Liver X receptors are regulators of adipocyte gene expression but not differentiation: identification of apoD as a direct target[S]

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Abstract The liver X receptors α and β (LXRα and LXRβ) have been shown to play important roles in lipid homeostasis in liver and macrophages, however, their function in adipose tissue is not well defined. Both LXRs are highly expressed in fat, and the expression of LXRα increases during adipogenesis. Furthermore, LXRα expression is induced by peroxisome proliferator-activated receptor γ (PPARγ), the master regulator of fat cell differentiation. Here we investigate the role of LXRs in adipocyte differentiation and gene expression and their potential crosstalk with the PPARγ pathway. We demonstrate that LXR agonists have no significant effect on the differentiation of 3T3-F442A or 3T3-L1 preadipocytes in vitro and do not alter the expression of differentiation-linked PPARγ target genes in vivo. Moreover, retroviral expression of LXRα in NIH-3T3 cells does not alter the adipogenic potential of these cells and neither augments nor inhibits the action of PPARγ. However, transcriptional profiling studies reveal that LXRs are important regulators of adipocyte gene expression. We identify the multifunction lipid carrier protein apolipoprotein D and the lipogenic protein Spot 14 as LXR responsive genes both in vitro and in vivo.[jlr] Thus, although LXRs do not influence adipocyte differentiation per se, these receptors are likely to play an important role in the modulation of lipid metabolism in adipocytes.—Hummasti, S., B. A. Laffitte, M. A. Watson, C. Galardi, L. C. Chao, L. Ramamurthy, J. T. Moore, and P. Tontonoz. **Liver X receptors are regulators of adipocyte gene expression but not differentiation: identification of apoD as a direct target. *J. Lipid Res.* 2004.** 45: **616–625.**

Supplementary key words adipocyte • apolipoprotein • differentiation • liver X receptor • nuclear receptor • peroxisome proliferator-activated receptor

Adipose tissue plays a central role in energy homeostasis, storing energy in times of nutritional abundance and releasing it in times of nutritional deprivation (1). Obesity, the excessive accumulation of adipose tissue, is an established risk factor for heart disease and noninsulin-dependent diabetes mellitus. On the other hand, lipodystrophy, too little adipose tissue, also results in diabetes and aberrant lipid metabolism. Therefore, proper regulation of adipogenesis is required not only for appropriate lipid storage, but also for systemic energy and lipid homeostasis. Indeed, alterations in triglyceride storage and FA release in adipose tissue have been shown to affect glucose metabolism in other tissues, such as liver and skeletal muscle (2). Elucidating the regulatory pathways that control adipocyte differentiation is likely to identify novel opportunities for intervention in metabolic diseases.

Over the past 10 years, several key regulators of adipogenesis have been identified. These include the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) and members of the C/EBP family of transcription factors (3, 4). Extensive investigation of PPARγ has established an essential role for this protein in both adipogenesis and adipocyte function. Ectopic expression of PPARγ is sufficient to drive the adipogenic program, and the loss of PPARγ expression renders cells incapable of becoming adipocytes (5–8). In addition, PPARγ has been shown to regulate the expression of several secreted cytokines, including leptin and adiponectin, which display sys-

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Abbreviations: apoD, apolipoprotein D; FACoA, FA coenzyme A; GARG-16, glucocorticoid attenuated response gene 16; GLUT4, glucose transporter-4; OSBP, oxysterol-binding protein; LXR, liver X receptor; LXRE, LXR response element; PGAR, PPARγ angioprotein related; PPARγ, peroxisome proliferator-activated receptor γ; RARγ, retinoic acid receptor γ; RXRα, retinoid X receptor α; SC5D, sterol-C5-desaturase; SREBP-1c, sterol-regulatory element binding protein 1c.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional table.

temic effects as signaling molecules (3, 9). While the role of PPARγ in adipose tissue has been firmly established, other factors working independently or in conjunction with PPARγ remain unidentified.

The liver X receptors (LXR) α and β are oxysterol-activated nuclear receptors with largely overlapping functions. Studies in macrophages have identified a number of genes involved in reverse cholesterol transport whose expression is controlled by LXR, including ABCA1, ABCG1, apolipoprotein E (apoE), LPL, and phospholipid transfer [as reviewed in ref. (10)]. In the liver, LXR has been shown to regulate expression of CYP7A1, the rate-limiting enzyme in conversion of cholesterol to bile acids, and sterol-regulatory element binding protein 1c (SREBP-1c) and FAS, important lipogenic proteins (11–14). Recently, LXR has also been implicated in control of glucose metabolism in the liver through regulation of glucokinase and gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (15–17). Thus, LXRs appear to play an important role in both lipid and glucose homeostasis.

While LXRβ is ubiquitously expressed, LXRα has a more restricted expression pattern. In addition to macrophages and liver, LXRα is also highly expressed in adipose tissue, and its expression increases during adipogenesis and is regulated by PPARγ (18–21). However, the function of LXRs in adipose tissue is poorly understood. In fact, conflicting reports have suggested that LXRs function as both positive and negative regulators of adipocyte differentiation and lipid accumulation (18, 19). In the present study, we have used retroviral expression systems and synthetic ligands to probe the function of LXR in fat cells. We demonstrate that expression and ligand activation of LXRα has no significant effect on adipogenesis or lipid accumulation and does not modulate the adipogenic activity of PPARγ. However, the identification of novel LXR adipocyte target genes in cultured cells and in vivo points to an important role for LXR in adipose tissue function that is distinct from PPARγ.

MATERIALS AND METHODS

Reagents and stable cell lines

GW3965, GW7845, and T0901317 were provided by Jon Collins and Timothy Wilson (GlaxoSmithKline). Ligands were dissolved in dimethyl sulfoxide before use in cell culture. Expression constructs containing full-length cDNAs for PPARγ and LXRα (22, 23) were packaged into retrovirus by transient transfection of Phoenix E cells as previously described (6). NIH-3T3 cells were infected at 50% confluence with approximately equal titers of virus. Stable cell lines were selected with either 2 μg/ml puromycin or 50 μg/ml hygromycin. For cell lines expressing both PPARγ and LXRα, hygromycin-resistant PPARγ cell lines were first selected and subsequently infected with puromycin-resistant LXRα expression vector.

Cell culture

3T3-L1, 442A, and NIH-3T3 cell lines were maintained in DMEM containing 10% bovine calf serum. 3T3-L1 cells were differentiated by treatment at confluence with dexamethasone (1 μM), methylisobutylxanthine (0.5 μM), and insulin (5 μg/ml), in DMEM containing 10% FBS, for 2 days. Cells were subsequently cultured in DMEM containing 10% FBS and insulin. 442A cells were differentiated as described for 3T3-L1 cells without the addition of differentiation cocktail. For time course studies, ligand was first added at confluence, and media with fresh ligand was added every 1–2 days. For gene expression studies in fully differentiated adipocytes, cells were differentiated into mature adipocytes (8–10 days) and subsequently treated with ligand for 24 h. Stably expressing NIH-3T3 cell lines were switched to DMEM containing 10% FBS at confluence and treated with ligand for 24 h.

RNA analysis

RNA was isolated using Trizol reagent (Life Technologies, Inc.). Sybrgreen and Taqman real-time quanitative PCR assays were performed using an Applied Biosystems 7700 sequence detector as described (20). Results show averages of duplicate experiments normalized to 36B4. Primer and probe sequences are available on request.

Animals

Ten-week-old female C57Bl/6 mice were maintained on standard rodent chow and gavaged with GW3965 (20 mg/kg/day) daily or with vehicle (0.5% methylcellulose) for three days prior to sacrifice. All mice were sacrificed during mid-light cycle after a 12 h fast. All mice received their final dose of GW3965 by gavage 2–4 h prior to sacrifice. Tissues were harvested for RNA with Trizol reagent. Animal experiments were approved by the Institution Animal Care and Research Advisory Committee of the University of California, Los Angeles.

DNA microarray analysis

Differentiated 3T3-L1 adipocytes were cultured in DMEM containing 10% FBS, insulin, and either vehicle or GW3965 (1 μM) for 24 h. Total RNA was isolated using Trizol reagent and further purified with a Qiagen RNeasy total RNA isolation kit. Total RNA was reverse transcribed using a T7-(dT)24 primer (Genset Corp.) and the Superscript Choice system (Life Technologies). Biotin-labeled cRNA was generated using a bioarray high-yield transcript labeling kit (Enzo). Fragmentation of cRNA was performed using 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C. Samples were hybridized to Affymetrix murine U74Av2 microarrays and visualized by the PAN Facility at Stanford University. The results of the microarrays were analyzed with Genespring and GeneChip Analysis Suite software (Affymetrix).

Electrophoretic mobility-shift assays

DNA binding was analyzed using a radiolabeled oligonucleotide probe corresponding to the LXR response element (LXRE) from the human apoD promoter. Competitor oligonucleotides were added at 5- or 25-fold molar excess (rat CYP7A oligonucleotide added only at 25-fold molar excess). The binding reactions were resolved on a preelectrophoresed 0.25 X TBE, 4% polyacrylamide gel at room temperature. Human LXRα and retinoid X receptor α (RXRα) proteins were synthesized from pSG5-h LXRα and RXRα using the TNT T7 coupled reticulocyte system (Promega, Madison, WI). The oligonucleotides used were as follows (sense strand only, with overhang and mutated nucleotides in lower case and underlined, respectively): Rat CYP7A1; 5′-gatcCTTTGGTCACTCAAGTTCAAGT-3′, apoD LXRE; 5′- agctGGTGGATCACCTGAGGTCAGGA-3′, Mut apoDLXRE; 5′- agctGGTGGCACACCTGAGAACAGGA-3′.

Transfection assays

HEK 293 cells were plated in 96-well plates at a density of 25,000 cells per well in high glucose DMEM supplemented with 10% charcoal/dextran-treated FBS (HyClone Laboratories, Lo-

gan, UT). Transfection mixes contained 2 ng of expression vector (containing full-length human LXRα or RXRα) and 8 ng of apoD×3-thymidine kinase-luciferase. A Renilla luciferase construct was added to the transfection mix to provide an internal control for transfection efficiency (carrier DNA was used to bring the total DNA per transfection to 65 ng/well). Transfections were performed with Fugene transfection reagent (Roche, Nutley, NJ) in OPTI-MEM medium (Life Technologies) according to manufacturer's instructions. The lipid-to-DNA ratio used in the transfections was 4:1. Cells were incubated in the transfection mix for 24 h followed by an additional 24 h in DMEM supplemented with 10% charcoal-stripped and delipidated serum (Sigma, St. Louis, MO) ± 1 μM LG100268, 1 μM T0901317, or both. At the end of the incubation, reporter activities were measured using a Stop-and-Glow dual luciferase assay kit according to manufacturer's instructions (Promega, Madison WI).

RESULTS

Based on the role of LXRs in lipid metabolism and the expression pattern of LXRα during adipogenesis, we investigated the impact of LXR signaling on adipocyte differentiation. 3T3-L1 preadipocytes were treated at confluence for 9 days with a differentiation cocktail (see Materials and Methods) and vehicle or nuclear receptor ligands as indicated (**Fig. 1**). Oil red O staining revealed that treatment with the synthetic LXR agonist GW3965 had no significant effect on lipid accumulation and morphologic differentiation when compared with control cells (Fig. 1). In contrast, treatment with the PPARγ-specific ligand GW7845 resulted in a marked potentiation of lipid accumulation and differentiation as expected. Treatment of cells with LXR agonist in combination with PPARγ agonist neither enhanced nor diminished the effect of PPARγ ligand alone. Similar results were obtained with the structurally unrelated LXR agonist T1317 (data not shown). LXR ligands also had no effect on the differentiation of 3T3-F442A adipocytes (data not shown). Thus, under the conditions used here, LXR activation has no significant effect on lipid accumulation or morphologic differentiation of murine preadipocytes in vitro.

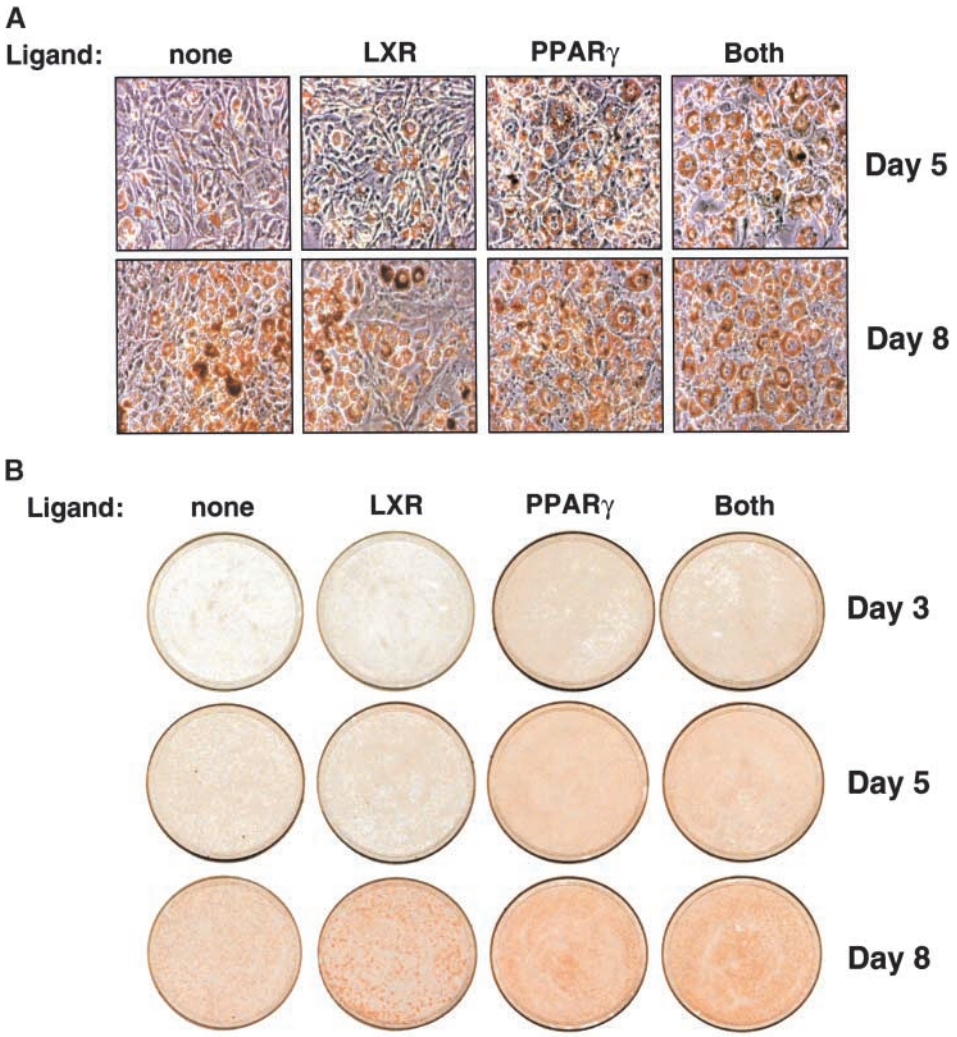

Fig. 1. Liver X receptor (LXR) agonists do not affect lipid accumulation during 3T3-L1 cell differentiation. 3T3-L1 cells were treated with differentiation media and LXR agonist (GW3965), peroxisome proliferator-activated receptor γ (PPARγ) agonist (GW7845), or both. At the indicated time points, cells were fixed and stained with oil red O. A: Microscopic view; objective magnification 20×. B: Macroscopic view of oil red O-stained 10 cm dishes.

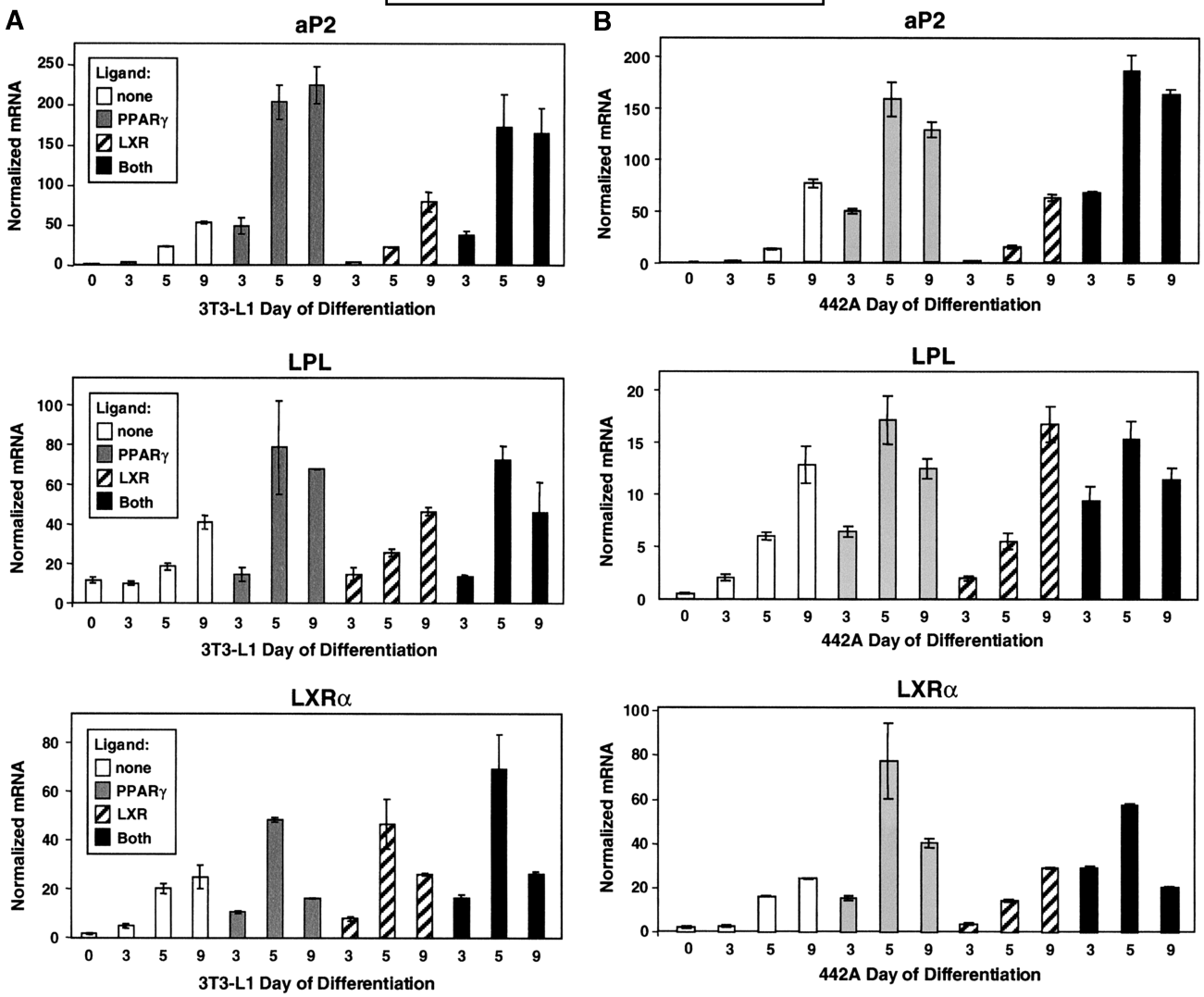

Fig. 2. LXR agonists do not affect adipocyte-associated gene expression in preadipocyte cell lines. 3T3-L1 (A) and 3T3-F442A (B) cells were treated with differentiation cocktail and LXR ligand (GW3965), PPARγ ligand (GW7845), or both. RNA was isolated at the indicated time points and analyzed by real-time quantitative PCR assays. Expression of the adipocyte-associated genes aP2 and LPL is increased by PPARγ agonist treatment, but not LXR agonist treatment. Expression of LXRα mRNA increases during differentiation and is further increased by PPARγ ligand in both 3T3-L1 (A) and 3T3-F442A cells (B).

To confirm these observations on a molecular level, we examined the expression of adipocyte differentiation-linked genes in preadipocyte cell lines. 3T3-L1 and 3T3-442A cells were treated with GW3965 (LXR) and/or GW7845 (PPARγ) ligands throughout the differentiation time course as above. RNA was isolated and gene expression analyzed at 3, 5, and 9 days post confluence. LXR agonist had no significant effect on expression of the adipocyte genes aP2 and LPL in either 3T3-L1 cells (**Fig. 2A**) or 3T3-442A cells (Fig. 2B). As expected, GW7845 strongly enhanced expression of these differentiation markers. The very slight reduction in expression of aP2 and LPL in 3T3-L1 cells in the presence of both GW7845 and GW3965 (Fig. 2A) was not statistically significant and not reproducible in three independent experiments. The LXR signaling pathway is functional in these cells, however, because established LXR target genes such as ABCA1 were increased by GW3965 as expected (data not shown and see below). Furthermore, expression of LXRα itself increased during differentiation and was modestly increased by PPARγ ligand in both cell lines.

We next used a defined system to examine the effect of ectopic expression of LXRα on the adipogenic potential of NIH-3T3 cells. These cells express LXRβ, but not LXRα (22). NIH-3T3 cells do not normally undergo adipogenesis, but can be induced to accumulate lipid and to undergo an adipocyte conversion when PPARγ is ectopically expressed (6). We transduced NIH-3T3 cells with retroviral expression vectors for LXRα, PPARγ, or both (see Materials and Methods). Stable cell lines were isolated and then treated at confluence with LXR and/or PPARγ ligands. A low level of LXR ligand responsiveness was observed in control cell lines due to the presence of endogenous LXRβ (**Fig. 3**). Expression of the LXR target gene

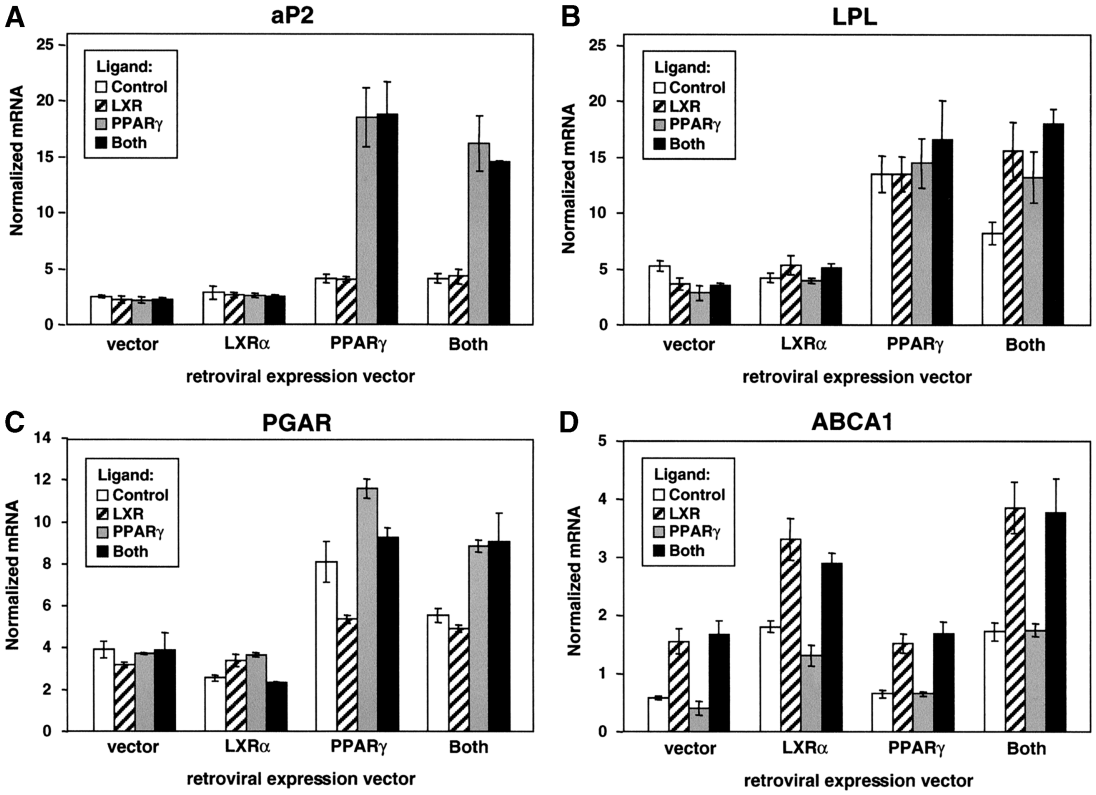

Fig. 3. Ectopic expression of LXRα does not affect adipocyte-associated gene expression in NIH-3T3 fibroblasts. NIH-3T3 murine fibroblasts were infected with retroviral expression vectors to create stable cell lines expressing either vector alone, PPARγ, LXRα, or both PPARγ and LXRα. mRNA expression was analyzed by real-time quantitative PCR assays. Treatment of PPARγ-expressing cells with PPARγ agonist (GW7845) induces expression of the known target genes aP2 (A), LPL (B), and PPARγ angioprotein related (PGAR) (C) as expected. In contrast, LXRα expression and ligand (GW3965) activation has no effect on the expression of aP2 (A), LPL (B), or PGAR (C), either alone or in combination with PPARγ. The LXR target gene ABCA1 is induced by LXR expression and agonist treatment as expected (D).

ABCA1 was increased by the expression of LXRα and further induced in response to LXR ligand, confirming that LXR signaling was activated. However, in agreement with our results in 3T3-L1 and F442A cells, expression and activation of LXRα did not significantly affect mRNA levels of the adipocyte-associated genes aP2, LPL, or PPARγ angioprotein related (Fig. 3) and failed to induce lipid accumulation (data not shown). Consistent with previous work (6, 24), expression of PPARγ triggered adipocyte differentiation and induction of these genes. Moreover, the induction of adipogenic markers in cell lines stably expressing both PPARγ and LXRα was comparable to those expressing PPARγ alone. Thus, in the NIH-3T3 system, LXR signaling neither augments nor inhibits PPARγ-driven adipogenesis.

The results presented above fail to support a role for LXR signaling in the process of adipocyte differentiation per se; however, these results do not preclude a role for LXR in the function of mature adipocytes. We, therefore, performed transcriptional profiling on fully differentiated 3T3-L1 adipocytes that had been treated for 24 h with vehicle or GW3965. RNA from these cells was used to hybridize to Affymetrix U74Av2 arrays. Selected results are shown in **Table 1** (the entire results are provided as supplementary data). Established targets, such as ABCA1 and apoC-I, were highly induced in this experiment, validating the approach. A number of potentially novel LXR responsive genes were also identified, including: apoD, a member of the lipocalin superfamily of carrier proteins that transport small hydrophobic molecules (25); Spot 14, a gene implicated in lipogenesis (26); and GARG-16, the glucocorticoid attenuated response gene 16 (27).

In order to confirm the microarray results, we examined the effects of LXR ligand treatment on gene expres-

TABLE 1. Results of Affymetrix murine U74Av2 microarrays

Gene	Activation (fold) by GW3965
GARG-16	4.5
SC5D	4.5
ABCA1	4.1
apoD	3.6
FACoA ligase	3.5
Spot 14	3.1
apo C-I	3.1
Estrogen-responsive zinc finger protein	2.8
Solute carrier family 38, member 2	2.5
RARγ	2.0
OSBP	2.0

apoD, apolipoprotein D; FACoA, FA coenzyme A; GARG-16, glucocorticoid attenuated response gene 16; OSBP, oxysterol-binding protein; RARγ, retinoic acid receptor γ; SC5D, sterol-C5-desaturase.

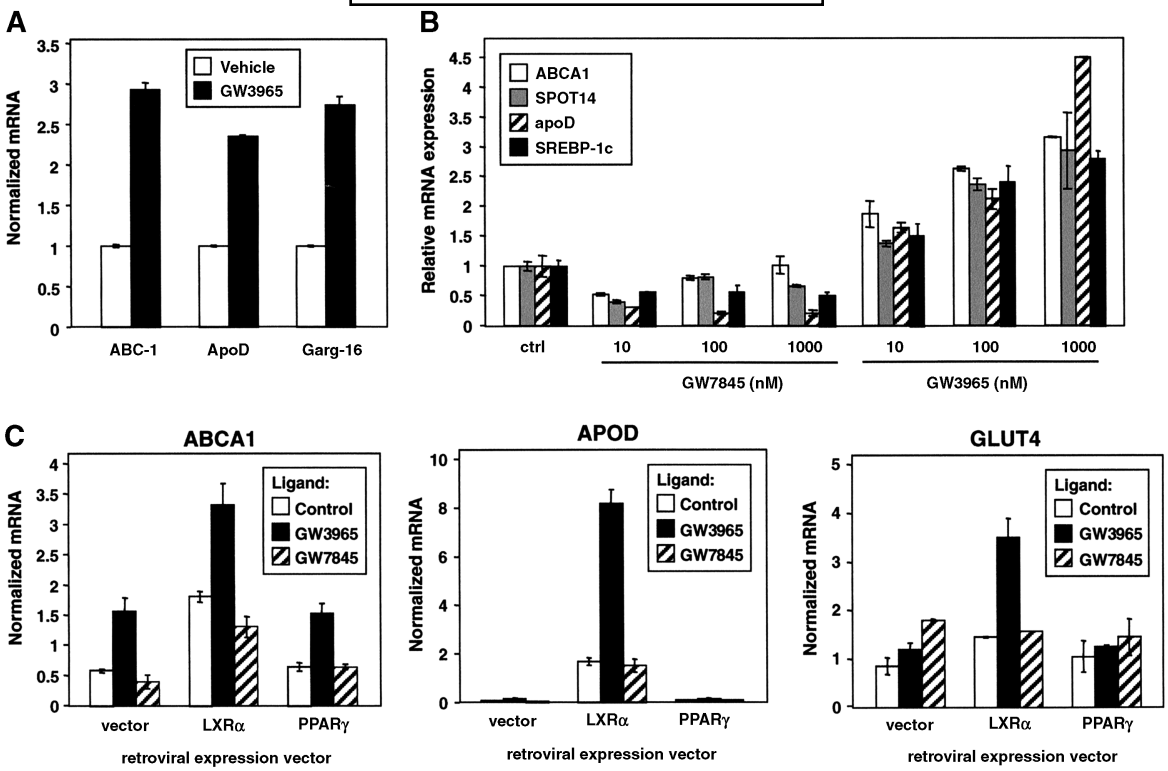

Fig. 4. Regulation of novel adipocyte target genes in vitro by LXR ligands. mRNA expression was analyzed by real-time quantitative PCR assays. A: Differentiated 3T3-L1 adipocytes were treated with LXR agonist (GW3965) for 24 h. B: Differentiated F442A adipocytes were treated with the indicated concentration of PPARγ (GW7845) or LXR (GW3965) agonist for 24 h. C: NIH-3T3 cells expressing vector, LXRα, or PPARγ were treated with GW7845 or GW3965 for 24 h as indicated.

sion in differentiated adipocytes. Real-time quantitative PCR analysis revealed LXR-dependent increases in the expression of apoD, GARG-16, and the established target ABCA1 in 3T3-L1 adipocytes (**Fig. 4A**). apoD, Spot 14, ABCA1, and SREBP-1c, but not GARG-16, were also specifically regulated by LXR ligand in a dose-dependent manner in 442A adipocytes (Fig. 4B). To verify that induction of these genes by GW3965 was mediated by LXRs rather than another pathway, we again turned to the NIH-3T3 system. We compared the ability of synthetic receptor ligands to induce gene expression in NIH-3T3 cells expressing vector, LXRα, or PPARγ. Ectopic expression of LXRα induced both basal- and ligand-inducible expression of the known targets, ABCA1 and glucose transporter-4 (GLUT4) (Fig. 4C). In addition, expression of the novel target apoD was markedly responsive to LXR expression. The response of all three genes is specific for LXR because expression and activation of PPARγ had no effect on their expression. Expression of Spot 14 and GARG-16 was not inducible by LXR ligand in NIH-3T3 cells (data not shown). In the case of Spot 14, the lack of induction may relate to the very low level of SREBP-1c expression in this cell type or to the need for additional adipose-tissue specific regulatory factors.

Next, we endeavored to determine the mechanism whereby LXRs control expression of the apoD gene. Sequence analysis of the human apoD promoter identified a potential LXRE located at position −2524 bp relative to the transcriptional start site (**Fig. 5A**). Electrophoretic mobility shift assays using in vitro translated LXRα and RXRα proteins and radiolabeled apoD LXRE oligonucleotide confirmed the ability of LXR/RXR heterodimers to bind this element in a sequence-specific manner (Fig. 5B). In addition, the apoD LXRE was an effective competitor for LXR/RXR binding to the previously identified CYP7A LXRE.

We further analyzed the ability of the apoD LXRE element to drive transcription in transfection assays. Expression vectors for LXRα and RXRα were transiently transfected into CV-1 cells along with a luciferase reporter construct containing three tandem copies of the apoD LXRE. After transfection, cells were treated with vehicle or 1 μM T1317 for 24 h. As shown in **Fig. 6**, this reporter was strongly activated by transfected LXR/RXR in a ligand-dependent manner. Both LXR and RXR ligands stimulated reporter activity, and the combination of both ligands had an additive effect. Taken together, the results of Figs. 5 and 6 identify the apoD gene as a direct transcriptional target of LXR.

The established function of apoD and Spot 14 in lipid transport and lipogenesis suggests that their regulation by LXR might impact the physiologic function of adipose tissue. We therefore endeavored to determine whether these genes were regulated by the LXR signaling pathway in vivo. C57BL/6 mice (n = 9/group) were treated with vehicle or the synthetic LXR agonist GW3965 (20 mg/kg/

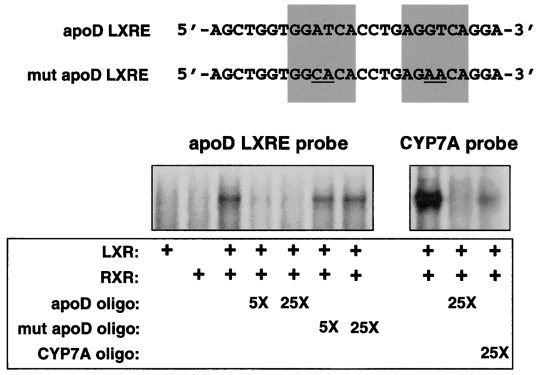

Fig. 5. Electrophoretic mobility-shift analysis of apolipoprotein D (apoD) LXR response element (LXRE). Competition experiments were performed using a radiolabeled oligonucleotide probe corresponding to the LXRE from the human apoD promoter or the LXRE from the rat CYP7A gene. In addition to the probe, binding reactions contained in vitro-translated human LXRα and/or retinoid X receptor α (RXRα). Some reactions also contained competitor oligonucleotides corresponding to either the apoD LXRE from the human apoD promoter, the mutated human apoD LXRE, or rat CYP7A1 DR4 as indicated. Competitor oligonucleotides were added at 5- or 25-fold molar excess over the radiolabeled probe.

day) for 3 days. Animals were fasted overnight prior to tissue and RNA isolation. As shown in **Fig. 7A**, white adipose tissue from LXR agonist-treated mice showed a significant increase in both apoD and Spot 14 gene expression compared with control mice. Interestingly, both genes were also induced by LXR ligand in skeletal muscle (Fig. 7C), while only Spot 14 was induced in liver (Fig. 7D). Thus, the control of apoD expression by LXR appears to be tissue-specific. Similar results have previously been reported for apoE (28). In contrast, expression of GARG-16 was not regulated by LXR in vivo, suggesting that this gene may not be physiologically relevant to adipose tissue (data not shown). It remains possible that GARG-16 may be regulated by LXR in other target tissues such as macrophages. We also addressed the issue of whether LXR activation alters the expression of PPARγ-dependent differentiation markers in adipose tissue. Consistent with the in vitro studies in Figs. 2 and 3, the LXR agonist did not alter the expression of either CD36 or aP2 in vivo (Fig. 7B). Thus, LXR activation neither blocks nor augments PPARγ function in adipose tissue. Taken together, these results indicate that although LXRs do not actively promote adipogenesis, they are potent regulators of lipid metabolic gene expression in mature adipose tissue in vivo.

DISCUSSION

The nuclear receptors LXRα and LXRβ have been shown to play an important role in lipid metabolism in liver, intestine, and macrophages. Several lines of evidence point to a role for LXRs in adipose tissue as well, including the high level of expression of LXRα and LXRβ in fat, the increase in LXRα mRNA levels during adipogenesis, and the regulation of LXRα by PPARγ. We have, therefore, investigated the role of LXRs in fat cell differentiation and gene expression. We show here that highly specific synthetic ligands have no effect on lipid accumulation or differentiation-linked gene expression in preadipocyte cells in vitro and do not alter the expression of PPARγ target genes in vivo. Furthermore, forced expression of LXRα in NIH-3T3 fibroblasts has no effect on differentiation, either alone or in conjunction with PPARγ. Despite the lack of evidence for an obligatory role in adipogenesis, the identification of novel LXR target genes by microarray analysis supports a role for LXRs in mature fat cell function.

Our findings that LXRs do not influence differentiation or lipid accumulation of murine preadipocyte cell lines in vitro contrast with two recent studies. Juvet et al. (18) reported increased lipid accumulation upon LXR ligand (T1317 or 22(R)-hydroxycholesterol) treatment of 3T3-L1 cells. The basis for these differing results is not clear, but subtle differences in cell culture conditions or cell line variations cannot be excluded. Although the use of different LXR agonists (22(R)-hydroxycholesterol and T1317 compared with GW3965) could potentially lead to different results, we have not observed any significant effect of T0901317 on lipid droplet size (data not shown) or differentiation-linked gene expression (Fig. 2) in either 3T3-L1 or F442A cells. We have also been unable to confirm the observation that 22(R)-hydroxycholesterol promotes differentiation (18), because in our hands, this compound is cytotoxic to cells when administered chronically at micromolar concentrations. Because small mole-

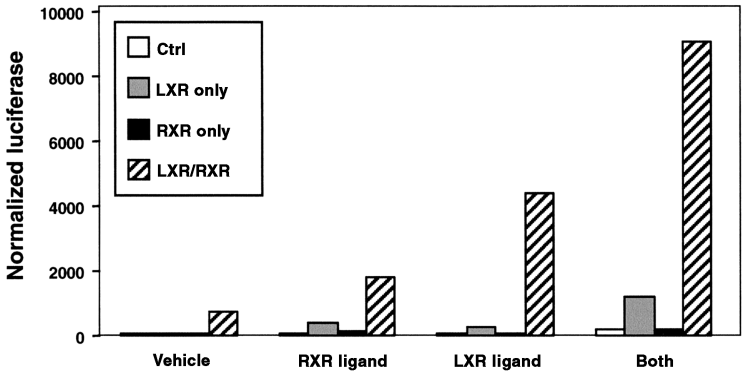

Fig. 6. Activation of the apoD LXRE by the LXR/RXR heterodimer. HEK 293 cells were transiently transfected with a reporter gene containing three copies of the apoD LXRE cloned upstream of a mininal thymidine kinase promoter and luciferase reporter gene. Cells were also cotransfected with or without expression vectors for LXR, RXR, or both, as indicated. Cells were subsequently treated with or without ligands for LXR (T1317) and/or RXR (LG268) for 24 h before harvesting. Cell lysates were assayed for luciferase activity.

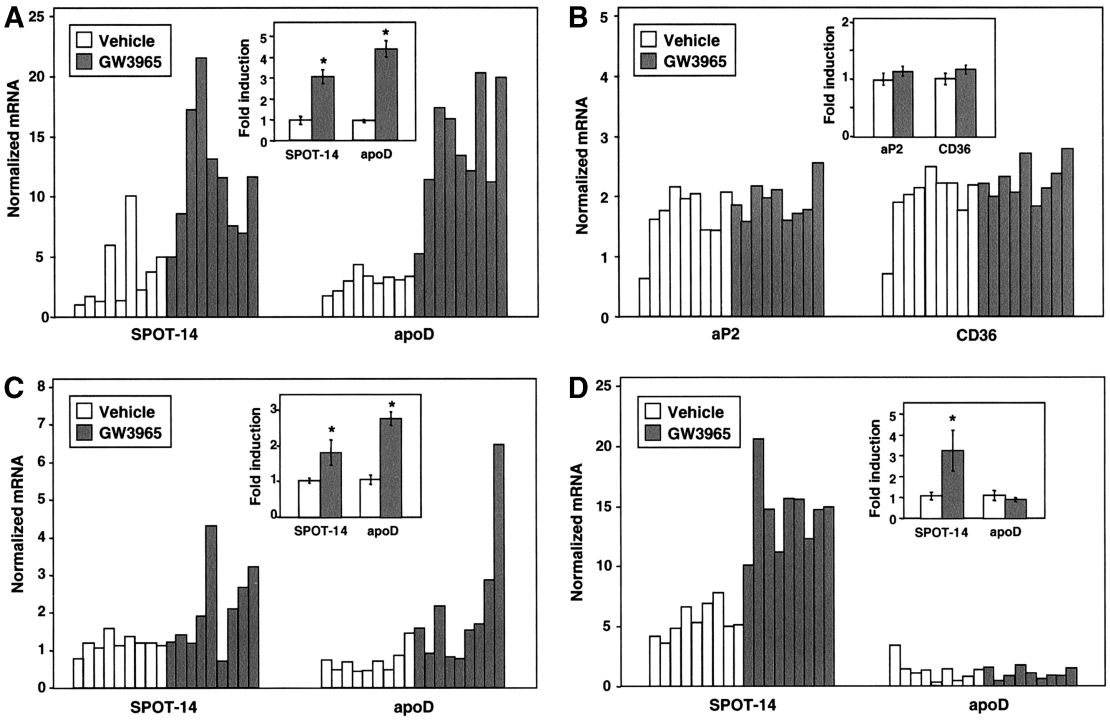

Fig. 7. Regulation of apoD and Spot 14 by LXR agonist in vivo. C57BL/6 mice (n = 9 per group) were gavaged daily with GW3965 (20 mg/kg) or vehicle. At the end of the treatment period, total RNA was isolated from liver, adipose tissue, and skeletal muscle. Gene expression for individual animals was determined by real-time quantitative-PCR assays. Insets show the average expression for each group ± SD. A, B: White adipose tissue. C: Skeletal muscle. D: Liver. * $P < 0.05$ by Student's *t*-test.

cule activators of nuclear receptors often possess receptor-independent effects, we further explored the effect of ectopic expression of LXRα in cells that lack this receptor. Using the NIH-3T3 cell system, which was originally used to define the adipogenic activity of PPARγ (6), we showed that expression of LXRα does not influence the adipogenic potential of these cells and does not act cooperatively with PPARγ.

In contrast to Juvet et al. (18), Ross et al. (19) have reported that LXR activity inhibits adipocyte differentiation and lipid accumulation in cultured cells. They showed that ectopic expression of a constitutively active VP16-LXRα fusion protein inhibited the differentiation of 3T3-L1 cells. Our results do not support the suggestion that LXRs are physiologic inhibitors of adipocyte differentiation, lipid accumulation, or PPARγ-dependent gene expression. We have not observed significant inhibition of differentiation-dependent gene expression in either the 3T3-L1 system or the F442A system using two different synthetic LXR ligands. We also found that expression of physiologically relevant levels of wild-type LXRα in cells lacking this receptor does not inhibit PPARγ-driven differentiation of 3T3-L1 cells. Finally, we have shown that activation of LXR in adipose tissue by LXR ligand in vivo does not alter the expression of PPARγ-dependent differentiation markers.

Although it is clear that LXR cannot be required for adipose tissue development because LXRαβ null mice have fat (18, 29), the possibility that LXR signaling modulates lipid accumulation in vivo is not excluded by our studies. Older LXRαβ null mice exhibit reduced adipose tissue [(18) and our unpublished observations)]; however, the basis of this phenotype is not known. One prominent difference between preadipocyte cell lines and adipose tissue in vivo is the relatively low levels of SREBP-1c expression in cultured cell lines. Previous work has shown that LXR agonists are potent regulators of SREBP-1c expression in vivo. However, one cannot extrapolate from such observations that ligand activation of LXR would necessarily lead to a net increase in lipid accumulation in adipose tissue. In fact, transgenic overexpression of SREBP-1c from an adipocyte-specific promoter in mice results in the paradoxical loss of adipose tissue (30). Clearly, additional studies will be required to define the function of LXRs in adipose tissue and systemic lipid metabolism.

Together with previous work, our data points to an important role for LXRs in the control of gene expression in fat. LXRα expression is induced by PPARγ as a consequence of adipocyte differentiation (18, 20, 21), and LXRs regulate a specific gene expression program that is largely distinct from that of PPARγ. Previously identified targets of LXRs in adipose tissue include the lipogenic transcription factor SREBP-1c, lipogenic enzymes such as FAS and stearoyl-CoA desaturase 1, and the insulin-sensitive GLUT4 (11, 12, 14, 15, 29). In the present work, we have identified apoD and Spot 14 as new LXR-regulated genes in both cell-culture systems and in vivo. We have also shown that regulation of apoD is mediated by direct

binding of LXR/RXR heterodimers to the apoD promoter. apoD is a member of the lipocalin family of transporters (25). Among its potential physiological ligands are cholesterol and arachidonic acid. apoD appears likely to play a role in lipid transport, perhaps transporting ligands for LXR or PPAR or participating in LXR-dependent reverse cholesterol transport. Spot 14, a liver- and adipose-specific protein, is involved in fatty acid synthesis and lipogenesis (26). LXR has previously been implicated in lipogenesis through transcriptional control of SREBP-1c and FAS expression. The identification of Spot 14 as an LXR-regulated gene in adipocytes supports a role for LXR in lipogenesis in fat as well. It is likely that the effect of LXR on Spot 14 is due, at least in part, to induction of SREBP-1c expression, as Spot 14 is an established target of SREBP-1c (31).

Recent reports have linked LXR agonist treatment with improved glucose tolerance in diabetic rats and a mouse model of diet-induced obesity and insulin resistance (15, 17). While the mechanistic basis of this effect is not yet clear, it may involve suppression of hepatic gluconeogenesis or induction of GLUT4 expression in adipose tissue. It is also possible that the newly identified targets Spot 14 and apoD participate in these effects. For example, arachidonic acid, a potential ligand for apoD, has recently been reported to stimulate glucose uptake by regulating GLUT1 and GLUT4 expression at the plasma membrane (32). In addition, Spot 14 has been shown to be both insulin and glucose responsive, suggesting a role for the regulation of Spot 14 in glucose metabolism (33). Finally, genetic studies have found a significant association of an apoD polymorphism with type II diabetes, obesity, and hyperinsulinemia (34, 35). Thus, further characterization of the role of Spot 14 and apoD in adipocyte biology is an important area of future research, with possible therapeutic implications for treatment of metabolic disorders such as diabetes and obesity.

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