A Novel Pathway to Enhance Adipocyte Differentiation of 3T3-L1 Cells by Up-regulation of Lipocalin-type Prostaglandin D Synthase Mediated by Liver X Receptor-activated Sterol Regulatory Element-binding Protein-1c

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Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is expressed in adipocytes and is proposed to be involved in the regulation of glucose tolerance and atherosclerosis in type 2 diabetes, because L-PGDS gene knock-out mice show abnormalities in these functions. However, the role of L-PGDS and the regulation mechanism governing its gene expression in adipocytes remain unclear. Here, we applied small interference RNA of L-PGDS to mouse 3T3-L1 cells and found that it suppressed differentiation of these cells into adipocytes. Reporter analysis of the mouse L-PGDS promoter demonstrated that a responsive element for liver receptor homolog-1 (LRH-1) at −233 plays a critical role in preadipocytic 3T3-L1 cells. Moreover, we identified two sterol regulatory elements (SREs) at −194 to be cis-elements for activation of L-PGDS gene expression in adipocytic 3T3-L1 cells. L-PGDS mRNA was induced in response to synthetic liver X receptor agonist, T0901317, through activation of the expression of SRE-binding protein-1c (SREBP-1c) in the adipocytic 3T3-L1 cells. The results of electrophoretic mobility shift assay and chromatin immunoprecipitation assay revealed that LRH-1 and SREBP-1c bound to their respective binding elements in the promoter of L-PGDS gene. Small interference RNA-mediated suppression of LRH-1 or SREBP-1c decreased L-PGDS gene expression in preadipocytic or adipocytic 3T3-L1 cells, respectively. These results indicate that L-PGDS gene expression is activated by LRH-1 in preadipocytes and by SREBP-1c in adipocytes. Liver X receptor-mediated up-regulation of L-PGDS through activation of SREBP-1c is a novel pathway to enhance adipocyte differentiation.

Adipocytes play a critical role in lipid homeostasis and energy balance. Their major role is storage of large amounts of lipid metabolites during periods of energy excess and utilization them during nutritional deprivation (1). Adipocytes are also known as endocrine cells that secrete various adipocytokines (2, 3). Disorders of lipid metabolism are associated with diseases such as obesity and diabetes (4). Adipocyte differentiation (adipogenesis) is a complex process involving coordinated changes in hormone sensitivity and gene expression.

Mouse 3T3-L1 cells are well used in vitro model for adipogenesis (5). Previous studies demonstrated that lipocalin-type prostaglandin (PG)D synthase (L-PGDS) gene was expressed in adipocytes (6, 7). L-PGDS is the member of the lipocalin gene family to be recognized as an enzyme that catalyzes the isomerization of PGH2, a common precursor of various prostanoids, to PGD2, a potent endogenous somnogen (8). Alternatively, L-PGDS binds small lipophilic molecules such as retinal and retinoic acid (9), biliverdin and bilirubin (10), gangliosides (11), and amyloid β peptides (12). L-PGDS knock-out mice did not show SeCl4-induced insomnia (13) and accelerated amyloid β-deposition (12). Ragolia et al. (14) demonstrated that L-PGDS knock-out mice become glucose-intolerant and insulin-resistant and that their adipocytes were significantly larger than those of wild-type mice. The L-PGDS expression level was shown to be high in the omental adipose tissue (15). A polymorphism in the 3′-untranslated region of the human L-PGDS gene appears to be associated with carotid atherosclerosis in Japanese individuals with hypertension (16). The balance between L-PGDS and PGE synthase levels was proposed to be a major determinant for stability/instability of atherosclerotic plaques (17). However, the role of L-PGDS and the regulation mechanism controlling its gene expression in adipocytes remain unclear.

In this study, we showed that L-PGDS gene expression was enhanced during differentiation of mouse 3T3-L1 cells into adipocytes and that small interference RNA (siRNA)-mediated suppression of L-PGDS mRNA decreased the lipid accumulation in 3T3-L1 cells. Reporter analysis of the mouse L-PGDS promoter demonstrated that L-PGDS gene expression was mediated by liver receptor homolog-1 (LRH-1) in preadipo-

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3 The abbreviations used are: PG, prostaglandin; L-PGDS, lipocalin-type PGD synthase; LRH-1, liver receptor homolog-1; LRH-RE, LRH-1-responsive element; SRE, sterol-regulatory element; SREBP-1c, SRE-binding protein-1c; LXR, liver X receptor; LXRE, LXR-responsive element; IDH, insulin, deoxymethasone, and 3-isobuty-1-methykanthine; EIA, enzyme immunoassay; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay; PPAR, peroxisome proliferator-activated receptor; N.C., negative control; siRNA, small interference RNA; CMV, cytomegalovirus.
cytes and by sterol regulatory element (SRE)-binding protein-1c (SREBP-1c) in adipocytes. Each cis-element was identified as an LRH-1-responsive element (LRH-RE) or two SREs, respectively, in the proximal region of L-PGDS promoter. The liver X receptor (LXR) agonist T0901317 enhanced the expression of LXRα and SREBP-1c as well as that of the L-PGDS gene, thus indicating that LXR-activated SREBP-1c enhanced L-PGDS gene expression during adipocyte differentiation. Our findings demonstrate a novel mechanism for the enhancement of adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse 3T3-L1 cells were purchased from Health Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco’s modified Eagle’s medium supplemented with charcoal-treated fetal calf serum and antibiotics. Adipocyte differentiation of 3T3-L1 cells was induced by incubation of the cells for 2 days in differentiation medium containing insulin (10 μg/ml), 1 μm dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IDX). On day 2, the medium was replaced with growth medium containing insulin (10 μg/ml) alone and changed every 2 days. For visualization of lipid accumulation, the cells were stained with Oil Red O as described earlier (18).

**RNA Analysis**—Extraction of total RNA and first-strand cDNA synthesis were performed by using SuperScript III Reverse Transcriptase (Invitrogen) primed by random-hexamer as described previously (19). PCR was carried out under the following condition; initial denaturation at 96 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 30 s. The gene-specific primer sets used were 5’-CAGCCGGCCCTGCTCTCAACTC-3’ and 5’-GGGTTGCCCATTGCGGAAGTTCTC-3’ for L-PGDS, and 5’-ACCAAGTCTCA-TGCCATCAC-3’ and 5’-TCAACCACCTGTTGCTGA-3’ for glyceraldehyde-3-phosphate dehydrogenase. Resultant PCR products were analyzed by agarose-gel electrophoresis.

Quantification of mRNA levels was measured by using a real-time PCR system (Applied Biosystems, Foster City, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems) with the following gene-specific primer sets: 5’-GGAAAAACCGACTGTTGACACCA-3’ and 5’-ACTGACACGGAGTTGATTGCT-3’ for L-PGDS, 5’-GGAGCCATGGATTGAGCAT-3’ and 5’-GCTTCCAGAGGAGGCGCAGC-3’ for SREBP-1c, and 5’-TCACTGGGCTTTCCAC-3’ and 5’-GGCAGATGATGACCCCTTTC-3’ for glyceraldehyde-3-phosphate dehydrogenase. The primers for LXRα and LRH-1 were synthesized as described previously (20).

**Plasmids, Transfection, and Luciferase Assay**—The plasmid for mouse L-PGDS promoter-luciferase containing the promoter region from -1500 to +76 was constructed by using the pGL4.10(luc2) vector (Promega, Madison, WI) as described previously (21). Site-directed mutagenesis was carried out by the use of a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instruction. All constructs were subjected to the nucleotide sequencing to verify their correct sequences and orientation.

For transfection, cells were cotransfected with each construct (0.9 μg) and pRL-CMV (0.1 μg, Promega) in 24-well plates, the latter carrying the *Renilla* luciferase gene under the control of the cytomegalovirus promoter as the transfection control, by use of FuGENE Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the method prescribed by the manufacturer. The cells were cultured for further 48 h. The luciferase activities were measured by using a Dual-Glo Luciferase Reporter Assay Kit (Promega). The reporter activity was calculated relative to that of pGL4.10(luc2) vector, and was defined as 1. All data were obtained from at least three independent experiments, and each experiment was performed in duplicate. The relative promoter activities were reported as the mean ± S.D.

**Electrophoretic Mobility Shift Assay and Chromatin Immuno precipitation Assay**—Preparation of nuclear extracts and EMSA were carried out by the method described previously (21). Oligonucleotides used in this experiment were 5’-TTTGC-CGGCAGGAGTTGGGCAAGTTCTGAGCCAGTTCGCCC-3’ for LRH-RE and 5’-AGTTCTCTGCTGGAGCTTGGGA-TGGGCCAGCGCA-3’ for SRE, and modified at their

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**FIGURE 1. Roles of L-PGDS during differentiation of mouse 3T3-L1 cells into adipocytes.** A. Oil-Red O staining of 3T3-L1 cells. 3T3-L1 cells were incubated in the differentiation medium for the indicated number of days. B. expression level of L-PGDS and PPARγ genes was measured by quantitative PCR. C. RNA interference-mediated suppression of L-PGDS mRNA level during differentiation of 3T3-L1 cells. The cells were transfected with either of two distinct siRNAs for L-PGDS (#1 and #2) or negative control (N.C.) siRNA (Invitrogen) at 2 days after the start of differentiation. D. cells were cultured for more 6 days, and then stained with Oil-Red O to visualize the lipid-droplets. E. PGD2 production was measured by EIA. Preadipocytic and adipocytic cells were treated with A23187 for 10 min at 37 °C, followed by collecting medium to measure PGD2 level. The data represent the mean ± S.D. from three independent assays.
Fluorescence signals were detected with an Odyssey infrared imaging system (LI-COR, Lincoln, NE). The ChIP assay was performed as described earlier (22). Antibodies specific for LRH-1 and SREBP-1c were obtained from Perseus Proteomics (Tokyo, Japan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Immunoprecipitated DNA-protein complexes were reverse-cross-linked, and the purified DNA fragments were utilized for subsequent PCR amplification with the following specific primer sets: 5'-GGA-TGATAGTGAGCTCGTGGAG-3' and 5'-CTCAAGGGGTGAAGCTCTCAGT-3' for LRH-RE and 5'-GTCTCAGCGAGTTCTGCCC-3' and 5'-CAGGCAACCTCCTGCTCAGCAA-3' for SRE. PCR was conducted under the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 96 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s.

**SiRNA-mediated Knockdown Experiment**—Stealth siRNA for L-PGDS, LRH-1, and SREBP-1c, and Stealth Negative Control siRNA were obtained from Invitrogen as follows: L-PGDS siRNA#1, 5'-GAAACUAGUGACGAGUGGACUAU-3'; L-PGDS siRNA#2, 5'-GACUUCCGCAUGGCAACCUCUCA-3'; LRH-1 siRNA #1, 5'-CAGUGGACCUUAGGACUAUACCU-3'; LRH-1 siRNA #2, 5'-GGACAGACCCUGUUCUCAUU-3'; SREBP-1c siRNA#1, 5'-GCAAGAGACGAGAAGAUGGCUCUAAUU-3'; SREBP-1c siRNA#2, 5'-CCCUGCACUUCUUGACACGUUU-3'. Cells were transfected with each siRNA or negative control siRNA (5 nM) by using TransIT-TKO transfection reagent (Mirus Bio, Madison, WI). After 48 h of transfection, the RNA was extracted as described (19), and the mRNA level was estimated by quantitative PCR as described above.

**Measurement of PGD2**—PGD2 level was measured by enzyme immunoassay (EIA, Cayman Chemical, Ann Arbor, MI) as described previously (23). In brief, 3T3-L1 cells were washed twice with phosphate-buffered saline, followed by treatment with calcium ionophore, A23187 (5 μM) for 10 min at 37 °C. The culture medium was collected and utilized for measurement of PGD2 by EIA.

**RESULTS**

**Enhancement of L-PGDS Gene Expression and Role of L-PGDS during Adipocyte Differentiation of Mouse 3T3-L1 Cells**—To investigate the role of L-PGDS in adipocyte differentiation, we first examined the expression profile of the L-PGDS gene during the differentiation of 3T3-L1 cells into adipocytes. 3T3-L1 cells were treated with a differentiation medium containing IDX for 2 days and further cultured in the medium containing insulin. Oil Red O staining of 3T3-L1 cells demonstrated that these cells accumulated lipid in a differentiation time-dependent manner (Fig. 1A). The L-PGDS gene was expressed even in preadipocytic 3T3-L1 cells (0 days, preadipocytes, Fig. 1B). During the differentiation of the 3T3-L1 cells into adipocytes, the expression of L-PGDS mRNA increased to 3.5-fold that in the preadipocytes under the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 96 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s.

5'-end with Alexa680. Fluorescence signals were detected with an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

The ChIP assay was performed as described earlier (22). Antibodies specific for LRH-1 and SREBP-1c were obtained from Perseus Proteomics (Tokyo, Japan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Immunoprecipitated DNA-protein complexes were reverse-cross-linked, and the purified DNA fragments were utilized for subsequent PCR amplification with the following specific primer sets: 5'-GGAGTGAATGGGCTGGTGAAG-3' and 5'-TCCAGAGGCTAGAAGCTCTCAGT-3' for LRH-RE and 5'-GTCTGAGCGAGTTCTGCCC-3' and 5'-CAGGCAACCTCCTGCTCAGCAA-3' for SRE. PCR was conducted under the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 96 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s.
pendent manner (Fig. 1B). Peroxisome proliferator-activated receptor (PPAR) γ mRNA level, a marker of adipocyte differentiation (24), also gradually increased during adipocyte differentiation (Fig. 1B). 

To further investigate the importance of L-PGDS in adipocyte differentiation, we transfected 3T3-L1 cells on day 2 with either of two siRNAs for L-PGDS. The L-PGDS mRNA level at 2 days after transfection was significantly decreased by transfection with L-PGDS siRNA#1 or #2 as compared with that obtained with the negative control (N.C.) siRNA (Fig. 1C, upper panel) or without transfection (data not shown). Glyceraldehyde-3-phosphate dehydrogenase mRNA levels were almost unchanged in all samples (Fig. 1C, lower panel).

We then examined the relationship between the L-PGDS level and lipid-accumulation. 3T3-L1 cells were incubated with IDX for 2 days, transfected with either of the two siRNAs for L-PGDS or with N.C. siRNA, and cultured for a further 6 days in the presence of insulin. When lipid accumulation in the cells was visualized with Oil-Red O staining (Fig. 1D), the lipid accumulation was significantly decreased in the cells transfected with L-PGDS siRNA #1 or #2, as compared with that in the cells transfected with N.C. siRNA. We measured PGD₂ level in both preadipocytic and adipocytic cells. These cells were treated with A23187 for 10 min, and PGD₂ level in the medium was measured by EIA. PGD₂ level in adipocytic cells were slightly enhanced as compared with that in preadipocytic cells (Fig. 1E). These results indicate that the L-PGDS and PGD₂ levels correlated well with the level of differentiation of 3T3-L1 cells to adipocytes and that L-PGDS acts as an accelerator of adipocyte differentiation.

**Regulation of L-PGDS Gene Expression in the Preadipocytic 3T3-L1 Cells**—Next we examined the regulation of L-PGDS gene expression during differentiation of 3T3-L1 cells to adipocytes. To identify transcription factors that enhance L-PGDS gene expression in the preadipocytic or adipocytic cells, we constructed a series of luciferase reporter plasmids carrying various lengths of promoter region of the mouse L-PGDS gene, transfected both the preadipocytic and adipocytic cells with these plasmids, and measured the reporter-luciferase activity (Fig. 2A). When the promoter-luciferase reporter construct carrying the promoter region from −1500 to +76 was used for transfection, efficient reporter activity was detected in both preadipocytic and adipocytic 3T3-L1 cells, indicating that this region contained some cis-element(s) for the regulation of L-PGDS gene expression. Deletion of the region from −1500 to −300 did not significantly change the promoter activity in either preadipocytic or adipocytic cells.
cytic cells. On the contrary, further deletion to −190 resulted in a strong decrease in the promoter activity in both types of 3T3-L1 cells, which activity was ∼50% of that obtained with the −300/+76 construct. Further deletion analysis demonstrated that, when the region from −190 to −100 was deleted, the reporter activity was not detected any more, thus indicating that this region was required for the basal promoter activity (Fig. 2A). Therefore, these results indicate that cis-elements critical for the transcriptional activation of the mouse L-PGDS gene were located in the regions from −300 to −190 in both preadipocytic and adipocytic 3T3-L1 cells. This region contained several potential cis-acting elements capable of binding with the transcription factors that might play critical roles in the regulation of the mouse L-PGDS gene expression, including the LXR-responsive element (LXRE) for LXR at −248, LRH-RE for LRH-1 at −233, and two SREs for SREBP, one at −201 and the other at −194 (Fig. 2B).

Next, we examined the mRNA expression profiles of LXRα, LRH-1, and SREBP-1c in 3T3-L1 cells during differentiation to adipocytes (Fig. 2C). Gene expression of LXRα and SREBP-1c was increased during the adipocyte differentiation, whereas that of LRH-1 was detected in the preadipocytic cells but was negligible in the adipocytic cells. These results indicate that LRH-1 gene expression was downregulated, whereas LXRα and SREBP-1c mRNA levels were increased during the adipocyte differentiation.

Role of LRH-RE in Activation of L-PGDS Gene Expression in the Preadipocytic 3T3-L1 Cells—To confirm the role of LRH-RE in the activation of L-PGDS gene expression in preadipocytic 3T3-L1 cells, we prepared a mutant LRH-RE by site-directed mutagenesis (Fig. 3A) and measured its reporter activity (Fig. 3B). The luciferase activity of the promoter containing the mutation at the LRH-RE was significantly decreased as compared with that of wild-type construct carrying the promoter region from −300 to +76 and was almost the same as that of the −190/+76 construct (Fig. 3B). These results indicate that mouse L-PGDS gene expression
was activated through the LRH-RE in the preadipocytic 3T3-L1 cells.

EMSA with the synthetic oligonucleotide containing the LRH-RE of the L-PGDS promoter showed a shifted DNA-protein complex, when the nuclear extracts prepared from the preadipocytic 3T3-L1 cells was utilized (Fig. 3C, lane 2). Formation of this DNA-protein complex was not detected when the nuclear extracts were omitted from this incubation mixture (Fig. 3C, lane 1) and was decreased by the addition of an excess amount (5- or 10-fold) of unlabeled oligonucleotide containing LRH-RE (Fig. 3C, lanes 3 and 4). Furthermore, the DNA-protein complex was not observed when the nuclear extracts were prepared from the adipocytic 3T3-L1 cells (Fig. 3D), indicating that the binding of nuclear factor to the LRH-RE occurred only in the preadipocytic 3T3-L1 cells.

The results of the ChIP assay revealed that an amplicon (170 bp) was produced by the gene-specific primer sets for the L-PGDS promoter when either total input DNA or purified DNA fragments obtained with the anti-LRH-1 antibody were used for PCR (Fig. 3E). However, the primers for the region without the LRH-RE did not produce the amplicon (Fig. 3E). These results, taken together, indicate that LRH-RE is critical for activation of L-PGDS gene expression in the preadipocytic 3T3-L1 cells and that LRH-1 binds specifically to the LRH-RE in the L-PGDS promoter both in vitro and in vivo.

LRH-1 Affects L-PGDS Gene Expression in the Preadipocytic 3T3-L1 Cells—We then investigated the relationship between the LRH-1 level and L-PGDS gene expression level in preadipocytic 3T3-L1 cells, by knockdown of LRH-1 expression with LRH-1 siRNAs (Fig. 3F). Either of the two LRH-1 siRNAs suppressed both LRH-1 and L-PGDS mRNA production in the preadipocytic 3T3-L1 cells, whereas N.C. siRNA did not affect the expression level of either gene, which was almost the same as that for the non-transfected control (vehicle). These results indicate that the LRH-1 level was associated with the L-PGDS gene expression level in preadipocytic 3T3-L1 cells.

Role of LXRE and SRE in Activation of L-PGDS Gene Expression in the Adipocytic 3T3-L1 Cells—As described above (Fig. 2B), we found the −300/−190 region of the L-PGDS promoter containing LXRE at −248 and the SREs at −201 and −194 to be important for the transcriptional activation of L-PGDS gene expression in the adipocytic 3T3-L1 cells. So we examined whether LXRE and/or these two SREs were involved in the activation of L-PGDS gene expression in the 3T3-L1 cells by performing luciferase reporter assays with the mutated inactive LXRE- or SREs-containing con-
treatment with T0901317 in adipocytic cells, indicating that induction of SREBP-1c precedes that of L-PGDS (Fig. 5B). Furthermore, when the adipocytic cells were treated with siRNA for SREBP-1c, mRNAs for both SREBP-1c and L-PGDS were decreased (Fig. 5C). The transfection with N.C. siRNA did not change the level of either mRNA. These results indicate that LXRα-activated SREBP-1c regulated the expression of L-PGDS in the adipocytic 3T3-L1 cells.

**DISCUSSION**

Obesity is a major risk factor in metabolic diseases such as diabetes, hypertension, and cardiovascular diseases (25–27). Much research is currently being conducted regarding the molecular mechanisms that regulate the lipid metabolism.

However, few investigators have reported on the involvement of L-PGDS in energy intake, lipid metabolism, adipocyte differentiation, and obesity. In our previous study (14), L-PGDS knock-out mice showed hypertrophy of adipocytes, indicating that L-PGDS is involved in adipocyte differentiation.

In this study, we elucidated the molecular mechanisms of L-PGDS gene expression in mouse 3T3-L1 cells during their differentiation into adipocytes and found that the expression was activated by LRH-1 in the preadipocyte stage yet by SREBP-1c, in an LXRα-dependent manner, in the adipocyte stage, indicating that L-PGDS gene expression was differentially regulated during the differentiation of 3T3-L1 cells. Fig. 6 summarizes the switching of molecular mechanisms of L-PGDS gene expression between preadipocytes and adipocytes of 3T3-L1 cells demonstrated in this study.

The L-PGDS gene was expressed in pre-adipocytic 3T3-L1 cells, and its expression was enhanced during their differentiation. We showed that preadipocytes, but not adipocytes, expressed LRH-1, which bound to and activated the L-PGDS promoter (Figs. 2C, 3E, and 4). This finding is consistent with previous reports showing that LRH-1 mRNA was expressed in preadipocytic 3T3-L1 cells but not in adipocytic ones (20, 28). We transfected preadipocytes with LRH-1 siRNA and then treated with IDX. The cells accumulated lipid droplets and expressed two adipocyte-differentiation marker genes, stearoyl-CoA desaturase and PPARγ, to the same extent as those in negative control siRNA-transfected cells (data not shown). Therefore, LRH-1-mediated activation of L-PGDS is not involved in the adipocytic differentiation. The roles of L-PGDS in preadipocytes remain to be clarified. LRH-1 is an orphan receptor and is expressed in various tissues, including preadipocytes (28). Natural agonists for LRH-1 have not yet been identified, although crystals of the ligand binding domain of LRH-1 were found to contain phospholipids (29).

**FIGURE 6.** Proposed regulatory mechanism of mouse L-PGDS gene expression during differentiation of 3T3-L1 cells from the preadipocytic state to the adipocytic state. L-PGDS gene expression is activated by LRH-1 in the preadipocyte stage and by LXRα-activated SREBP-1c in the adipocyte stage.

Association between SREBP-1c and L-PGDS Levels in the Adipocytic 3T3-L1 Cells—When the adipocytic 3T3-L1 cells were treated with T0901317, an LXR agonist, mRNAs for LXRα, SREBP-1c, and L-PGDS genes were increased (Fig. 5A), indicating that these three genes were up-regulated by the agonist. Western blot analysis revealed that SREBP-1c and L-PGDS production was increased after 12 and 24 h, respectively, of the

struct (Fig. 4A). The −300/+76 construct with the mutated LXRE showed almost the same promoter activity as the wild-type construct, whereas the −300/+76 construct with the mutated SREs gave a decreased reporter activity similar to that obtained with the −190/+76 construct (Fig. 4B), thus indicating that the SREs functioned as active cis-elements in the expression of L-PGDS gene in the adipocytic cells.

EMSA with a synthetic oligonucleotide containing the SREs of the L-PGDS promoter revealed that two DNA-protein complexes were formed in the presence of nuclear extracts prepared from the adipocytic 3T3-L1 cells (Fig. 4C, lane 2). These DNA-protein complexes were not detected in the absence of the nuclear extracts (Fig. 4C, lane 1), and their amount was decreased by adding an excess amount (5-fold or 10-fold) of unlabeled oligonucleotide carrying the SREs (Fig. 4C, lanes 3 and 4). These two DNA-protein complexes were detected only when the nuclear extracts had been prepared from the adipocytic cells (Fig. 4D). The results of the ChIP assay with anti-SREBP-1c antibody showed that the region containing the two SREs in the DNA from adipocytic 3T3-L1 cells was amplified but that the amplification was negligible in the case of preadipocytic cells (Fig. 4E). On the contrary, no amplified signal was observed, when the region without the conserved SREs was amplified in the same DNA sample. The input control was positive in all samples. These data, taken together, provide evidence that SREBP-1c bound to SREs of the proximal promoter region of the mouse L-PGDS gene in the adipocytic 3T3-L1 cells in vitro and in vivo.
During adipocyte differentiation, several nuclear receptors, including LXRα and PPARγ, are expressed and regulate the expression of various genes (20). LXR forms a heterodimer with retinoid X receptor, and this heterodimer binds to the LXRE (30). The synthetic LXR agonists such as T0901317 and natural ligands such as 22(R)-hydroxycholesterol are able to bind to LXRβ and activate their function (31). Activation of LXRα expression in 3T3-L1 cells increases the adipocyte phenotype such as lipid accumulation and expression of various lipogenic genes such as fatty acid synthase and SREBP-1c (32). However, the role of LXR in adipocyte differentiation is controversial. We demonstrated that LXRs expression was increased during adipocyte differentiation, and LXR-mediated up-regulation of L-PGDS was involved in the activation of this differentiation process (Fig. 6, and B). Seo et al. (33) suggested that LXR-stimulate adipocyte differentiation through PPARγ, and Juvet et al. (32) reported enhancement of lipid accumulation by activation of LXR in 3T3-L1 cells. In contrast, Ross et al. (34) reported that the activation of LXR might negatively function in adipocyte differentiation. Hummasti et al. (35) also indicated that LXRβ are not involved in the lipid accumulation in mouse 3T3-F442A and 3T3-L1 cells. Further studies are needed to provide a clear explanation for this discrepancy.

SREBP-1c belongs to the SREBP family of basic helix-loop-helix leucine-zipper transcription factors that play important roles in lipid metabolism (36, 37). SREBP-1c is involved in adipocyte differentiation, insulin sensitivity, and fatty acid synthesis (37–39). The stimulation of SREBP-1c enhances the expression of various genes involved in lipogenesis and adipogenesis, including PPARγ, fatty acid synthase (39, 40), lipoprotein lipase (39), acetyl-CoA carboxylase (41), and resistin (42). In the present study, we demonstrated that L-PGDS gene expression was enhanced by the LXRα agonist through binding of SREBP-1c to the SREs in L-PGDS promoter (Figs. 4E, 5A, and 5B). SREBP-1c is dominantly expressed in adipose tissue (38). Although the one or more functional roles of L-PGDS remain to be elucidated, the present results support the notion that SREBP-1c regulates L-PGDS gene expression upon adipogenesis. Previously, LXR-mediated activation of adipocytes was shown to occur through induction of PPARγ (42).

In summary, L-PGDS enhanced lipid accumulation in adipocytes. L-PGDS gene expression was activated by LRH-1 in preadipocytes and by SREBP-1c in adipocytes. Thus, we propose an alternative pathway to activate adipocyte differentiation, i.e. LXRα-activated L-PGDS through induction of SREBP-1c. Further characterization of the role of L-PGDS as well as that of PGD2 in adipocytes is an important goal, with possible therapeutic implications for treatment of metabolic disorders such as diabetes and obesity.

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REFERENCES

1. Spiegelman, B. M., and Flier, J. S. (2001) Cell 104, 531–543
2. Mohamed-Ali, V., Pinkney, J. H., and Coppack, S. W. (1998) Int. J. Obes. Relat. Metab. Disord. 22, 1145–1158
3. Nadler, S. T., and Attie, A. D. (2001) J. Nutr. 131, 2078–2081
4. Berg, A. H., and Scherer, P. E. (2005) Circ. Res. 96, 939–949
5. Green, H., and Kehinde, O. (1975) Cell 2, 19–27
6. Iwase, I. R., Murdock, P. R., Moore, G. B., Murphy, G. I., Smith, S. A., and Hayes, J. D. (2003) Prostaglandins Other Lipid. Mediat. 70, 267–284
7. Xie, Y., Kang, X., Ackerman, W. E., Belury, M. A., Koster, C., Rovin, B. H., Landon, M. B., and Kniss, D. A. (2006) Diabetes Obes. Metab. 8, 83–93
8. Urade, Y., Eguchi, N., and Hashida, O. (2006) Lipocalin-type Prostaglandin D Synthase as an Enzymic Lipocalin, pp. 99–109, Landes Bioscience/Eurekah.com, Austin, TX
9. Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A., and Hayashi, O. (1997) J. Biol. Chem. 272, 15789–15795
10. Beuckmann, C. T., Aoyagi, M., Okazaki, L., Hiroike, T., Toh, H., Hayashi, O., and Urade, Y. (1999) Biochemistry 38, 8006–8013
11. Mohri, I., Tanikie, M., Okazaki, I., Kagitani-Shimoto, K., Aritake, K., Kanekiyo, T., Yagi, T., Takikita, S., Kim, H., Urade, Y., and Suzuki, K. (2006) J. Neurochem. 97, 641–651
12. Kanekiyo, T., Ban, T., Aritake, K., Huang, Z. L., Qu, W. M., Okazaki, I., Mohri, I., Murayama, S., Ozono, K., Tanikie, M., Goto, Y., and Urade, Y. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 6412–6417
13. Qu, W. M., Huang, Z. L., Xu, X. H., Aritake, K., Eguchi, N., Namba, F., Narumiya, S., Urade, Y., and Hayashi, O. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 17949–17954
14. Regalia, L., Palaisa, T., Hall, C. E., Maesaka, J. K., Eguchi, N., and Urade, Y. (2005) J. Biol. Chem. 280, 29946–29955
15. Quinnell, M., Bjujska, L. J., Tomlinson, J. W., Smith, D. M., and Stewart, P. M. (2006) (Gene (Amst.) 380, 137–143
16. Miwa, Y., Takuchi, S., Kamide, K., Yoshii, M., Horio, T., Tanaka, C., Banno, M., Miyata, T., Sasaguri, T., and Kawano, Y. (2004) Biochem. Biophys. Res. Commun. 322, 428–433
17. Cipollone, F., Fazia, M., Iezzi, A., Ciabattoni, G., Pini, B., Cucurullo, C., Ucchino, S., Spiganoardo, F., De Luca, M., Frontiera, C., Chiarelli, F., Cucurullo, F., and Mezzetti, A. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 1259–1265
18. Ramirez-Zacarias, J. L., Castro-Munozledo, F., and Kuri-Harcwuch, W. (1992) Histochemistry 97, 493–497
19. Fujimori, K., Okada, T., and Urade, Y. (2002) J. Biochem. (Tokyo) 131, 383–389
20. Fu, M., Sun, T., Bookout, A. L., Downes, M., Yu, R. T., Evans, R. M., and Mangelsdorf, D. J. (2005) Mol. Endocrinol. 19, 2437–2450
21. Fujimori, K., Kanaoka, Y., Sakaguchi, Y., and Urade, Y. (2000) J. Biol. Chem. 275, 40511–40516
22. Fujimori, K., Fujitani, Y., Kadowai, K., Kumanohaguh, H., Ishikawa, K., and Urade, Y. (2003) J. Biol. Chem. 278, 6018–6026
23. Aritake, K., Kado, Y., Inoue, T., Miyano, M., and Urade, Y. (2006) J. Biol. Chem. 281, 15277–15286
24. Rosen, E. D., Walkey, C. J., Puigserver, P., and Spiegelman, B. M. (2000) Genes Dev. 14, 1293–1307
25. Kahn, B. B., and Flier, J. S. (2000) J. Clin. Invest. 106, 473–481
26. Kopelman, P. G. (2000) Nature 404, 635–643
27. Zimmel, P., Alberti, K. G., and Shaw, J. (2001) Nature 414, 782–787
28. Clyne, C., Speed, C. J., Zhou, J., and Simpson, E. R. (2002) J. Biol. Chem. 277, 20591–20597
29. Krylova, I. N., Sabin, E. P., Moore, J., Xu, R. X., Waitt, G. M., MacKay, J. A., Juvet, L. K., Andresen, S. M., Schuster, G. U., Dalen, K. T., Tobin, K. A., Hollung, K., Haugen, F., Jacinto, S., Ulven, S. M., Bamberg, K., Gustafsson, J. A., and Neub, H. I. (2003) Mol. Endocrinol. 17, 172–182
30. Seo, J. B., Moon, H. M., Kim, W. S., Lee, Y. S., Jeong, H. W., Yoo, E. J., Han, J., Kang, H., Park, M. G., Steffensen, K. R., Stulning, T. M., Gustafsson, J. A., Park, S. D., and Kim, J. B. (2004) Mol. Cell. Biol. 24, 3430–3444
31. Ross, S. E., Erickson, R. L., Gerin, I., DeRose, P. M., Bajnok, L., Longo, K. A.,
LXR-SREBP-1c-mediated L-PGDS Induction in Adipocytes

Misek, D. E., Kuick, R., Hanash, S. M., Atkins, K. B., Andresen, S. M., Nebb, H. I., Madsen, L., Kristiansen, K., and MacDougald, O. A. (2002) Mol. Cell. Biol. 22, 5989–5999
35. Hummasti, S., Laffitte, B. A., Watson, M. A., Galardi, C., Chao, L. C., Ramamurthy, L., Moore, J. T., and Tontonoz, P. (2004) J. Lipid Res. 45, 616–625
36. Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towlé, H. C., and Spiegelman, B. M. (1995) Mol. Cell. Biol. 15, 2582–2588
37. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) J. Clin. Invest. 101, 1–9
38. Tontonoz, P., Kim, J. B., Graves, R. A., and Spiegelman, B. M. (1993) Mol. Cell. Biol. 13, 4753–4759
39. Kim, J. B., and Spiegelman, B. M. (1996) Genes Dev. 10, 1096–1107
40. Boizard, M., Le Liepvre, X., Lemarchand, P., Foufelle, F., Ferre, P., and Dugail, I. (1998) J. Biol. Chem. 273, 29164–29171
41. Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M., and Osborne, T. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1049–1053
42. Seo, J. B., Noh, M. J., Yoo, E. J., Park, S. Y., Park, J., Lee, I. K., Park, S. D., and Kim, J. B. (2003) Mol. Endocrinol. 17, 1522–1533