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**Ca<sup>++</sup>-Independent Phospholipase A2 (iPLA2) is required for  $\alpha$ 2-adrenergic-induced preadipocyte spreading.**

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Running Title: iPLA2 and preadipocyte spreading

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**Abstract**

In the present study, we studied the involvement of A2 phospholipases (PLA2) in  $\alpha$ 2-adrenergic receptor-control of preadipocyte actin cytoskeleton. For that, various PLA2 inhibitors were tested on the ability of the selective  $\alpha$ 2-adrenergic agonist UK14304 to induce the spreading in  $\alpha$ 2AF2 preadipocytes. We observed that, whereas several  $\text{Ca}^{++}$ -dependent PLA2 blockers were ineffective, the  $\text{Ca}^{++}$ -independent phospholipase A2 (iPLA2) inhibitor, broenolactone (BEL), specifically blocked  $\alpha$ 2-adrenergic-dependent preadipocyte spreading without affecting the spreading activity of lysophosphatidic acid (LPA) or serum. BEL-inhibition was completely restored by lysophosphatidic acid, not by arachidonic acid or other fatty acids. The presence of the lysophospholipase (phospholipase B) suppressed LPA effect on preadipocyte spreading, but had no influence on  $\alpha$ 2-adrenergic-induced spreading. Thus, the extracellular production of LPA or fatty acids are not involved in iPLA2-dependent preadipocyte spreading. iPLA2 protein was found in preadipocytes but, conversely to cPLA2, did not exhibit any modification of its electrophoretic mobility after  $\alpha$ 2-adrenergic stimulation. We concluded that iPLA2 is involved in  $\alpha$ 2-adrenergic-control of preadipocyte actin cytoskeleton.

## Introduction

$\alpha$ 2-adrenergic receptors are membrane G-protein coupled receptors that mediate the action of catecholamines (epinephrine and norepinephrine) in a wide range of tissues (1). In white adipose tissue,  $\alpha$ 2-adrenergic receptors belong to the  $\alpha$ 2A-subtype and are expressed in both adipocytes and preadipocytes. In adipocytes  $\alpha$ 2-adrenergic receptors mediate inhibition of lipolysis through a Gi/Go-dependent coupling to adenylyl cyclase (2). Stimulation of  $\alpha$ 2-adrenergic receptors also increases preadipocyte proliferation. This effect is associated with a Gi/Go-dependent activation of the ras/MAPK (Mitogen Activated Protein Kinases) pathway (3). In parallel,  $\alpha$ 2-adrenergic stimulation leads to a rapid spreading of preadipocytes on their growing substratum. This is associated with a reorganization of actin cytoskeleton in stress fibers, the formation of adhesive pseudopodes (lamellipodia and filipodia), and tyrosyl-phosphorylation of the Focal Adhesion Kinase (FAK) (4). These regulations result from the existence of a positive coupling between  $\alpha$ 2-adrenergic receptors and the small GTPase p21rhoA via the heterotrimeric G proteins Gi/Go (5). Our objective is to go further in elucidating the transduction pathways involved in  $\alpha$ 2-adrenergic-control of preadipocyte cytoskeleton and adhesion.

This study was undertaken to analyse the involvement of phospholipase A2 (PLA2) in  $\alpha$ 2-adrenergic-control of preadipocyte cytoskeleton. Among the various existing PLA2, only the  $\text{Ca}^{++}$ -dependent PLA2 (cPLA2) has been shown to be involved in cytoskeleton rearrangements in several cell types and adhesion including platelets (6), neurite (7), macrophages (8). Thus the influence of various PLA2 inhibitors were tested on the ability of a specific  $\alpha$ 2-adrenergic agonist UK14304 to promote preadipocyte spreading. This study was undertaken in a preadipose cell line ( $\alpha$ 2AF2) derived from 3T3F442A preadipocyte permanently expressing the human  $\alpha$ 2A-adrenergic receptor (3). The present data show for the first time that  $\alpha$ 2-adrenergic-dependent preadipocyte spreading specifically requires the activity of a  $\text{Ca}^{++}$ -independent PLA2 (iPLA2) with no involvement of the cPLA2. The mechanisms involved in iPLA2 control of preadipocyte spreading is discussed.

## Material and Methods

### Reagents

Bromo-enol lactone (BEL; Biomol), amino methoxyflavone (PD098059; Calbiochem Novabiochem), indomethacin (SIGMA) were solubilized and diluted in DMSO. Methyl arachidonyl fluorophosphonate (MAPF; Biomol), arachidonyltrifluoromethyl ketone (AACOCF<sub>3</sub>; Biomol), arachidonic acid (SIGMA), palmitic acid (SIGMA), oleic acid (SIGMA) and chlorobenzyl isopropyl butylthionindol dimethylpropanoic acid (MK886; Calbiochem Novabiochem) were solubilized and diluted in ethanol. Propranolol (SIGMA) was solubilized and diluted in water. Lysophosphatidic acid oleoyl (SIGMA) was solubilized and diluted in PBS buffer containing 1% fatty acid free bovine albumine. Phospholipase B (SIGMA) was reconstituted in PBS buffer. Rabbit iPLA2 polyclonal antiserum was purchased from Cayman Chemical and mouse anti-cPLA2 monoclonal antibody was purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-linked anti-mouse antibody was from Amersham Pharmacia Biotech, and (HRP)-linked anti-rabbit antibody from SIGMA.

### Culture and Spreading analysis

The  $\alpha$ 2AF2 cell line was obtained after permanent transfection of the human  $\alpha$ 2C10-adrenergic receptor gene in 3T3F443A preadipose cell line followed by G418-selection (3). Preconfluent  $\alpha$ 2AF2 preadipocytes were washed twice with PBS and placed in serum free DMEM for 30 to 60 min to induce cell retraction which is characterized by an increase in cell body refringency. Retracted cells were then exposed to various agents (UK14304, LPA) for 15 min. The intensity of cell spreading was quantified by determining the proportion of refringent cells under a microscope connected to video camera and an image analysis program (Visiolab).

### RT-PCR analysis

Total  $\alpha$ 2AF2 preadipocytes RNA was extracted using RNeasy mini kit (Qiagen). One microgram of total RNA was reverse transcribed for 60 min at 42°C using Omniscript Reverse Transcriptase (QIAGEN) and subject to amplification. Polymerase chain reaction (PCR) was carried out in a final volume of 50  $\mu$ l containing 3  $\mu$ l of cDNA, 1  $\mu$ l dNTP (10 mM), 5  $\mu$ l 10x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3  $\mu$ l MgCl<sub>2</sub> (25 mM), 1.5  $\mu$ l sense and antisense specific oligonucleotide primers (10  $\mu$ M) and 1.25 unit of Taq DNA polymerase (Promega). Conditions for the PCR reaction were an initial denaturation step of 94°C for 5 min, followed by 28 cycles of 94 °C for 1 min 10 s, 57°C (HPRT) or 60 °C (cPLA2 and iPLA2) for 1 min, 72 °C for 1 min 30 s and a final extension at 72°C for 6 min. PCR products were separated on 1.5% agarose gel and labelled with ethidium bromide. Primers used for PCR were: cPLA2 (sense, 5'-TTGGGTTTCAGGTGGGGTTTC-3' and antisense, 5'-CCAATCGGCAAACATCAGCTC-3'); iPLA2 (sense, 5'-CTCATCGCCATCGAGAAGGC-3' and

antisense, 5'-CAGTGTCCCTGTCAGCATCA-3'); HPRT (sense, 5'-TCCCAGCGTCGTGATTAGC-3' and antisense, 5'-GCATTGTTTTACCAGTGTC-3').

### Western blot analysis

After stimulation  $\alpha$ 2AF2 cells were lysed 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, and 2 mM phenylmethylsulfonylfluoride] and passed several times through a fine needle. Insoluble debris and the fat cake were removed by centrifugation (13,000 rpm for 5 min). The protein content was determined using the Lowry protein assay kit (Bio-Rad).

Total protein from each samples (80  $\mu$ g) of  $\alpha$ 2AF2 cells were separated on a 11% SDS-PAGE for 15 h at 10 mA, and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Nonspecific sites were blocked with Tris-buffer solution containing 0.2 % Tween-20 (TBST) and 2% nonfat milk for 1 h at room temperature. Membranes were incubated with primary antibodies against cPLA2 (1/1000) or iPLA2 (1/750) overnight at 4°C . Immunes complexes were revealed by an HRP-linked secondary antibody (1/1000 for cPLA2 and 1/2000 for iPLA2) and visualized using the Amersham ECL system. After detection of specific PLA2 signal, membranes were stripped in a solution containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10 mM 2-mercaptoethanol for 30 min at 55 °C. After extensive washing, membranes were reblocked prior to reprobing. The intensities of the bands was measured using Image'quant software. The band-shift was quantified by measuring the ratio between the intensity of the upper (hyperphosphorylated form) versus the lower (hypophosphorylated form) bands.

## Results and Discussion

Fifteen min exposure of retracted  $\alpha$ 2AF2 preadipocytes to 10  $\mu$ M of the selective  $\alpha$ 2-adrenergic agonist UK14304 lead to a dramatic decrease in the proportion of retracted preadipocytes (figure 1) resulting from their spreading on the growing substratum (4). In order to precise the transduction pathways involved in  $\alpha$ 2-adrenergic-control of preadipocyte spreading,  $\alpha$ 2AF2 preadipocytes were pretreated with various inhibitors before addition of UK14304. UK14304-induced preadipocyte spreading was strongly and significantly reduced by 30 min pretreatment of retracted preadipocytes to 10  $\mu$ M of the iPLA2 inhibitor bromoenol lactone (BEL) (Figure 1). BEL is known as a suicide substrate inhibitor of iPLA2 (9), but can also exert potential inhibitory activities on other type of PLA2 as well as on the phosphatidate phosphohydrolase PAP (10). As shown in figure 1, the inhibitory effect of BEL on UK14304-induced preadipocyte spreading was not observed with cPLA2 inhibitors such as MAPF (40  $\mu$ M) or AACOCF3 (40  $\mu$ M), nor by the PAP inhibitor propranolol (50  $\mu$ M). A potential toxic effect of BEL on  $\alpha$ 2AF2 preadipocyte was also excluded since the spreading effect induced by serum (10%) or lysophosphatidic acid (LPA) (10  $\mu$ M) were not altered by BEL (Figure 2). Those observations strongly suggested that the blocking effect of BEL was due to its selective action on iPLA2.

iPLA2 is known to play an important role in phospholipid turn-over. It acts at sn-2 position on phospholipids leading to the production of fatty acids and lysophospholipids (11). Those lipid metabolites have previously shown to act on cell spreading (7, 8, 12). As shown in figure 2, arachidonic acid, palmitic acid, or oleic acid exerted no influence  $\alpha$ 2AF2 preadipocyte spreading. This showed that extracellular production of fatty acids, at least those we have tested, was not responsible for the iPLA2-dependent control of preadipocyte spreading. Fatty acid metabolites were also excluded since, as shown in figure 2, UK14304-induced preadipocyte spreading was not altered by cyclooxygenase inhibitor indomethacin (10  $\mu$ M), nor by the 5-lipoxygenase inhibitor MK886 (1  $\mu$ M).

We previously demonstrated that  $\alpha$ 2-adrenergic stimulation in adipocytes leads to an extracellular production of lysophosphatidic acid (LPA) which in turn is able to activate preadipocyte spreading and proliferation (13). Therefore we hypothesized that LPA could play a role in iPLA2-dependent preadipocyte spreading. This hypothesis was in agreement with the fact that one of the best substrate of iPLA2, at least in vitro, is the 1-2-acyl-phosphatidic acid, the immediate precursor of LPA (14). As shown in Figure 1, LPA was indeed a powerful activator of  $\alpha$ 2AF2 preadipocyte spreading. In addition, the blocking effect of BEL on UK14304-induced preadipocyte spreading was completely reversed by further addition of 1  $\mu$ M LPA (Figure 3). This suggested that BEL could have blocked  $\alpha$ 2-adrenergic production of LPA, thus blocking LPA-induced preadipocyte spreading. However, whereas the spreading effect of LPA could be completely suppressed by the presence in the culture medium of 3U/ml of a lysophospholipase, phospholipase B (PLB), the spreading effect induced by UK14304 was not

altered by PLB (Figure 4). Therefore the involvement of extracellular LPA in  $\alpha$ 2-adrenergic-induced preadipocyte spreading could be excluded.

The blocking effect of BEL on  $\alpha$ 2-adrenergic-dependent preadipocyte spreading suggested the existence of an  $\alpha$ 2-adrenergic regulation of iPLA2. In order to test this hypothesis, the expression of iPLA2 was studied in  $\alpha$ 2AF2 preadipocytes in parallel to the expression of the  $\text{Ca}^{++}$ -dependent PLA2 (cPLA2). As analysed by RT-PCR, both cPLA2 and iPLA2 mRNAs are present in  $\alpha$ 2AF2 preadipocytes (Figure 5). Western blot analysis performed on total homogenate proteins from retracted  $\alpha$ 2AF2 preadipocytes with a polyclonal antibody directed against iPLA2 protein revealed the existence of a major signal with a molecular weight around 80 kDa (Figure 6A). In parallel, western blot analysis performed with a monoclonal antibody directed against cPLA2 protein revealed the presence of two closely migrating bands around 90 kDa (Figure 6B). These two bands correspond to the hypo (inactive)- and hyper (active)-phosphorylated forms of cPLA2 (15). Following subcellular fractionation of retracted  $\alpha$ 2AF2 preadipocytes, and as previously observed in other cell types (16), whereas cPLA2 was found to be similarly expressed in both membrane and cytosolic fractions, iPLA2 was found to be exclusively expressed in membrane fraction (not shown). Seven minutes exposition of retracted  $\alpha$ 2AF2 preadipocytes to 10  $\mu\text{M}$  UK14304 led to an increase in the intensity of the upper band (3.2 fold increase in the ratio of intensity between the upper and the lower band) which was not observed after pretreatment with the Mitogen Activated Protein Kinase Kinase inhibitor PD098059 (50  $\mu\text{M}$  for 90 min) (Figure 6B). In parallel, seven minutes exposure of retracted  $\alpha$ 2AF2 preadipocytes to 10  $\mu\text{M}$  UK14304 had no significant influence on iPLA2 migration (Figure 6A). cPLA2 is known to be activated by several G-protein coupled receptors including  $\alpha$ 2-adrenergic receptors (17, 18). According to the literature, and conversely to cPLA2, iPLA2 was not reported to be regulated by phosphorylation (19). This could explain the absence of alteration in iPLA2 electrophoretic mobility following  $\alpha$ 2-adrenergic stimulation. Nevertheless, another mechanism of regulation of iPLA2 by  $\alpha$ 2-adrenergic receptor cannot be excluded and will be the purpose of further investigations.

In conclusion, our data demonstrate the involvement of iPLA2 in  $\alpha$ 2-adrenergic control of preadipocyte spreading. The mechanisms by which iPLA2 could control actin cytoskeleton in preadipocyte exclude the extracellular action of iPLA2 products such as fatty acids and LPA. We cannot completely rule out a role of intracellularly released fatty acids or LPA. Indeed, a recent report demonstrates the existence of a potential intracellular action of LPA which could control actin polymerization by binding on gelsolin, a protein which caps actin filaments and blocks their polymerization (20).

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## Legend of the figures

**Figure 1:** Influence of various inhibitors on UK14304-induced  $\alpha$ 2AF2-preadipocyte spreading.  $\alpha$ 2AF2 preadipocytes were previously retracted by serum deprivation, pretreated to 10  $\mu$ M BEL (30 min) or 30  $\mu$ M MAFP (30 min) or 40  $\mu$ M AACOCF3 (30 min) or 50  $\mu$ M propranolol (15 min) or 1  $\mu$ M MK886 (30 min) or 10  $\mu$ M indomethacin (90 min) or 50  $\mu$ M PD098059 (90 min), prior addition of 10  $\mu$ M UK14304. Intensity of cell spreading was evaluated by the proportion of refringent cells after 15 min. Data correspond to the mean  $\pm$  SEM from three separate experiments.

**Figure 2:** Influence of bromoenol lactone on  $\alpha$ 2AF2-preadipocyte spreading.  $\alpha$ 2AF2 preadipocytes were previously retracted by serum deprivation, pretreated with 10  $\mu$ M of bromoenol lactone (BEL) for 30 min, prior addition of 10  $\mu$ M UK14304 (UK), or 10  $\mu$ M lysophosphatidic acid (LPA), 10% serum, 100  $\mu$ M arachidonic acid (ARA), 80  $\mu$ M palmitic acid (PALM) or 80  $\mu$ M oleic acid (OLEA). Intensity of cell spreading was evaluated by the proportion of refringent cells after 15 min. Data correspond to the mean  $\pm$  SEM from three separate experiments.

**Figure 3:** Influence of arachidonic acid and LPA on UK14304-induced  $\alpha$ 2AF2 preadipocyte spreading.  $\alpha$ 2AF2 preadipocytes were previously retracted by serum deprivation, pretreated to 10  $\mu$ M BEL for 30 min, then to 10  $\mu$ M UK14304 for 15 min and then to 100  $\mu$ M arachidonic acid or 10  $\mu$ M LPA. Intensity of cell spreading was evaluated by the proportion of refringent cells 15 min later. Data correspond to the mean  $\pm$  SEM from three separate experiments.

**Figure 4:** Influence of phospholipase B on LPA- and UK14304-induced  $\alpha$ 2AF2 preadipocyte spreading.  $\alpha$ 2AF2 preadipocytes were previously retracted by serum deprivation and exposed for 15 min to 10  $\mu$ M UK14304 or 10  $\mu$ M LPA in the presence or not of 3U/ml phospholipase B. Intensity of cell spreading was evaluated by the proportion of refringent cells after 15 min. Data correspond to the mean  $\pm$  SEM from three separate experiments.

**Figure 5:** Expression of cPLA2, iPLA2 mRNA in  $\alpha$ 2AF2 preadipocytes. RT-PCR analysis were performed with specific oligonucleotides on total RNA from  $\alpha$ 2AF2 preadipocytes as described in Material and Methods. Hypoxanthine phosphoribosyl transferase (HPRT) gene was determined in parallele as a control.

**Figure 6:** Influence of UK14304 and PD098059 on cPLA2 and iPLA2 in retracted  $\alpha$ 2AF2 preadipocytes. Serum-deprived  $\alpha$ 2AF2 preadipocytes were pretreated or not with 50  $\mu$ M PD098059 for 90 min before being exposed for 7 min to 10  $\mu$ M UK14304. iPLA2 (A) and cPLA2

(B) were resolved by SDS-PAGE as described in Material and Methods. (cPLA2-P) and (cPLA2) correspond to hyper- and hypo-phosphorylated forms of cPLA2.