizes with retinoid X receptors (RXRs), binds specific sequence called

Abbreviations: PPARγ, peroxisome proliferator-activated receptor-γ; VLDLR, very low density lipoprotein receptor; L-FABP, liver fatty acid binding protein; RXR, retinoid X receptor; FATP, fatty acid transport protein; aP2, adipocyte protein 2; ACRP30, adiponectin; HSL, heparin sensitive lipase; LPL, lipoprotein lipase; CD36, fatty acid transporter/cluster of differentiation 36; TZD, thiazolidinedione; PIO, pioglitazone; 15d-PGJ_2, 15-deoxy-delta-12,14-prostaglandin J2; DMSO, dimethyl sulfoxide.

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doi:10.1016/j.bcp.2011.09.003

PPAR response elements (PPREs), and induces the expression of target genes [8,9]. Typically, PPRE is a direct repeat of two-hexameric nucleotide (AGGTCA) spaced by one nucleotide (DR-1) located within the 5′-flanking region of target genes [8]. Recent investigations identified new PPREs in numerous target genes [8]. In adipocytes, PPARγ controls the expression and function of genes implicated in intra- and extracellular lipid metabolism including lipoprotein lipase (LPL) [13], adipocyte fatty acid binding protein aP2 [14], low density lipoprotein receptor-related protein (LRP1) [15], Fatty acid translocase (FAT/CD36) [16], fatty acid transport protein (FATP) [17], and heparin sensitive lipase (HSL) [18]. Discovering new PPARγ target genes will help to understand the role of genes involved in adipocyte differentiation and uncover new potential targets for pharmacological and nutritional therapies.

Recently, we reported that VLDLR expression is upregulated by synthetic PPARγ agonist pioglitazone in adipocyte culture and *in vivo* in mouse adipose tissue [6]. 15d-PGJ_2, a naturally occurring metabolite of arachidonic acid, is also a potent activator of PPARγ and strong inducer of adipocyte differentiation and lipid deposition [11,12]. PPARγ ligands such as the TZDs and 15d-PGJ_2 have been shown to elicit a diverse range of shared and distinct biological effects, some of which appear to be mediated through pathways that are independent of PPARγ [19]. These studies were designed to examine the effects of PPARγ agonists on VLDLR-dependant adipogenesis and VLDL uptake in adipocytes.

2. Material and methods

2.1. Materials

Pioglitazone (PIO) was obtained from Cayman Chemical (Ann Arbor, MI). Bovine insulin, dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), and 15-deoxy-delta^{2,14}-prostaglandin J_2 were purchased from Sigma–Aldrich (St. Louis, MO). Free fatty acid bovine serum albumin (BSA) was from BioPharm laboratories, LLC (Alpine, UT). 3T3-L1 cells were purchased from the American Tissue Culture Collection (Rockville, MD). Anti-PPARγ antibody and mouse IgG were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibodies for VLDLR and β actin were purchased from R&D systems (Minneapolis, MN) and Sigma, respectively. Expression plasmid mouse pSV Sport PPARγ2 and human pSV Sport RXRα were obtained from Addgene Inc. (Cambridge, MA). The pSV-SPORT and pRL-CMV vectors were from Promega (Madison, WI) and Invitrogen (Carlsbad, California), respectively. Small interfering RNA (siRNA) against PPARγ and control scrambled siRNA were obtained from Dharmacon, Inc. (Lafayette, CO).

2.2. Cell culture and differentiation

3T3-L1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) with 10% delipidated fetal calf serum (FCS). Differentiation of 3T3-L1 to mature adipocytes was performed as described previously [6]. Briefly, after 2 days of confluence, cells were induced by incubation in DMEM with 10% FCS and differentiation cocktail containing 0.5 mM IBMX, 1 μg/ml dexamethasone, and 10 μg/ml insulin for 48 h. Then, cells were incubated in DMEM with 10% FCS and 10 μg/ml insulin for another 7 days. Medium was replaced every 2 days.

2.3. 15d-PGJ_2 treatment and VLDLR expression

Fully differentiated 3T3-L1 cells were incubated overnight with DMEM plus 0.2% serum-free BSA. To examine the effects of 15d-PGJ_2 on VLDLR expression, adipocytes were cultured with 15d-PGJ_2 for various doses (0.1, 1, and 10 μM) and times (12, 24, and 48 h). At the end of the experiments, cells were harvested in assay buffer as described previously [6], and used for western blot protein analysis or mRNA extraction and quantitative polymerase chain reaction (qPCR).

2.4. RNA interference and PPARγ silencing

RNA interference by siRNA was performed according to the manufacturer instructions and as described by Kershaw et al. [20]. Briefly, 3T3-L1 adipocytes on day 7 of differentiation were detached from culture dishes with 0.25% trypsin (Invitrogen) and 0.5 mg/ml collagenase D (Roche Diagnostics), washed twice, and re-suspended in phosphate-buffered saline (PBS). Control (Scrambled non-interfering pool; Dharmacon) or murine PPARγ-specific (5′-CAACAGGCCTCATGAAGAATT; Dharmacon) siRNAs were delivered into adipocytes (2 nmol of each siRNA/1 million cells) by electroporation. Adipocytes were then mixed with DMEM containing 10% FBS and reseeded onto multiwell plates. Then 48 h later, corresponding to day 9 of differentiation, cells were collected for determination of mRNA and protein expression. The conditions of differentiation and transfection were chosen based on previous studies [20]. The choice of transfection of adipocytes on day 7 and analysis of gene expression on day 9 of differentiation was selected on the basis of prior optimization experiments demonstrating effectiveness of this method for siRNA-mediated gene knockdown in adipocytes at this stage of differentiation [20]. The mRNA abundance of PPARγ was assessed by qPCR using specific previously tested primers [6]. PPARγ and VLDLR protein levels were examined by western blots.

2.5. Immunofluorescence analyses

3T3-L1 cells were differentiated and transfected with siControl or siPPARγ RNA on coverslips and then fixed with 3.7% formaldehyde at room temperature for 45 min and permeabilized at room temperature with 0.1% Triton X-100. Subsequently, coverslips were incubated with primary anti-VLDLR antibody (1:250 dilution) in PBS containing 2% BSA (2 h), followed by 1 h of incubation with secondary Alexa Fluor^{488} secondary antibody (1:1000; Santa Cruz). Coverslips were mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA) and images were acquired with a fluorescence microscope (Axioskop 40/40 FL; Zeiss, Goettingen, Germany).

2.6. Protein analysis by western blot

Measurement of protein concentration in cell lysate and western blot analysis were performed as described elsewhere [6]. Briefly, 60–80 μg of proteins were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes (Amersham, Piscataway, NJ) and blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Triton X-100. Proteins were probed with primary antibodies specific for VLDLR (R&D systems), PPARγ (Santa Cruz Inc.), and β actin (Sigma) by incubation with horseradish peroxidase-conjugated secondary antibody. Blots were visualized with an ECL Plus (Pierce, Rockford, IL), and the membrane was exposed to Hyperfilm. Densitometric units were determined for each band by means of a ChemiDoc apparatus and Quantity One software (Bio-Rad, Hercules, CA). Relative density for each optic density was optimized to β actin density.

2.7. Analysis of gene expression by quantitative PCR qPCR

Total RNA was extracted using a Total RNA fatty and fibrous tissue pack (Bio-Rad) according to the manufacturer's protocol.

Table 1
Primers used for real time RT-PCR.

Gene	Forward	Reverse	Genbank no.
VLDLR	5′-GATGATGACGCAGACTGTTC-3′	5′-CACTGGATCTCACTGGTAGG-3′	NM_013703
PPARγ	5′-ATGGTTGACACAGAGATGC-3′	5′-GAATGCGAGTGGTCTTCC-3′	NM_011146
aP2	5′-GAACCTGGAAGCTTGTC-3′	5′-CGTGACTTCCACAAGAGT-3′	NM_024406
ACRP30	5′-ACTGCAACATTCCGGGACTCTACT-3′	5′-AGAGAACGGCCTTGTCCTTCTTGA-3′	NM_009605
β actin	5′-AGGGAAATCGTGCGTGACAT-3′	5′-CGTTGCCAATAGTGATGACC-3′	NM_007393

Complementary DNA was synthesized from 1 μg of total RNA with iScript reverse transcriptase (Bio-Rad). Reactions of qPCR were performed using SYBR Green Supermix (Bio-Rad) with iTaqDNA polymerase (Bio-Rad) on Bio-Rad's IQ5 thermocylcer, as described previously [21]. Oligonucleotides (Table 1) were designed, optimized, and efficiently tested before use [6,21]. For each primer set, annealing temperature was optimized and formation of a solitary product was confirmed through melt curve analysis in each PCR run. The PCR program includes 36 cycles of heat, denaturing at 95 °C for 30 s and annealing at 52–62 °C for 30 s, followed by the melt curve cycle. The qPCR data were obtained as C_T values, where C_T was defined as the threshold cycle of PCR at which products amplify exponentially. As an internal control, β actin expression was measured in parallel. The difference in the C_T values (ΔC_T) was derived from the specific gene tested and C_T of the control gene (β actin) according to the equation $2^{C_T \text{ actin} - C_T \text{ target gene}}$ [6].

2.8. Effects of pioglitazone and 15d-PGJ$_2$ on VLDL uptake in adipocytes

In order to trace the uptakes of both VLDL-derived fatty acids and remnant particles, VLDL were double labeled with ^{3}H as a tracer for lipids and ^{125}Iodine (^{125}I) as a tracer for proteins. Mouse VLDL particles were labeled with ^{3}H–TG palmitate (TG) and iodine (^{125}I-proteins) as described previously [6]. Briefly, mice were injected intravenously into the tail vein with 100 μCi of [^{3}H] palmitate complexed with BSA as described elsewhere [6]. Then, blood was collected 60 min after the injection, and serum samples were ultracentrifuged to obtain the VLDL fraction [6]. Lipids were separated by thin layer chromatography (TLC) as described previously [6,22], and VLDL-bound radioactivity was counted to ensure that the bulk of the label was in the TG fraction. Then, ^{3}H–TG labeled VLDL were iodinated in vitro with ^{125}I as described elsewhere [6,22]. Double labeled VLDL were extensively dialyzed and used for the in vitro incubation within the following 48 h. VLDL uptake was examined in 3T3-L1 adipocytes with or without PPARγ siRNA and treated with pioglitazone, 15d-PGJ$_2$ or vehicle (DMSO). The objective was to test if these agonists exert an effect on VLDL uptake and whether it requires PPARγ expression. Accordingly, mature 3T3-L1 adipocytes (day 7 of differentiation) were transfected with control scrambled siRNA (siControl) or PPARγ siRNA (siPPARγ) and then preincubated with pioglitazone (10 μM), 15d-PGJ$_2$ (1 μM) or DMSO for 24 h prior to testing VLDL uptake. Incubation with double labeled VLDL was performed on day 9 and for 4 h as described elsewhere [6]. Then, cells were extensively washed with PBS and harvested in lysis buffer. Aliquots (20 μl) were used to measure ^{125}I counts in a gamma counter [6]. Additional aliquots were used to extract lipids and count ^{3}H in a scintillation counter as described previously [6].

2.9. Isolation and differentiation of primary mouse preadipocytes

Preadipocytes were prepared from epididymal fat of wild type (WT) and VLDLR null (*vldlr*$^{-/-}$) mice as described previously [6]. Briefly, 1–2 g of white adipose tissue was minced and digested in Hank's buffered salt solution with 2% BSA containing 1 mg/ml collagenase type I (Sigma–Aldrich) at 37 °C with shaking for 60 min. The digested material was filtered and centrifuged at 500 × *g* for 10 min. The resulting pellets were resuspended in erythrocyte lysis buffer (Lonza, Wakersville, MD). After a wash with PBS, the cells were suspended in DMEM-F12 with 10% delipidated FCS and used for cell culture at passage 3 to eliminate non-preadipocyte cell contamination [6]. For differentiation of primary mouse preadipocytes, confluent cells were differentiated in DMEM-F12 with 10% FCS, 0.5 mM IBMX, 0.1 μM corticosterone, and 10 μg/ml insulin for 4 days and then maintained in culture in medium DMEM-F12 with 10% FCS, 10 μg/ml insulin [6]. Culture medium was changed every 3 days and differentiated adipocytes reached maturity after approximately 10 days. At maturity, experiments were performed to determine the effects of pioglitazone, 15d-PGJ$_2$ and VLDLR expression on VLDL uptake.

2.10. Assessment of adipogenesis

The objective of these experiments was to test the effects of pioglitazone and 15d-PGJ$_2$ and VLDLR on adipogenesis in the presence of a source of exogenous lipids, and VLDL particles are the natural ligands for VLDLR [23]. Thus, we added VLDL particles and tested whether induction of adipogenesis by 15d-PGJ$_2$ was affected by VLDLR expression according to our previously published procedure [6]. VLDL particles were isolated from mouse plasma by ultracentrifugation as described earlier [6]. Preadipocytes of *vldlr*$^{+/+}$ and *vldlr*$^{-/-}$ mice were differentiated in the presence of VLDL (100 μg of triglycerides per well) and without or with the addition of 15d-PGJ$_2$ at a final concentration of 1 μM. Oil red staining was examined in preadipocytes (before differentiation) and mature adipocytes (day 10). On the indicated day, cells were stained with Oil red O as described previously [6]. In brief, cell monolayers were washed three times with PBS and fixed with 10% formalin in PBS (pH 7.4) for 1 h. Then, cells were stained with freshly prepared 0.3% Oil red O in 60% isopropanol for 30 min. After being rinsed twice with distilled water, stained cells were viewed and photographed using a microscope with phase contrast optics and a digital camera. For quantification, TG content in adipocytes was also tested enzymatically as described previously [6]. In addition, adipogenesis was evaluated by testing the expression of aP$_2$ and adiponectin genes in mature adipocytes. Abundance of mRNA was examined with qPCR using specific primers (Table 1) and normalized to β actin [6].

2.11. VLDL uptake in *vldlr*$^{+/+}$ and *vldlr*$^{-/-}$ adipocytes

On day 10 of differentiation, double labeled VLDL particles were added to *vldlr*$^{+/+}$ and *vldlr*$^{-/-}$ adipocytes for 4 h and ^{3}H and ^{125}I uptake was measured as described above for 3T3-L1 adipocytes.

2.12. TESS computer program search

A computerized search using the TESS program (http://www.cbil.upenn.edu/cgi-bin/tess/tess) [24] for putative PPRE was performed using the 2 kb mouse *vldlr* promoter sequence (GenBank Nucleotides: NM_013703) as a template. The consensus sequence TGACCT X TGACCT (PPRE) was used to search sites in the promoter with 75% minimum core similarity

2.13. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to previously published procedures [25]. Differentiated 3T3-L1 adipocytes were cultured in the presence of 10 μM pioglitazone for 24 h. To cross-link proteins to DNA, culture medium containing formaldehyde (final concentration 1%) was added to the differentiated adipocytes and incubated for 10 min at room temperature. Then, a final concentration of 0.125 M glycine was added to stop the reaction and cells were scraped and collected by centrifugation at 700 × *g* for 5 min at 4 °C. The cell pellets were treated with SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1) containing protease inhibitors. Aliquots of cell lysates were sonicated to shear DNA and cellular debris was removed by centrifugation at 13,000 × *g* for 15 min at 4 °C. Chromatin aliquots were pre-cleared with 60 μl of 50% protein A/G agarose suspension (Santa Cruz Biotechnology Inc.). Samples were then incubated with either monoclonal anti-PPARγ antibody or mouse IgG (as a background control) (Santa Cruz Biotechnology Inc.) for 12 h at 4 °C with gentle mixing. Immune-complexes were mixed with 80 μl of 50% protein A/G agarose suspension followed by incubation for 1 h at 4 °C with gentle mixing. Beads were collected by brief centrifugation and the immunocomplexes were eluted by freshly prepared elution buffer (100 mM $NaHCO_3$, 1% SDS). Chromatin was then de-cross-linked for 5 h at 65 °C. After treatment with proteinase K, DNA was purified with Qiaquick PCR Purification Kits (Qiagen, Valencia, CA) and finally eluted in 50 μl of Tris EDTA (TE) buffer. An aliquot (2 μl) of each sample was subjected to PCR analysis using Taq DNA polymerase (35 cycles). In order to determine regions of specific binding for PPARγ, a pair of primers (F1/R1) covering the putative PPRE in the *vldlr* promoter was designed and used for the ChIP assay (forward 5′-TTGAGTGTAGAGCCATAACTTGG-3′; reverse 5′-TGTATAATCAGAGGGTAGGAGATGG-3′). Liver fatty acid binding protein (L-FABP) is a known target gene for PPARγ, and a binding site within its promoter has been identified [26]. Therefore, a pair of primers covering the known PPRE site of the L-FABP promoter was used as a positive control for these experiments. These primer sequences are: forward 5′-GCATATGTGCATTGCTGGAG-3′; reverse 5′-AGGTCACCCACTGTTGCC-3′. Also a pair of primers used as a negative control were designed to cover a region of the *vldlr* promoter located 1500 bp far away from the putative PPRE forward 5′-CTGAG CTGCTGGGCTGAG-3′; reverse 5′-TGCAACTTGCACACTCTACTCC-3′). In addition, a pair of primers designed from mouse β actin gene promoter was used as negative primer control (forward 5′-AAGTTG AACCCCAACACACC-3′; reverse 5′-ATGAAGAGTTTTGGCGATGG-3′). Three experiments of ChIP assays were performed with triplicates for each condition.

2.14. Plasmid construction

Genomic fragments of the *vldlr* promoter, created by digestion with selected restriction enzymes, were cloned into the upstream region of luciferase cDNA in the pGL3-basic reporter vector (Promega, City, State) as described by Jitrapakdee et al. [25]. Briefly, the 5′-upstream region of the promoter of the mouse *vldlr* gene was amplified by Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) using mouse genomic DNA as a template. The following two primers containing Kpn I/Xho I sites were applied for the amplification: forward primer: 5′-AAACCGAGACTCTTGAGTGTAGAGCCATAACTTGG-3′, and reverse primer: 5′-AAACCGCTCGAGTGCAACTTGCACACTCTACTCC-3′, which spans the −1604 to +412 of the 5′-upstream region of the promoter and 5′-UTR of the mouse *vldlr* gene. The PCR product was cloned into the pCR2.1 TA-TOPO cloning vector (Invitrogen) and a nested family of 5′ deletion clones was generated by the same strategy. The five promoter constructs used in this analysis of promoter function were: −1604Luc (sequence: −1604/+412), −1291Luc (sequence: −1291/+412), −886Luc (sequence: −886/+412), −549Luc (sequence: −549/+412), and −220Luc (sequence: −220/+412) (Fig. 2A). Numbers in the parentheses were cited according to the rank of the last nucleotide position in the DNA upstream of the start. To verify that the correct sequences were present, all constructs were sequenced by the DNA sequence core of Vanderbilt University.

To test the activity of the presumed PPRE site, a point mutation was introduced in the promoter sequence using Stratagene's Site-Directed Mutagenesis kit (La Jolla, CA). Compared to the initial sequence, six base pairs of the PPRE were mutated according to PPAR algorithm [27]. Mutated sequence 5′-TTATTGACTTC**G**C**A**-**G**A**C**GT**TA**CCCACCCATTCATCAGG-3′ was verified by DNA sequencing, and mutated bases are indicated in bold and underlined.

2.15. Transfection and transactivation studies

3T3-L1 preadipocytes less than 6 passages old were plated in 24-well dishes and grown to 75% confluence in media with 10% FCS [6]. Transient transfection was performed using LipofectAMINE plus (Invitrogen) according to the manufacturer's instruction. Briefly, 300 ng/well of *vldlr* luciferase promoter constructs and 30 ng/well of pRL-CMS renilla vector were co-transfected into preadipocytes. In addition, 300 ng/well pSV Sport PPARγ2 and pSV Sport RXRα were co-transfected with the promoter constructs; and a similar amount of empty vectors was used for negative control (Mock). Transfection was allowed to proceed for 6 h. Then, PPARγ agonist pioglitazone was added for treated wells, while 0.01% DMSO was added in control wells. The luciferase activity was read 24 h later in the Monolight 3010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Activity results of wells containing PPARγ activators and the *vldlr* reporter were calculated as a fold increase of luciferase activity compared with the control group. The renilla luciferase signal served as a control for transfection efficiency in the data normalization. Experiments were performed at least 3 times with triplicates for each condition.

2.16. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from 3T3-L1 preadipocytes and fully differentiated adipocytes treated with pioglitazone for 24 h using Nuclear Extract Kit (Panomics, Freemont, CA). EMSA was performed using EMSA "Gel Shift" System (Panomics) according to manufacturer's instruction and as described elsewhere [28,29]. The sequences of duplex oligonucleotides used for EMSA are (only sense strand shown): mouse wild type *vldlr* PPRE (WT): 5′-TTATTGACT**TCACCTATGTCCC**CCACCCATT-3′ mutant PPRE (MUT): 5′-TTATTGACT**TC**G**C**A**G**A**C**G**TTAC**CCACCCATT-3′. Six base pairs of the initial PPRE were mutated according to PPAR algorithm [27]. The biotin-labeled single strand oligonucleotides were annealed to form double-strand segments according to the manufacturer's protocol (Panomics). EMSA assays were carried out using 10 μg of nuclear extracts. DNA-protein complexes were resolved by electrophoresis through 6% polyacrylamide gels at 4 °C and transferred onto the positive charged nylon membrane (Pall Corporation, Ann Arbor, MI). Then the biotinylated nucleotides were detected using horseradish peroxidase-conjugated streptavidin and Chemiluminescent Reagent Plus (Panomics). For the competition assay, 20-fold and 200-fold amounts of wild type and mutant unlabeled double-stranded DNAs were added into binding reaction. For the supershift assay, the reaction mixture was incubated for 1 h at 20 °C with 2 μg of mouse monoclonal anti-PPARγ antibody (Santa Cruz Biotechnology).

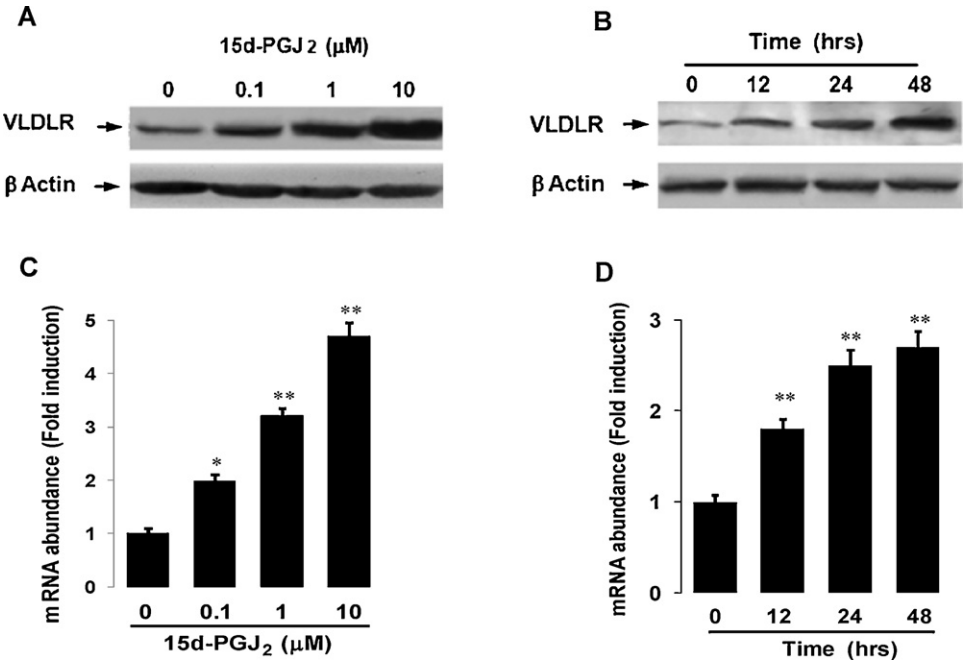


Fig. 1. Dose- and time-dependent effects of 15-deoxy-Delta^{12,14}-prostaglandin J_2 (15d-PGJ_2 on VLDLR expression in 3T3-L1 adipocytes. Representative western blots of VLDLR and β actin (A and B) and mRNA abundance of VLDLR (C and D) in 3T3-L1 adipocytes treated with 15d-PGJ_2 at indicated time and doses as described in Section 2. Data are means ± SE (n = 3 experiments performed in triplicate). Cell treatments and western blot and qPCR analyses were described in Section 2. Statistical differences between treated and untreated adipocytes are indicated with asterisks: $^{*}P < 0.05$, $^{**}P < 0.001$.

2.17. Statistics

Results are expressed as means ± SEM. Statistical differences between groups were assessed by the Student's *t*-test, or by one-way analysis of variance (ANOVA) followed by Mann–Whitney *U*-test for multiple groups.

3. Results

3.1. 15d-PGJ_2 up-regulates vldlr expression in 3T3-L1 adipocytes

To test the possibility of regulation by 15d-PGJ_2, VLDLR expression was investigated in 3T3-L1 adipocytes treated with 15d-PGJ_2 at various doses and periods (Fig. 1). 15d-PGJ_2 increased VLDLR protein levels in a dose-dependent manner (Fig. 1A) with about 7-fold induction ($P < 0.001$) at the maximum dose (mean ratios of VLDLR-to-β actin were 0.32 ± 0.05, 0.82 ± 0.11, 1.23 ± 0.15, and 2.53 ± 0.45 for 0, 0.1, 1, and 10 μM of 15d-PGJ_2). Induction of VLDL expression by 15d-PGJ_2 was also time-dependent, as shown by the protein levels (Fig. 1B). Similarly, 15d-PGJ_2 treatments significantly induced mRNA abundance of VLDLR in a dose- (Fig. 1C) and time- (Fig. 1D) dependent manners suggesting that 15d-PGJ_2 acted at the transcriptional level to upregulate VLDLR expression.

3.2. 15d-PGJ_2-induction of vldlr expression is dependent on PPARγ

Previous investigations have demonstrated that 15d-PGJ_2 acts with PPARγ dependent and independent mechanisms [19], while pioglitazone is a specific PPARγ agonist [9]. To determine whether PPARγ mediates 15d-PGJ_2 regulation of VLDLR expression, experiments were performed in adipocytes with suppressed PPARγ using specific siRNA procedures (Fig. 2). First, PPARγ siRNA strongly suppressed PPARγ mRNA (Fig. 2A). Western blot analysis of the protein levels showed a significant decrease ($P < 0.001$) in PPARγ protein after siRNA (1 ± 0.019 siControl versus 0.20 ± 0.01 siPPARγ) and VLDLR protein levels (1 ± 0.02 *vs* 0.30 ± 0.01) (Fig. 2B). Suppression of VLDLR protein levels in siPPARγ treated adipocytes was also confirmed by immunofluorescence imaging (Fig. 2C). These results show that PPARγ expression is required to mediate 15d-PGJ_2 induction of VLDLR expression.

3.3. Pioglitazone and 15d-PGJ_2 induction of vldlr expression increased VLDL uptake in 3T3-L1 adipocytes

To test the effects of PPARγ agonists and VLDLR expression on VLDL metabolism in siRNA treated adipocytes, uptake experiments were performed using double labeled VLDL. [^{3}H, ^{125}I]-double-labeled VLDL were added to adipocyte culture to examine whether uptake of VLDL-derived lipids occurred through whole remnant particle uptake or as free fatty acid after lipolysis. Uptake of both ^{3}H and ^{125}I was significantly increased (+82%, $P < 0.01$) in adipocytes expressing PPARγ and treated with 15d-PGJ_2 (Fig. 2D). In addition, these results show that silencing PPARγ (siPPARγ) without 15d-PGJ_2 treatment strongly reduced the uptake of ^{3}H and ^{125}I (5–6-fold). Combined treatments of PPARγ siRNA and 15d-PGJ_2 resulted in a modest increase of the uptake of both labels (approximately 2-fold compared to adipocytes treated only with siPPARγ) (Fig. 2D). Pioglitazone treatment also induced VLDL uptake in adipocytes expressing PPARγ (Fig. 2E). Moreover, the action of pioglitazone was blunted by PPARγ silencing but was not completely eliminated. These results show that 15d-PGJ_2 and pioglitazone enhanced VLDL uptake and their action required normal expression of PPARγ and VLDLR.

3.4. 15d-PGJ_2 induction of adipogenesis is dependent of vldlr expression

Previous studies have reported that PPARγ agonists enhance the rate of adipocyte differentiation [11,12]. Hence, we questioned whether VLDLR mediates the proadipogenic effects of 15d-PGJ_2. Preadipocytes isolated from adipose tissue of *vldl*$^{+/+}$ and *vldlr*$^{-/-}$ mice were cultured and differentiated in vitro. Lipid accumulation was estimated by Oil red O staining of adipocyte differentiation in

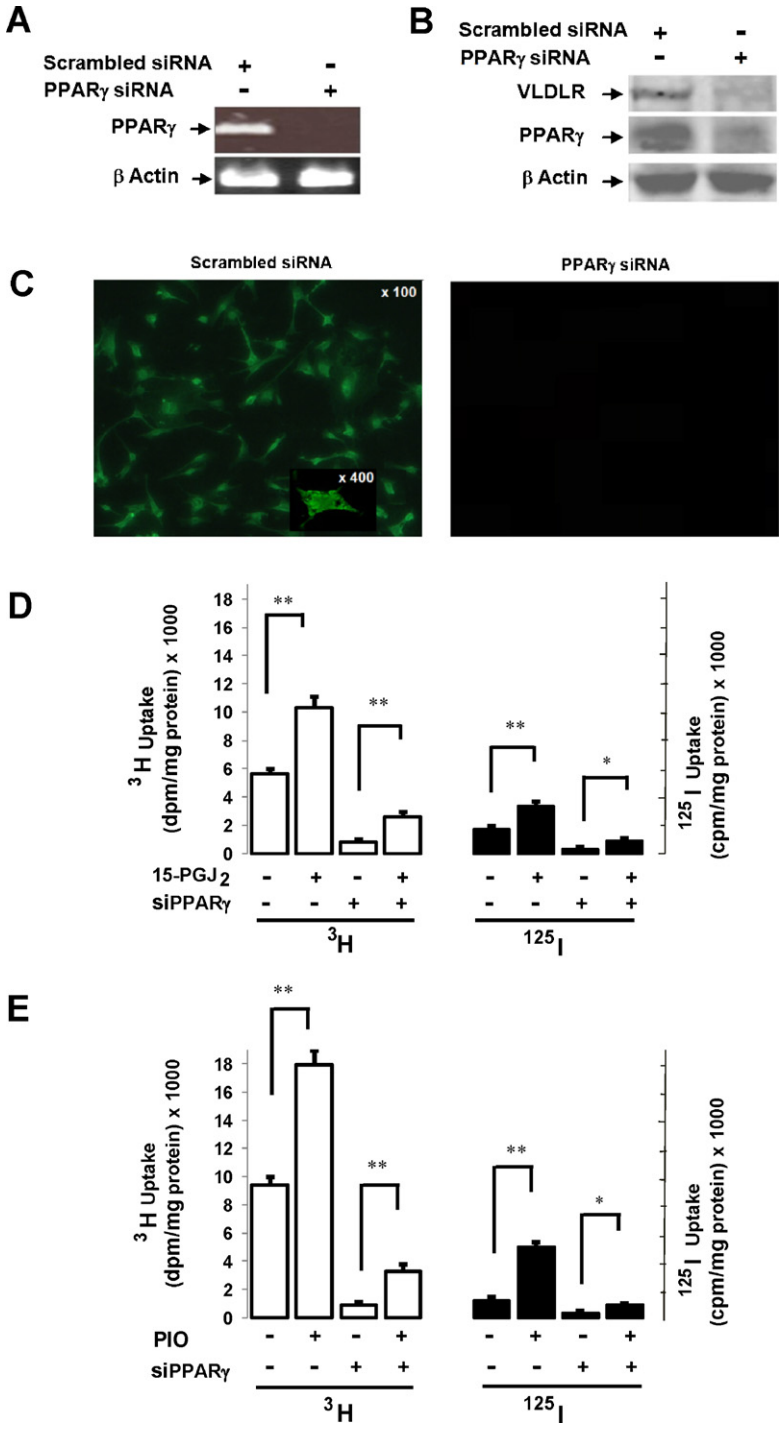


Fig. 2. Effects of small interfering RNA (siRNA)-mediated knockdown of PPARγ and PPARγ agonists on VLDLR expression and VLDL uptake in 3T3-L1 adipocytes. (A) Representative agarose gel of qPCR products showing PPARγ and β actin abundance and (B) representative western blots of VLDLR, PPARγ, and β actin proteins. 3T3-L1 adipocytes were electroporated in the presence of either control (siControl) or PPARγ-specific siRNAs (siPPARγ) on day 7 of differentiation. Then, wells were collected 48 h later (day 9 of differentiation) for determination of mRNA and proteins (3 experiments with $n = 3$ each). Adipocyte culture and western blot and qPCR analyses were performed as described in Section 2. (C) Immunofluorescence images of cells transfected with siControl or siPPARγ. (D) Uptake of ^{3}H and ^{125}I double labeled VLDL in adipocytes transfected with control or PPARγ-specific siRNAs with or without 15d-PGJ$_2$ treatment. (E) Uptake of ^{3}H and ^{125}I double labeled VLDL in adipocytes transfected with control or PPARγ-specific siRNAs with or without pioglitazone treatment. Cell transfection was performed on day 7 of differentiation and treated with 15d-PGJ$_2$ (1 μM), pioglitazone (10 μM) or vehicle (DMSO). Forty eight hours later (day 9), cells were incubated with double labeled [^{3}H, ^{125}I]-VLDL for 4 h and then cells were collected for radioactivity counting. Rates of radioactivity uptake were normalized for protein content. Data are means ± SEM ($n = 3$ experiments performed in triplicate) and statistical differences between treated and untreated adipocytes are indicated with asterisks: $^*P < 0.05$, $^{**}P < 0.001$.

the presence of VLDL and with or without 15d-PGJ$_2$ treatment (Fig. 3). In the absence of 15d-PGJ2, the intensity of Oil red O lipid staining was stronger in *vldlr*$^{+/+}$ adipocytes than in *vldlr*$^{-/-}$ adipocytes (Fig. 3A and C). Addition of 15d-PGJ$_2$ strongly increased lipid staining in *vldlr*$^{+/+}$ but had less effect in *vldlr*$^{-/-}$ adipocytes (Fig. 3B and D). Analysis of TG content corroborated these observations and showed that TG content was about 50% lower in *vldlr*$^{-/-}$ adipocytes without 15-d PGJ$_2$ treatment (Fig. 3E). Treatment with 15d-PGJ$_2$ strongly increased lipid content in *vldlr*$^{+/+}$ adipocytes (+130% compared to untreated) but was less effective

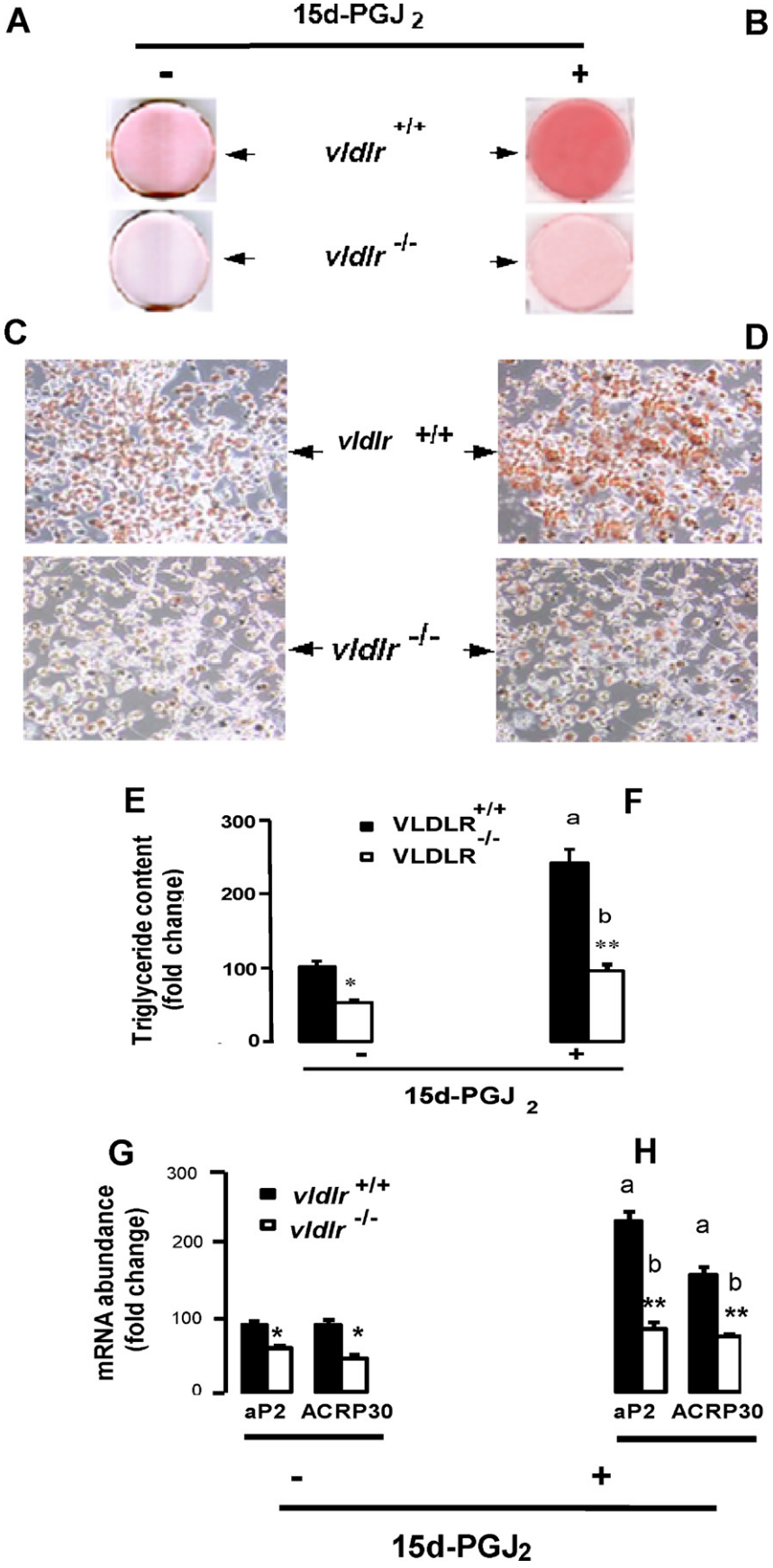


Fig. 3. Effects on adipogenesis in control (*vldlr*$^{+/+}$) and *vldlr* null (*vldlr*$^{-/-}$) mouse adipocytes. Preadipocytes were isolated from mice and differentiated in vitro with or without the presence of 15d-PGJ_2. Cells were then fixed and stained with Oil red O as described in Section 2. Photos were taken for whole plates (A and B) and for cells at initial magnifications ×200 (C and D). Triglyceride (TG) content (E and F) was measured in differentiated adipocytes with or without 15d-PGJ_2 treatment. Abundance of adipocyte fatty acid binding protein (aP2) and adiponectin (ACRP30) mRNA (G and H) was determined by qPCR using specific primers according to Section 2. Data are means ± SEM

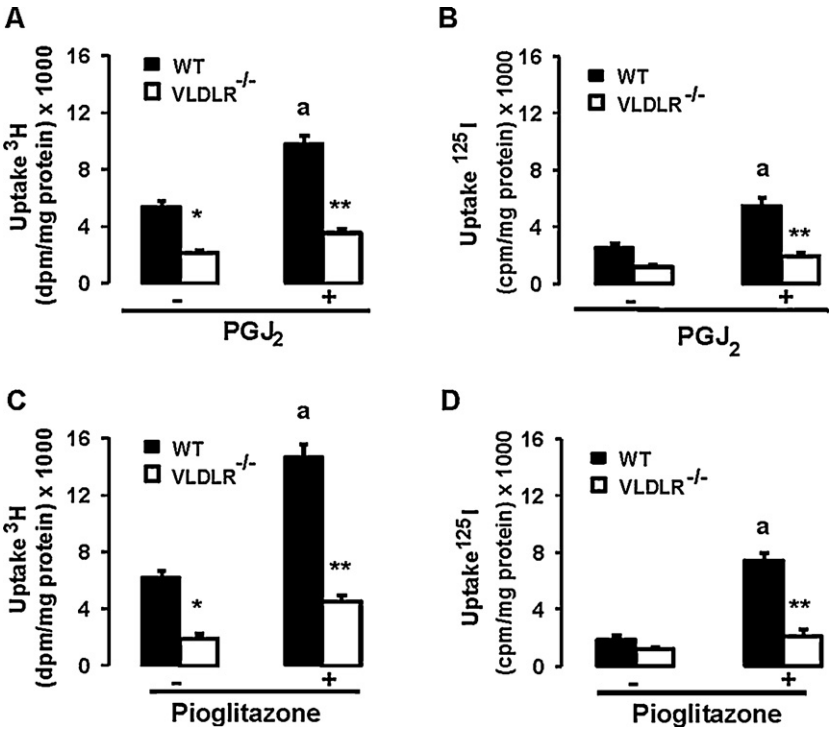


Fig. 4. Effects of pioglitazone and 15d-PGJ_2 on VLDL uptake in control ($vldlr^{+/+}$) and *vldlr* null ($vldlr^{-/-}$) adipocytes. (A) Uptake of ^3H and (B) ^{125}I double labeled VLDL in adipocytes with or without 15d-PGJ_2 treatment. (C) Uptake of ^3H and (D) ^{125}I double labeled VLDL in adipocytes with or without pioglitazone treatment. Preadipocytes were isolated from mice and differentiated in vitro in presence of pioglitazone, 15d-PGJ_2 or vehicle (DMSO). Differentiated mature adipocytes (day 10) were incubated with double labeled [^3H, ^{125}I]-VLDL for 4 h and then cells were collected for radioactivity counting. Rates of radioactivity uptake were normalized for protein content. Data are means ± SEM ($n = 3$ experiments performed in triplicate). Statistical differences between $vldlr^{+/+}$ and $vldlr^{-/-}$ adipocytes are indicated with asterisks: $^*P < 0.05$, $^{**}P < 0.001$. Statistical differences between treated and untreated adipocytes are indicated with alphabetic letter: $^aP < 0.001$.

in $vldlr^{-/-}$ adipocytes (+60% compared to untreated) (Fig. 3F). Taken together, these results show that VLDLR deficiency greatly reduced 15d-PGJ_2-induction of preadipocyte differentiation and lipid deposition. These results are also in agreement with the differentiation markers of adipocytes (Fig. 3G and H), which show lower expression of aP2 and adiponectin in $vldlr^{-/-}$ adipocytes compared to $vldlr^{+/+}$ with or without 15d-PGJ_2 treatment. Using the same experimental set up, we also tested the effects of pioglitzone on $vldlr^{-/-}$ and $vldlr^{+/+}$ adipocyte differentiation, and the results were comparable to those of 15d-PGJ_2.

3.5. VLDLR is required for pioglitazone and 15d-PGJ_2-induced VLDL uptake in adipocytes

Because VLDL particles are the natural ligands of *vldlr* [1,2] we questioned if VLDLR expression is required to mediated the induction of VLDL uptake by pioglitazone and 15d-PGJ_2. Accordingly, VLDL uptake was measured in $vldlr^{+/+}$ and $vldlr^{-/-}$ adipocytes using double-labeled VLDL (Fig. 4). In absence of 15d-PGJ_2, uptake of ^3H (Fig. 4A) and ^{125}I (Fig. 4B) was about 90% lower in $vldlr^{-/-}$ adipocytes compared to $vldlr^{+/+}$ adipocytes. 15d-PGJ_2 treatment significantly increased ^3H and ^{125}I uptake (+68%) in $vldlr^{+/+}$ adipocytes, but failed to induce similar effects (only 30% increase) in $vldlr^{-/-}$ adipocytes (Fig. 4A and B). Similarly, pioglitazone-induced VLDL uptake was strongly suppressed in $vldlr^{-/-}$ adipocytes (Fig. 4C and D). These results suggest that *vldlr* is crucial to mediate the effects of PPARγ agonists 15d-PGJ_2 and pioglitazone.

3.6. Computer search identified a putative PPRE sequence in mouse vldlr promoter

Because 15d-PGJ_2-induction of VLDLR expression required the expression of PPARγ (Fig. 2) we asked whether PPARγ acts through PPAR-responsive element (PPRE) sites in *vldlr* promoter. To identify putative PPRE sites, the internet-based transcription element search system (TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess) was used to search for the motif in the 2000 bps of mouse *vldlr* promoter and 5′-UTR by the PPAR algorithm. This search yielded only one candidate sequence with high similarity to the consensus sequence (76%). The location of this site included bases −1432 through −1420 of the *vldlr* promoter (Fig. 5A). This putative site is different than previously reported PPRE site ([37] located at −2307 (Fig. 5A).

3.7. Endogenous PPARγ binds with vldlr promoter region containing PPRE site

We also tested the binding of endogenous PPARγ to the PPRE site with the ChIP assay. The immunoprecipitates containing PPARγ-bound DNA fragments were subjected to PCR using sets of primers that flanked either the FABP PPRE site, identified *vldlr* PPRE (F1/R1), or another region in the *vldlr* promoter outside the PPRE site (F2/R2) (Fig. 5B). As expected, primers designed for the FABP PPRE site resulted in a strong amplicon indication of reaction with PPARγ (Fig. 5C, lane 2). The specificity of the primers was determined by the negative reaction with β actin primers (lane 2)

($n = 3$ experiments performed in triplicate). Statistical differences between $vldlr^{+/+}$ and $vldlr^{-/-}$ adipocytes are indicated with asterisks: $^*P < 0.05$, $^{**}P < 0.001$. Statistical differences between treated and untreated adipocytes are indicated with alphabetic letter: $^aP < 0.001$, $^bP < 0.05$.

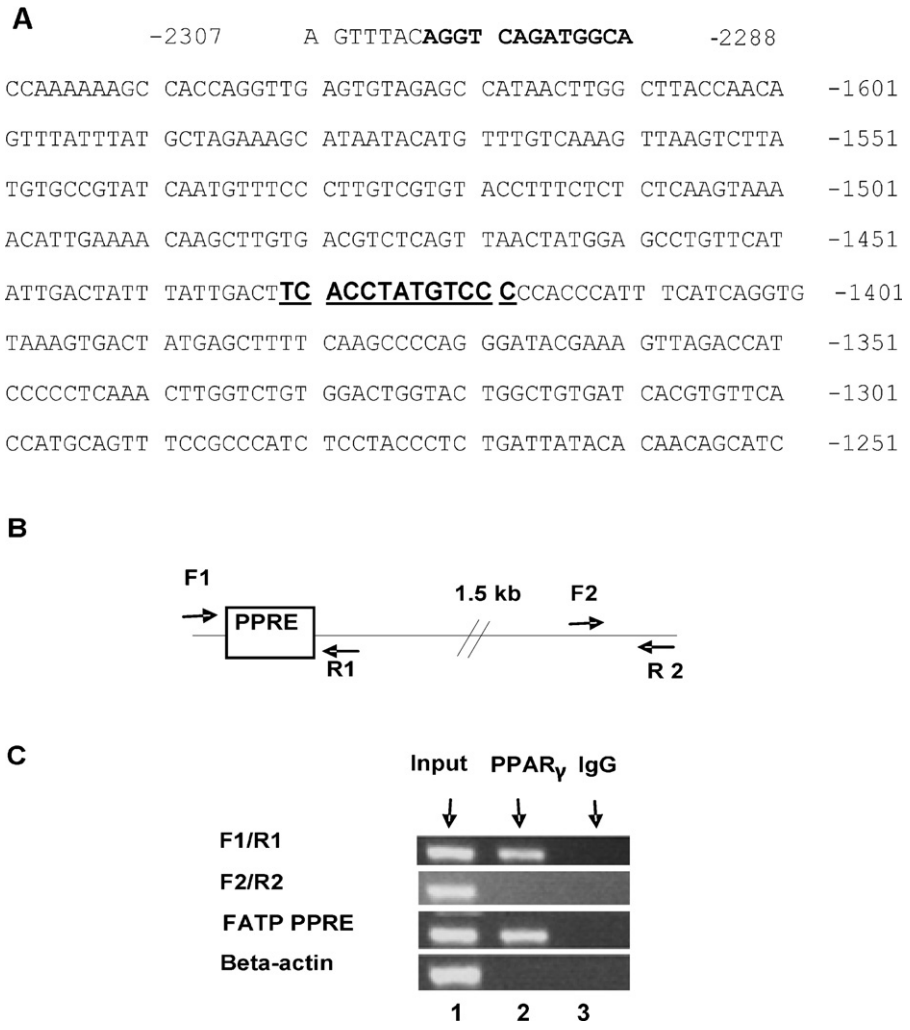


Fig. 5. Identification of a putative PPRE site and chromatin immunoprecipitation (ChIP) of PPARγ binding sites in mouse *vldlr* promoter in 3T3-L1 adipocytes. (A) *vldlr* Promoter sequence showing the putative PPRE sequence (bold) located between −1432 and −1420. An identified PPRE site reported earlier by Kadowaki et al. [37] at the position −2307/−2288 is also indicated in panel A. Search of putative PPRE sequences was performed with computer-assisted transcription factor search system (TESS) of the 2000 bps of 5′-flanking region of mouse VLDLR promoter and 5′-UTR (GenBank no. NM_013703) from the translation start site. ChIP experiments were performed as described in Section 2. Soluble chromatin was prepared from fully differentiated 3T3-L1 adipocytes treated with 10 μM pioglitazone for 24 h. The PPARγ-associated DNA fragments were immunoprecipitated with mouse monoclonal antibody against PPARγ. (B) Positions of the primers (F1/R1) relative to putative PPRE sequence of the mouse *vldlr* promoter and (F2/R2) outside PPRE region are shown. (C) Agarose gel analysis of PCR products obtained following PCR amplification using F1/R1 and F2/R2 primers, and primers designed to cover the fatty acid binding protein (FABP) PPRE (positive control), or a region in the β actin promoter (negative control). Lane 1: products of PCR amplification of DNA input using all sets of primers before immunoprecipitation (IP). Products of PCR amplification obtained after IP with anti-PPARγ antibody (lane 2) or IgG antibody (lane 3). Three independent experiments were conducted with triplicates for each condition.

and IgG (lane 3). The amplicon resulting from *vldlr* PPRE primer set (F1/R1) was detected in input DNA (Fig. 5C, lane 1) and in the DNA fraction that was immunoprecipitated with PPARγ antibody (lane 2), but not in a DNA fraction that was immunoprecipitated with mouse IgG antibody (lane 3). In contrast, no amplicon was detected in the DNA fraction pull-down with PPARγ antibody after using the primer F2/R2 set designed outside the PPRE region of the *vldlr* promoter. These results indicate that PPARγ was associated with the DNA fragments containing the putative PPRE of the mouse *vldlr* promoter.

3.8. Determination of transcriptional regulatory region in mouse vldlr promoter

To determine the region in the *vldlr* promoter responsible for the interaction with PPARγ, serial 5′-deletions of the mouse *vldlr* promoter were linked to the pGL3 basic luciferase reporter gene (Fig. 6A) and co-transfected into 3T3-L1 preadipocyte cells with PPARγ/RXRα exogenously. These cells were chosen because of the low expression of endogenous PPARs. As shown in Fig. 6B, the full vector −1604Luc of *vldlr* promoter significantly induced luciferase activity (5-fold in cells trasnsfected with PPARγ/RXRα and 18.2-fold when cells were simultaneously transfected with PPARγ/RXRα and treated with pioglitazone). In contrast, truncated vectors −1291Luc and −886Luc were less efficient in inducing luciferase activity with only 2.4- and 2.6-fold increases, respectively. Luciferase activity was not induced with −549Luc and −220Luc vectors. These results suggest that the transcriptional regulatory region responding to PPARγ is located between positions −1604 and −1291 in the mouse *vldlr* promoter, which matches the result of the computer-assisted search reported above.

3.9. The −1432/−1420 site is required for transactivation of vldlr promoter by PPARγ in preadipocytes

To examine the role of the identified PPRE site in *vldlr* transactivation, we performed reporter assays using wild type and mutated constructs. Mutations were created within the putative PPRE site as shown in Fig. 6C. Ability of the wild type and mutant

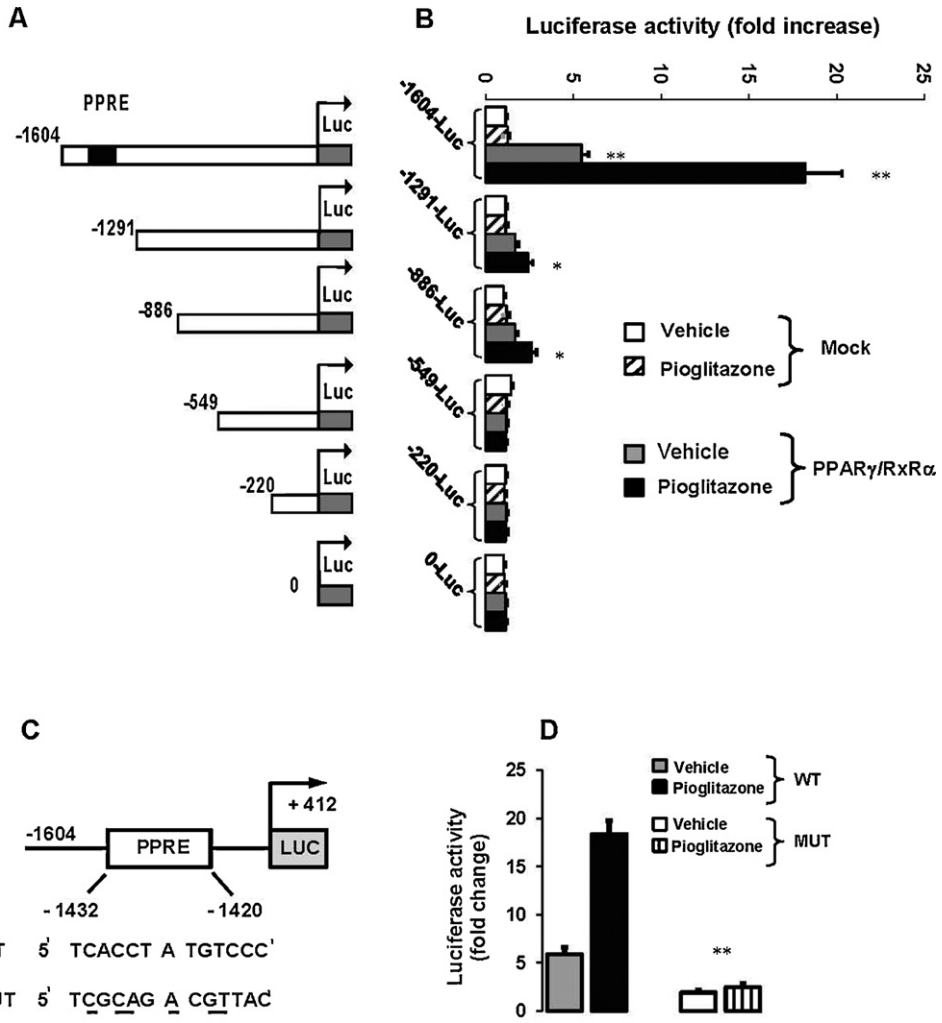


Fig. 6. Effects of serial 5′-flanking deletion PPRE mutations of *vldlr* promoter on promoter transactivation in 3T3-L1 preadipocytes. (A) presentation of constructs with serial 5′-deletions of the mouse *vldlr* promoter linked to the pGL3 basic luciferase reporter vector and (B) results of dual luciferase assays performed after co-transfection with *vldlr* promoter constructs and treated with pioglitazone (10 μM) or vehicle (DMSO). Results obtained for full length of (−1604Luc), truncated constructs (−1291Luc, −886Luc, −549Luc, and 220Luc), and pGL3 basic vector (B) are presented as fold increase of treated cells compared with mock control. Three independent experiments were conducted in triplicate for each condition. Data are mean ± SEM and statistical differences are performed compared to empty vector and indicated with asterisks: $^{**}P < 0.01$, $^{*}P < 0.05$. (C) Sequence of wild type (WT) and mutated (MUT) putative PPRE sites in *vldlr* promoter and (D) results of dual luciferase assays after co-transfection with plasmids of WT and MUT and PPARγ. 3T3-L1 preadipocytes were transiently co-transfected with 300 ng of 1.6-kb of wild type (WT) or mutant (MUT) mouse *vldlr* promoter construct and PPARγ/RXRα. Co-transfection with empty vector and PPARγ/RXRα was used for mock control. 30 ng of pRL-CMV also was transfected for normalization. Cells were also treated with 10 μM of pioglitazone or vehicle (DMSO) for 24 h. Then dual luciferase assay was used for transaction activity measure. The results are means ± SEM of triplicate determination. The results are presented as fold increase of treated cells compared with mock control. Three independent experiments were conducted in triplicate for each condition. Data are mean ± SEM and statistical differences between WT and MUT are indicated with asterisks: $^{**}P < 0.01$.

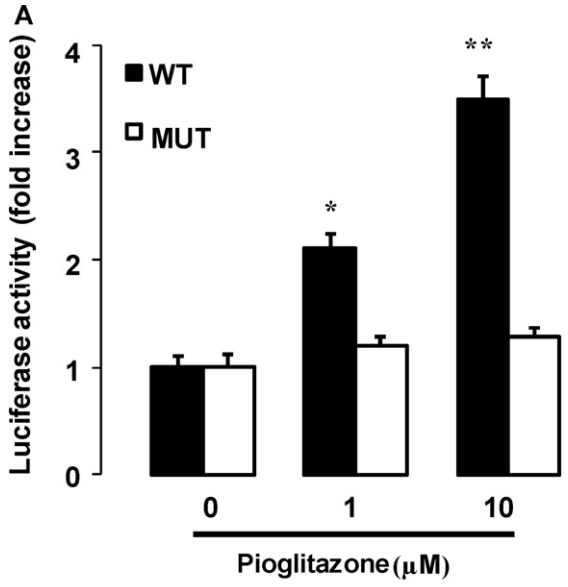
(MUT) constructs to induce luciferase activity was tested after transfection into 3T3-L1 preadipocytes with or without PPARγ/RXRα constructs and induction with the PPARγ agonist pioglitzaone. Mutations within the putative PPRE site (−1432/−1420) induced a drastic reduction ($P < 0.001$) of luciferase activity (15-fold from WT) and pioglitazone treatment was erased (Fig. 6D). These data demonstrate that the identified PPRE site is functional for *vldlr* transactivation and provide evidence that preservation of this sequence is required for PPARγ induction of the *vldlr* promoter.

3.10. The −1432/−1420 PPRE is responsive to endogenous PPARγ

To test if the identified PPRE site is also responsive to endogenous PPARγ, we compared the luciferase activity in transfected mature adipocyte culture with PPARγ agonists. In these experiments, endogenous PPARγ was the only driver of the transactivation, and pioglitazone and 15d-PGJ_2 were used to compare the effects of pharmacological and natural ligands. Following the transfection with WT vector, pioglitazone increased luciferase activity by 2.2- and 3.8-fold for the concentrations 1 and 10 μM, respectively (Fig. 7A). Moreover, addition of 15d-PGJ_2 induced luciferase activity by 1.6- and 2.4-fold for the concentrations 0.1 and 1 μM, respectively (Fig. 7B). Transfection with MUT vector significantly reduced luciferase induction by both pioglitazone and 15d-PGJ_2 (Fig. 7A and B). These data indicate that the −1432/−1420 site is responsive to stimulation of endogenous PPARγ with both synthetic and natural ligands of PPARγ.

3.11. PPARγ specifically binds to the −1432/−1420 PPRE

To determine whether PPARγ is capable of binding specifically at the −1432/−1420 site in mouse VLDLR promoter, an electrophoretic mobility shift assay was performed using the wild type VLDLR-PPRE-(5′-TTATTGACT**TCACCTATGTCCC**CCACCCATT-3′) as a probe. These experiments were performed with nuclear extracts from differentiated adipocytes with endogenous expression of PPARγ. Nuclear extracts from preadipocytes without PPARγ expression were used as negative control. The EMSA results are



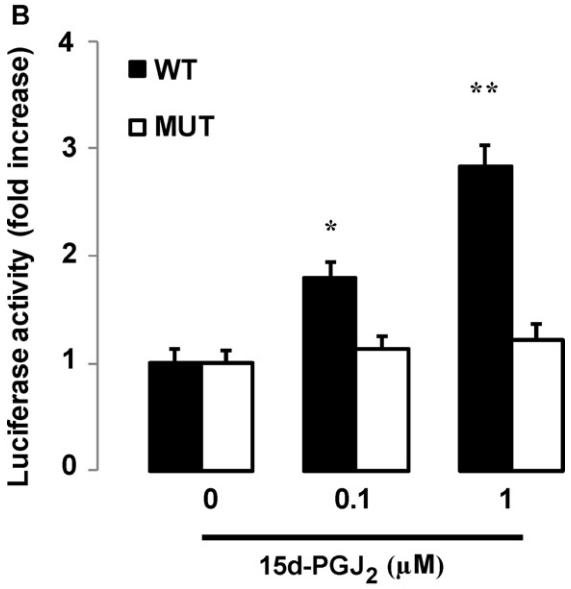


Fig. 7. Effect of PPRE mutation on promoter transactivation with endogenous PPARγ in adipocytes. Differentiated 3T3-L1 adipocytes were transfected with WT or MUT VLDLR promoter luciferase reporter plasmids and treated with either pioglitazone or 15d-15D-PGJ_2 or their vehicle as mock control. After 24 h of treatment, luciferase report assay was performed as described in Section 2. The results show that pioglitazone and 15d-PGJ_2 elevated transcriptional activity on wild type *vldlr* promoter in dose-dependent manner with respective 3.6- and 2.4-fold maximal effects, but failed to elevate activity on mutant *vldlr* promoter. The results are presented as fold increase of pioglitazone (A) or 15d-PGJ_2 (B) treated cells compared with mock control. Three independent experiments were conducted in triplicate for each condition. Data are mean ± SEM and statistical differences between WT and MUT are indicated with asterisks: $^{**}P < 0.01$, $^{*}P < 0.05$.

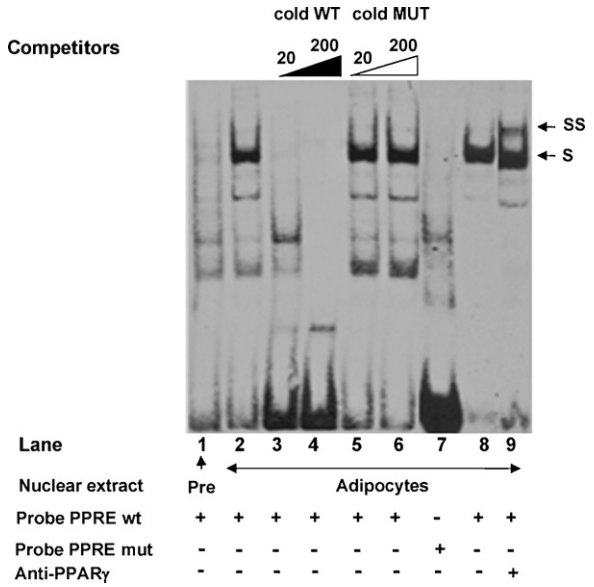


Fig. 8. Electrophoretic mobility shift and supershift assays of PPRE (−1432/−1420) of the mouse *vldlr* promoter. Nuclear proteins were isolated from 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipoctyes that were treated with 10 μM pioglitazone for 24 h. The mouse VLDLR wild type PPRE-wt (5′-TTATTGACTTCACCTATGTCCCCCACCCATT-3′) and mutant PPRE-mut (5′-TTATTGACT**TCGCAGACGTTAC**CCACCCATT-3′) were labeled with biotin. All EMSA assays were carried out using 10 μg of nuclear extracts. DNA-protein complexes were resolved by electrophoresis through 6% polyacrylamide gels in 0.5 × TBS at 4 °C and transferred onto the nylon membrane. Then the biotinylated nucleotides were detected using horseradish peroxidase-conjugated streptavidin and Chemiluminescent Reagent Plus on film. For competition assay, 20- and 200-fold amount of wild type and mutant unlabeled double-stranded DNA fragments were added into the binding reaction. For supershift assay, the reaction mixture was incubated for 1 h at 20 °C with 2 μg of mouse monoclonal anti-PPAR antibody. Nuclear extract from adipocytes formed DNA-protein complex and resulted in shifted band (lane 2 and 8), but nuclear extract from preadipocytes failed to form the shift band (lane 1). 20- and 200-fold cold competitor almost completely abolished the shift bands (lane 3 and 4), but the mutant 20- and 200-fold cold competitor failed to induce a similar effect (lane 5 and 6). The mutant PPRE fragments did not form a stable complex shift (lane 7). However, monoclonal antibody formed a super-shift (lane 9).

presented in Fig. 8. When incubated with nuclear extract from differentiated 3T3-L1 cells, the WT double-stranded probe formed a distinct band of DNA-protein complex called "shift" (S) (Fig. 8, lane 2 and 8), but failed to form a clear band with nuclear extract from preadipocytes (Fig. 8, lane 1). We also tested the specificity and affinity of PPARγ binding to the PPRE site using a competitive assay with excess unlabeled double-stranded wild type or mutated (MUT) forms. As shown in Fig. 8 (lane 3 and 4), the PPRE/PPARγ complex could be competed off with the addition of 20- and 200-fold excess of unlabeled WT sequence. However, addition of similar amounts (×20 and ×200) of mutated sequence failed to abolish the binding with PPARγ, and strong bands were detected (lane 5 and 6). This suggests that the competitive efficiency requires the WT sequence. The specificity of this site for PPARγ binding was also tested using the mutated sequence as a probe (Fig. 8, lane 7). In this case the band S "shift" was not detected (lane 7) as the case for the WT sequence (lane 2 and 8), indicating that PPARγ binding to this site was virtually abolished by the mutation. Moreover, incubation of the 3T3-L1 nuclear extract with monoclonal antibody against PPARγ formed a "supershift" (SS) band with higher molecular weight (Fig. 8, lane 9) indicating the presence of PPARγ in this complex as an additional proof for the binding specificity.

4. Discussion

Pioglitoane and 15d-PGJ_2 are potent activators of PPARγ and promote adipocyte differentiation and lipid deposition [11,12,30]. These studies were undertaken to examine if *vldlr* expression is regulated by PPARγ agonists and whether it mediates their proadipogenic effects. Here, we show that *vldlr* expression is induced by 15d-PGJ_2 in dose- and time-dependent manners through mechanisms involving PPARγ and that *vldlr* expression is crucial to mediate the induction of adipocyte differentiation and VLDL uptake by PPARγ agonists. We also report the identification of a new functional PPRE site in the *vldlr* promoter, which is responsive to PPARγ agonist treatment.

Adipogenesis occurs in response to signals that promote the expression and activation of PPARγ. Subsequently, PPARγ induces the expression of an array of genes important for lipid metabolism and adipogenesis [8]. Our results demonstrate that 15d-PGJ_2, similar to pioglitaozne, up-regulates *vldlr* expression, induce adipocyte differentiation and promotes VLDL uptake. These results are consistent with the pro-adipogenic effects of pioglitazone and 15d-PGJ_2 reported previously [11,12], and also demonstrate for the first time that VLDLR plays a crucial role in mediating the effects of PPARγ ligands. To promote adipogenesis, VLDLR could act through multiple mechanisms. First, *vldlr* is required for optimal functioning of LPL either by increasing LPL and TG-rich lipoprotein interactions at the capillary surface [23,31] or by serving as a helper for the transcytosis of LPL [32]. Second, VLDLR binds and internalizes TG-rich lipoprotein particles through specific binding of apolipoprotein E, as has been shown in isolated cells [33]. Through these mechanisms, *vldlr* participates at multiple stages of VLDL processing in adipoytes and could regulate the uptake of FFAs as well as remnant particles. The low recovery of ^{125}I, which is the label for the remnant particle, and ^{3}H, the label of VLDL-derived lipids, indicate that both pathways were disrupted by *vldlr* deficiency in adipocytes. The expression of CD36 and FATP, proteins known to facilitate FA uptake [34], and LRP1, known receptor for remnant lipoproteins, were induced in *vldlr*$^{-/-}$ adipocytes [6] which could explain a residual uptake of VLDL in the absence of VLDLR. It also indicates that these proteins did not fully compensate for deficiency of VLDLR in adipocytes.

Silencing PPARγ with siRNA abolished the induction of VLDL uptake by pioglitazone and 15d-PGJ_2 (Fig. 2) and indicates that PPARγ is involved in *vldlr* expression. Computational site prediction led to the identification of a putative PPRE site at −1432/−1420 of the 5′-flanking region, and EMSA and ChIP binding assays provided evidence of the specificity and affinity of PPARγ to this site. Moreover, reporter assays demonstrated that this site supports promoter transactivation when the sequence is preserved, while site-directed mutation abolished PPARγ binding and transactivation. Taken together, these results demonstrate that the −1432/−1420 is a functional PPRE site that binds PPARγ and mediates promoter transactivation. Because the expression of PPARγ is very low in preadipocytes [6], this cell model was used to test the efficiency of the putative site to respond to exogenous PPARγ introduced by co-transfection. The construct containing the −1432/−1420 of the *vldlr* promoter (−1604Luc vector) was greatly efficient ($P < 0.001$) to promote transactivation (18-fold increase of luciferase activity). Induction of luciferase activity with −1291Luc and −886Luc constructs was very low, but was about 2-fold above the basal value. The computer search did not show any PPRE matching sequence within −1291 or −886 sequences, but we cannot exclude the possibility of weak binding motifs outside of the −1432/−1420 PPRE site that could explain this low transactivation. The sequence of the *vldlr* PPRE reported here shows a high compatibility to the consensus PPRE sequence (10/13) and to other PPRE sequences of known PPARγ target genes including LPL [13], aP2 [14], CD36 [16], and HSL [18] (Table 2). Previously, Takazawa et al. [37] have reported another PPRE site within VLDLR promoter located at −2307 bp outside of 2 kb region where we performed our analysis. Compared to our results, luciferase activity of the −2307 PPRE site reported earlier [37] seems to be different that the activity of the site reported here. In addition, this −1432/−1420 PPRE has more compatibility (10/13 bases matching) with the PPRE consensus sequence (Table 2) than the −2307 PPRE site (9/13) reported by Takazawa et al. [37].

Table 2
Comparative PPRE sequences of known PPAR target genes.

Gene	Species	DR-1	Number of matches	Reference
Consensus		5′-AGGTCA A/T AGGTCA-3′		[14]
VLDLR	Mouse	5′-GGGACA T AGGTGA-3′	10/13	
FATP	Mouse	5′-GGGGCA A AGGGCA-3′	10/13	[17]
aP2	Mouse	5′-GGATCA G AGGTCA-3′	10/13	[14]
HSL	Mouse	5′-GGGACA A AGGTAG-3′	9/13	[18]
LPL	Mouse	5′-GGATCA G AGGTCA-3′	10/13	[13]
FAT/CD36	Mouse	5′-AAGTCA C ACTTCA-3′	9/13	[16]
L-FABP	Rat	5′-AGGCCA A AGGTCA-3′	12/13	[26]

Using mature 3T3-L1 adipocytes, we also showed that this identified PPRE site efficiently responded to endogenous PPARγ after induction with natural (15d-PGJ_2) as well as synthetic (pioglitazone) PPARγ ligands. Pioglitazone and 15d-PGJ_2 are potent activators of PPARγ and promote adipocyte differentiation and lipid deposition [11,12]. The fact that the newly identified PPRE is responsive to both ligands provides a possible mechanism through which natural and synthetic agonists of PPARγ induce *vldlr* expression and promotes adipogenesis.

Among the PPAR family, PPARγ is the main isoform expressed in adipocytes and the key driver of adipogenesis [8,9]. Therefore, these studies focused on the expression of *vldlr* by PPARγ in adipocytes. However, we cannot exclude the possibility that *vldlr* is also responsive to other PPARs according to the tissue expression. In agreement with this hypothesis, a previous study showed that PPARδ knockdown reduced *vldlr* expression in mouse liver [35]. Similarly, treatments with PPARα agonist WY-14643 induced VLDLR expression in liver of control wild type mice and not in PPARα null mice [36].

In conclusion, the current data demonstrate that VLDLR expression is crucial to mediate PPARγ induction of the adipocyte differentiation and VLDL uptake. The identification of a functional PPRE site in mouse *vldlr* promoter provides a possible mechanism through which activation of PPARγ by pioglitazne and 15d-PGJ_2 induces the expression of *vldlr* in adipocytes.

Acknowledgements

This work was supported by Development Award no. AHA0730356N from the American Heart Association (T. Hajri) and a Development Grant no. 1047089103 from the Department of Surgery, Vanderbilt University (T. Hajri)

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