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**Expression of ecto-lipid phosphate phosphohydrolases in 3T3F442A preadipocytes and adipocytes: involvement in the control of lysophosphatidic acid production.**

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Running-title

Fat cell ecto-lysophosphatidic acid phosphatase

## SUMMARY

Because of its production by adipocytes and its ability to increase preadipocyte proliferation, lysophosphatidic acid (LPA) could participate to the paracrine control of adipose tissue development. The aim of the present study was to determine which enzyme activities are involved in exogenous LPA hydrolysis by preadipocytes and adipocytes. Using a quantitative method, we observed that extracellular LPA rapidly disappeared from the culture medium of 3T3F442A preadipocytes. This disappearance was strongly slowed down in the presence of the phosphatase inhibitors, sodium vanadate and sodium pervanadate. By using [<sup>33</sup>P]LPA on intact 3T3F442A preadipocytes, we found that 90% of LPA hydrolysis resulted from LPA phosphatase activity biochemically related to previously described ecto-lipid phosphate phosphohydrolases (LPP). Quantitative real time RT-PCR revealed that 3T3F442A preadipocytes expressed mRNAs of three known LPP subtypes (-1, -2 and -3), with a predominant expression of LPP-1 and LPP-3. Differentiation of 3T3F442A preadipocytes into adipocytes led to 80% reduction in ecto-LPA-phosphatase activity, with a concomitant down-regulation in LPP-1, LPP-2, and LPP-3 mRNA expression. In spite of this regulation, treatment of 3T3F442A adipocytes with sodium vanadate increased LPA production in the culture medium, suggesting the involvement of ecto-LPA phosphatase activity in the control of extracellular production of LPA by adipocytes. In conclusion, these data demonstrate that hydrolysis of extracellular LPA by preadipocytes and adipocytes mainly results from a dephosphorylation activity. This activity: (i) occurs at the extracellular face of cell membrane; (ii) exhibits similar biochemical characteristics than to the LPP; (iii) is negatively regulated during adipocyte differentiation, (ii) and plays an important role

in the control of extracellular LPA production by adipocytes. Ecto-LPA-phosphatase activity represents a potential target to control adipose tissue development.

## INTRODUCTION

Obesity corresponds to the enlargement of adipose tissue, resulting from both an excessive accumulation of triglycerides in adipocytes (hypertrophy), and the recruitment of new fat cells (adipogenesis) via proliferation and differentiation of adipocyte precursors (preadipocytes). Throughout life, preadipocytes are present in adipose tissue closely associated to adipocytes (1). Adipogenesis can be regulated by circulating hormones and growth factors (insulin, catecholamines, glucocorticoids, thyroid hormones, etc...), as well as by paracrine/autocrine factors (tumor necrosis factor, angiotensinogen, leptin, fatty acids, monobutyrin, eicosanoids, lysophosphatidic acid, etc...) produced locally in the adipose tissue, particularly by adipocytes (2).

Our group has demonstrated that adipocytes are able to produce lysophosphatidic acid in their environment (culture media, extra-cellular fluid of adipose tissue) (3). This bioactive phospholipid is able to activate preadipocyte proliferation by interacting preferentially with a specific G-protein coupled receptor: the Endothelial Differentiation Gene Receptor-2 (EDG-2) (4) (EDG-2 is also named LPA<sub>1</sub>-Receptor according to IUPHAR Nomenclature Committee recommendation). Based upon these findings, LPA may participate, with other factors, to the paracrine/autocrine control of adipose tissue development.

One way to test the physiological relevance of this hypothesis *in vivo* would be to act on the LPA concentration in adipose tissue and analyze the consequences on adipose tissue development. In order to achieve such a strategy, it is first necessary to understand the mechanisms involved in the control of LPA bioavailability in adipose tissue.

According to the literature, the origin of extracellular LPA remains controversial (5). LPA can be synthesized by secreted phospholipase A2 (6) or soluble lysophospholipase D (7,8). Alternatively, LPA could also be synthesized intracellularly by a glycerol-3-phosphate acyl-transferase (9) or monoacyl glycerol kinase (10). Whether intracellular LPA can be externalized by passive or active diffusion remains a matter of debate.

Recent data from our group show that one important pathway of LPA synthesis by adipocytes is the hydrolysis of lysophosphatidylcholine by a lysophospholipase D secreted by adipocytes <sup>1</sup> (manuscript in press).

In parallel, extra-cellular LPA can be hydrolyzed by an ecto-lipid phosphate phosphatase (LPP) leading to the formation of monoacylglycerol, inactive on LPA-receptors (11). LPP are integral membrane glycoproteins with six transmembrane domains, exhibiting a catalytic site on the extracellular face of the cells, and able to degrade exogenously added glycerol- or sphingosyl- phosphate lipids (12-15). Conversely to another class of lipid phosphatase localized in intracellular compartments (class 1 phosphatidic acid phosphatases or PAP-1), LPP do not require Mg<sup>++</sup> for full activity and are insensitive to N-ethylmaleimide (NEM) (13,16). At least three genes encoding LPP isoenzymes (LPP-1, -2, and -3) have been identified in human and rodents (12,14,17,18). In human two mRNAs issued from alternate splicing are transcribed from LPP1 gene (LPP1 and LPP1a) (14). An over-expression of some LPP genes leads to attenuation of LPA-induced cell responses (19-21), showing that these enzymes serve as regulator of strength and duration of LPA signal.

The presence of NEM-insensitive lipid phosphatase has previously been reported in rat adipocyte membranes (22,23), but their contribution in the bio-availability of LPA

has never been studied. The aim of the present study was to investigate the contribution of LPP in the catabolism of LPA (exogenous or produced by adipocytes) by intact preadipocytes or adipocytes of the mouse cell line 3T3F442A.

## EXPERIMENTAL PROCEDURES

*Materials*—Dulbecco's minimum essential medium (DMEM), penicillin, streptomycin, were from Life Technologies and fetal calf serum (FCS) donor calf serum (DCS) from BioWhittaker. Solvents came from PROLABO, [ $\gamma$ - $^{33}\text{P}$ ] ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech. Diacylglycerol Kinase was from Calbiochem. Oleoyl-lysophosphatidic acid was from Cayman chemical company. Fatty acid-free bovine serum albumin (fatty acid-free BSA) was from ICN. Monooleoylglycerol and other chemicals were from Sigma.

*Cell Culture*—The mouse preadipose cell line 3T3F442A used in this study was initially derived from Swiss mouse fibroblast embryo, and were selected for their ability to spontaneously differentiate into adipocytes (24). Cells were grown in 24-well plate at 37°C in a humidified atmosphere containing 7% CO<sub>2</sub> in the presence of DMEM supplemented with 10% DCS. In some experiments differentiation of preadipocyte into adipocyte was achieved by cultivating confluent preadipocytes in DMEM supplemented with 10% FCS and 50 nM insulin for 10 days as reported previously (25).

*Extracellular LPA Concentration Measurement*—After 1-butanol extraction of LPA present in the medium, the concentration of remaining lipid was determined according to the radioenzymatic method described in reference (26) using specific LPA acyl transferase activity.

*Substrate Preparation*—Oleoyl-lysophosphatidic acid, dioleoyl-phosphatidic acid, C<sub>8</sub>-ceramide phosphate used in this study were enzymatically synthesized from their respective monoacyl-, diacyl-glycerol or C<sub>8</sub>-ceramide precursors. They were incubated in the presence of diacylglycerol Kinase and [ $\gamma$ - $^{33}\text{P}$ ] ATP. Sphingosine-1-

phosphate was obtained by acidic hydrolysis of ceramide phosphate according to reference (27). Radioactive compounds after chromatographic separation on C/M/Aceton/ Acetic acid/Water (50:10:20:10:5 v/v) purification and elution from silica using C/M/W (1:2:0.8 v/v) were solubilized in ethanol then mixed with cold corresponding lipid in order to obtain a specific activity of 5 000-20 000 cpm/nmole.

*LPP Activity Assay*—Cells were grown for at least 24 h, deprived of serum for 18 h then washed with DMEM before measuring activity in HEPES-buffer (118 mM NaCl, 6 mM KCl, 6 mM glucose, 1 mM CaCl<sub>2</sub>, 12.4 mM HEPES, 0.1% fatty acid-free BSA pH 7.4). LPP activity in cell culture was determined by measuring [<sup>33</sup>P] production from [<sup>33</sup>P]-labeled LPA dispersed in buffer containing 0.1% fatty acid-free BSA. LPA concentration was adjusted to 5 μM and incubation was stopped 10 min after substrate addition. Lipids present in the extracellular medium were extracted using 1 volume *n* butanol. After phase separation radioactivity was measured in each layer.

*Analysis of Water-soluble [<sup>33</sup>P]-LPA Degradation Product(s)*—This was done according to (28). Briefly 100 μl aqueous phase were mixed with 10 μl concentrated HCl, 10 μl PBS, 30 μl 5% ammonium molybdate. Then 200 μl isobutyl alcohol/toluene (1:1 v/v) were added and the sample mixed vigorously. The upper organic phase contains phosphomolybdate complex and lower phase glycerol-3-phosphate. Each phase was collected and radioactivity determined.

#### *Non quantitative RT-PCR analysis*

Total RNA were isolated using RNeasy kit from Qiagen. Total RNA (500 ng) were reverse transcribed for 60 min at 37°C using Superscript II reverse transcriptase (Life Technology) in the presence of oligo dT primers. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. Polymerase Chain Reaction (PCR) was carried out in a final volume of 50 μl

containing 1.5 µl RT reaction, 1 µl dNTP (10 mM), 5 µl 10x PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl and 0.1 % Triton X-100), 3 µl MgCl<sub>2</sub> (25 mM), 1.5 µl sense and antisense specific oligonucleotide primers (10 µM) and 1.25 unit of Taq DNA polymerase (Promega). Conditions for PCR reaction were: initial denaturation step at 94°C, 1 min at 60°C, and 72°C for 90 s. After a final extension at 72°C for 6 min, PCR amplification products were separated on 1.5 % agarose gel, and visualized by ethidium bromide staining.

Primers used for mouse LPP1 were designed from the GenBank sequence D84376, positions 691 to 939 (249 bp): sense 5'- CCCAGACTGGTCAAAAATCAA-3'; antisense 5'-ACTCGAGAAAGGCCACATA-3'.

Primers used for mouse LPP2 were designed from the GenBank sequence AF123611, positions 396 to 618 (249pb): sense 5'-GCTGCCATCTACAAGGTGCT-3'; antisense 5'-CATGCAATACATGCCAAAGG-3'

Primers used for mouse LPP3 were designed from the GenBank sequence AK011276, positions 583 to 785 (202pb): sense 5'-TCATCTGCCTGGACCTCTTC-3' antisense 5'-CCTGTAATGATCGCCAGGAT-3'.

A splice variant of LPP-1 gene was described in human (14). If such a splice variant exist in mouse, the designed primers do not discriminate between the two mRNAs.

*Quantitative Real Time RT-PCR*—Total RNAs were isolated using Rneasy kit from Qiagen. Total RNA (1 µg) were 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 RT-PCR was performed starting with 25 ng cDNA with 300 nM (LPP1) or 900 nM (LPP2 and LPP3) concentration of both sense

and antisense primers in a final volume of 25  $\mu$ l using the SYBR green TaqMan Universel 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. Standard curves were determined after amplification of gel purified PCR amplification products ( $5.5 \cdot 10^{-5}$  à  $5.5 \cdot 10^{-7}$  ng/ $\mu$ l) generated from 3T3F442A cDNA by non-quantitative RT-PCR (see previous paragraph). Each RT-PCR quantification experiment was performed twice using duplicate samples from two independently generated cDNA templates. The mRNA quantity present in each assay was determined by comparison with the standard curves.

Oligonucleotide primers used to quantify LPP1, LPP2, and LPP3 mRNAs by real time RT-PCR were designed within the sequence of the PCR amplification product generated by non-quantitative RT-PCR (see previous paragraph). Primer design was optimized by using the Primer Express software (Perkin Elmer Life Sciences). Oligonucleotides used were: LPP-1: sens 5'-GGGAGACTGGGCAAGACTCTT-3', antisense: 5'-CACTCGAGAAAGGCCACAT-3'; LPP-2 sense: 5'-CGCGATCCAACCTTCAACAAC-3', antisense: 5'-CAGCCCCGAACAGAAAGGT-3'; LPP-3: sense 5'-CCATCCTGGCGATCATTACAG-3', antisense 5'-AAAGGAAGCATCCCCTTGCT-3'.

*Protein Determination*—After complete removal of incubation medium, total cell protein were solubilized in NaOH 0.5N and quantified using DC protein assay kit (Bio-Rad) according to the manufacturer instructions.

*Mouse Adipocytes Preparation*—Perigonadic mouse adipose tissue was carefully dissected out and adipocytes isolated using collagenase as previously described

(29). Floating cells were washed in Krebs-Ringer bicarbonate, RNA isolated or enzyme activity measured as previously described.

## RESULTS

*Half-life of Exogenous LPA in Preadipocyte Culture Medium*— In order to determine the ability of preadipocytes to hydrolyze exogenous LPA, 5  $\mu\text{M}$  of 1-oleoyl-LPA was added to intact serum-starved 3T3F442A preadipocytes, and the changes in LPA concentration in the culture medium was determined using a radioenzymatic assay (26).

As shown in Fig. 1, LPA progressively disappeared from the culture medium with an initial rate of disappearance of  $22.7 \pm 8.0$  nmoles/h/mg protein ; 50% of initial concentration of LPA remaining after 2 to 3 h incubation. Similar kinetic of disappearance (half-life of 2 h) was observed when LPA was brought in serum (initial concentration of 0.3  $\mu\text{M}$ ) (not shown).

These results showed that when exposed to intact preadipocytes exogenous LPA rapidly disappeared from the culture medium, suggesting the existence of a LPA catabolic pathway in these cells.

*LPA-phosphatase Activity Results from an Ecto-lysophospholipid Phosphatase*— In order to precise the metabolic pathways involved in disappearance of exogenous LPA, intact serum-starved 3T3F442A preadipocytes were incubated with 5  $\mu\text{M}$  LPA mixed with traces of [ $^{33}\text{P}$ ]-LPA. At different incubation times, the culture medium was removed and extracted with 1-butanol in order to separate [ $^{33}\text{P}$ ]-LPA (butanol phase) from water-soluble [ $^{33}\text{P}$ ]-labeled hydrolysis-products (aqueous phase).

As shown in the Fig. 2, a time-dependent decrease in [ $^{33}\text{P}$ ]-LPA concentration paralleled with a proportional increase in water soluble [ $^{33}\text{P}$ ]-labeled hydrolysis products was observed. After 30 minutes incubation, [ $^{33}\text{P}$ ]-LPA and water-soluble [ $^{33}\text{P}$ ]-labeled products represented 42% and 54% of the initial concentration of [ $^{33}\text{P}$ ]-

LPA respectively. Moreover, only 5% of [<sup>33</sup>P]-LPA were associated with the cells strongly suggesting that most of [<sup>33</sup>P]-LPA hydrolysis occurred extracellularly (Table I). Based upon TLC analysis, [<sup>33</sup>P] present in butanol phase was exclusively in the form of LPA (not shown).

In order to determine whether LPA hydrolysis was due to a membrane- or a soluble-bound enzyme, the culture medium was separated from the cells and subjected to centrifugation (20 000 g) in order to discard cell debris. Whereas [<sup>33</sup>P]LPA hydrolysis was detected in the pellet (cell debris), no detectable hydrolysis of [<sup>32</sup>P]LPA was observed in the supernatant (not shown). This result showed that LPA-phosphatase activity only results from a membrane-bound enzyme activity.

Formation of water-soluble [<sup>33</sup>P]-labeled products was inhibited by sodium vanadate or sodium pervanadate in a dose-dependent manner (Fig. 3) (IC<sub>50</sub> of 5 μM and maximal effect reached at 100 μM), suggesting the involvement of a phosphatase activity in this formation. This hypothesis was confirmed by analysis of the water-soluble [<sup>33</sup>P]-labeled products (see Experimental Procedures) which revealed that almost 90% corresponded to [<sup>33</sup>P]-inorganic phosphate and about 10% corresponded to [<sup>33</sup>P]-glycerol phosphate (Table I). Finally, the initial rate of appearance of water-soluble [<sup>33</sup>P]-labeled products (25.3±2.4 nmoles/h/mg of protein) was very close to the initial rate of disappearance of non-labeled 1-oleoyl-LPA (22.7±8.0 nmoles/h/mg protein).

Above results showed that LPA-phosphatase activity was predominantly (about 90% of total hydrolysis) involved in hydrolysis of exogenous LPA by 3T3F442A preadipocytes.

*Preadipocyte Ectophosphatase Activity belongs to the PAP-2/LPP Family—*

According to the literature, LPA can be dephosphorylated by two classes of phosphatases so called: phosphatidic acid phosphatase (PAP)-1 and -2. PAP-1 is an intracellular enzyme sensitive to magnesium, sulfhydryl-reactive reagent such as NEM. PAP-2, also called lysophospholipid phosphatase (LPP), are ecto-enzymes that, conversely to PAP-1, are insensitive to magnesium and NEM (11,13). As shown in Table II, 3T3F443A preadipocyte LPA-phosphatase activity was insensitive to magnesium and NEM. In addition, preadipocyte LPA-phosphatase was not sensitive to *para* nitro phenyl phosphate, or glycerol phosphate showing that it cannot correspond to an alkaline phosphatase (result not shown). These results suggested that preadipocyte LPA-phosphatase activity could be classified in the PAP-2/LPP family. Previous report showed that LPP-1 was sensitive to  $\text{Ca}^{++}$  (21). In 3T3F442A preadipocytes, neither  $\text{Ca}^{++}$  nor EDTA were able to significantly modify ecto-LPA phosphatase activity (Table II). In parallel to [ $^{33}\text{P}$ ]-LPA, preadipocytes were also able to hydrolyze [ $^{33}\text{P}$ ]-sphingosine-1-phosphate, and to a much lower extend [ $^{33}\text{P}$ ]-phosphatidic acid (Fig. 4). This result was in agreement with previous reports showing that several phospholipids can be hydrolyzed by ecto-phosphatases (30,31).

*RT-PCR Analysis of LPP mRNA—* At least three genes encoding LPP isoenzymes (LPP-1, -2, and -3) have been identified in human (14,17). The mouse homologue of LPP-1 and LPP-2 have been reported (12,18). A mouse sequence exhibiting 88% identity with human LPP3 was found in GenBank (accession number AK011276) and we hypothesized that it corresponded to mouse LPP-3. This expression of LPP isoenzyme mRNA in mouse 3T3F442A preadipocytes was successively evaluated by non-quantitative and quantitative RT-PCR. Non-quantitative RT-PCR analysis (see

Experimental procedures) of total RNA extracted from 3T3F442A preadipocytes revealed the presence LPP1, 2, and 3 mRNAs (Fig. 5). In order to determine the relative proportion of each LPP subtype mRNA, quantitative real time RT-PCR was performed (see Experimental procedures). This analysis revealed a predominant expression of LPP-1 and LPP-3 mRNAs, and a weaker expression of LPP-2 mRNAs (see Table III).

*Down-regulation of LPA-phosphatase Activity and Expression in Adipocytes*—When cultured in appropriate conditions (see Experimental Procedure), confluent 3T3F442A preadipocytes can differentiate into adipocytes. We tested whether ecto-LPA phosphatase activity and expression could be different between preadipocytes and adipocytes. As shown in Fig. 6 and Table IV, LPA-phosphatase specific activity was 80% lower in 3T3F442A adipocytes as compared to 3T3F442A preadipocytes. Ecto-LPA phosphatase activity measured in 3T3F442A adipocytes was close to that measured in mature adipocytes isolated from mouse adipose tissue (Table IV). In parallel, the kinetic of disappearance of non-labeled 1-oleoyl-LPA was much slower in adipocytes than in preadipocytes (Fig. 6), with a 88% reduction in the initial rate of disappearance. In parallel, the biochemical characteristics of adipocyte ecto-LPA phosphatase (sensitivity to vanadate, insensitivity to magnesium, NEM, EDTA and  $\text{Ca}^{++}$ ) were not significantly altered when comparing with preadipocytes (data not shown).

By using quantitative real time RT-PCR, LPP-1, LPP-2, and LPP-3 mRNA appeared less abundant (57%, 75%, and 72% respectively) in 3T3F442A adipocytes as compared with 3T3F442A preadipocytes. LPP-1 and LPP-2 mRNA levels determined in 3T3F442A adipocytes were close to that determined in mature adipocytes isolated

from mouse adipose tissue (Table III). LPP3 mRNA level was higher in mature adipocytes isolated from mouse adipose tissue than in 3T3F442A adipocytes (Table III).

These results revealed that differentiation of preadipocytes into adipocytes was associated with a strong down-regulation of both ecto-LPA-phosphatase activity and LPP gene expression.

*Influence of LPA-phosphatase Activity on Extracellular Production of LPA by Adipocytes*—Our laboratory has previously demonstrated the existence of an extracellular production of LPA by adipocytes (3). Despite the lower ecto-LPA phosphatase activity in adipocytes compared to preadipocytes, the activity was still significant. We therefore tested the influence of adipocyte ecto-LPA phosphatase on LPA production. As shown in Fig. 7, 18 h incubation of 3T3F442A adipocytes led to a significant release of LPA in the incubation medium (serum free DMEM supplemented with 1% fatty acid-free BSA). Treatment of the adipocytes with 100  $\mu$ M sodium vanadate between the 17<sup>th</sup> and the 18<sup>th</sup> hours of incubation, led to an 8-fold increase in LPA release. This result suggested that ecto-LPA phosphatase activity plays a crucial role in regulation of extracellular production of LPA by adipocytes.

## DISCUSSION

The present study shows that preadipocytes possess an ecto-LPA phosphatase activity belonging to the lipid-phosphate phosphohydrolase (LPP) family, which is predominantly involved in overall hydrolysis of exogenous LPA by preadipocytes. The present study also shows that LPP expression and activity are down-regulated after differentiation of preadipocytes into adipocytes, and that its inhibition in adipocytes increases extracellular production of LPA by these cells.

Our results show that most (about 90%) of exogenous [<sup>33</sup>P]-LPA hydrolysis by intact preadipocytes leads to the formation of water-soluble [<sup>33</sup>P]-inorganic phosphate. In addition, [<sup>33</sup>P]LPA hydrolysis by preadipocytes appears to result from a membrane-bound enzyme activity. These observations strongly suggested that the major part of exogenous LPA hydrolysis corresponds to its dephosphorylation, and that this reaction takes place mainly at the extracellular face of plasma membrane by an ecto-phosphatase. It is noticeable that about 10% of [<sup>33</sup>P]LPA hydrolysis leads [<sup>33</sup>P]glycerol phosphate. This result indicates that a minor proportion of LPA hydrolysis could result from lysophospholipase activity.

Preadipocyte LPA-phosphatase activity shares biochemical characteristics (insensitivity to magnesium and NEM) with the lipid phosphate phosphohydrolases (LPP) which are ecto-phosphatases able to dephosphorylate exogenous LPA. LPP are also able to hydrolyze other exogenous bioactive lipids such as LPA, sphingosine-1-phosphate, ceramide-phosphate, and phosphatidic acid (30,31), as we also observed in intact preadipocytes.

The LPP family is composed of at least three members: LPP-1, LPP-2, and LPP-3 (11,13). We found that LPP-1, -2 and -3 mRNA are present in preadipocytes. Since

LPP protein levels were not determined in our study, we cannot conclude about the relative involvement of each subtype in preadipocyte LPP-activity. Nevertheless we found that LPP activity in preadipocytes is not calcium sensitive. Previous report showed that LPP-1 subtype is inhibited by calcium (20,21), so we propose that LPP-1 is likely not involved in preadipocyte ecto-LPA phosphatase activity. Further investigations will be necessary to determine the relative contribution of LPP2 and LPP3.

Based upon the literature LPPs are present in numerous cell types (14), but their relative contribution in overall hydrolysis of exogenous LPA has never clearly been evaluated. By using a quantitative radioenzymatic assay of LPA, we have observed that the rate of disappearance of exogenous non-labeled LPA from preadipocyte culture medium was very close to the one measured with [<sup>33</sup>P]-LPA. These data show that LPP-activity is almost exclusively involved in overall hydrolysis of exogenous LPA by preadipocytes.

What could be the functional consequences of the presence of the LPP-activity in preadipocytes? We previously showed that LPA is able to activate preadipocyte proliferation, a biological response predominantly mediated by LPA-1/EDG-2 receptor (4). LPP-activity being mainly involved in LPA hydrolysis by preadipocytes, it very likely exerts an inhibitory effect on the proliferative activity of exogenous LPA as the result of tonic inactivation of the bioactive phospholipid. This hypothesis is in complete agreement with previous reports showing that over-expression of LPP decreased LPA effects in different cell types (19-21,32).

Another important finding of the present study is the down-regulation of extracellular LPA-phosphatase activity after differentiation of preadipocytes into adipocytes. This down-regulation is paralleled by a down-regulation of LPP-1, -2, and -3 mRNA levels,

reinforcing the hypothesis that LPPs could be involved in extracellular LPA-phosphatase activity. Adipocyte differentiation corresponds to the conversion of proliferating preadipocytes into quiescent adipocytes. This conversion is associated with the emergence of adipocyte specific genes through a coordinate program of transcription. Further investigations will be necessary to identify the factors and the mechanisms (transcription or mRNA stability) involved in the differentiation-dependent down-regulation of LPP-gene expression.

Despite its down-regulation, LPP-activity is still present in adipocytes. Adipocytes are quiescent cells, which have lost their capacity to proliferate in response to growth factors such as LPA. Moreover, adipocyte differentiation is accompanied by a strong down-regulation of LPA receptors (4) suggesting that adipocytes are poor targets for LPA. In this condition, the role of LPP-activity in adipocytes is questionable. Our group have demonstrated that adipocyte is able to release LPA in its incubation medium (3), as the result of hydrolysis of lysophosphatidylcholine by a secreted lysophospholipase D<sup>1</sup>. Here we observed that inhibition of LPP-activity by sodium vanadate increases extracellular production of LPA by adipocytes. This suggests that LPP-activity exerts a tonic inhibitory effect on the extracellular production of LPA by adipocytes. Taking into account the fact that sodium vanadate is not specific of LPP, and could act on many other targets, our hypothesis needs to be tested by another approach such as LPP-gene invalidation. Nevertheless, this is, to our knowledge, the first demonstration of the contribution of LPP in the control of extracellular production of LPA.

In conclusion, hydrolysis of extracellular LPA by preadipocytes and adipocytes mainly results from an ecto-LPA-phosphatase. This activity shares similar biochemical characteristics with the LPP, and plays an important role in the control of

both the biological activity and the extracellular production of LPA in adipose tissue. Ecto-LPA-phosphatase activity therefore constitutes a potential pharmacological and/or genic target to control the development of this tissue.

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## Footnotes

<sup>1</sup> S. Gesta, M. F. Simon, D. Sibrac, A. Girard, M. Lafontan, P. Valet, J. S. Saulnier-Blache. Secretion of a lysophospholipase D activity by adipocytes: involvement in lysophosphatidic acid synthesis. *J. Lipid Res* (in press).

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## FIGURE LEGENDS

**FIG. 1. Time-dependent hydrolysis of extracellular LPA by intact 3T3F442A preadipocytes.** Mouse 3T3F442A preadipocytes were exposed to 5  $\mu\text{M}$  LPA. The concentration of LPA in the culture media was followed using a radioenzymatic assay (see Experimental Procedures). Results represent means  $\pm$  sem from three separated experiments.

**FIG. 2. Time dependent hydrolysis of extracellular [ $^{33}\text{P}$ ]-labeled LPA by intact 3T3F442A preadipocytes.** Cells were incubated in the presence of 5  $\mu\text{M}$  [ $^{33}\text{P}$ ]-labeled LPA added in conditioned medium (see Experimental Procedures). At the indicated time, the supernatant was removed, lipid extracted using butanol. Water soluble [ $^{33}\text{P}$ ] compounds (dotted line) and remaining LPA in butanol (full line) were determined. Results represent means  $\pm$  sem from three separated experiments.

**FIG. 3. Inhibition of 3T3F442A preadipocyte phosphatase activity by vanadate or pervanadate ions.** Preadipocytes were incubated for 5 min at 37°C in the presence of various concentration of vanadate black squares or pervanadate white squares. Pervanadate was obtained by mixing 200  $\mu\text{l}$  12 mM vanadate and 3  $\mu\text{l}$  3%  $\text{H}_2\text{O}_2$  as stock solution. Then 5  $\mu\text{M}$  [ $^{33}\text{P}$ ]-LPA were added for 10 min and water soluble [ $^{33}\text{P}$ ] was determined. Results represent means  $\pm$  sem from three separated experiments

**FIG. 4. Substrate specificity of 3T3F442A preadipocyte ectophosphatase activity.** 5  $\mu\text{M}$  [ $^{33}\text{P}$ ]-substrates were dispersed in 0.1 mg/ml BSA and incubated for 30 min in the presence of intact preadipocytes as described for LPA. Water soluble

[<sup>33</sup>P] was determined. Results represent means ± sem from three separated experiments.

**FIG. 5. Non quantitative RT-PCR analysis of LPP subtype mRNA expression in preadipocytes.** Total RNA from 3T3F442A were reverse-transcribed and amplified by PCR using specific primers designed from mouse LPP-1, 2-, and 3 cDNA sequences. Amplification products were separated on agarose gel and stained with ethidium bromide. A 100pb ladder is shown.

**FIG. 6. Hydrolysis of extracellular LPA by intact preadipocytes and adipocytes.** Preadipocytes (circle) and adipocytes (square) were obtained from the mouse 3T3F442A cell line as described in Experimental Procedure, and exposed to 5 μM LPA. The concentration of LPA in the culture media was followed using a radioenzymatic assay (see Experimental Procedures). Results represent means ± sem from three separated experiments.

**FIG. 7. Influence of a phosphatase inhibitor of LPA production by adipocytes.** 3T3F442A adipocytes were obtained after 10 days of differentiation and maintained for 18 h in a serum-free media supplemented with albumin in the absence (control) or the presence of 100 μM vanadate during the last hour of incubation. At the end of the incubation period, the concentration of LPA present in the incubation medium was determined by using a radioenzymatic assay (see Experimental Procedures). Results represent means ± sem from four different determinations.

## TABLES

**Table I**

*Analysis of radioactivity repartition after [<sup>33</sup>P]-LPA contact with 3T3F442A preadipocyte.* Preadipocytes were incubated for 10 min at 37°C in the presence of 5 μM [<sup>33</sup>P]-LPA, then radioactivity remaining on cell surface or remaining in conditioned medium were analysed. water soluble [<sup>33</sup>P] compounds were separated as described in Experimental Procedures. Results represent means ± sem from three separated experiments.

<b>RADIOACTIVITY REPARTITION (%)</b>		
Butanol (LPA)	41.8 ± 1.3	
Aqueous phase	53.6 ± 1.4	
Pi		88.1 ± 1.2
Glycerol phosphate		11.9 ± 1.2
Cell	4.6 ± 0.6	

**Table II**

*Characterisation of 3T3F442A preadipocyte ecto-phosphatase activity.* Preadipocytes in HEPES-buffer described in Experimental Procedure but lacking  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , were incubated for 5 min at 37°C in the presence of the various agents before adding 5  $\mu\text{M}$  [ $^{33}\text{P}$ ]-LPA for 10 min, then water soluble [ $^{33}\text{P}$ ] was measured and enzyme specific activity determined. Results represent means  $\pm$  sem from three separated experiments.

	<b>Specific Activity</b> <b>nmoles / min / mg of protein</b>
Control	0.623 $\pm$ 0.049
$\text{Mg}^{++}$ 2 mM	0.669 $\pm$ 0.046
NEM 2 mM	0.805 $\pm$ 0.071
$\text{Ca}^{++}$ 1 mM	0.800 $\pm$ 0.078
EDTA 0.5 mM	0.683 $\pm$ 0.047

**Table III**

*Ecto-phosphatases mRNA expression in 3T3F442A preadipocytes and adipocytes, and mouse perigonadic isolated adipocytes..* Cells and mRNA extract were obtained as described in Experimental Procedures. mRNA levels of the known ecto-phosphatases were assessed by real-time quantitative RT-PCR as described in Experimental Procedures. The expression of each gene was quantified and normalised using simultaneous amplification of known quantities of the corresponding cDNA and determination of the concentration of 18 S in each sample. Results represent means  $\pm$  sem from four different RNA preparations.

mRNA $10^{-7}$ ng / 25 ng RNA			
3T3F442A			
	Preadipocyte	Adipocyte	Perigonadic mouse tissue
LPP1	28 $\pm$ 5	12 $\pm$ 3	12 $\pm$ 1
LPP2	8 $\pm$ 1	2 $\pm$ 1	0.5 $\pm$ 0.1
LPP3	37 $\pm$ 8	10 $\pm$ 3	36 $\pm$ 4

#### Table IV

*Ecto-phosphatase specific activity determined on intact 3T3F442A preadipocytes and adipocytes or mouse perigonadic isolated adipocytes. Cells obtained as described in Experimental Procedures were incubated in the presence of 5  $\mu$ M [ $^{33}$ P]-LPA for 10 min, then water soluble [ $^{33}$ P] was measured and enzyme specific activity determined. Results represent means  $\pm$  sem from three separated experiments.*

	<b>Specific Activity</b> <b>nmoles / min / mg of protein</b>
<b>3T3F442A</b>	
<b>Preadipocyte</b>	0.863 $\pm$ 0.161
<b>Adipocyte</b>	0.178 $\pm$ 0.005
<b>Perigonadic adipose tissue</b>	0.263 $\pm$ 0.017

Fig 1

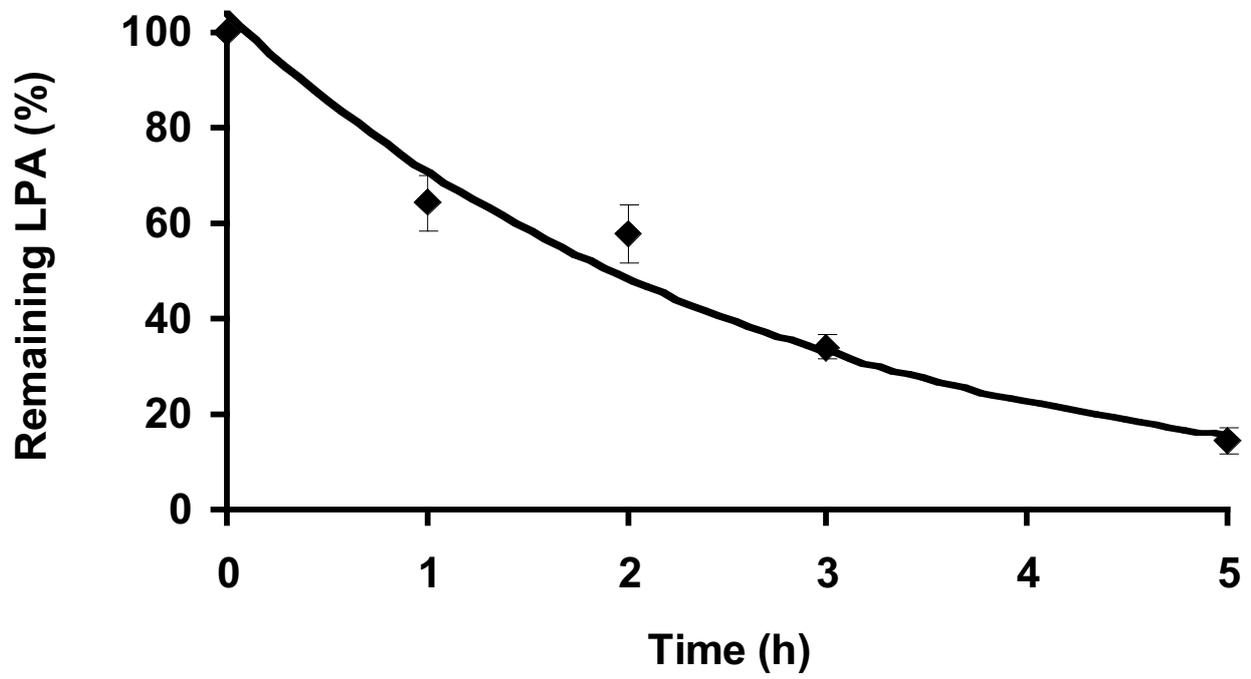


Fig 2

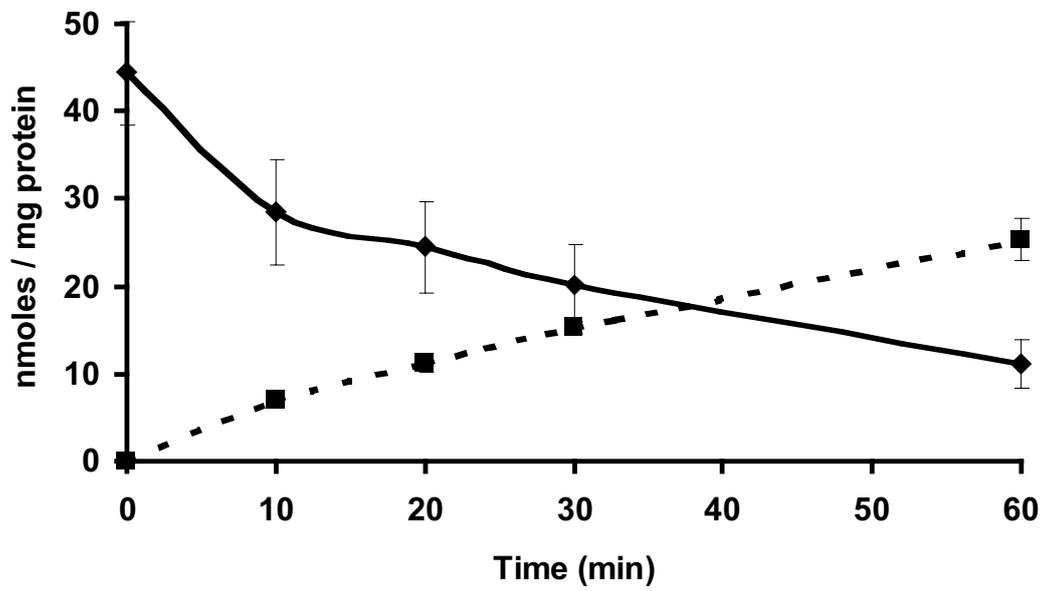


Fig 3

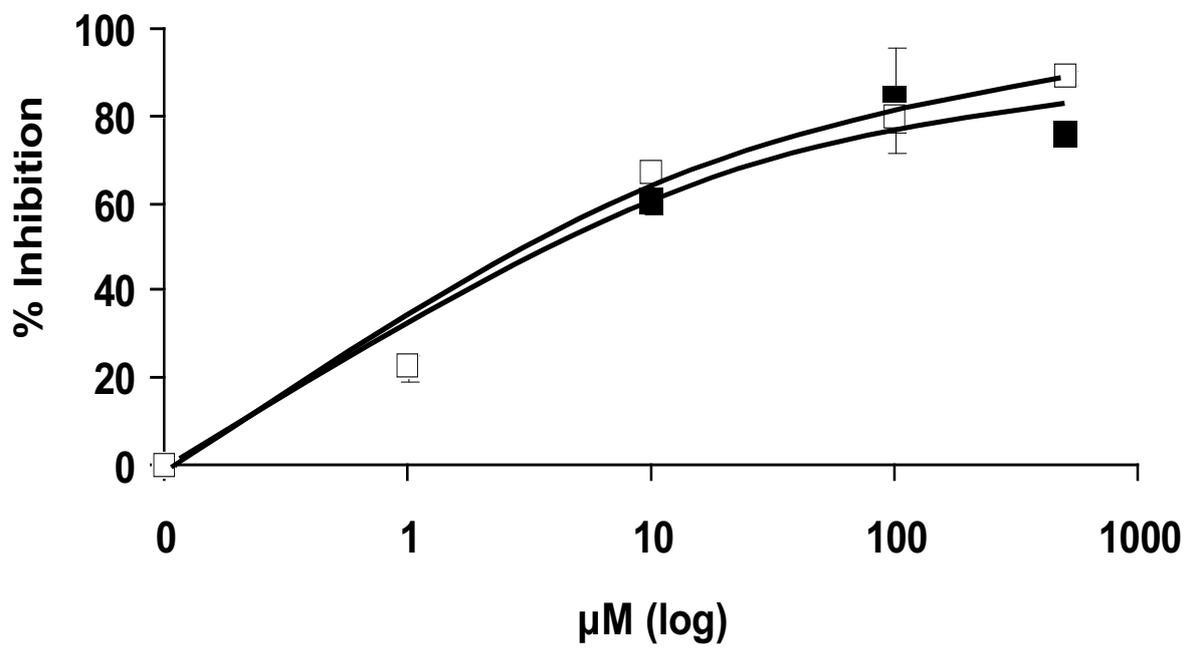


Fig 4

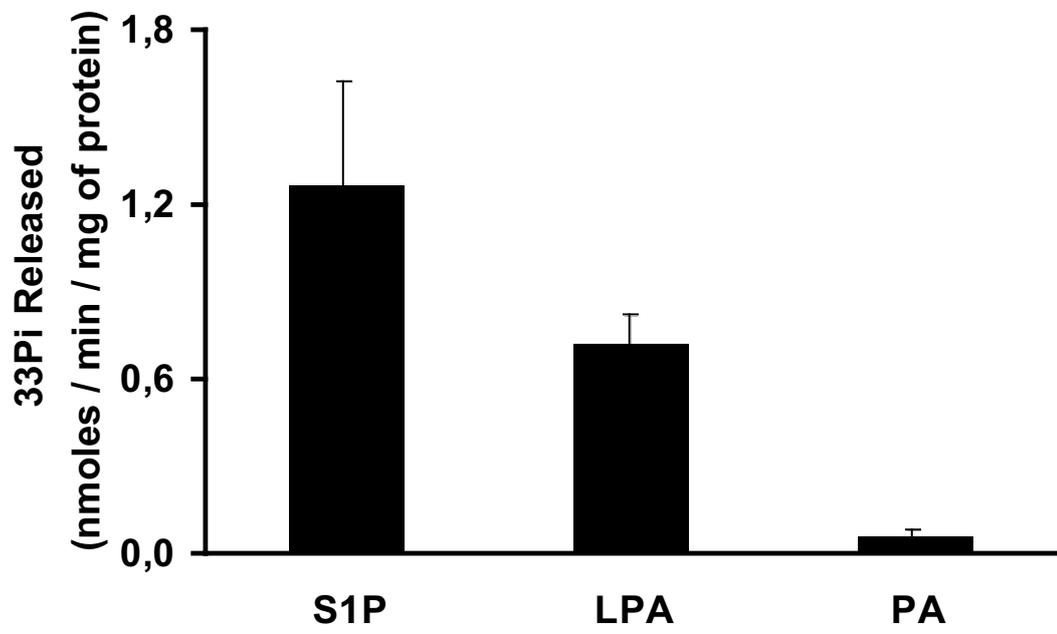


Fig 5

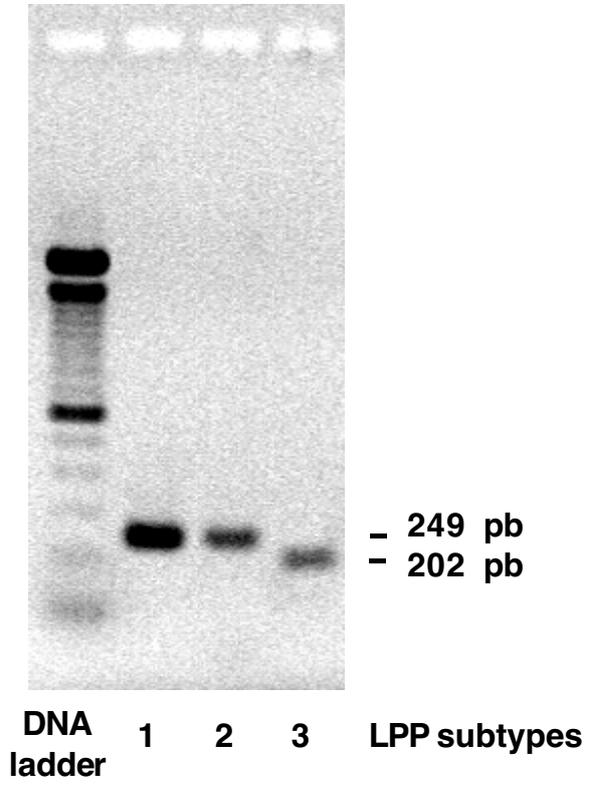


Fig 6

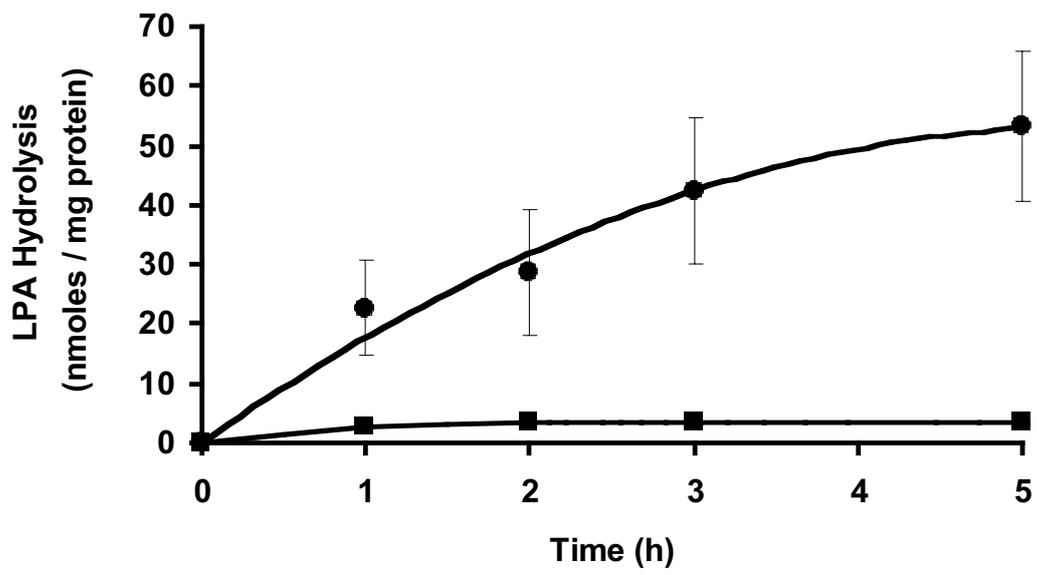


Fig 6

