Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. A paracrine signal for preadipocyte growth.

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Alpha$_2$-adrenergic Receptor–mediated Release of Lysophosphatidic Acid by Adipocytes

A Paracrine Signal for Preadipocyte Growth

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Abstract

In the search for the existence of adrenergic regulation of the autocrine/paracrine function of the white adipose tissue, it was observed that conditioned media from isolated adipocytes or dialysates obtained by in situ microdialysis of human subcutaneous adipose tissue increased spreading and proliferation of 3T3F442A preadipocytes. These effects were amplified when an alpha$_2$-adrenergic agonist was present during the obtention of conditioned media and microdialysates. This alpha$_2$-adrenergic–dependent trophic activity was completely abolished by pretreatment of the conditioned media or microdialysates with the lysophospholipase, phospholipase B. Among the different lysophospholipids tested only lysophosphatidic acid (LPA) was able to induce spreading and proliferation of 3T3F442A preadipocytes. Moreover, previous chronic treatment of 3T3F442A preadipocytes with LPA which led to a specific desensitization of LPA responsiveness, abolished the alpha$_2$-adrenergic–dependent trophic activities of the conditioned media and microdialysates. Finally, alpha$_2$-adrenergic stimulation led to a rapid, sustained, and pertussis toxin–dependent release of [^{32}P]LPA from [^{32}P]-labeled adipocytes. Based upon these results it was proposed that in vitro and in situ stimulation of adipocyte alpha$_2$-adrenergic receptors provokes the extracellular release of LPA leading, in turn, to regulation of preadipocyte growth. (*J. Clin. Invest.* 1998. 101:1431–1438.) Key words: human adipose tissue • microdialysis • alpha$_2$-adrenergic receptor agonists • lysophospholipids • phospholipases

Introduction

Adipose tissue development results from both adipocyte enlargement resulting from triglyceride accumulation, and recruitment of new fat cells resulting from proliferation and/or differentiation of adipocyte precursors (preadipocytes) present in the fat deposit. Formation of new fat cells from preadipocytes can take place throughout life in both rodents and humans where dormant precursor cells can be isolated in very old people of both sexes (1–3). Determining the identity of the factors regulating the formation of new fat cells is a major point in understanding normal and pathologic growth of adipose tissue, but is far from being completely clarified. Circulating hormones, growth factors, and cytokines, such as insulin, IGF-I, growth hormone, glucocorticoids, and thyroid hormone are known to be positive effectors that promote proliferation and/or differentiation of preadipocytes (1, 2). In addition, the production of paracrine and autocrine factors within adipose tissue could also play an important role in its development. Indeed, adipocytes are able to release several peptidergic factors (TNF-α, adipsin, angiotensinogen, or leptin), and also lipidic factors (fatty acids, prostaglandins) which are all involved directly or indirectly in preadipocyte growth or differentiation (2, 4, 5).

Lysophosphatidic acid (LPA)[1] (1-acyl-*sn*-glycerol-3-phosphate) has emerged as a potent and pleiotropic bioactive phospholipid known to regulate, through a recently described plasma membrane receptor, a number of cellular events such as platelet aggregation, actin cytoskeleton activation, fibroblast proliferation, and neurite retraction. LPA, which is relatively abundant in plasma in an albumin-bound form, was proposed to originate from thrombin-induced platelet aggregation as the result of a phospholipase A$_2$–catalyzed deacylation of phosphatidic acid (6, 7).

Alpha$_2$-adrenergic receptors are highly expressed in adipocytes, particularly human adipocytes, and play an important role in the adrenergic control of lipolysis (8). Because these receptors have been shown to promote phospholipase A$_2$ activation in platelets (9) and Chinese hamster ovary cells (10), we postulate the involvement of alpha$_2$-adrenergic receptors in LPA release from adipocytes.

In this study, we show that alpha$_2$-adrenergic stimulation promotes LPA release from adipocytes and that this production can influence the reorganization of actin cytoskeleton (characterized by cell spreading) and the proliferation of the 3T3F442A preadipose cell line. Moreover, an LPA-like activity was also found in dialysates from human adipose tissue stimulated by an alpha$_2$-adrenergic agonist.

1. *Abbreviations used in this paper:* CMC, control conditioned medium; CMU, conditioned medium prepared in the presence of UK14304; CMU+RX, conditioned medium prepared in the presence of UK14304 plus RX821002; LPA, lysophosphatidic acid; LPAd, lysophosphatidic acid desensitization.

Methods

Chemicals. L-α-LPA acid oleoyl, L-α-lysophosphatidylcholine oleoyl, L-α-lysophosphatidylethanolamine oleoyl, L-α-lysophosphatidylinositol, L-α-lysophosphatidyl-L-serine palmitoyl, platelet activating factor, prostaglandin E_2, sphingosine 1-phosphate, epinephrine, isoproterenol, pertussis toxin, and phospholipase B were obtained from Sigma Chemical Co. (Poole, UK). [^{3}H]RX821002 was from NEN (Boston, MA). [^{32}P]H$_3$PO$_4$ was from ICN (High Wycomb, UK). UK14304 was from Pfizer (Sandwich, UK). Clonidine was from Boehringer Ingelheim (Mannheim, Germany).

Isolation of human adipocytes. Human subcutaneous adipocytes were obtained from healthy, drug-free women having a body mass index (kg/m^2) of 26±2 and undergoing plastic surgery. Adipose tissue was carefully dissected out and adipocytes were isolated using collagenase as described previously (11). Isolated adipocytes were washed three times in Krebs-Ringer bicarbonate buffer and used for preparation of conditioned media and for [^{32}P]-labeling. Based on binding assays using [^{3}H]RX821002 as a radioligand (12) the density of alpha$_2$-adrenergic receptors expressed in the human adipocytes used in this study was 620±60 fmol/mg protein.

Cell lines. Two different preadipose cell lines were used in this study: the wild-type 3T3F442A preadipose cell line which exhibits no endogenous expression of alpha$_2$-adrenergic receptors (13), and the α$_2$AF2 preadipose cell line which is a 3T3F442A-derived cell line stably expressing the human alpha$_2$A-adrenergic receptor gene (13). Based on binding assay using [^{3}H]RX821002 as a radioligand (12) the density of alpha$_2$-adrenergic receptors expressed in the α$_2$AF2 cell line used in this study was 2,227±687 fmol/mg protein. Wild-type 3T3F442A and α$_2$AF2 preadipocytes were grown at 37°C in a humidified atmosphere containing 7% CO$_2$ in DME supplemented with 10% donor calf serum as reported previously (13). Differentiation of preadipocytes into adipocytes was achieved by cultivating confluent α$_2$AF2 preadipocytes in DME supplemented with 10% FCS plus 50 nM insulin as reported previously (14).

Preparation of the conditioned media. Isolated human adipocytes or 5-wk–differentiated α$_2$AF2 adipocytes were washed twice with PBS supplemented with 1% BSA (fraction V; Sigma Chemical Co.) and incubated at 37°C in the absence (control conditioned medium; CMC) or the presence of 1 μM of the selective alpha$_2$-adrenergic agonist UK14304 (conditioned medium prepared in the presence of UK14304) (CMU) in Krebs-Ringer buffer containing 10 mM Hepes and 1% albumin for human adipocytes, or DME supplemented with 1% albumin for α$_2$AF2 adipocytes. After incubation (16 h for human adipocytes and 48 h for α$_2$AF2 adipocytes), conditioned media were collected, centrifuged (5,000 g, 10 min, 4°C) to eliminate cell debris, treated or not treated with 3 U/ml of Vibrio phospholipase B (EC 3.1.1.5; Sigma Chemical Co.), and tested for their trophic activity on 3T3F442A preadipocytes.

In situ microdialysis in human subcutaneous adipose tissue. After having obtained the agreement of the ethical committee of the hospital, four healthy, adult volunteers (mean body mass index of 23±1 kg/m^2), participated in the experiments. After an overnight fast, in situ adipose tissue microdialysis was performed as described previously (15). A microdialysis probe was implanted in the subcutaneous adipose tissue and perfused at 2 μl/min with a sterile Ringer's solution containing 30 g/liter of albumin. After an equilibration period of 30 min, a 60-min fraction of the outgoing dialysate was collected to obtain control dialysate. Then the perfusate was supplemented with 10 μM clonidine (the only alpha$_2$-adrenergic agonist available for clinical investigation) and an additional 60-min fraction of outgoing dialysate was collected to obtain stimulated dialysate. Collected dialysates were treated or not treated with 3 U/ml of phospholipase B, and tested for their activity on 3T3F442A preadipocyte spreading.

Measurement of 3T3F442A preadipocyte spreading and proliferation. The trophic activity of conditioned media and dialysates was measured by their ability to generate short-term cytoskeleton activation and long-term proliferation of 3T3F442A preadipocytes. Cell spreading, which was used as an index of actin cytoskeleton activation, was measured on 3T3F442A preadipocytes preretracted as described previously (16). Preconfluent 3T3F442A preadipocytes were washed twice with PBS and replaced in DME without serum for 30–60 min to induce cell retraction which is characterized by an increase in cell body refringency. The spreading activity of the conditioned media or of the microdialysis perfusates was measured by determination of the proportion of refrigent cells after 20 min of exposure. Proliferation was measured by cell number determination after 48 h of exposure of retracted 3T3F442A preadipocytes to the conditioned media.

Analysis of labeled phospholipids released by adipocytes. After washing with PBS, human and α$_2$AF2 adipocytes were incubated at 37°C in a phosphate-deprived Hepes buffer (118 mM NaCl, 6 mM KCl, 1 mM CaCl$_2$, 1.2 mM MgSO$_4$, 12.4 mM Hepes, and 6 mM glucose, pH 7.4) containing 100 μCi/ml [^{32}P]H$_3$PO$_4$. After 90 min of incubation, adipocytes were washed three times with the Hepes buffer to eliminate excess free [^{32}P]H$_3$PO$_4$ and incubated at 37°C with various pharmacological compounds and for various periods of time in Hepes buffer containing 3.5% BSA. In the case of pertussis toxin treatment, α$_2$AF2 adipocytes were exposed to the toxin (100 ng/ml) 18 h before the experiment.

Lipids present in the extracellular medium were extracted with 2 vol of chloroform/methanol/12 M HCl (20:20:0.13) according to Fourcade et al. (17). After phase separation, the organic layer was dried under nitrogen and dissolved in chloroform/methanol (1:1). Lipids were then separated by two-dimensional TLC using chloroform/methanol/28% ammonia (65:25:5) for the first dimension and chloroform/methanol/acetic acid/water (45:20:5:0.5) for the second dimension. Labeled lipids were detected and quantified using a phosphorImager (Image Quant software; Molecular Dynamics, Sunnyvale, CA) and depicted in arbitrary units. The identity of each [^{32}P]-labeled phospholipid was achieved by comigration with cold internal standards revealed with iodine vapors.

Results

Production of phospholipase B–sensitive trophic factor(s) by alpha$_2$-adrenergic–stimulated adipocytes. Conditioned media were prepared either in the absence (CMC) or in the presence of 1 μM of the selective alpha$_2$-adrenergic agonist UK14304 (CMU) or in the presence of UK14304 plus 10 μM of the selective alpha$_2$-adrenergic antagonist RX821002 (conditioned medium prepared in the presence of UK14304+RX821002) (CMU+RX). Two different types of alpha$_2$-adrenergic receptor-expressing adipocytes were used for preparation of the conditioned medium: adipocytes isolated from human subcutaneous adipose tissue, endogenously expressing α$_2$A-adrenergic receptors (13); or α$_2$AF2 adipocytes (a 3T3F442A-derived cell line stably transfected with the human α$_2$A-adrenergic receptor gene [13]).

Short-term and long-term trophic activities of the conditioned media were tested on undifferentiated 3T3F442A preadipocytes. This cell line was shown to be refractory to the direct action of alpha$_2$-adrenergic agonists due to the absence of an endogenous expression of alpha$_2$-adrenergic receptors (13). Therefore, a direct trophic activity of the alpha$_2$-adrenergic agonists used for the preparation of the conditioned medium could be avoided. The so-called short-term trophic activity of the conditioned medium corresponded to their ability to generate cell spreading as the result of a reorganization of actin cytoskeleton (16). The so-called long-term trophic activity of the conditioned media corresponded to their ability to increase cell number in the absence of other growth factors (13).
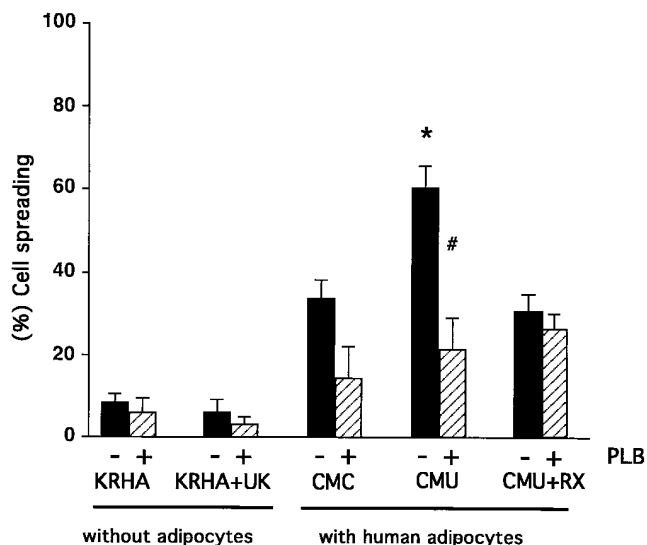
Figure 1. Induction of 3T3F442A-preadipocyte spreading by conditioned media from alpha$_2$-adrenergic–stimulated human adipocytes. Conditioned media were prepared (see Methods) from isolated human adipocytes incubated in Krebs-Ringer albumin buffer (*KRHA*) for 16 h at 37°C in the absence (*CMC*) or the presence of 1 µM of UK14304 without (*CMU*) or with 10 µM RX821002 (*CMU+RX*). The spreading activity was determined (see Methods) after 20 min of exposure of retracted 3T3F442A preadipocytes to conditioned media pretreated (+) or not (−) with phospholipase B (*PLB*) (see Methods). Data represent means±SEM (*n* = 3). Statistical analysis was performed using the Student's *t* test: *P < 0.05 when comparing CMC and CMU without phospholipase B treatment; #P < 0.05 when comparing the effect of CMU with and without phospholipase B treatment.

20 min of exposure to CMC from human adipocytes induced a moderate spreading ($\sim$ 35% of the cells) of 3T3F442A preadipocytes. This spreading was significantly amplified with CMU ($\sim$ 60% of the cells) but not with CMU+RX (Fig. 1). Similar results were obtained with conditioned media prepared from α_2AF2 adipocytes (Fig. 2 *A*). In the absence of adipocytes KRHA or DME buffers supplemented or not supplemented with UK14304 (Figs. 1 and 2 *A*) exhibited no significant spreading activity. 30-min pretreatment of the conditioned media from human (Fig. 1) and α_2AF2 adipocytes (Fig. 2 *A*) with 3 U/ml phospholipase B before their addition to 3T3F442A preadipocytes, completely abolished the alpha$_2$-adrenergic–dependent spreading effect generated by CMU without affecting the effects of CMC and CMU+RX (Figs. 1 and 2 *A*).

48 h of exposure of 3T3F442A preadipocytes to CMC and CMU+RX from α_2AF2 adipocytes generated a significant but moderate increase in cell number ($\sim$ 80% > basal) which was not modified by phospholipase B treatment (Fig. 2 *B*). In contrast, CMU promoted a stronger proliferation (200% > basal), and this additional effect was suppressed by phospholipase B treatment.

These results reveal that under alpha$_2$-adrenergic stimulation, adipocytes can release phospholipase B–sensitive factor(s) exhibiting the capacity to activate both spreading and proliferation of preadipocytes.

LPA activates the reorganization of actin cytoskeleton and the proliferation in 3T3F442A preadipocytes. Because phospholipase B possesses a lysophospholipase activity (18), the trophic activity of different lysophospholipids was tested on 3T3F442A preadipocytes. 20 min of exposure of retracted 3T3F442A preadipocytes to 10 µM of lysophosphatidylinositol, lysophosphatidylcholine, lysophosphatidylserine, or lysophosphatidylethanolamine did not lead to any significant spreading effect. Conversely, 20 min of exposure to 10 µM of LPA led to a dramatic spreading effect concerning almost 100% of the cells. This effect was suppressed by pretreatment of LPA with phospholipase B (Fig. 3 *A*). Another phospholipid, sphingosine-1-phosphate, was also able to activate 3T3F442A preadipocyte spreading but its activity was not influenced by phospholipase B treatment (Fig. 3 *A*). Platelet activating factor and prostaglandin PGE$_2$ exhibited no activity (Fig. 3 *A*). 48 h of exposure of retracted 3T3F442A preadipo-
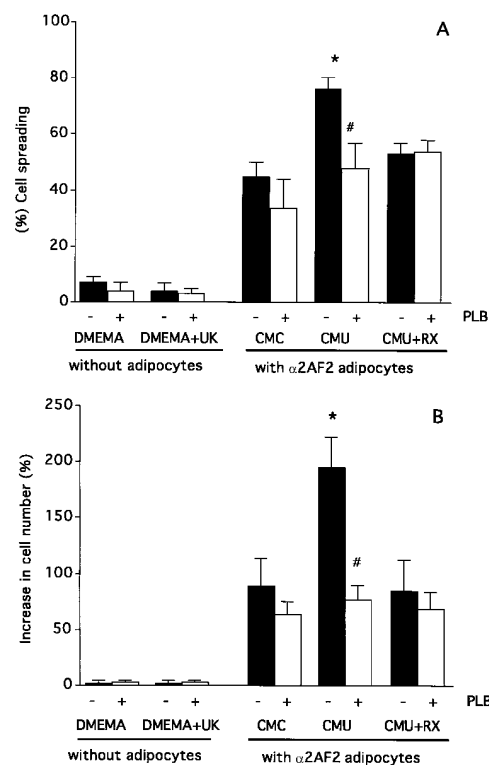

Figure 2. Activation of 3T3F442A-preadipocyte spreading (*A*) and proliferation (*B*) by conditioned media from alpha$_2$-adrenergic–stimulated α_2AF2 adipocytes. Conditioned media were prepared (see Methods) from α_2AF2 adipocytes incubated in DME containing albumin (*DMEMA*) for 48 h at 37°C in the absence (*CMC*) or the presence of 1 µM of UK14304 without (*CMU*) or with 10 µM RX821002 (*CMU+RX*). (*A*) The spreading activity was determined (see Methods) after 20 min of exposure of retracted 3T3F442A preadipocytes to conditioned media pretreated (+) or not (−) with phospholipase B (*PLB*) (see Methods). (*B*) The proliferative activity was determined after 48 h of exposure of retracted 3T3F442A preadipocytes to conditioned media pretreated or not with phospholipase B (*PLB*) (see Methods). Data represent means±SEM (*n* = 5). Statistical analysis was performed using the Student's *t* test: *P < 0.05 when comparing CMC and CMU without phospholipase B treatment; #P < 0.05 when comparing the effect of CMU with and without phospholipase B treatment.
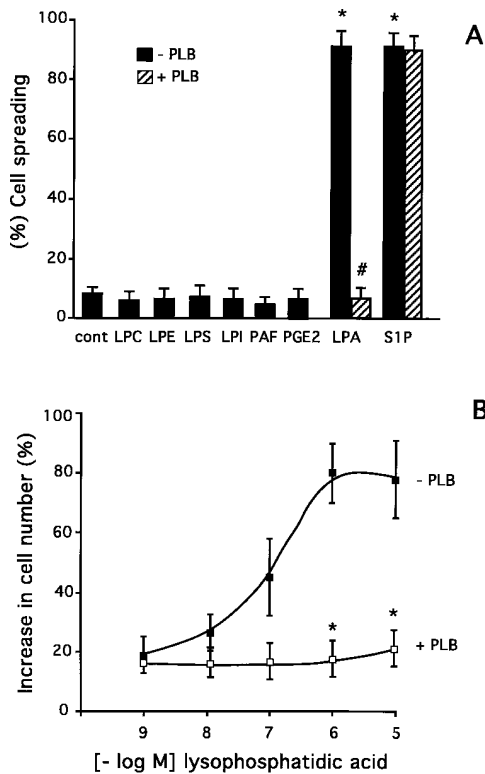
Figure 3. (*A*) Induction of 3T3F442A-preadipocyte spreading by LPA and sphingosine-1-phosphate. Spreading was determined (see Methods) after 20 min of exposure of retracted 3T3F442A preadipocytes to 10 μM of lysophosphatidylcholine (*LPC*), lysophosphatidylethanolamine (*LPE*), lysophosphatidylserine (*LPS*), lysophosphatidylinositol (*LPI*), LPA, platelet activating factor (*PAF*), prostaglandin E_2 (*PGE2*), and sphingosine-1-phosphate (*S1P*). Each compound was pretreated or not with phospholipase B (see Methods). Values are means±SEM from three separate experiments. Statistical analysis was performed using the Student's *t* test: *$P < 0.05$ when comparing with control (*cont*); #$P < 0.05$ when comparing with and with-out phospholipase B treatment. (*B*) Proliferative effect of LPA in 3T3F442A preadipocytes. Cell number was measured after 48 h of exposure of retracted 3T3F442A preadipocytes to increasing concentrations of LPA pretreated or not with phospholipase B (+ or − phospholipase B) and counted (see Methods). Data represent means±SEM from six separate experiments.

cytes to LPA also led to a significant and dose-dependent increase in cell number with an EC_{50} of 150 nM. This proliferative effect was suppressed by phospholipase B treatment (Fig. 3 *B*).

The spreading of 3T3F442A preadipocytes induced by LPA was dose-dependent with an EC_{50} of 4±1 nM (Fig. 4 *A*). Chronic pretreatment (48 h, twice a day) of 3T3F442A preadipocytes with 10 μM LPA, led to a strong attenuation of the spreading effect generated by LPA itself (12-fold increase of the EC_{50} and 36% reduction of the maximal effect) (Fig. 4 *A*). In parallel, the dose–response curve of sphingosine-1-phosphate was not significantly modified (Fig. 4 *B*). In these conditions, the spreading effect of CMU from human and α_2AF2 adipocytes was completely abolished without significant modification of the spreading effect of CMC (Fig. 5).

These results suggest that a specific LPA responsiveness of 3T3F442A preadipocytes was required to detect the alpha$_2$-adrenergic–dependent trophic activity of the conditioned media.

Production of LPA by adipocytes. The direct production of LPA by adipocytes was tested after [^{32}P]-labeling according to a method previously used in platelets (17) (Methods). After a prelabeling period, adipocytes were exposed to the alpha$_2$-adrenergic agonist UK14304 and the phospholipids present in the extracellular medium were extracted and separated by two-dimensional TLC. As shown in Fig. 6, after 5 h of incubation of human (Fig. 6 *left*) or α_2AF2 (Fig. 6 *right*) adipocytes in the absence of alpha$_2$-adrenergic agonist, the extracellular medium contained numerous [^{32}P]-labeled phospholipids such as PC, PS, PI, PE, PA, and LPC. In the presence of 1 μM UK14304 an additional spot appeared just under PS, comigrating with the cold LPA internal standard. 30 min of treatment of the extracellular medium with 3 U/ml of phospholipase B before phospholipid extraction led to a complete disappearance of the LPA spot (data not shown).

1 μM UK14304 induced a rapid (already detected after 5 min) and sustained (maintained for $\geq$ 5 h) release of [^{32}P]LPA into α_2AF2 adipocyte extracellular medium (Fig. 7 *A*). [^{32}P]LPA
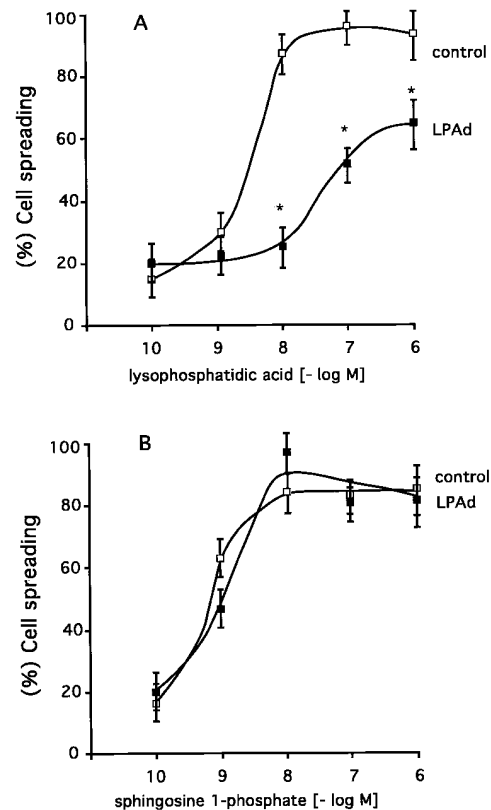

Figure 4. Desensitization of LPA-induced spreading of 3T3F442A preadipocytes. The spreading activity of increasing concentrations of LPA and sphingosine-1-phosphate was determined (see Methods) after 20 min of exposure of 3T3F442A preadipocytes which had been LPA-desensitized (*LPAd*) or not (*control*) with 10 μM LPA twice a day for 48 h before retraction. Values represent means±SEM from three different experiments. Statistical analysis was performed using Student's *t* test: *$P < 0.05$ when comparing LPAd with control.
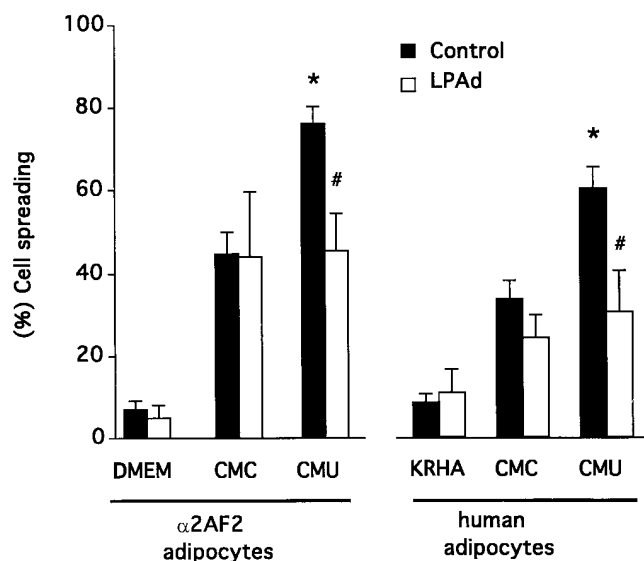
Figure 5. Suppression of the trophic activities of conditioned media by previous desensitization of 3T3F442A preadipocyte LPA receptivity. 3T3F442A preadipocytes were chronically treated (*LPAd*) or not (*Control*) with LPA before retraction, exposed for 20 min to conditioned media from α_2AF2 (*left*) or human (*right*) adipocytes prepared in the presence (*CMU*) or not (*CMC*) of UK14304 (see Methods) and spreading was measured as described in Methods. Data represent the means±SEM from five (α_2AF2 adipocytes) and three (human adipocytes) separate experiments. Statistical analysis was performed using Student's *t* test: *$P < 0.05$ when comparing with control (*DMEM* or *KRHA*); #$P < 0.05$ when comparing LPAd and control.

release promoted by 5 h of incubation with UK14304 was dose-dependent and was completely blocked by coincubation of UK14304 with 10 μM of the selective alpha$_2$-adrenergic antagonist RX821002 (Fig. 7 *B*). 18 h of pretreatment of the α_2AF2 adipocytes by 100 ng/ml of pertussis toxin completely abolished UK14304-induced [^{32}P]LPA release (Fig. 7 *C*). UK14304-mediated release of [^{32}P]LPA was also reproduced by 1 μM of the physiological agonist epinephrine or 10 μM of

the partial alpha$_2$-adrenergic agonist clonidine but not by 1 μM of the beta-adrenergic agonist isoproterenol. The effect of epinephrine was blocked by coincubation with 10 μM of RX821002 but not with 100 μM of the beta-adrenergic antagonist propranolol (Fig. 7 *C*).

These results showed that the stimulation of alpha$_2$-adrenergic receptors leads to direct release of LPA by adipocytes through a transduction pathway involving the heterotrimeric G$_i$ or G$_o$ proteins.

In situ production of LPA by human adipose tissue. To determine whether this alpha$_2$-adrenergic–dependent release of LPA could be revealed in vivo, we used in situ microdialysis, a technique allowing the collection of metabolites and other agents present in the extracellular fluid of the adipose tissue (15, 19). Microdialysis probes were implanted in human subcutaneous adipose tissue and perfused with albumin-containing Ringer's solution supplemented or not supplemented with 10 μM clonidine. Dialysates were collected and the presence of LPA was tested by a bioassay measuring their ability to generate 3T3F442A preadipocyte spreading. As shown in Fig. 8, 20 min of exposure of retracted 3T3F442A preadipocytes to the dialysates collected after 60 min of infusion with 10 μM of clonidine generated a spreading effect which was significantly stronger than that obtained with dialysate collected after the same time in the absence of clonidine. The additional effect generated by microdialysate obtained in the presence of clonidine was completely suppressed by pretreatment with 3 U/ml of phospholipase B as well as by previous desensitization of 3T3F442A preadipocyte LPA responsiveness (LPAd). Because of the limited volume of collected microdialysates it was impossible to determine their proliferative effect on 3T3F442A preadipocytes.

These results show that in situ alpha$_2$-adrenergic receptor stimulation of the human subcutaneous adipose tissue leads to the release of an LPA-like factor able to generate the activation of 3T3F442A preadipocyte actin cytoskeleton.

Discussion

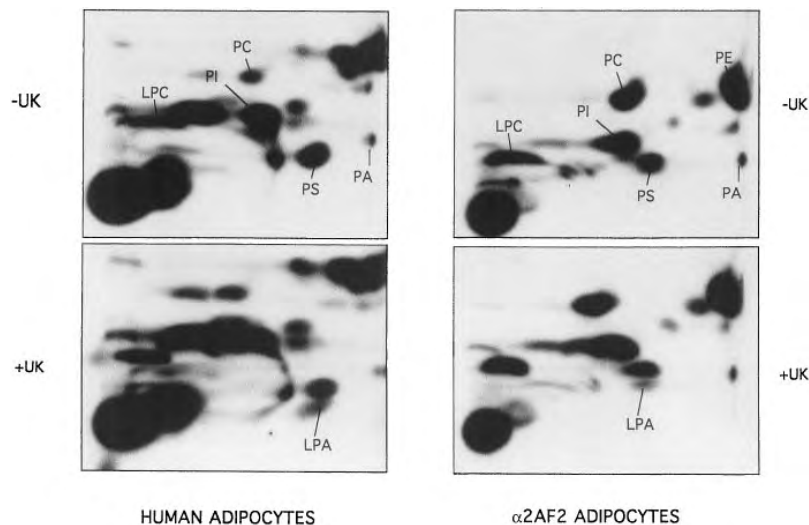In this study it was demonstrated that in vitro and in vivo exposure of adipocytes to an alpha$_2$-adrenergic stimulus led to the

HUMAN ADIPOCYTES α2AF2 ADIPOCYTES

Figure 6. Typical separation of [^{32}P]-labeled phospholipids from human and α_2AF2 adipocyte conditioned media. [^{32}P]-labeled human (*left*) and α_2AF2 (*right*) adipocytes (see Methods) were exposed (+UK) or not (−UK) to 1 μM UK14304 for 5 h and [^{32}P]-labeled phospholipids present in the extracellular medium were extracted (see Methods) and separated by two-dimensional TLC using chloroform/methanol/28% ammonia (65:25:5) for the first dimension and chloroform/methanol/acetic acid/water (45:20:5:0.5) for the second dimension. Each [^{32}P]-labeled phospholipid was identified by comigration with cold internal standards: phosphatidylcholine (*PC*), phosphatidylethanolamine (*PE*), phosphatidylinositol (*PI*), phosphatidylserine (*PS*), lysophosphatidylcholine (*LPC*), phosphatidic acid (*PA*), and LPA.
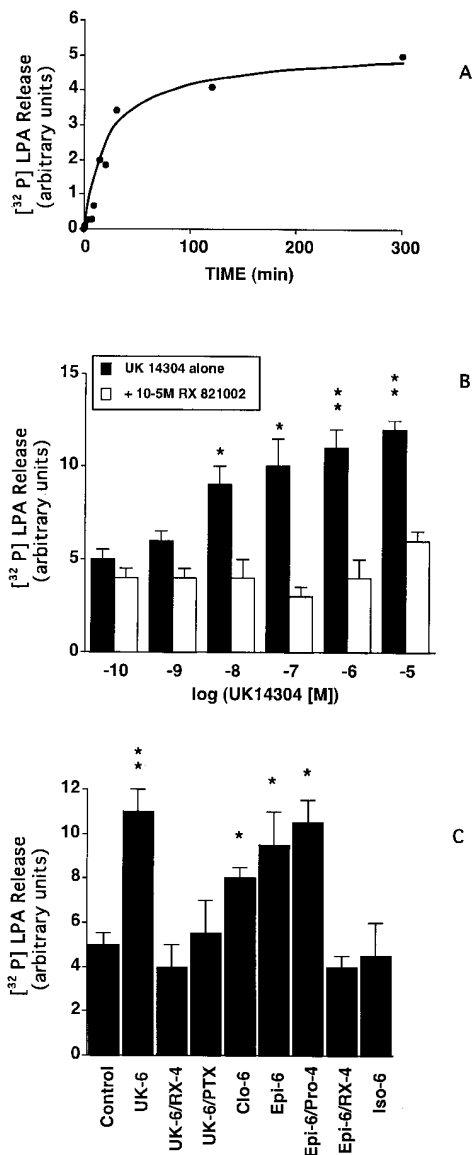
The proposal of the involvement of LPA in the alpha$_2$-adrenergic–dependent trophic activities of adipocyte extracellular media came from three sets of experiments, demonstrating: (*a*) that the spreading and proliferating activities of both conditioned media and dialysates were suppressed by phospholipase B and by agonist-induced desensitization of LPA receptivity of 3T3F442A preadipocytes; (*b*) that among various lysophospholipids, LPA was the only one able to activate spreading and proliferation of 3T3F442A preadipocytes; and (*c*) the direct alpha$_2$-adrenergic–dependent release of [^{32}P]-LPA from [^{32}P]-labeled adipocytes.

Phospholipase B is known to exhibit a strong lysophospholipase activity (18) and its use is precisely at the origin of the demonstration of the involvement of LPA in biological activities generated by serum (20, 21). Our results make it clear that LPA is the only lysophospholipid able to activate preadipocytes. The ability of phospholipase B to suppress both LPA and alpha$_2$-adrenergic–dependent effects of the conditioned media on the preadipocytes constitutes interesting but insufficient indications for the production of LPA by adipocytes. In certain conditions, phospholipase B can also exhibit partial phospholipase A activity (22). The involvement of another phospholipid, other than lysophospholipids, cannot completely be ruled out. Sphingosine-1-phosphate has been shown to be produced by some cell types (23) and to exert similar cell regulations as LPA, but through a distinct receptor (24, 25). Sphingosine-1-phosphate promotes spreading of 3T3F442A preadipocytes, but this phospholipid cannot be responsible for the alpha$_2$-adrenergic–dependent trophic activities of the conditioned media since it is not sensitive to phospholipase B.

Since the biological activity of LPA was being mediated by a G protein–coupled receptor (26–29), it was expected that its prolonged activation in 3T3F442A preadipocytes would lead

Figure 7. Production of [^{32}P]-labeled LPA in conditioned media from α$_2$AF2 adipocytes. [^{32}P]-labeled α$_2$AF2 adipocytes were exposed to various agents and for various period of time. [^{32}P]-labeled phospholipids present in the extracellular medium were extracted (see Methods) and separated by two-dimensional TLC as described in Fig. 6 and the [^{32}P]LPA spot was quantified using a PhosphorImager (see Methods). (*A*) Time course of the LPA release stimulated by 1 μM UK14304. (*B*) LPA release induced by 5 h of exposure to increasing concentrations of UK14304 with or without 10 μM RX821002 (*n* = 3). (*C*) LPA release induced by 5 h of exposure to various agents (concentration in log M): UK14304 (*UK*), RX821002 (*RX*), pertussis toxin (*PTX*) (see Methods), clonidine (*Clo*), epinephrine (*Epi*), propranolol (*Pro*), and isoproterenol (*Iso*) (*n* = 4). Values are means±SEM. Statistical analysis was performed using Student's *t* test: *P < 0.05, **P < 0.01 when comparing with control.

extracellular production of LPA. Because of its ability to regulate both actin cytoskeleton activity and proliferation of 3T3F442A preadipocytes, this bioactive phospholipid could be an important regulator in the autocrine/paracrine control of preadipocyte growth in adipose tissue.
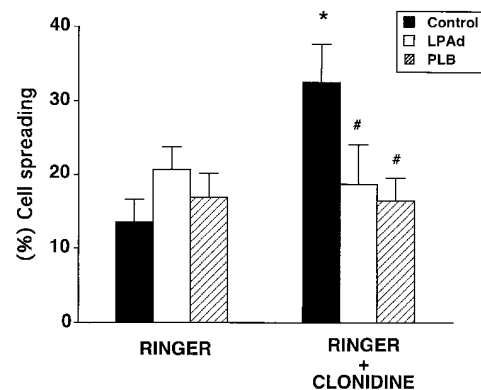

Figure 8. Induction of 3T3F442A preadipocyte spreading by microdialysates from human subcutaneous adipose tissue. Dialysates obtained after perfusion of human subcutaneous adipose tissue with a Ringer's solution supplemented (*RINGER+CLONIDINE*) or not (*RINGER*) with 10 μM clonidine (see Methods) were diluted 1:8, pretreated (*PLB*) or not with phospholipase B (*Control*) and tested for their ability to induce the spreading (see Methods) of normal 3T3F442A preadipocytes (*Control* and *PLB*) or 3T3F442A preadipocytes previously exposed for 48 h to 10 μM LPA to induce LPAd. Values are means±SEM from four different individuals. Statistical analysis was performed using the Student's *t* test: *P < 0.05, when comparing with control Ringer, #P < 0.05 when comparing LPAd or phospholipase B with control Ringer+clonidine.

to its specific desensitization, as shown previously in the N1E-115 neurite cell (24). This was indeed the case, since chronic pretreatment of 3T3F442A preadipocytes with LPA leads to a strong reduction of its own spreading effect without alteration of the response generated by sphingosine-1-phosphate, shown previously to act through a distinct G protein–coupled receptor (24, 25). In the same condition the spreading induced by conditioned medium and dialysates prepared in the presence of alpha$_2$-adrenergic agonist was completely abolished. This strongly suggests the requirement of LPA receptors in the mediation of trophic activity of the conditioned media and dialysates in 3T3F442A preadipocytes. This strategy of agonist-induced LPA receptor desensitization, along with phospholipase B treatment, strongly suggests that LPA is responsible for the alpha$_2$-adrenergic–dependent trophic activity of the conditioned media and dialysates in preadipocytes. This conclusion could be reinforced by the development of specific antagonists of the LPA receptor.

The demonstration of the release of [^{32}P]LPA from [^{32}P]-labeled adipocytes stimulated with an alpha$_2$-adrenergic agonist provided further strong evidence for the involvement of LPA. However, such labeling experiments give good indications concerning the turnover of LPA phosphorylation but give very poor information concerning the real amount of LPA produced. No methods are currently available to assay unlabeled LPA in mass, so it is not yet possible to confirm that the amount of LPA produced by adipocytes is in agreement with its EC$_{50}$ for 3T3F442A preadipocyte spreading and proliferation.

Though not quantified, the production of LPA by adipocytes was demonstrated for the first time. This conclusion is important since, so far, the only cell type known to produce LPA was the thrombin–activated platelet (7). Some other cell types such as the PDGF-activated fibroblasts (30) or IL-1–stimulated mesangial cells (31, 32) have been suspected to be able to produce LPA, but no clear demonstration has been provided. Considering the high mass of adipose tissue in the organism, particularly in obese patients, the physiological relevance of the production of LPA by adipocytes is an open question. Our results demonstrate that LPA is produced not only from cultured adipocytes, but can also be produced in intact adipose tissue, as demonstrated by microdialysis experiments. In the adipose tissue, adipocytes have a close relationship with other cell types such as preadipocytes, endothelial cells, blood cells, and nervous cells, all being potential targets for LPA. LPA has been shown to regulate the morphology and the growth of some of these cell types by activation of specific transduction pathways such as the ras/MAPK pathway and/or the pathway involving low molecular weight GTPases of the *rho* family (6). In this study, we demonstrated that LPA was able to regulate both proliferation and spreading of 3T3F442A preadipocytes. We also have strong evidence that LPA is able to regulate spreading and proliferation of preadipocytes in primary culture obtained from rodents (Valet, P., unpublished data). Moreover, regulation of cytoskeleton activity and proliferation can affect both preadipocyte growth and differentiation and, consequently, the potential recruitment of new fat cells in the adipose tissue.

It is noticeable that, as shown previously (33), in the absence of alpha$_2$-adrenergic agonist, conditioned media has an effect, per se, suggesting that trophic activities, unrelated to alpha$_2$-adrenergic–dependent LPA release, exist. It is obvious that LPA is not the only trophic agent present in the conditioned media. It is even possible that the full expression of the LPA effect could require the presence of another unknown agent existing in CMC. Synergism between different pathways is rather common in the control of cell growth. Therefore, LPA could be an additional member of the autocrine/paracrine factors controlling adipose tissue alongside nonesterified fatty acids and prostaglandin metabolites, also produced by adipocytes, and operating as *trans*-acting regulators of preadipocyte growth and differentiation (2, 4, 5).

This study also reveals that the production of LPA from adipocytes specifically occurs under an adrenergic stimulus mediated by alpha$_2$-adrenergic receptors, probably through heterotrimeric G$_i$ or G$_o$ proteins. Although it was not intended, in this study, to clarify the metabolic pathways responsible for the alpha$_2$-adrenergic–dependent release of LPA by adipocytes, a possible scenario could be proposed. In platelets, thrombin-induced LPA release was proposed to result from a phospholipase A$_2$–catalyzed deacylation of phosphatidic acid (7). Possible coupling of alpha$_2$-adrenergic receptors to phospholipase A$_2$ activation has been proposed in different cell types including platelets (9) and Chinese hamster ovary cells transfected with the human alpha$_2$-adrenergic receptor gene (10). To our knowledge, even though there is evidence for a phospholipase A$_2$ activity in adipocytes (34, 35), its alpha$_2$-adrenergic control has never been demonstrated. Alpha$_2$-adrenergic receptors are abundantly expressed in adipocytes where they are involved in the adrenergic control of lipolysis (8). Previous studies from our group have shown that alpha$_2$-adrenergic receptors are also present in preadipocytes and that their stimulation increases both the proliferation and cytoskeleton activity of these cells (13, 16). Therefore, in the context of the intact adipose tissue, the alpha$_2$-adrenergic–dependent release of LPA from adipocytes could amplify the trophic activity generated by direct alpha$_2$-adrenergic stimulation of preadipocytes in close contact with adipocytes. Therefore, the adrenergic control of preadipocyte growth could result from the combined direct activation of preadipocyte alpha$_2$-adrenergic receptors and alpha$_2$-adrenergic–dependent release of LPA from adipocytes. Additionally, it is now clear that the expression of the alpha$_2$-adrenergic receptors in adipocytes is positively correlated with adipocyte hypertrophy in both rodents and human (36–38). Therefore, it will be of considerable interest to determine whether the level of LPA produced by adipocytes is dependent on the level of adipose tissue hypertrophy, particularly in obese patients.

To conclude, the results support the idea that LPA release induced by the selective stimulation of alpha$_2$-adrenergic receptors in mature adipocytes promotes preadipocyte activation and proliferation. This LPA-dependent paracrine loop, operating under sympathetic nervous system control, could act synergistically with other adipogenic agents. Exploration of this pathway will be considerably facilitated by improvement of LPA determination in biological fluids and when reliable and selective LPA antagonists become available.

Acknowledgments

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