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**LYSOPHOSPHATIDIC ACID INHIBITS ADIPOCYTE DIFFERENTIATION VIA LPA₁ RECEPTOR-DEPENDENT DOWN-REGULATION OF PPAR γ 2.**

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Running title: Anti-adipogenic activity of lysophosphatidic acid

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Abstract:

Lysophosphatidic acid (LPA) is a bioactive phospholipid acting via specific G-protein coupled receptors, and which is synthesized at the extracellular face of adipocytes by a secreted lysophospholipase D (autotaxin). Preadipocytes mainly express LPA₁ receptor subtype and LPA increases their proliferation. In monocytes and CV1 cells LPA was recently reported to bind and activate PPAR γ , a transcription factor also known to play a pivotal role in adipogenesis. Here we show that, conversely to the PPAR γ agonist rosiglitazone, LPA was unable to increase transcription of PPAR γ -sensitive genes (PEPCK and ALBP) in the mouse preadipose cell line 3T3F442A. In contrast, treatment with LPA decreased PPAR γ 2 expression, impaired the response of PPAR γ -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 (WT) mice. Conversely, the anti-adipogenic activity of LPA was not observed in primary preadipocytes from LPA₁ receptor knockout mice (LPA₁-KO) which, in parallel, exhibited a higher adiposity than WT mice. In conclusion, LPA does not behave as a potent PPAR γ -agonist in adipocytes, but

conversely inhibits PPAR γ expression and adipogenesis via LPA₁ receptor activation. The local production of LPA may exert a tonic inhibitory on development of adipose tissue.

Introduction :

Enlargement of adipose tissue is conditioned by the ability of adipocytes to store triglycerides, as well as 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 (PPAR γ), mainly the PPAR γ 2 isoform (11). Identifying the factors that control and/or regulate PPAR γ activity and adipogenesis is of major interest to understand normal and pathologic growth of adipose tissue. Many circulating factors (insulin, IGF-I, growth hormone, glucocorticoids and thyroid hormone, ...) are known to promote proliferation and/or differentiation of preadipocytes (see reference 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, adiponectin, adiponectin, angiotensinogen, ...), proteins (lipoprotein lipase, autotaxin, ...) or lipids (fatty acids, prostaglandins, lysophosphatidic acid, ...)

involved in preadipocyte growth and/or differentiation (see reference 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: LPA₁, LPA₂, LPA₃, and LPA₄ (3). LPA₁ (*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 LPA₁-R 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). Conversely to adipocytes, preadipocytes do not produce LPA (6, 7). Extracellular LPA activates the mitogen-activated protein kinases ERK1 and ERK2, and increases proliferation of growing 3T3F442A preadipocytes, which mainly expresses LPA₁ receptor subtype (5, 8). Since 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 preadipocyte number 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 PPAR γ agonist (9, 10). Such a conclusion was based on the ability of LPA to compete the binding of the classical PPAR γ -agonist rosiglitazone on purified PPAR γ protein, as well as on its ability to increase the transcription of a PPRE-reporter gene after transfection in RAW 264.7 monocytes and CV1 cells.

Knowing the pivotal role of PPAR γ in adipogenesis, the initial objective of the present study was to determine whether LPA could regulate PPAR γ -activity in adipocytes and whether this could influence adipogenesis. We observed that LPA does not activate PPAR γ in adipocytes but conversely down-regulates PPAR γ 2 expression and impairs adipogenesis via LPA₁ 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.

Material and Methods

Animals :

LPA₁-receptor null male mice (LPA₁-KO) and their wild type (WT) littermates (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), humidity (50-60%) and with a 12:12h light/dark cycle (lights on at 8:00 am). All mice had free access to food (energy contents in % kcal: 20% protein, 70% carbohydrate and 15% fat, from UAR, France) 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 concentration of insulin (Diagnostics Pasteur, Paris, France) and leptin (Linco) were determined with an RIA kit. Plasma concentrations of triglycerides and free-fatty acids were determined using a colorimetric kit (Wako, Germany).

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 previously described (12). Adipose tissue was minced and incubated in 5 ml of Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Paisley, UK) supplemented with 1 mg/ml collagenase and 1% BSA for 30 to 45 min at 37 °C under shaking. Digestate was filtrated through a 100- μ m screen and centrifuged at 2100 rpm for 10 minutes in order to separate adipocytes as a floating fat cake, and stroma-vascular cells in the 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 previously described (14). They were grown to confluence in DMEM supplemented with 10% donor calf serum (Invitrogen life technology) then cultured in an adipogenic medium consisting in DMEM supplemented with 10% fetal calf serum plus 50 nM insulin for 4 or 7 days (the medium was changed

every two days). Triglyceride content was quantified using a colorimetric kit (Wako Chemicals, Germany). Proteins were quantified with a Bradford assay kit (Protassay, Biorad). LPA present in adipogenic medium was quantified using a radioenzymatic assay as previously described (15). For LPA treatments, 1-oleoyl-LPA (18:1) solubilized in PBS containing 1% fatty acid-free BSA was used.

Adipocyte differentiation of primary preadipocytes from adipose tissue was induced in serum-free medium as previously described (12). Briefly, stroma-vascular cells were prepared (see previous section) from 4 weeks-old mice. Pelleted cells were suspended in 1 ml erythrocyte-lysis buffer (Tris-HCl 16 mM, NH₄Cl 0.08%, pH 7.65) for 2 minutes and then diluted in 50 ml DMEM before centrifugation. Pelleted cells were filtrated through a 100- μ m screen and 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 10mg/ml transferrin, 33 mM biotin, 66 mM insulin, 1 nM triiodothyronin and 17 mM panthothenate) for 7 days. After 7 days culture in these conditions the proportion of newly differentiated adipocytes was evaluated by counting the number of lipid-laden (light refringent) cells under microscope.

Preadipocytes from Simpson-Golabi-Behmel syndrome (SGBS) were grown and differentiated as previously described (16). They were grown to confluence in DMEM/Ham's F12 (1:1) medium supplemented with 10% fetal calf serum (Invitrogen life technology) then transferred in a serum-free adipogenic medium (DMEM/Ham's F12 (1:1) medium supplemented with 10 mg/ml transferrin, 15 mM NaHCO₃, 15 mM HEPES, 33 mM biotin, 17 mM panthothenate, 10 nM insulin, 200 pM triiodothyronine, 2 μ M rosiglitazone and 1 mM cortisol) for 4 days, 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 previously described (7). Total RNA (1 μ g) was reverse-transcribed for 60 min at 37°C using Superscript II reverse transcriptase (Life Technology) in the presence of random

hexamer. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. Real time PCR was performed starting with 25 ng cDNA and 900 nM concentration of both sense and antisense primers in a final volume of 25 μ l using the SYBR green TaqMan Universal PCR Master Mix (Applied Biosystem). Fluorescence was monitored and analyzed in a GeneAmp 5700 detection system instrument (Applied Biosystems). Analysis of the 18S ribosomal RNA was performed in parallel using the Ribosomal RNA control Taqman Assay Kit (Applied Biosystem) in order to normalize gene expression. Results are expressed as following: $2^{-(Ct^{18S}-Ct^{gene})}$ where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. Oligonucleotide primers were designed using the Primer Express software (Perkin Elmer Life Sciences).

Oligonucleotides for mouse gene expression studies were:

LPA₁ receptor : sense : 5'-CATGGTGGCAATCTACGTCAA-3';

antisense : 5'-AGCCAATCCAGCGAAGAA-3'

LPA₂ receptor : sense : 5'-TGTCTGACTGCACAGCTTGGA-3';

antisense : 5'-CTCATGGAGTTTTCTGGTGCC-3'

LPA₃ receptor: sense : 5'-TGGGCCATCGCCATTTT-3'; antisense : 5'-GAGCAGGCAGAGATGTTGCA-3'

LPA₄ receptor (also known as p2y9/GPR23): sense : 5'-CCTTACCAACATCTATGGGAGCAT -3'; antisense : 5'-TGGCCAGGAAACGATCCA -3'

ALBP (adipocyte lipid binding protein) : sens : 5'-TTCGATGAAATCACCGCAGA-3' ; antisense : 5'-GGTCTGACTTTCCATCCCACTT-3'

PPAR γ 2 (peroxisome proliferator activated receptor gamma-2) : sense : 5'-CTGTTTTATGCTGTTATGGGTGAAA-3' ;

antisense : 5'-GCACCATGCTCTGGGTCAA-3'

HSL (hormone sensitive lipase) : sense : 5'-GGCTTACTGGGCACAGATACCT-3' ;

antisense : 5'-CTGAAGGCTCTGAGTTGCTCAA-3'.

PEPCK (phosphoenol pyruvate carboxykinase): sense : 5'-

ATGTTTCGGGCGGATTGAAG-3'; antisense:
5'- TCAGGTTCAAGGCGTTTTCC- 3'

Oligonucleotides for human gene expression were:

LPA₁-R : sense : 5' -
TGGGCCATTTTCAACTTGGT-3' ;

antisense : 5' -
TCTGGCGAACATAGCCAAAGA-3'

LPA₂-R : sense : 5' -
TCATCATGGGCCAGTGCTACT-3' ;

antisense : 5' -
GTGGGAGCTGAGCTCTTTC-3'

LPA₃-R : sense : 5' -
TGGGCCATCGCCATTTT-3' ; antisense : 5' -

GAGCAGGCAGAGATGTTGCA-3'

LPA₄-R : sense : 5' -
CCTTACCAACATCTATGGGAGCAT -3' ;

antisense : 5' - TGGCCAGGAAACGATCCA -

3'

ALBP : sens : 5' -
GCATGGCCAAACCTAACATGA-3' ;

antisense : 5' -
CCTGGCCAGTATGAAGGAAA-3'

HSL : sense : 5' -
GTGCAAAGACGGAGGACCACTCCA-3' ;

antisense : 5' -
GACGTCTCGGAGTTTCCCCTCAG-3'.

Western blot analysis: Cells were homogenized in RIPA 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, 2 mM phenylmethylsulfonylfluoride], and 40 µg of protein were separated on 8% SDS-PAGE and transferred on nitrocellulose membrane. The blot was pre-incubated for 1 hour at room temperature in TBS/Tween 1% containing 10% dry-milk, and overnight at 4°C in TBS/Tween 0.5% containing 1% dry-milk supplemented (1/5000 dilution) with rabbit polyclonal PPAR γ antibody raised against an N-terminal PPAR peptide (amino acids 20-104) as previously described (25). After washing in PBS/Tween 0.5%, PPAR γ was visualized by enhanced chemiluminescence detection system (ECL, Amersham Biosciences) using an anti rabbit-HRP antibody (SIGMA).

Results

Expression of LPA receptor subtypes and PPAR γ 2 in 3T3F442A cells

Experiments were carried out in the mouse 3T3F442A cell line previously characterized for its ability to differentiate into adipocytes when cultured in an appropriate adipogenic medium (see Material and Methods). Confluent cells expressed PPAR γ 2, LPA₁ receptor and LPA₄ receptor mRNAs. LPA₂ and LPA₃ were not detected (Figure 1 and Table 1). PPAR γ 2 mRNA level increased 4 fold between confluence and 8 days post-confluence adipocytes (Figure 1). In contrast LPA₁ receptor mRNA level decreased by 14 fold (Figure 1). LPA₄ receptor mRNA level did not change during the course of adipocyte differentiation (Figure 1).

LPA inhibits agonist-mediated activation of PPAR γ 2 in 3T3F442A cells

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

PPAR γ activity was studied in 3T3F442A cells cultured in an adipogenic medium for 4 days (Material and Methods). At that stage the cells exhibited maximal expression of PPAR γ 2 mRNA and minimal expression of LPA₁ receptor (Figure 1). As a positive control of PPAR γ -activation the influence of rosiglitazone on PEPCK mRNA was tested. Rosiglitazone led to a dose-dependent increase in PEPCK mRNA level with an EC50 of 10 nM with a maximal effect to 17 fold when compared to control (Figure 2A). In contrast, LPA (10 µM in 1%BSA as vehicle) did not significantly modify PEPCK mRNA level (Figure 2, curve +LPA, rosiglitazone 0). When using other vehicles (ethanol, methanol, DMSO 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 EC₅₀ to 20 nM without modification of the maximal effect (Figure 2A). When the adipogenic medium was supplemented with 10 μ M LPA from confluence to day 4, the dose-response of rosiglitazone on PEPCK mRNA level was almost completely annealed (Figure 2B). Similar results were obtained when analyzing the Adipocyte Lipid Binding Protein (ALBP) mRNA level as another PPAR γ -sensitive gene (not shown).

These results suggested that LPA was not able to activate PPAR γ in 3T3F442A cells, but conversely inhibits agonist-dependent activation of PPAR γ .

LPA inhibits PPAR γ 2 expression in 3T3F442A cells

In order to test whether LPA-inhibition of agonist-mediated activation of PPAR γ could result from a down-regulation of PPAR γ 2 expression, PPAR γ 2 mRNA level was measured. In 4 days post confluent 3T3F442A cells, 24 h treatment with 10 μ M LPA led to a significant reduction (38%) in PPAR γ 2 mRNA level when compared to control cells (Figure 3A). When LPA was chronically present in the culture medium from confluence to day 4, PPAR γ 2 mRNA level was further down-regulated (74%) when compared to control (Figure 3A). In parallel, PPAR γ protein level was reduced after 4 days treatment with LPA (Figure 3B). No alteration of PPAR γ protein level was observed after 24h LPA treatment.

These results suggested that LPA-mediated inhibition of agonist-mediated activation of PPAR γ resulted from down-regulation of PPAR γ 2 expression.

LPA inhibits adipocyte differentiation of 3T3F442A cells:

PPAR γ 2 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 concentration of LPA in the adipogenic medium, a dose-dependent

reduction in triglyceride accumulation was observed (Figure 4A). The effect of LPA was significantly detected at 4 μ M, and was maximal at 8 μ M. LPA treatment also led to a significant reduction in the level of PPAR γ 2, ALBP, and HSL mRNAs (Figure 4B).

These results revealed the anti-adipogenic activity of LPA.

LPA normally present in serum restrains adipocyte differentiation of 3T3F442A cells

As described in Material and Methods, the adipogenic medium used to allow differentiation of 3T3F442A cells contains 10% fetal calf serum, and serum is known to contain LPA (15). We tested whether LPA brought by serum could influence adipogenesis. As shown in the figure 5A, LPA concentration in fresh adipogenic medium measured using a radioenzymatic assay (15) was 0.45 μ M. As previously demonstrated LPA can be hydrolyzed and inactivated by treatment with the lysophospholipase: phospholipase B (PLB) (5, 7). As shown in Figure 5A, 30 min treatment with 0.5 U/ml PLB led to more than 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 U/ml PLB the amount of triglycerides stored into the cells was significantly increased when compared with a control adipogenic medium (Figure 5B). This was accompanied by a significant increase in the level of PPAR γ 2, ALBP, and HSL mRNAs (Figure 5C). 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 contain serum. Our observations could help to optimize the composition of serum-containing adipogenic medium.

The anti-adipogenic activity of LPA is mediated by LPA₁ receptor subtype:

In order to determine whether LPA₁ receptor was involved in the anti-adipogenic activity of LPA, adipocyte differentiation was analyzed in primary preadipocytes from LPA₁ receptor knockout (LPA₁-KO) mice and their wild type (WT) littermates (4). Preadipocytes are present in the stroma-vascular fraction of

the adipose tissue, and was isolated from adipocytes after collagenase dissociation (see Material and Methods). When prepared from WT mice, the stroma-vascular fraction expressed LPA₁, LPA₂, and LPA₄ receptor mRNAs, LPA₁ receptor subtype being predominantly expressed (Table 1). LPA₃ receptor mRNAs were undetectable. When the stroma-vascular fraction was prepared from LPA₁-KO mice, LPA₁ mRNAs were undetectable, and LPA₂ and LPA₄ mRNA levels were not different than in WT mice (Table 1).

The stroma-vascular fraction of adipose tissue is not only composed of preadipocytes but also of endothelial cells and macrophages. In order to evaluate the proportion of preadipocyte present, the stroma-vascular fraction was cultured in an adipogenic medium (see Material and Methods) for 7 days. In these conditions a certain proportion of cell become light refringent as the result of triglyceride droplets accumulation (Figure 6A) and the expression of adipocyte-specific genes (ALBP, PPAR γ 2, HSL) (not shown). Interestingly, the proportion of lipid-laden cells obtained with WT mice (24%) was significantly lower than that obtained with LPA₁-KO mice (Figure 6B). This 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 from LPA₁-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 μ M LPA) in the proportion of lipid-laden cells when compared to control cells (Figure 7). When starting with LPA₁-KO mice, no changes in the proportion of lipid-laden cells (Figure 7) was observed after LPA treatment. These data showed that the absence of LPA₁ receptor in preadipocytes led to the suppression of the anti-adipogenic activity of LPA.

Adipose tissue phenotype of LPA₁-KO mice:

In order to determine the possible consequences of LPA₁ invalidation on adipose tissue development, LPA₁-KO mice were analyzed and compared with that of WT mice.

Data presented were obtained with males, but similar results were obtained with females (not shown). LPA₁-KO mice exhibited lower body weight than WT mice whatever the age of the animals (Figure 5A). This was accompanied by no differences in the mean daily food intake measured over 10 weeks: 1.0 ± 0.2 and 0.8 ± 0.1 g of food/g body weight/day for WT (n=6) and LPA₁-KO (n=3) mice respectively. At 15 weeks of age, and despite the lower body weight, perigonadic (PG) adipose tissue weight was significantly higher in LPA₁-KO than in WT mice (Figure 5B). Inguinal (ING) adipose tissue weight also tended to be higher in LPA₁-KO mice than in WT mice, but this was not significant (Figure 5B). At the plasma level, LPA₁-KO mice exhibited significantly higher (2 fold) concentration of leptin when compared to WT mice (Table 2). In contrast, no differences in plasma concentration of insulin, glucose, triglycerides, and free-fatty acids were observed (Table 2). Taken together, these observations showed that, despite their lower body weight, LPA₁-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 Simpson-Golabi-Behmel syndrome (SGBS) (16). These cells have previously been described for their ability to differentiate into adipocytes in a serum free medium (Material and Methods). Confluent SGBS preadipocytes, expressed both LPA₁ and LPA₂ receptor mRNAs (Table 1), LPA₁ receptor being 150 fold more expressed than LPA₂ receptor (Table 1). In contrast, LPA₃-R and LPA₄-R remained undetectable.

After 10 days culture in appropriate serum-free adipogenic medium SGBS cells accumulate triglycerides droplets (Figure 8A) and expressed adipocyte-specific genes such as ALBP and HSL (Figure 8B). Supplementation of the adipogenic medium with LPA, led to a striking reduction in triglyceride droplets accumulation (Figure 8A) accompanied by a dose-dependent reduction in ALBP and HSL gene expression (figure 2B). 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), a process tightly controlled by the transcription factor PPAR γ 2. Factors locally produced in adipose tissue by adipocytes could contribute to 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 LPA₁ subtype) (8). We previously demonstrated that, in 3T3F442A preadipocytes, LPA was able to increase phosphorylation of the mitogen-activated protein kinases ERK1 and ERK2, and to increase 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 PPAR γ in monocytes and CV1 cells (9, 10). Since PPAR γ is known to play a pivotal role in the control of adipogenesis (17), determining whether LPA could activate PPAR γ in adipocyte was of main interest in the context of adipose tissue.

The first part of the present study shows that, conversely to the PPAR γ agonist rosiglitazone, LPA was unable to activate PPAR γ -activity in adipocytes evaluated by measuring the induction of PPAR γ -sensitive genes (PEPCK and ALBP). These results lead us to conclude that LPA does not behave as a potent activator of PPAR γ in adipocytes. This conclusion is not in agreement with that previously drawn from experiments performed in monocytes and CV1 cells (9, 10). Although LPA was demonstrated to bind to PPAR γ in an *in vitro* assay (9), the ability of LPA to activate PPAR γ obviously appears to be dependent on the cell-type. Possible activation of a nuclear receptor such as PPAR γ by exogenous LPA would require that enough amount of LPA penetrates into the cell and reach the nucleus. As previously demonstrated by our group, when preadipocytes are exposed to

radiolabeled LPA, virtually no radiolabelled LPA can be detected in the cells because of the presence of a high ecto-Lipid Phosphate Phosphohydrolase (LPP) activity which dephosphorylates and inactivates LPA (20). This could explain why we observed no activation of PPAR γ by LPA in adipocytes. This also suggest that LPP activity could be weaker in monocytes and CV1 cells than in adipocytes, allowing higher amount of LPA to enter into the cells and activate PPAR γ .

The most important result of the present study, was that chronic exposure of preadipocytes to LPA inhibits their differentiation into adipocytes, as attested by reduction in triglyceride accumulation and 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 LPA₁ knockout mice, indicating that LPA₁ receptor is fully responsible of the anti-adipogenic activity of LPA. These observations clearly support the concept that LPA is an anti-adipogenic mediator acting via a specific receptor.

PPAR γ 2 clearly plays a pivotal positive role in adipogenesis (17). Our data show that treatment with LPA leads to down-regulation of PPAR γ 2 expression and activity as expected for an anti-adipogenic factor. It is therefore very likely that the anti-adipogenic activity of LPA be due to its negative impact on PPAR γ 2. This conclusion is supported by previous reports showing that LPA is able to inhibit PPAR γ activity in THP-1 monocytes and in IMR-32 neuroblastoma (21, 22). In this last cell line, LPA inhibits the capacity of PGJ2 to activate the transcription of a PPAR γ responsive element-dependent reporter gene, and, by using specific pharmacological inhibitors it was shown that the effect of LPA involves activation of the MAPK pathway as well as the activation of the Rho-kinase (22). In addition, it has previously been shown that increasing Rho-GTPase activity decreases adipocyte differentiation and PPAR γ expression in mouse embryo-derived fibroblast (23). In preadipocytes, LPA activates the phosphorylation of ERK1 and ERK2 MAPKs, and activates the small G-protein rho (19). It can therefore be proposed that LPA-mediated inhibition of PPAR γ 2 activity and expression

may result from the LPA₁ receptor-dependent activation of the MAPKs and/or of the Rho-kinase.

What could be the physiological relevance of the anti-adipogenic activity of LPA? Because LPA₁-R was responsible for the anti-adipogenic activity of LPA in preadipocytes, phenotypic analysis of LPA₁-KO mice was an excellent opportunity to determine the possible involvement of LPA on adipose tissue development. As previously demonstrated LPA₁-KO mice exhibit reduced size and weight of their body (4). In the present study, we observed that despite their lower body weight, LPA₁-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). Since LPA₁-KO mice exhibited the same food intake than WT mice, the higher adiposity of LPA₁-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 LPA₁-KO mice

was higher than that of WT mice (Figure 6). It is therefore possible that increased adiposity of LPA₁-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. Since preadipocytes express LPA₁ 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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Legends of the figures :

Figure 1: Expression of LPA₁ receptor, LPA₄ receptor, and PPAR γ 2 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 LPA₁, LPA₄ and PPAR γ 2 mRNAs were quantified by real time RT-PCR as described in Material and Methods. Values are means \pm SE of three experiments.

Figure 2: Influence of LPA and rosiglitazone on PEPCK mRNA level in 3T3F442A cells: (A) Confluent 3T3F442A cells were cultured in an adipogenic medium (see Material and Methods) and, after 4 days of culture, the cells were serum-deprived and exposed for an additional 24h to increasing concentration of rosiglitazone in the presence (+ LPA) or not (-LPA) of 10 μ M lysophosphatidic acid (LPA). (B) Confluent 3T3F442A cells were cultured in an adipogenic medium for 4 days in the presence (+LPA) or not (-LPA) of 10 μ M lysophosphatidic acid, and serum-deprived cells were exposed for an additional 24h to increasing concentration of rosiglitazone for 24h. (A and B) After 24h, total RNA were extracted and PEPCK mRNAs were quantified by real time PCR quantification (see Material and Methods). Values are means \pm SE of three experiments. Comparison between (-LPA) and (+LPA) was performed using Student's paired t-test : * (P<0.05) ; ** (P<0.01).

Figure 3: Influence of LPA on PPAR γ 2 expression in 3T3F442A cells

Confluent 3T3F442A cells were cultured in an adipogenic medium (see Material and Methods) for 4 days in the presence or not of 10 μ M LPA from confluence or from the 3rd day. On the 4th day, total RNA were extracted and PPAR γ 2 mRNAs were quantified by real time RT-PCR (A). In parallel, PPAR γ protein level was determined by western blot analysis (B). Values are means \pm SE of three experiments. Comparison with control was performed using Student's paired t-test : * (P<0.05) ; ** (P<0.01).

Figure 4: Influence of LPA on adipocyte differentiation of 3T3F442A cells

Confluent 3T3F442A cells were cultured in an adipogenic medium (Material and Methods) in the presence or absence of increasing concentration of LPA. After 10 days the amount of triglycerides

accumulated in adipocytes (A) or the expression of adipocyte-specific genes (B) were measured as described in Material and Methods. Values are means \pm SE of three separate experiments. Comparison with control was performed using Student's paired t-test : * (P<0.05) ; ** (P<0.01).

Figure 5: Influence of phospholipase B on adipocyte differentiation of 3T3F442A cells: (A) The adipogenic medium was treated or not (cont) with 0.5 U/ml phospholipase B (PLB) for 30 min at 37°C before quantification of LPA concentration using a radioenzymatic assay (see Material and Methods). (B and C) Confluent 3T3F442A cells were cultured in an adipogenic medium (Material and Methods) in the presence or absence 0.5 U/ml PLB. After 10 days the amount of triglycerides accumulated in adipocytes (A) or the expression of adipocyte-specific genes (B) were measured as described in Material and Methods. Values are means \pm SE of three separate experiments. Comparison with control was performed using Student's paired t-test : * (P<0.05) ; ** (P<0.01); *** (P<0.001).

Figure 6: Differentiation of primary preadipocytes from wild type and LPA₁ receptor knockout mice. Stroma-vascular cells were isolated from wild type (WT) or LPA₁-receptor knockout male mice (LPA₁-KO) male mice and cultured in a serum-free adipogenic medium as described in Material and Methods. After 7 days of culture, the proportion of lipid-laden cells (A) were determined (B). Values are means \pm SE from 4 WT and 7 LPA₁-KO mice. Comparisons were performed using Student's t-test : * (P<0.05) .

Figure 7: Influence of LPA on differentiation of primary preadipocytes from wild type and LPA₁ (-/-) mice. Stroma-vascular cells were isolated from WT or LPA₁-KO male mice and cultured in a serum-free differentiating medium (see Material and Methods) in the absence (control) or in the presence of increasing concentrations of LPA. After 7 days of culture lipid-laden cells were counted under microscope. Values are means \pm SE of 4 and 7 separate experiments for WT and LPA₁-KO mice respectively. Comparison with control was performed using Student's t-test : * (P<0.05).

Figure 8: Body and fat pad weights of wild type and LPA₁ (-/-) mice. Wild type (WT) and LPA₁-receptor knockout (LPA₁-KO) male mice were housed and fed as described in Material and Methods and their body weight was followed from 4 weeks to 15 weeks of age (A). The animals were sacrificed at 15th week (s) in order to dissect out and weight inguinal (ING) and perigonadic (PG) adipose tissue from wild type (open bars) and LPA₁-receptor knockout (closed bars) mice (B). Values are means \pm SE of 8 and 5 separate experiments for WT and LPA₁-KO mice respectively. Comparisons between WT and LPA₁-KO were performed using Student's t-test : * (P<0.05).

Figure 9: Influence of LPA on adipocyte differentiation of human preadipocytes : Confluent SGBS preadipocytes were cultured in a serum-free differentiating medium (see Material and Methods) in the absence (cont) or the presence of increasing concentration of LPA. After 10 days culture, SGBS adipocytes were photographed (A) and the expression of adipocyte-specific gene expression (HSL : white column ; ALBP : black column) were measured as described in Material and Methods. Values are means \pm SE of four separate experiments. Comparison with control was performed using Student's t-test : * (P<0.05) ; ** (P<0.01).

Table 1: Expression of LPA₁-, 2-, 3-, and 4-receptor subtype genes in preadipocytes :

	LPA ₁	LPA ₂	LPA ₃	LPA ₄
3T3F442A preadipocytes	27 \pm 3	und	und	0.3 \pm 0.1
SGBS preadipocytes	45 \pm 3	0.3 \pm 0.1	und	und
WT-SVC	117 \pm 12	1.4 \pm 0.4	und	2.8 \pm 0.1
LPA ₁ -KO SVC	und	1.7 \pm 0.5	und	2.5 \pm 0.3

Gene expression was determined by real time PCR as the following formula : $2^{-(Ct_{18S}-Ct_{gene})} \times 10000$ as described in Material and Methods. Values are means \pm SE of 2 to 4 separate experiments.

Table 2: Blood parameters of WT and LPA₁-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 *

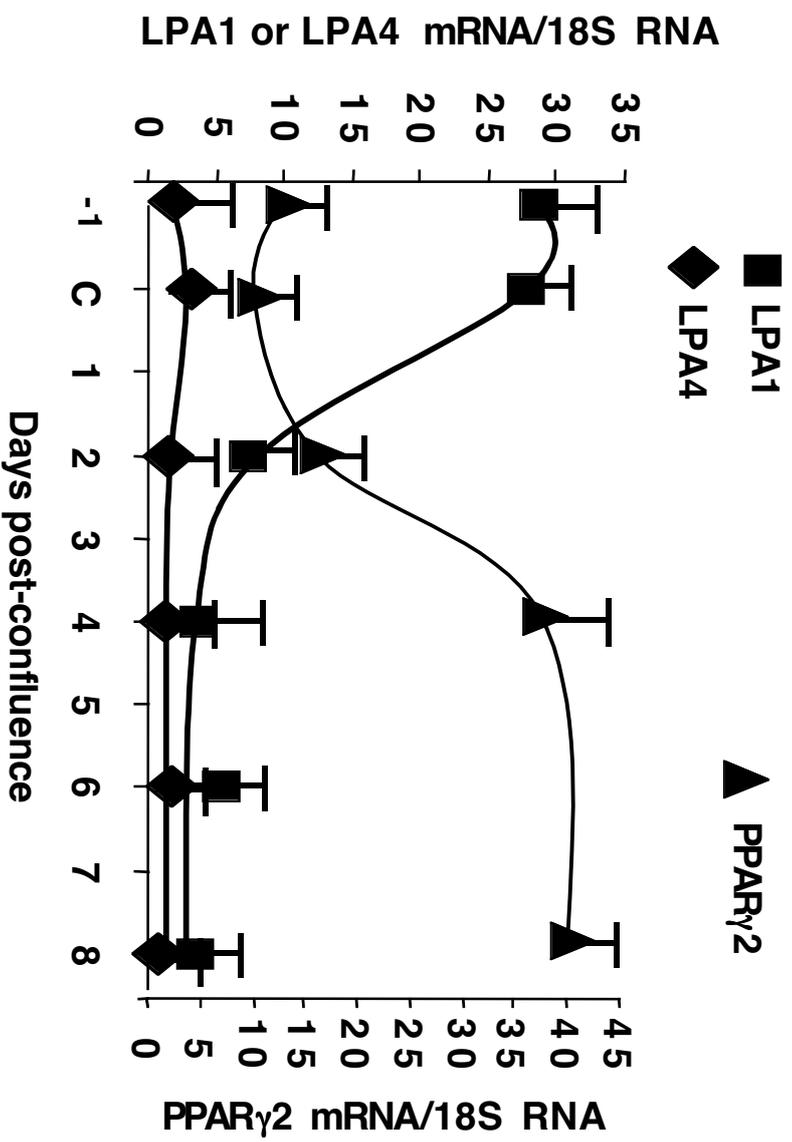
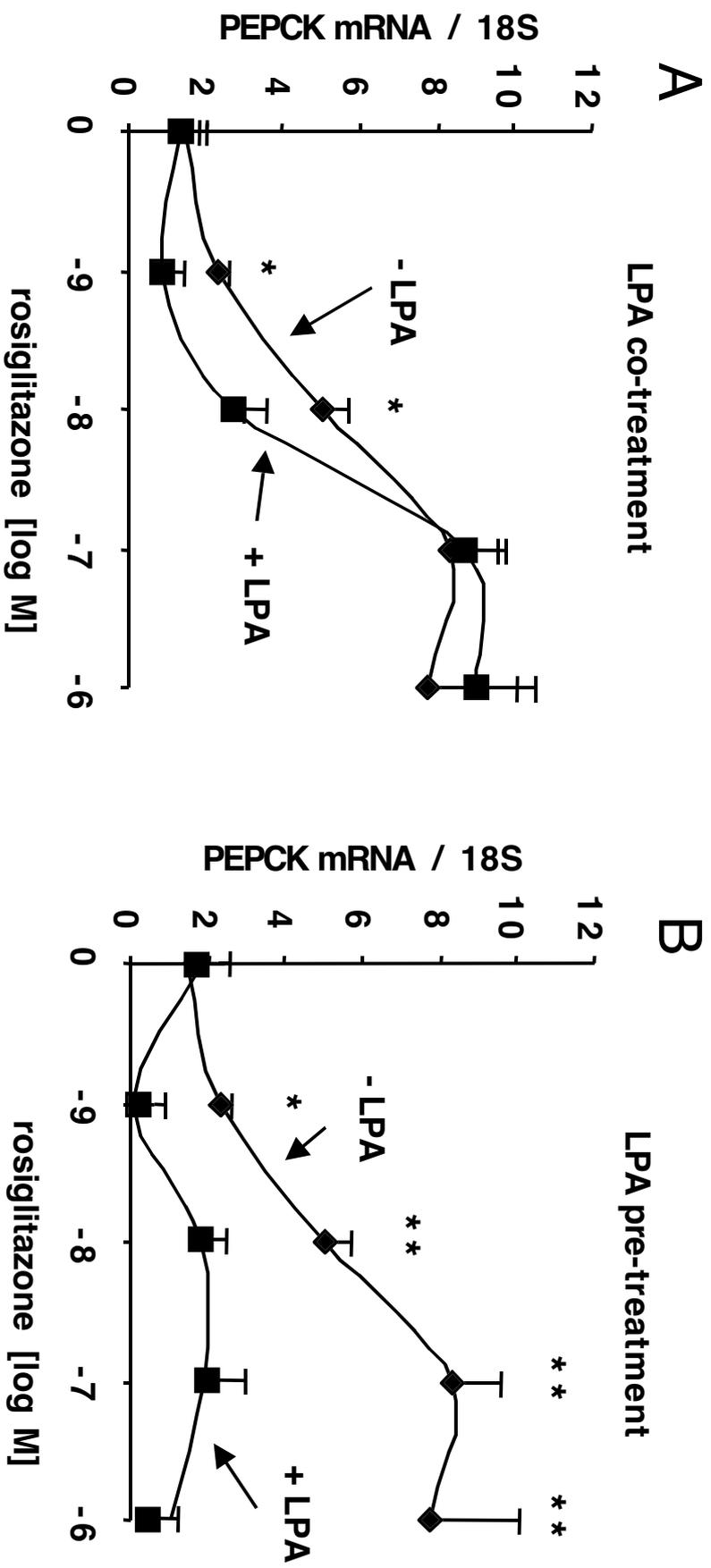


Figure 1

Figure 2



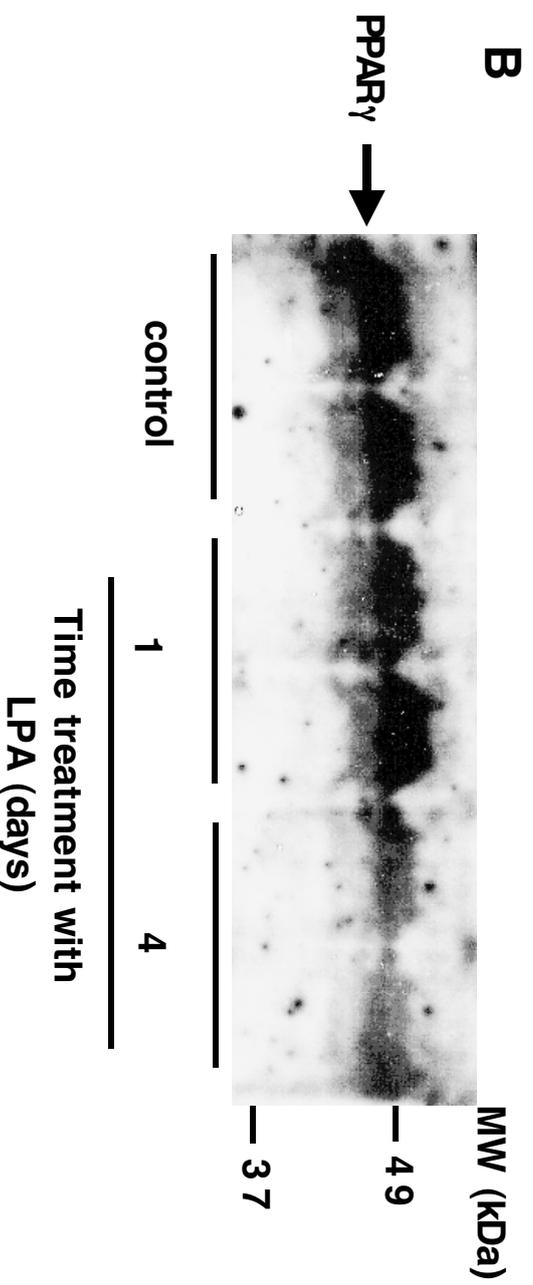
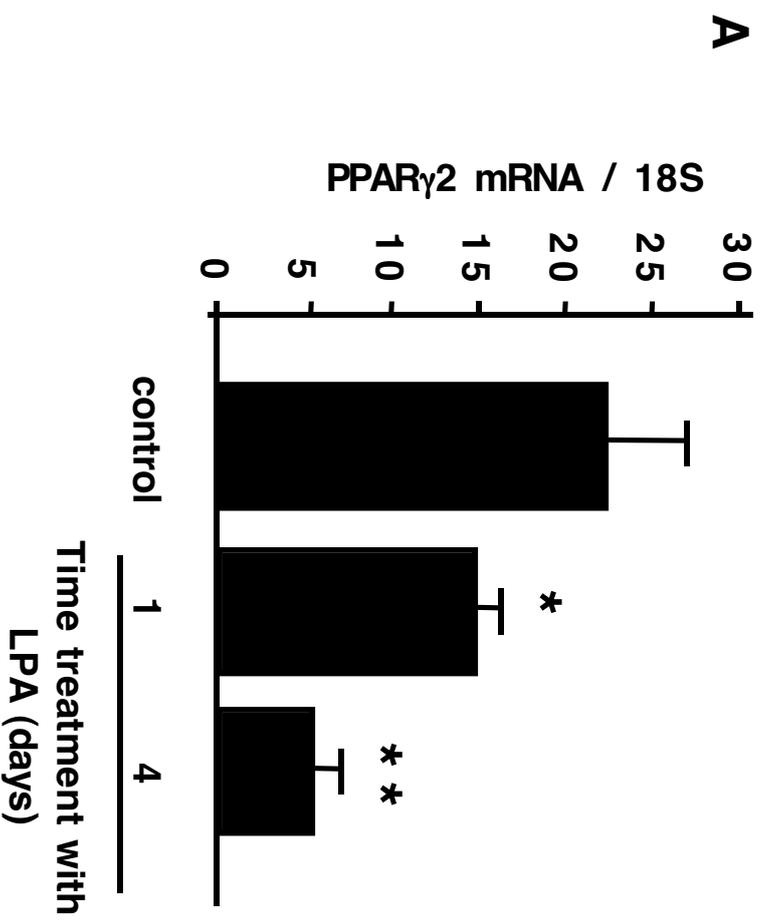


Figure 3

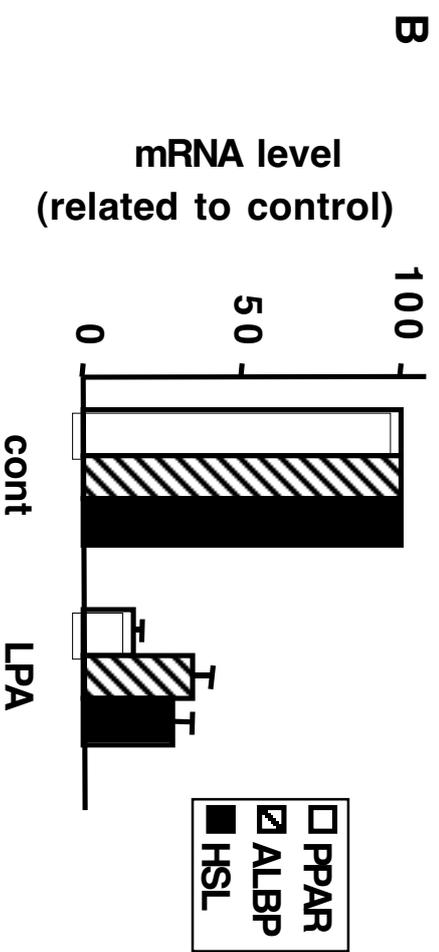
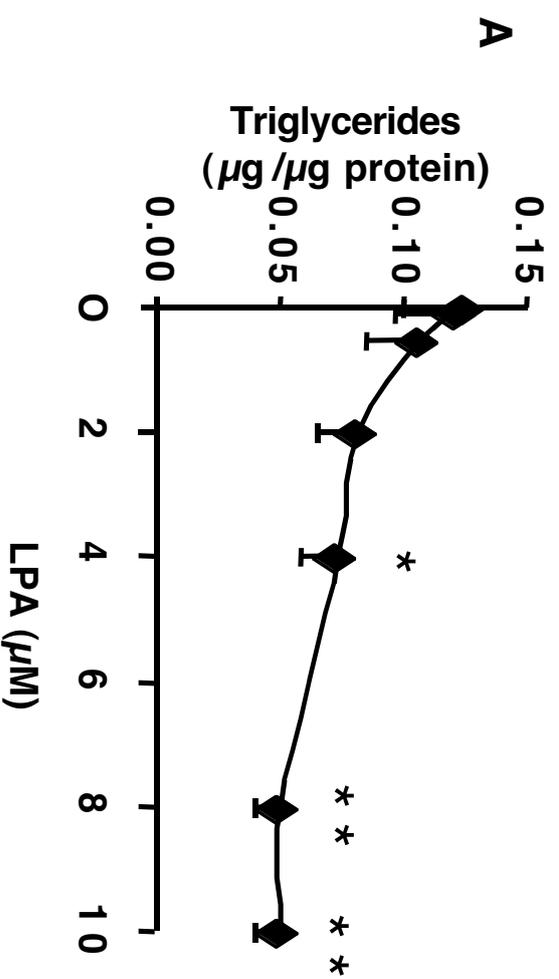
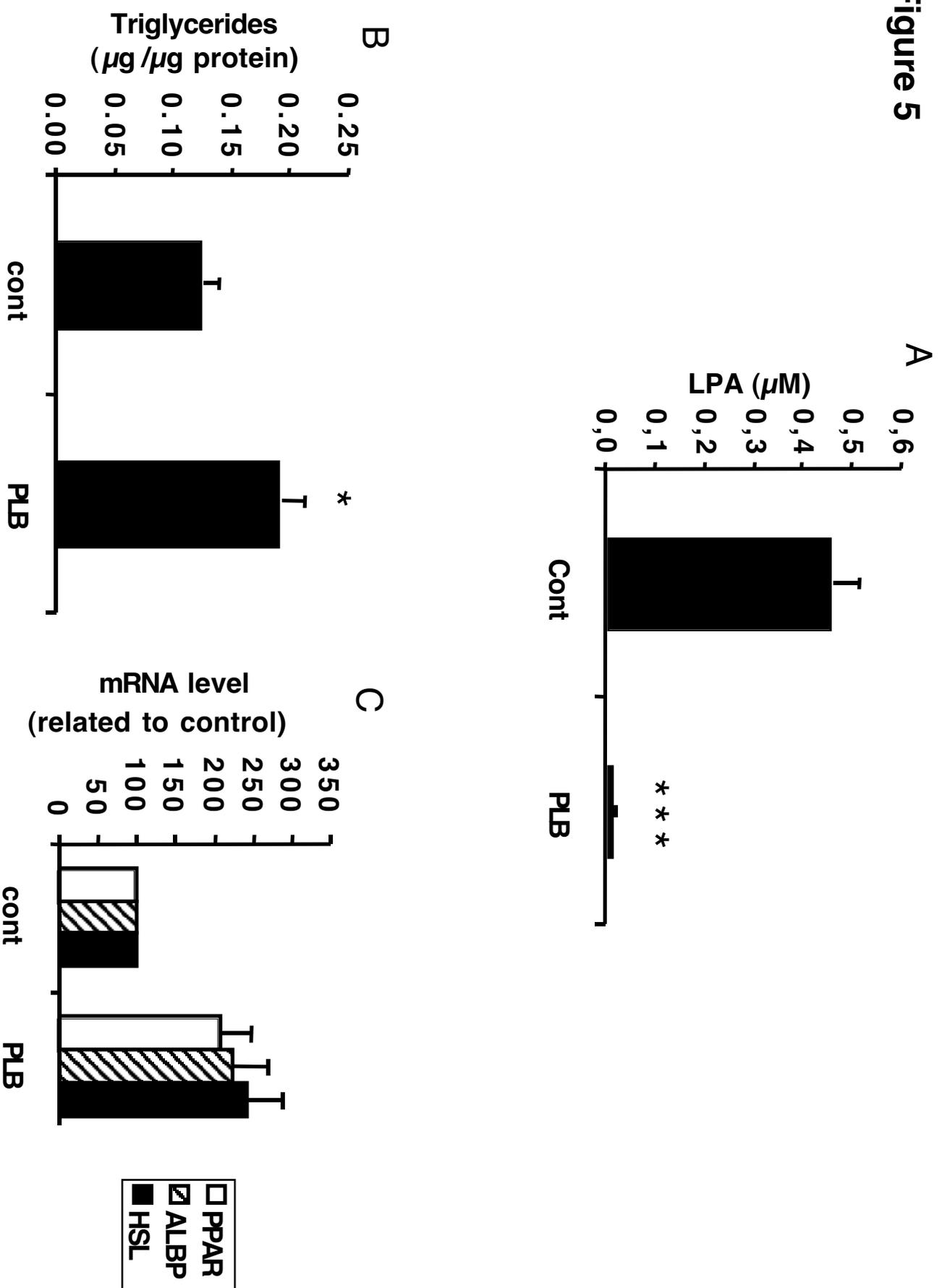


Figure 4

Figure 5



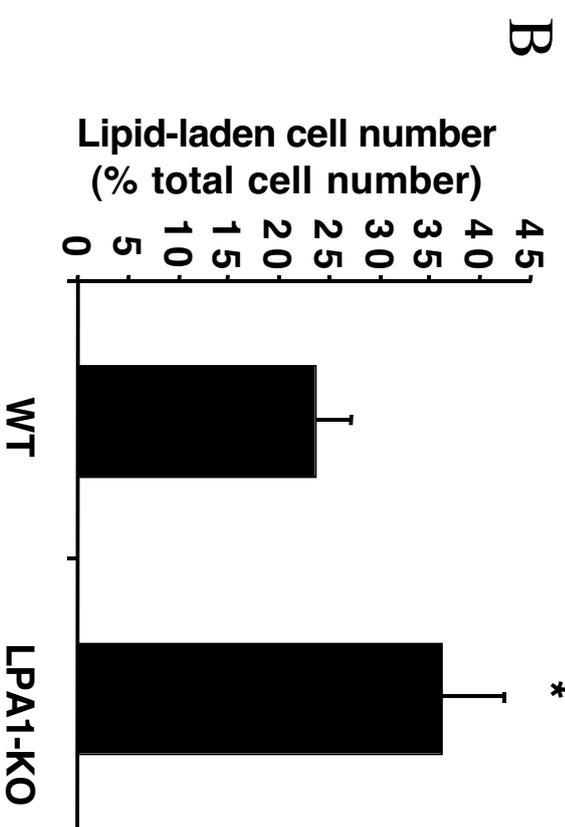
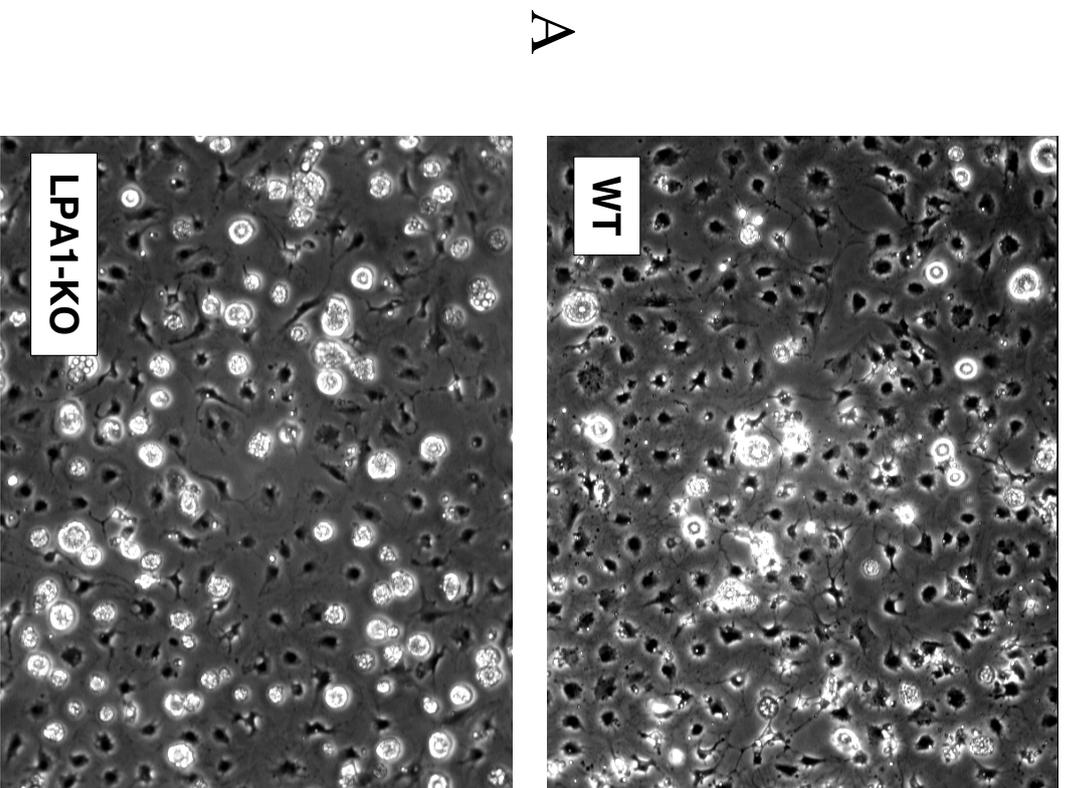


Figure 6

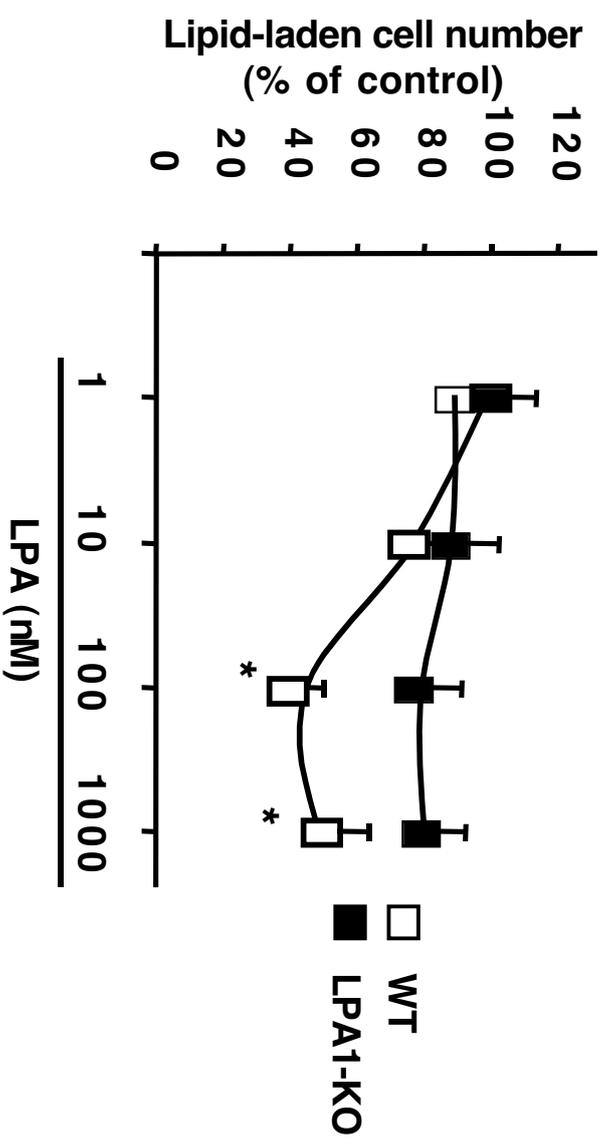


Figure 7

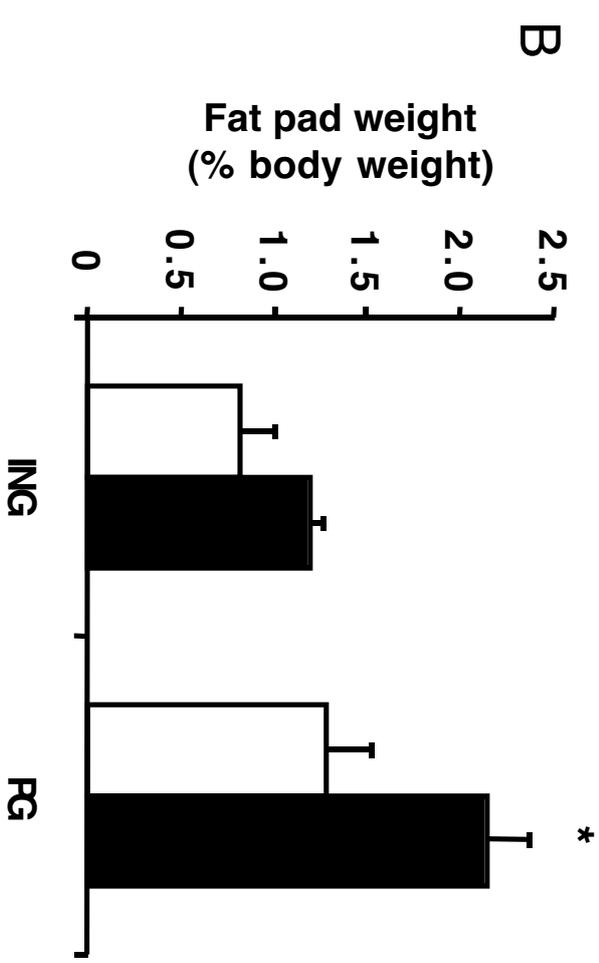
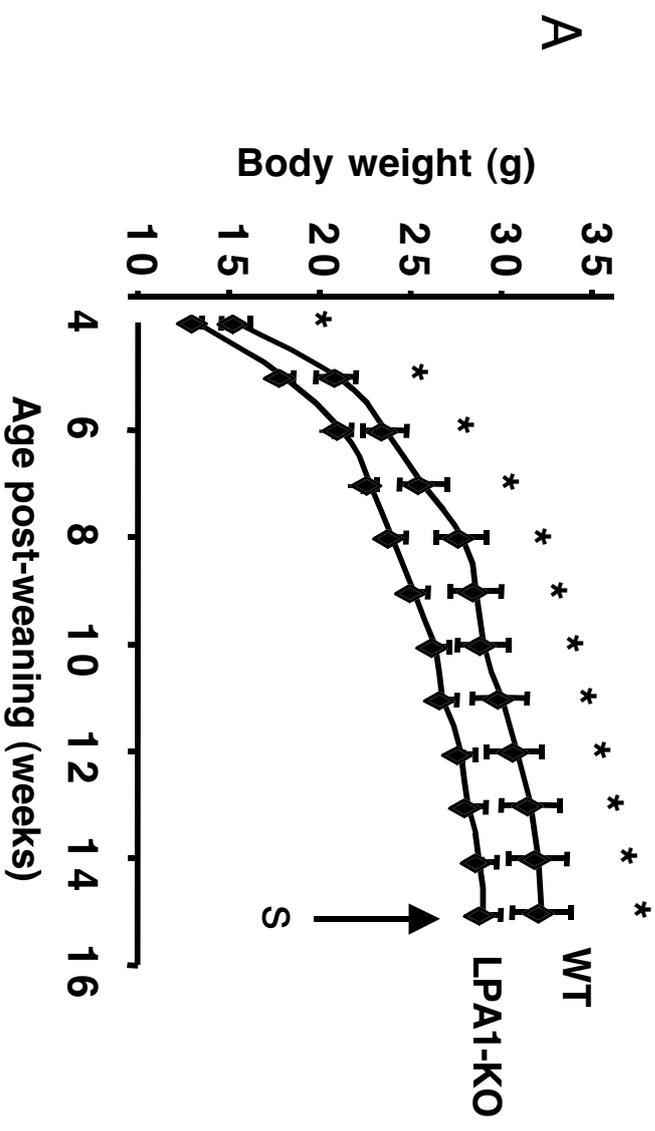


Figure 8

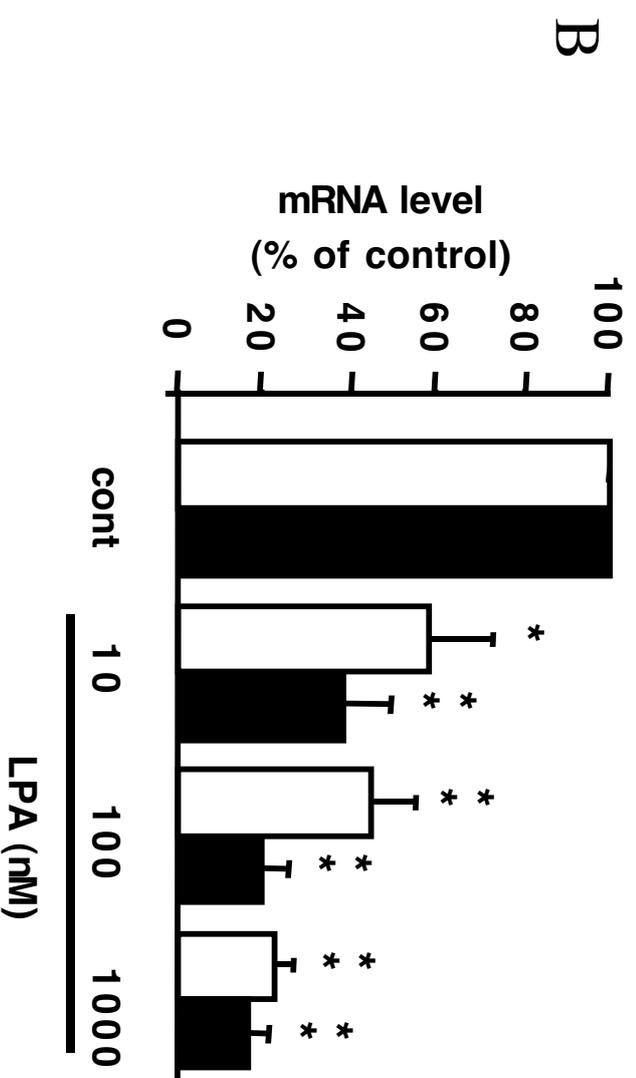
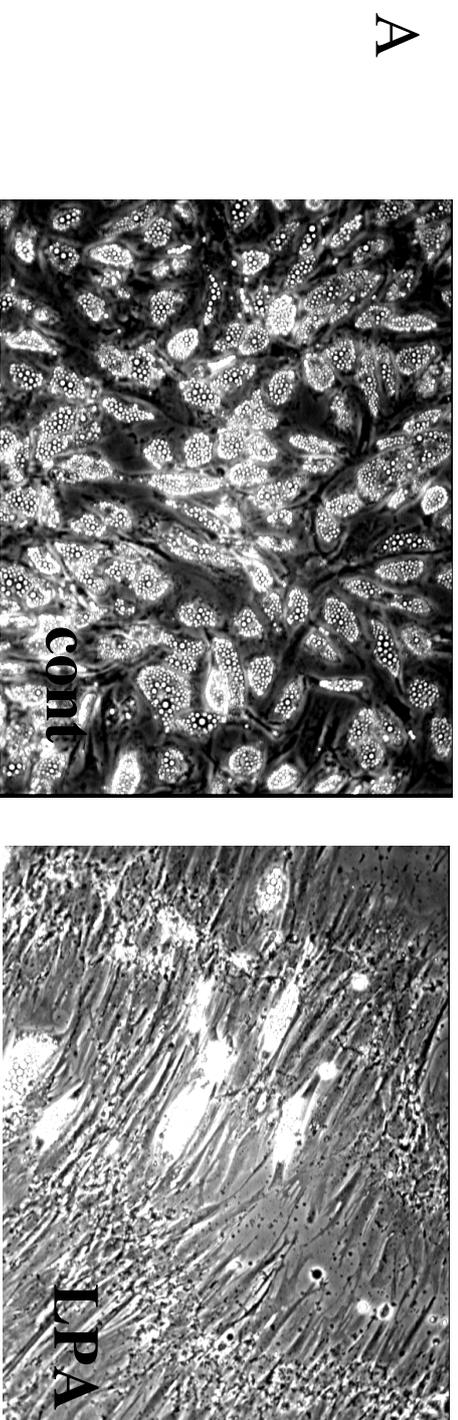


Figure 9