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Lysophosphatidic Acid Inhibits Adipocyte Differentiation via Lysophosphatidic Acid 1 Receptor-dependent Down-regulation of Peroxisome Proliferator-activated Receptor γ2*

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Lysophosphatidic acid (LPA) is a bioactive phospholipid acting via specific G protein-coupled receptors that is synthesized at the extracellular face of adipocytes by a secreted lysophospholipase D (autotaxin). Preadipocytes mainly express the LPA_1 receptor subtype, and LPA increases their proliferation. In monocytes and CV1 cells LPA was recently reported to bind and activate peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor also known to play a pivotal role in adipogenesis. Here we show that, unlike the PPARγ agonist rosiglitazone, LPA was unable to increase transcription of PPARγ-sensitive genes (*PEPCK* and *ALBP*) in the mouse preadipose cell line 3T3F442A. In contrast, treatment with LPA decreased PPARγ2 expression, impaired the response of PPARγ-sensitive genes to rosiglitazone, reduced triglyceride accumulation, and reduced the expression of adipocyte mRNA markers. The anti-adipogenic activity of LPA was also observed in the human SGBS (Simpson-Golabi-Behmel syndrome) preadipocyte cell line, as well as in primary preadipocytes isolated from wild type mice. Conversely, the anti-adipogenic activity of LPA was not observed in primary preadipocytes from LPA_1 receptor knock-out mice, which, in parallel, exhibited a higher adiposity than wild type mice. In conclusion, LPA does not behave as a potent PPARγ agonist in adipocytes but, conversely, inhibits PPARγ expression and adipogenesis via LPA_1 receptor activation. The local production of LPA may exert a tonic inhibitory effect on the development of adipose tissue.

Enlargement of adipose tissue is conditioned by the ability of adipocytes to store triglycerides as well as by the ability of preadipocytes to differentiate into adipocytes (adipogenesis). The genetic program set up for adipogenesis is tightly controlled by the coordinated interplay of several transcription factors, the most important being peroxisome proliferator-activated receptor γ (PPARγ)[1] (mainly the isoform PPARγ2) (11). Identifying the factors that control and/or regulate PPARγ activity and adipogenesis is of major interest for understanding the normal growth and the pathologic growth of adipose tissue. Many circulating factors (insulin, insulin growth factor I, growth hormone, glucocorticoids, thyroid hormone, *etc.*) are known to promote proliferation and/or differentiation of preadipocytes (see Ref. 1 for review). In addition, the production of paracrine and autocrine factors within adipose tissue could also play an important role in its development. Adipocytes release several peptides (leptin, adipsin, adiponectin, angiotensinogen, *etc.*), proteins (lipoprotein lipase, autotaxin, *etc.*), and lipids (fatty acids, prostaglandins, lysophosphatidic acid, *etc.*) involved in preadipocyte growth and/or differentiation (see Ref. 2 for review).

Lysophosphatidic acid (LPA) is a potent bioactive phospholipid able to regulate several cellular responses via activation of specific G protein-coupled receptors. Four LPA receptor subtypes have been identified, namely LPA_1, LPA_2, LPA_3, and LPA_4 (3). LPA_1 (edg-2 in the former nomenclature) was the first identified LPA receptor subtype. It is abundantly expressed in the central nervous system but is also present in numerous peripheral tissues. Invalidation of LPA_1 receptor in mouse is associated with impaired suckling behavior in neonate pups and reduced body size and weight of the adults (4).

Our group has demonstrated that LPA is produced in the extracellular medium of adipocytes (5) as the result of the secretion of lysophospholipase D (autotaxin) (6, 7). Unlike adipocytes, preadipocytes do not produce LPA (6, 7). Extracellular LPA activates the mitogen-activated protein kinases ERK1 and ERK2 and increases the proliferation of growing 3T3F442A preadipocytes, which mainly express the LPA_1 receptor subtype (5, 8). Because preadipocytes are known to be present in adipose tissue in the close environment of adipocytes, extracellular LPA produced by adipocytes could be involved in paracrine control of the number of preadipocytes in adipose tissue. However, the possible influence of this regulation on *in vivo* enlargement of adipose tissue is conditioned by the ability of preadipocytes to differentiate into adipocytes.

Interestingly, LPA was recently proposed to behave as a PPARγ agonist (9, 10). Such a conclusion was based on the

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[1] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; ALBP, adipocyte lipid-binding protein; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; HSL, hormone-sensitive lipase; KO, knock-out; LPA, lysophosphatidic acid; PEPCK, phosphoenolpyruvate carboxykinase; SGBS, Simpson-Golabi-Behmel syndrome; WT, wild type.

ability of LPA to compete the binding of the classical PPARγ agonist rosiglitazone on the purified PPARγ protein as well as on its ability to increase the transcription of a peroxisome proliferator response element reporter gene after transfection in RAW 264.7 monocytes and CV1 cells.

Knowing the pivotal role of PPARγ in adipogenesis, the initial objective of the present study was to determine whether LPA could regulate PPARγ activity in adipocytes and whether this could influence adipogenesis. We observed that LPA does not activate PPARγ in adipocytes but, conversely, down-regulates PPARγ2 expression and impairs adipogenesis via LPA_1 receptor activation. Therefore, the local production of LPA by adipocytes may exert a tonic inhibitory effect on the recruitment of new adipocytes into adipose tissue.

MATERIALS AND METHODS

Animals—LPA_1 receptor null male mice (LPA_1-KO) and their wild type (WT) litter mates (4) were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). They were housed conventionally in an animal room with constant temperature (20–22 °C) and humidity (50–60%) and with a 12-h light/12-h dark cycle (lights on at 8:00 am). All mice had free access to food from UAR, Epinay, France (energy contents in percentage of kilocalories: 20% protein, 70% carbohydrate, and 15% fat) and water throughout the experiment. On the day of sacrifice the blood was collected on heparin, and glucose was immediately measured with a glucose meter. Plasma concentrations of insulin (Diagnostics Pasteur, Paris, France) and leptin (Linco) were determined with a radioimmunoassay kit. Plasma concentrations of triglycerides and free fatty acids were determined using a colorimetric kit (Wako).

Separation of Adipocytes and Stroma-vascular Cells from Adipose Tissue—Adipose tissue was dissected out and weighed before separating adipocytes from stroma-vascular cells as described previously (12). Adipose tissue was minced and incubated in 5 ml of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 1 mg/ml collagenase and 1% bovine serum albumin for 30–45 min at 37 °C under shaking. The digestate was filtrated through a 100-μm screen and centrifuged at 2100 rpm for 10 min to separate adipocytes as a floating fat cake and stroma-vascular cells in a pellet. Pelleted cells were induced to differentiate into adipocytes in primary culture as described in the next section.

Adipocyte Differentiation in Culture—Mouse 3T3F442A preadipocytes (13) were grown and differentiated into adipocytes as described previously (14). The preadipocytes were grown to confluence in DMEM supplemented with 10% donor calf serum (Invitrogen) and then cultured in an adipogenic medium consisting of DMEM supplemented with 10% fetal calf serum plus 50 nM insulin for 4 or 7 days (the medium was changed every 2 days). Triglyceride content was quantified using a colorimetric kit (Wako Chemicals) Proteins were quantified with a Bradford assay kit (Protassay, Bio-Rad). LPA present in adipogenic medium was quantified using a radioenzymatic assay as described previously (15). For LPA treatments, 1-oleoyl-LPA (18:1) solubilized in phosphate-buffered saline containing 1% fatty acid-free bovine serum albumin was used.

Adipocyte differentiation of primary preadipocytes from adipose tissue was induced in serum-free medium as described previously (12). Briefly, stroma-vascular cells were prepared (see previous section) from 4-week-old mice. Pelleted cells were suspended in 1 ml of erythrocyte lysis buffer (16 mM Tris-HCl and 0.08% NH_4Cl, pH 7.65) for 2 min and then diluted in 50 ml of DMEM before centrifugation. Pelleted cells were filtrated through a 100-μm screen, seeded in 12-well plates (150,000 cells/well), and cultured in a serum-free adipogenic medium (DMEM/Ham's F12 (1:1) medium supplemented with 10 mg/ml transferrin, 33 mM biotin, 66 mM insulin, 1 nM triiodothyronine, and 17 mM pantothenate) for 7 days. After 7 days of culture under these conditions, the proportion of newly differentiated adipocytes was evaluated by counting the number of lipid-laden (light-refringent) cells under the microscope.

Preadipocytes from Simpson-Golabi-Behmel syndrome (SGBS) were grown and differentiated as described previously (16). They were grown to confluence in DMEM/Ham's F12 (1:1) medium supplemented with 10% fetal calf serum (Invitrogen) and then transferred into a serum-free adipogenic medium (DMEM/Ham's F12 (1:1) medium supplemented with 10 mg/ml transferrin, 15 mM $NaHCO_3$, 15 mM HEPES, 33 mM biotin, 17 mM pantothenate, 10 nM insulin, 200 pM triiodothyronine, 2 μM rosiglitazone, and 1 mM cortisol) for 4 days and then in the same medium without rosiglitazone for 6 more days.

Gene Expression—Total RNAs were extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Gene expression was analyzed using real time PCR as described previously (7). Total RNA (1 μg) was reverse-transcribed for 60 min at 37 °C using Superscript II reverse transcriptase (Invitrogen) in the presence of a random hexamer. A minus reverse transcriptase reaction was performed in parallel to ensure the absence of genomic DNA contamination. Real time PCR was performed starting with 25 ng of cDNA and a 900 nM concentration of both sense and antisense primers in a final volume of 25 μl using the SYBR Green TaqMan Universal PCR master mix (Applied Biosystems). Fluorescence was monitored and analyzed in a GeneAmp 5700 detection system instrument (Applied Biosystems). Analysis of 18 S ribosomal RNA was performed in parallel using the ribosomal RNA control TaqMan assay kit (Applied Biosystem) to normalize gene expression. Results are expressed as $2^{(Ct_{18\ S} - Ct_{gene})} \times 10{,}000$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold (Table I). Oligonucleotide primers were designed using the Primer Express software (PerkinElmer Life Sciences).

Oligonucleotides for mouse gene expression studies were as follows: LPA_1 receptor, 5′-CATGGTGGCAATCTACGTCAA-3′ (sense) and 5′-AGGCCAATCCAGCGAAGAA-3′ (antisense); LPA_2 receptor, 5′-TGTCTGACTGCACAGCTTGGA-3′ (sense) and 5′-CTCATGGAGTTTTCTGGTGCC-3′ (antisense); LPA_3 receptor, 5′-TGGGCCATCGCCATTTT-3′ (sense) and 5′-GAGCAGGCAGAGATGTTGCA-3′ (antisense); LPA_4 receptor (also known as p2y9/GPR23), 5′-CCTTACCAACATCTATGGGAGCAT-3′ (antisense) and 5′-TGGCCAGGAAACGATCCA-3′ (antisense); adipocyte lipid-binding protein (ALBP), 5′-TTCGATGAAATCACCGCAGA-3′ (sense) and 5′-GGTCGACTTTCCATCCCACTT-3′ (antisense); PPARγ2, 5′-CTGTTTTATGCTGTTATGGGTGAAA-3′ (sense) and 5′-GCACCATGCTCTGGGTCAA-3′ (antisense); hormone-sensitive lipase (HSL), 5′-GGCTTACTGGGCACAGATACCT-3′ (sense) and 5′-CTGAAGGCTCTGAGTTGCTCAA-3′ (antisense); and phosphoenolpyruvate carboxykinase (PEPCK), 5′-ATGTTCGGGCGGATTGAAG-3′ (sense) and 5′-TCAGGTTCAAGGCGTTTTCC-3′ (antisense).

Oligonucleotides for human gene expression were as follows: LPA_1 receptor, 5′-TGGGCCATTTTCAACTTGGT-3′ (sense) and 5′-TCTGGCGAACATAGCCAAAGA-3′ (antisense); LPA_2 receptor, 5′-TCATCATGGGCCAGTGCTACT-3′ (sense) and 5′-GTGGGAGCTGAGCTCTTTGC-3′ (antisense); LPA_3 receptor, 5′-TGGGCCATCGCCATTTT-3′ (sense) and 5′-GAGCAGGCAGAGATGTTGCA-3′ (antisense); LPA_4 receptor, 5′-CCTTACCAACATCTATGGGAGCAT-3′ (sense) and 5′-TGGCCAGGAAACGATCCA-3′ (antisense); ALBP, 5′-GCATGGCCAAACCTAACATGA-3′ (sense) and 5′-CCTGGCCCAGTATGAAGGAAA-3′ (antisense); and HSL, 5′-GTGCAAAGACGGAGGACCACTCCA-3′ (sense) and 5′-GACGTCTCGGAGTTTCCCCTCAG-3′ (antisense).

Western Blot Analysis—Cells were homogenized in radioimmune precipitation assay buffer (0.01 M Tris-HCl (pH 7.0), 0.15 M NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, and 2 mM phenylmethylsulfonylfluoride), and 40 μg of protein were separated on an 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blot was pre-incubated for 1 h at room temperature in Tris-buffered saline/Tween (1%) containing 10% dry milk and then incubated overnight at 4 °C in Tris-buffered saline/Tween (0.5%) containing 1% dry milk supplemented (1:5000 dilution) with a rabbit polyclonal PPARγ antibody raised against an N-terminal PPAR peptide (amino acids 20–104) as described previously (25). After washing in phosphate-buffered saline/Tween (0.5%), PPARγ was visualized by an enhanced chemiluminescence detection system (ECL, Amersham Biosciences) using an anti rabbit-horseradish peroxidase antibody (Sigma).

RESULTS

Expression of LPA Receptor Subtypes and PPARγ2 in 3T3F442A Cells—Experiments were carried out in the mouse 3T3F442A cell line characterized previously for its ability to differentiate into adipocytes when cultured in an appropriate adipogenic medium (see "Materials and Methods"). Confluent cells expressed PPARγ2, LPA_1 receptor, and LPA_4 receptor mRNAs. LPA_2 and LPA_3 were not detected (Fig. 1 and Table I). The PPARγ2 mRNA level increased 4-fold between confluence and 8-day post-confluence adipocytes (Fig. 1). In contrast, the LPA_1 receptor mRNA level decreased by 14-fold (Fig. 1). The LPA_4 receptor mRNA level did not change during the course of adipocyte differentiation (Fig. 1).

LPA Inhibits Agonist-mediated Activation of PPARγ2 in 3T3F442A Cells—LPA was reported for its ability to bind and activate the nuclear receptor PPARγ in monocytes and CV1 cells (9, 10). PPARγ is a transcription factor known for its pivotal role in adipogenesis (17). Our initial objective was to determine whether LPA could activate endogenously expressed PPARγ in 3T3F442A cells. One way to evaluate PPARγ activation was to measure the ability of a PPARγ-agonist such as rosiglitazone to increase the transcription of endogenously expressed genes containing a peroxisome proliferator response element in their promoters. As demonstrated previously (18), *PEPCK* is one of the most sensitive PPARγ-sensitive genes in 3T3F442A adipocytes.

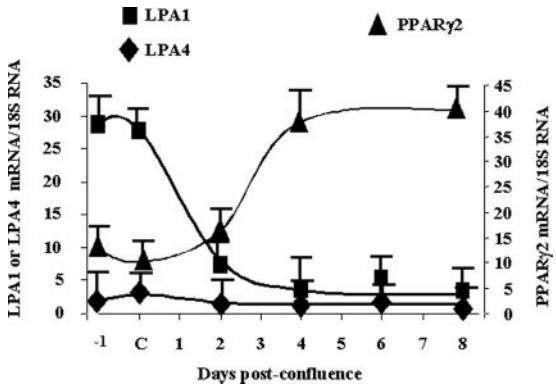

FIG. 1. **Expression of LPA_1 receptor, LPA_4 receptor, and PPARγ2 mRNAs during the course of differentiation of 3T3F442A cells.** Total RNA were extracted from 3T3F442A preadipocytes at different time points during the course of adipocyte differentiation, and LPA_1, LPA_4, and PPARγ2 mRNAs were quantified by real time reverse transcription PCR as described under "Materials and Methods." Values are means ± S.E. of three experiments.

TABLE I
Expression of LPA_1, LPA_2, LPA_3, and LPA_4 receptor subtype genes in preadipocytes

Gene expression was determined by real time PCR using the formula $2^{(Ct_{18\,S} - Ct_{gene})} \times 10{,}000$ as described under "Material and Methods." Values are means ± S.E. of two to four separate experiments. Und, undetermined; SVC, stroma-vascular cells.

	LPA_1	LPA_2	LPA_3	LPA_4
3T3F442A preadipocytes	27 ± 3	Und	Und	0.3 ± 0.1
SGBS preadipocytes	45 ± 3	0.3 ± 0.1	Und	Und
WT SVC	117 ± 12	1.4 ± 0.4	Und	2.8 ± 0.1
LPA_1-KO SVC	Und	1.7 ± 0.5	Und	2.5 ± 0.3

PPARγ activity was studied in 3T3F442A cells cultured in an adipogenic medium for 4 days (see "Materials and Methods"). At that stage the cells exhibited maximal expression of PPARγ2 mRNA and minimal expression of LPA_1 receptor (Fig. 1). As a positive control of PPARγ activation, the influence of rosiglitazone on PEPCK mRNA was tested. Rosiglitazone led to a dose-dependent increase in PEPCK mRNA level with an EC_{50} of 10 nM and a maximal effect of up to 17-fold when compared with control (Fig. 2*A*). In contrast, LPA (10 μM in 1% bovine serum albumin as vehicle) did not significantly modify the PEPCK mRNA level (Fig. 2, curve labeled *+LPA*, rosiglitazone at 0). When using other vehicles (ethanol, methanol, Me_2SO, or translocase-3) (9), LPA alone still had no significant effect on PEPCK mRNA (data not shown). Interestingly, when LPA was used in co-treatment with rosiglitazone, the dose-response of rosiglitazone was significantly shifted to the right, leading to an EC_{50} of up to 20 nM without modification of the maximal effect (Fig. 2*A*). When the adipogenic medium was supplemented with 10 μM LPA from confluence to day 4, the dose-response of rosiglitazone on the PEPCK mRNA level was almost completely annealed (Fig. 2*B*). Similar results were obtained when analyzing the ALBP mRNA level as another PPARγ-sensitive gene (not shown). These results suggested that LPA was not able to activate PPARγ in 3T3F442A cells but, conversely, inhibited agonist-dependent activation of PPARγ.

LPA Inhibits PPARγ2 Expression in 3T3F442A Cells—To test whether LPA-inhibition of agonist-mediated activation of PPARγ could result from a down-regulation of PPARγ2 expression, the PPARγ2 mRNA level was measured. In 4-day post-confluent 3T3F442A cells, 24 h of treatment with 10 μM LPA led to a significant reduction (38%) in PPARγ2 mRNA level when compared with control cells (Fig. 3*A*). When LPA was chronically present in the culture medium from confluence to day 4, PPARγ2 mRNA level was further down-regulated (74%) when compared with control (Fig. 3*A*). In parallel, the PPARγ protein level was reduced after 4 days of treatment with LPA (Fig. 3*B*). No alteration of PPARγ protein level was observed after 24 h of LPA treatment. These results suggested that LPA-mediated inhibition of agonist-mediated activation of PPARγ resulted from down-regulation of PPARγ2 expression.

LPA Inhibits Adipocyte Differentiation of 3T3F442A Cells—With PPARγ2 playing a pivotal role in adipocyte differentiation, the influence of LPA on adipocyte differentiation of 3T3F442A cells was studied. When 3T3F442A cells were differentiated for 7 days in the presence of increasing concentra-

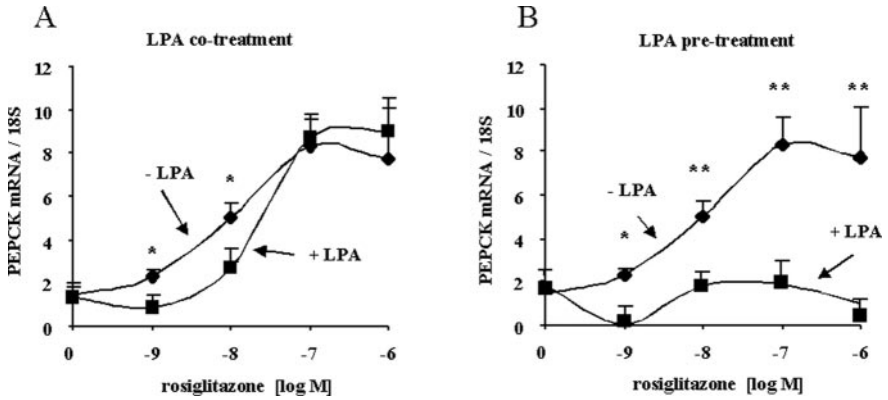

FIG. 2. **Influence of LPA and rosiglitazone on the PEPCK mRNA level in 3T3F442A cells.** *A*, confluent 3T3F442A cells were cultured in an adipogenic medium (see "Materials and Methods") and, after 4 days of culture, the cells were serum-deprived and exposed for an additional 24 h to increasing concentrations of rosiglitazone in the presence (+) or absence (−) of 10 μM LPA. *B*, confluent 3T3F442A cells were cultured in an adipogenic medium for 4 days in the presence (+) or absence (−) of 10 μM lysophosphatidic acid, and serum-deprived cells were exposed for an additional 24 h to increasing concentrations of rosiglitazone for 24 h. *A* and *B*, after 24 h total RNA was extracted, and PEPCK mRNAs were quantified by real time PCR quantification (see "Materials and Methods"). Values are means ± S.E. of three experiments. Comparison between *−LPA* and *+LPA* was performed using Student's paired *t* test; *, $p < 0.05$; **, $p < 0.01$.

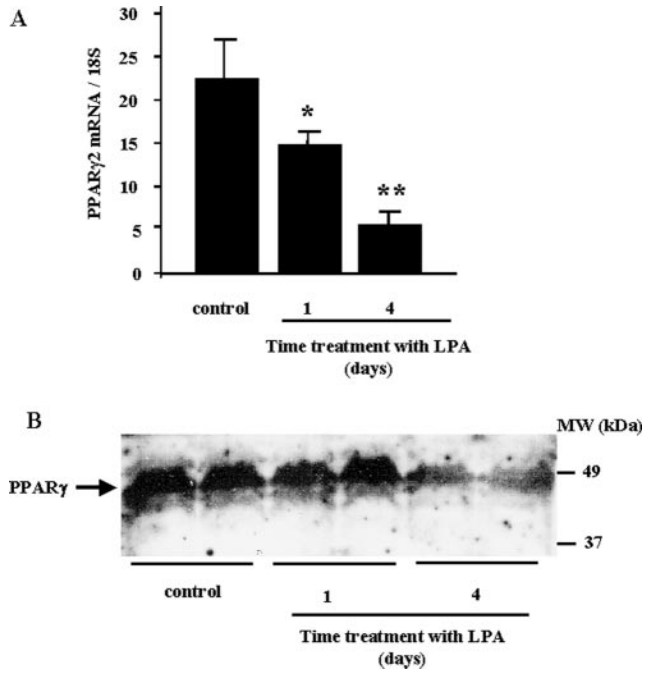

FIG. 3. **Influence of LPA on PPARγ2 expression in 3T3F442A cells.** *A* and *B*, confluent 3T3F442A cells were cultured in an adipogenic medium (see "Materials and Methods") for 4 days in the presence or absence of 10 μM LPA from confluence or from the 3rd day. On the 4th day total RNA was extracted, and PPARγ2 mRNAs were quantified by real time reverse transcription PCR (*A*). In parallel, PPARγ protein level was determined by Western blot analysis (*B*). Values are means ± S.E. of three experiments. Comparison with control was performed using Student's paired *t* test; *, $p < 0.05$; **, $p < 0.01$.

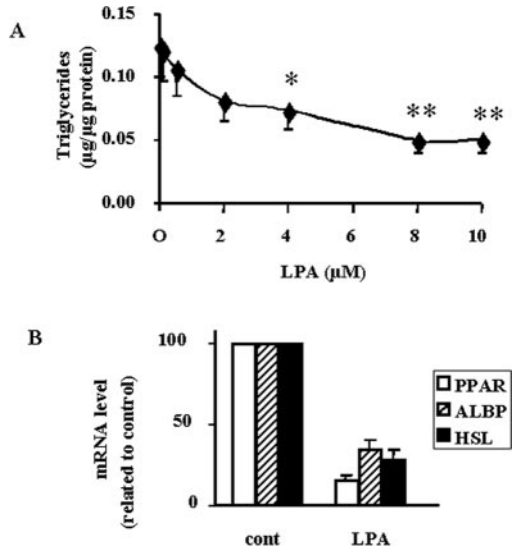

FIG. 4. **Influence of LPA on adipocyte differentiation of 3T3F442A cells.** *A* and *B*, confluent 3T3F442A cells were cultured in an adipogenic medium (see "Materials and Methods") in the presence or absence of increasing concentration of LPA. After 10 days the amount of triglycerides accumulated in adipocytes (*A*) and the expression of adipocyte-specific genes (*B*) were measured as described under "Materials and Methods." Values are means ± S.E. of three separate experiments. Comparison with control was performed using Student's paired *t* test; *, $p < 0.05$; **, $p < 0.01$.

tion of LPA in the adipogenic medium, a dose-dependent reduction in triglyceride accumulation was observed (Fig. 4*A*). The effect of LPA was significantly detected at 4 μM and was maximal at 8 μM. LPA treatment also led to a significant reduction in the level of PPARγ2, ALBP, and HSL mRNAs (Fig. 4*B*). These results revealed the anti-adipogenic activity of LPA.

LPA Normally Present in Serum Restrains Adipocyte Differentiation of 3T3F442A Cells—As described under "Materials and Methods," the adipogenic medium used to allow differentiation of 3T3F442A cells contains 10% fetal calf serum, and this serum is known to contain LPA (15). We tested whether LPA brought by serum could influence adipogenesis. As shown in Fig. 5*A*, LPA concentration in fresh adipogenic medium measured using a radioenzymatic assay (15) was 0.45 μM. As demonstrated previously, LPA can be hydrolyzed and inactivated by treatment with the lysophospholipase called phospholipase B (5, 7). As shown in Fig. 5*A*, 30 min of treatment with 0.5 units/ml phospholipase B led to a >90% reduction in LPA content of the adipogenic medium. When 3T3F442A cells were differentiated for 7 days in an adipogenic medium supplemented with 0.5 units/ml phospholipase B, the amount of triglycerides stored in the cells was significantly increased when compared with that for a control adipogenic medium (Fig. 5*B*). This was accompanied by a significant increase in the level of PPARγ2, ALBP, and HSL mRNAs (Fig. 5*C*). These results showed that LPA brought by serum in the adipogenic medium restrains adipocyte differentiation of 3T3F442A preadipocytes. Most of the adipogenic medium used to differentiate preadipose cell lines contains serum. Our observations could help to optimize the composition of serum-containing adipogenic medium.

The Anti-adipogenic Activity of LPA Is Mediated by the LPA_1 Receptor Subtype—To determine whether the LPA_1 receptor was involved in the anti-adipogenic activity of LPA, adipocyte differentiation was analyzed in primary preadipocytes from LPA_1-KO mice and their WT litter mates (4). Preadipocytes are present in the stroma-vascular fraction of the adipose tissue and were isolated from adipocytes after collagenase dissociation (see "Materials and Methods"). When prepared from WT mice, the stroma-vascular fraction expressed LPA_1, LPA_2, and LPA_4 receptor mRNAs, with the LPA_1 receptor subtype being predominantly expressed (Table I). LPA_3 receptor mRNAs were undetectable. When the stroma-vascular fraction was prepared from LPA_1-KO mice, LPA_1 mRNAs were undetectable, and LPA_2 and LPA_4 mRNA levels were not different from those in WT mice (Table I).

The stroma-vascular fraction of adipose tissue is not only composed of preadipocytes but also of endothelial cells and macrophages. To evaluate the proportion of preadipocytes present, the stroma-vascular fraction was cultured in an adipogenic medium (see "Materials and Methods") for 7 days. Under these conditions a certain proportion of cells became light-refringent as the result of triglyceride droplet accumulation (Fig. 6*A*) and the expression of adipocyte-specific genes (*ALBP*, *PPARγ2*, and *HSL*) (not shown). Interestingly, the proportion of lipid-laden cells obtained with WT mice (24%) was significantly lower than that obtained with LPA_1-KO mice (Fig. 6*B*). This finding was associated with a lower expression of adipocyte-specific genes (not shown). This observation suggested that the proportion of preadipocytes present in the stroma-vascular fraction from WT mice was lower than that from LPA_1-KO mice.

When starting with WT mice, supplementation of the adipogenic medium with increasing concentrations of LPA during the 7 days of culture led to a dose-dependent decrease (maximal inhibition of 50% at 0.1 μM LPA) in the proportion of lipid-laden cells when compared with control cells (Fig. 7). When starting with LPA_1-KO mice, no changes in the proportion of lipid-laden cells (Fig. 7) were observed after LPA treatment. These data showed that the absence of an LPA_1 receptor in preadipocytes led to the suppression of the anti-adipogenic activity of LPA.

Adipose Tissue Phenotype of LPA_1-KO Mice—To determine the possible consequences of LPA_1 invalidation on adipose tissue development, LPA_1-KO mice were analyzed and compared

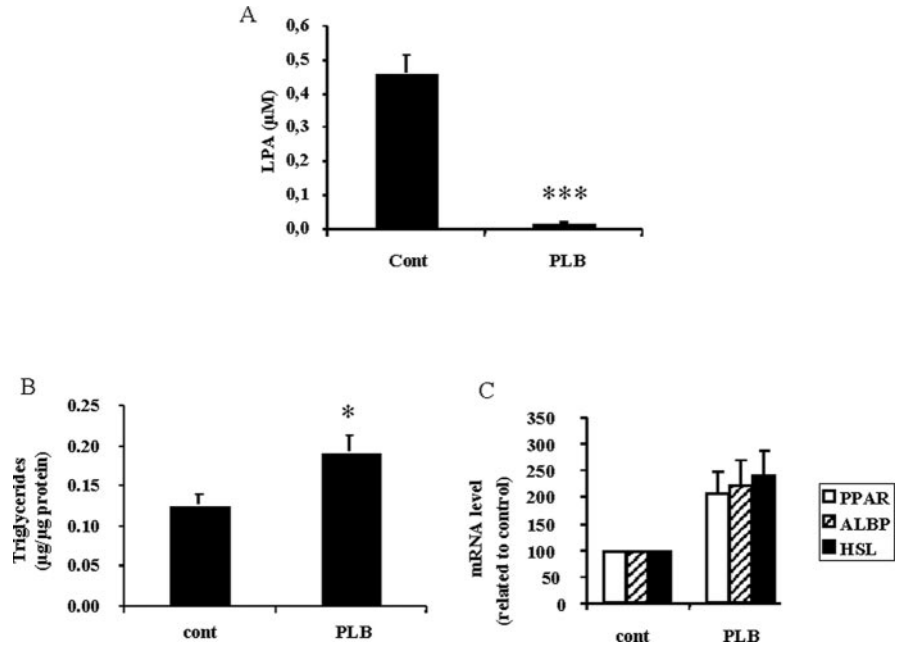

FIG. 5. **Influence of phospholipase B on adipocyte differentiation of 3T3F442A cells.** *A*, the adipogenic medium was treated or not treated (*Cont*) with 0.5 units/ml phospholipase B (*PLB*) for 30 min at 37 °C before quantification of LPA concentration using a radioenzymatic assay (see "Materials and Methods"). *B* and *C*, confluent 3T3F442A cells were cultured in an adipogenic medium (see "Materials and Methods") in the presence or absence of 0.5 units/ml phospholipase B (*PLB*). After 10 days the amount of triglycerides accumulated in adipocytes (*B*) and the expression of adipocyte-specific genes (*C*) were measured as described under "Materials and Methods." Values are means ± S.E. of three separate experiments. Comparison with control was performed using Student's paired *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

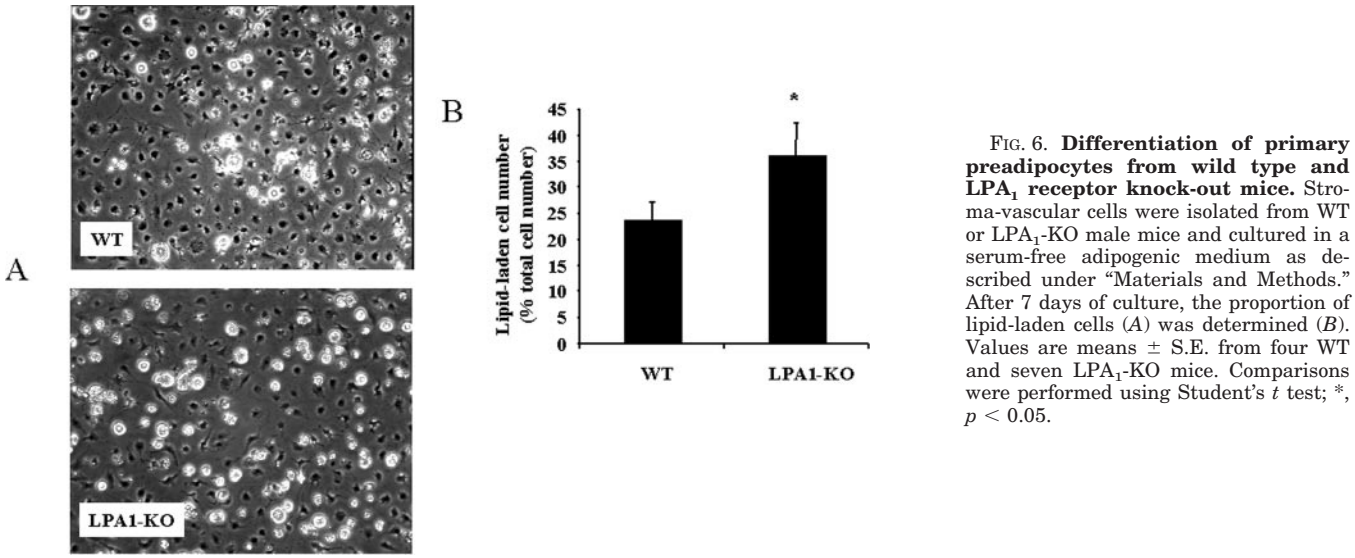

FIG. 6. **Differentiation of primary preadipocytes from wild type and LPA_1 receptor knock-out mice.** Stroma-vascular cells were isolated from WT or LPA_1-KO male mice and cultured in a serum-free adipogenic medium as described under "Materials and Methods." After 7 days of culture, the proportion of lipid-laden cells (*A*) was determined (*B*). Values are means ± S.E. from four WT and seven LPA_1-KO mice. Comparisons were performed using Student's *t* test; *, $p < 0.05$.

with WT mice. Data presented were obtained with males, but similar results were obtained with females (not shown). LPA_1-KO mice exhibited lower body weight than WT mice, whatever the age of the animals (Fig. 8*A*). This was accompanied by no differences in the mean daily food intake measured over 10 weeks, *i.e.* 1.0 ± 0.2 and 0.8 ± 0.1 gram of food per gram of body weight per day for WT ($n = 6$) and LPA_1-KO ($n = 3$) mice, respectively. At 15 weeks of age and despite the lower body weight, perigonadic adipose tissue weight was significantly higher in LPA_1-KO mice than in WT mice (Fig. 8*B*). Inguinal adipose tissue weight also tended to be higher in LPA_1-KO mice than in WT mice, but this was not significant (Fig. 8*B*). At the plasma level, LPA_1-KO mice exhibited a significantly higher (2-fold) concentration of leptin when compared with WT mice (Table II). In contrast, no differences in plasma concentration of insulin, glucose, triglycerides, and free-fatty acids were observed (Table II). Taken together, these observations showed that, despite their lower body weight, LPA_1-KO mice exhibited higher adiposity than WT mice.

LPA Inhibits Adipocyte Differentiation of Human Preadipocytes—The possible anti-adipogenic activity of LPA was tested in human preadipocytes. This was tested in the human preadipocyte cell strain from SGBS (16). These cells have previously been described for their ability to differentiate into adipocytes in a serum-free medium (see "Materials and Methods"). Confluent SGBS preadipocytes expressed both LPA_1 and LPA_2 receptor mRNAs (Table I), with the LPA_1 receptor RNAs being 150-fold more expressed than LPA_2 receptor RNAs (Table I). In contrast, LPA_3 receptor and LPA_4 receptor RNAs remained undetectable.

After 10 days of culture in an appropriate serum-free adipogenic medium, SGBS cells accumulated triglyceride droplets (Fig. 9*A*) and expressed adipocyte-specific genes such as *ALBP* and *HSL* (Fig. 9*B*). Supplementation of the adipogenic medium with LPA led to a striking reduction in triglyceride droplet accumulation (Fig. 9*A*) accompanied by a dose-dependent reduction in *ALBP* and *HSL* gene expression (Fig. 9*B*). These results showed that LPA was anti-adipogenic in human preadipocytes.

DISCUSSION

The recruitment of new fat cells in adipose tissue requires the differentiation of preadipocytes into adipocytes (adipogenesis),

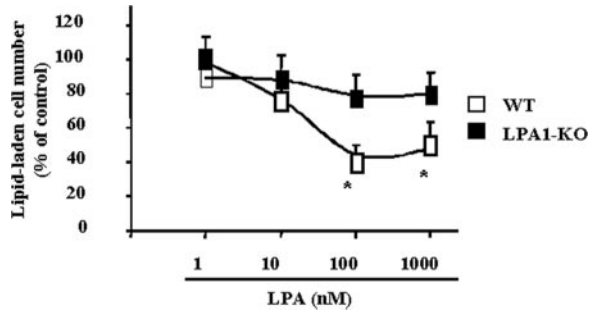

FIG. 7. **Influence of LPA on differentiation of primary preadipocytes from wild type and LPA_1 (−/−) mice.** Stroma-vascular cells were isolated from WT or LPA_1-KO male mice and cultured in a serum-free differentiating medium (see "Materials and Methods") in the absence (control) or presence of increasing concentrations of LPA. After 7 days of culture, lipid-laden cells were counted under the microscope. Values are means ± S.E. of four and seven separate experiments for WT and LPA_1-KO mice, respectively. Comparison with control was performed using Student's *t* test; *, $p < 0.05$.

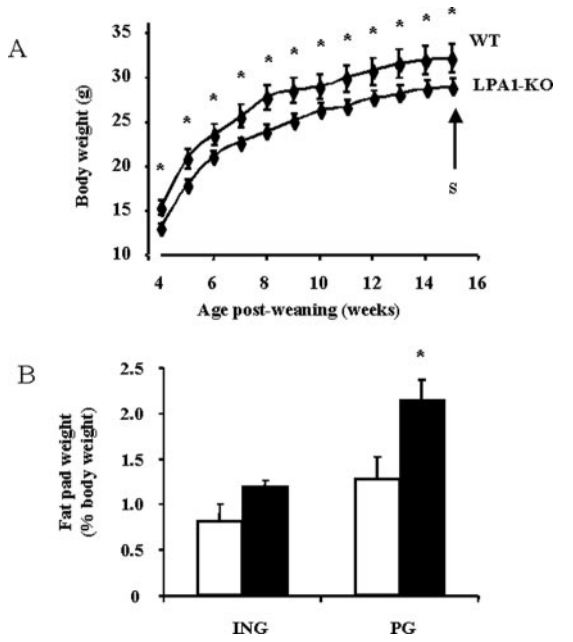

FIG. 8. **Body and fat pad weights of wild type and LPA_1 (−/−) mice.** *A* and *B*, WT and LPA_1-KO male mice were housed and fed as described under "Materials and Methods," and their body weight was followed from 4 to 15 weeks of age (*A*). The animals were sacrificed at the 15th week (*S* and *arrow* in *panel A*) to dissect out and weigh inguinal (*ING*) and perigonadic (*PG*) adipose tissue from wild type (*open bars*) and LPA1-receptor knock-out (*closed bars*) mice (*B*). Values are means ± S.E. of eight and five separate experiments for WT and LPA_1-KO mice, respectively. Comparisons between WT and LPA_1-KO were performed using Student's *t* test; *, $p < 0.05$.

TABLE II
Blood parameters of WT and LPA_1-KO mice

	WT mice	LPA_1-KO mice
Triglycerides (g/l)	0.36 ± 0.03	0.33 ± 0.03
Free-fatty acids (nM)	0.65 ± 0.06	0.77 ± 0.06
Glucose (g/l)	1.73 ± 0.13	2.02 ± 0.15
Insulin (μg/ml)	0.72 ± 0.17	0.65 ± 0.24
Leptin (ng/ml)	2.21 ± 0.22	4.57 ± 0.83

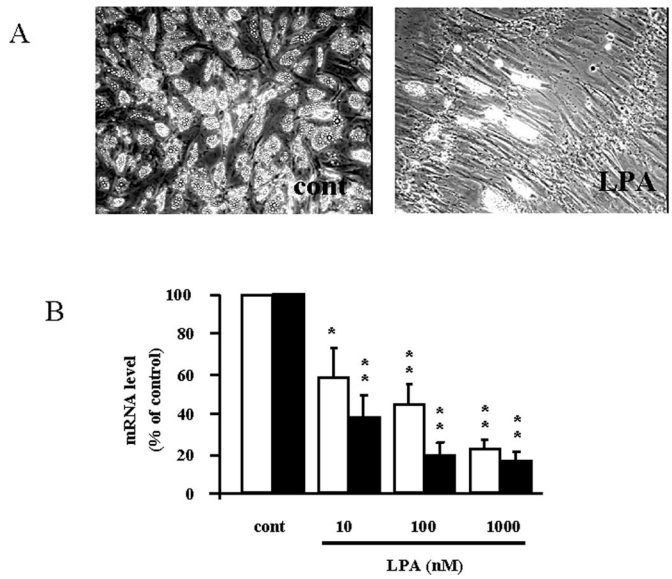

FIG. 9. **Influence of LPA on adipocyte differentiation of human preadipocytes.** *A* and *B*, confluent SGBS preadipocytes were cultured in a serum-free differentiating medium (see "Materials and Methods") in the absence (*cont*) or presence of increasing concentration of LPA. After 10 days of culture SGBS adipocytes were photographed (*A*), and adipocyte-specific gene expression (*HSL*, *white columns*; *ALBP*, *black columns*) was measured as described under "Materials and Methods." Values are means ± S.E. of four separate experiments. Comparison with the control was performed using Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

a process tightly controlled by the transcription factor PPARγ2. Factors locally produced in adipose tissue by adipocytes could contribute to the regulation of adipogenesis by exerting paracrine actions on preadipocytes. Among those paracrine factors LPA could play an important role, because it is produced at the extracellular face of adipocytes by autotaxin (7) and because preadipocytes express LPA receptors (mainly the LPA_1 subtype) (8). We demonstrated previously that, in 3T3F442A preadipocytes, LPA was able to increase phosphorylation of the mitogen-activated protein kinases ERK1 and ERK2 and to increases proliferation (8, 19), but the influence of LPA on adipogenesis was not studied until the present work. Such study was further motivated by recent reports showing that LPA could, in parallel to its ability to activate G protein-coupled membrane receptors, behave as an agonist to the nuclear transcription factor PPARγ in monocytes and CV1 cells (9, 10). Because PPARγ is known to play a pivotal role in the control of adipogenesis (17), determining whether LPA could activate PPARγ in adipocytes was of main interest in the context of adipose tissue.

The first part of the present study shows that, unlike the PPARγ agonist rosiglitazone, LPA was unable to activate PPARγ activity in adipocytes evaluated by measuring the induction of PPARγ-sensitive genes (*PEPCK* and *ALBP*). These results lead us to conclude that LPA does not behave as a potent activator of PPARγ in adipocytes. This conclusion is not in agreement with that drawn previously from experiments performed in monocytes and CV1 cells (9, 10). Although LPA was demonstrated to bind to PPARγ in an *in vitro* assay (9), the ability of LPA to activate PPARγ obviously appears to be dependent on the cell type. Possible activation of a nuclear receptor such as PPARγ by exogenous LPA would require that a high enough amount of LPA penetrated into the cell and reached the nucleus. As demonstrated previously by our group, when preadipocytes are exposed to radiolabeled LPA virtually no radiolabeled LPA can be detected in the cells because of the presence of a high ecto-lipid phosphate phosphohydrolase activity, which dephosphorylates and inactivates LPA (20). This could explain why we observed no activation of PPARγ by LPA in adipocytes. This finding also suggests that ecto-lipid phosphate phosphohydrolase activity could be weaker in monocytes and CV1 cells

than in adipocytes, allowing a higher amount of LPA to enter into the cells and activate PPARγ.

The most important result of the present study was the finding that chronic exposure of preadipocytes to LPA inhibits their differentiation into adipocytes, as attested by a reduction in triglyceride accumulation and a reduction in the expression of adipocyte specific genes. Therefore, LPA clearly behaves as an anti-adipogenic compound. In addition, the anti-adipogenic effect of LPA was not found in primary preadipocytes from LPA_1 knock-out mice, indicating that the LPA_1 receptor is fully responsible for the anti-adipogenic activity of LPA. These observations clearly support the concept that LPA is an anti-adipogenic mediator acting via a specific receptor.

PPARγ2 clearly plays a pivotal positive role in adipogenesis (17). Our data show that treatment with LPA leads to down-regulation of PPARγ2 expression and activity, as expected for an anti-adipogenic factor. It is therefore very likely that the anti-adipogenic activity of LPA is due to its negative impact on PPARγ2. This conclusion is supported by previous reports showing that LPA is able to inhibit PPARγ activity in THP-1 monocytes and IMR-32 neuroblastomas (21, 22). In this last cell line, LPA inhibits the capacity of PGJ2 to activate the transcription of a PPARγ responsive element-dependent reporter gene, and by using specific pharmacological inhibitors it was shown that the effect of LPA involves activation of the mitogen-activated protein kinase pathway as well as activation of the Rho kinase (22). In addition, it has previously been shown that increasing Rho-GTPase activity decreases adipocyte differentiation and PPARγ expression in mouse embryo-derived fibroblasts (23). In preadipocytes, LPA activates the phosphorylation of ERK1 and ERK2 mitogen-activated protein kinases and activates the small G-protein Rho (19). It can therefore be proposed that LPA-mediated inhibition of PPARγ2 activity and expression may result from the LPA_1 receptor-dependent activation of the mitogen-activated protein kinases and/or the Rho kinase.

What could be the physiological relevance of the anti-adipogenic activity of LPA? Because the LPA_1 receptor was responsible for the anti-adipogenic activity of LPA in preadipocytes, phenotypic analysis of LPA_1-KO mice presented an excellent opportunity for determining the possible involvement of LPA on adipose tissue development. As demonstrated previously, LPA_1-KO mice exhibit reduced the size and weight of their bodies (4). In the present study, we observed that despite their lower body weight, LPA_1-KO mice exhibited higher adiposity than WT mice. This was associated with a higher plasma concentration of leptin, a cytokine known to be tightly associated with adipose tissue mass (24). Because LPA_1-KO mice exhibited the same food intake as WT mice, the higher adiposity of LPA_1-KO mice cannot be explained by an alteration of their feeding behavior. Enlargement of adipose tissue not only results from increased accumulation of triglyceride accumulation in adipocytes but can also be influenced by the recruitment of new adipocytes resulting from adipogenesis. Interestingly, the proportion of preadipocytes present in adipose tissue from LPA_1-KO mice was higher than that of WT mice (Fig. 6). It is therefore possible that increased adiposity of LPA_1-KO mice may result from the suppression of the anti-adipogenic activity of LPA normally existing in WT mice.

In conclusion, LPA is produced in the extracellular medium of adipocytes as the result of the lysophospholipase D activity of autotaxin (6, 7). Adipocytes and preadipocytes are both present in adipose tissue in a close environment. Because preadipocytes express LPA_1 receptor and are sensitive to LPA, it is likely that local production of LPA by adipocytes could exert a paracrine anti-adipogenic activity on surrounding preadipocytes and therefore exert a negative paracrine effect on adipose tissue development.

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