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Sandrine Bétuing, Danièle Daviaud, Céline Pagès, Elisabeth Bonnard, Philippe Valet, et al.. Gbeta gamma-independent coupling of alpha2-adrenergic receptor to p21(rhoA) in preadipocytes.. Journal of Biological Chemistry, 1998, 273 (25), pp.15804-10. inserm-00110175

**HAL Id: inserm-00110175**

**<https://inserm.hal.science/inserm-00110175>**

Submitted on 8 Jan 2007

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**G $\beta$ -independent coupling of  $\alpha$ 2-adrenergic receptor to p21rhoA in preadipocytes.**

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Running title:  $\alpha$ 2-adrenergic receptor coupling to p21rhoA

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## ABSTRACT

In preadipocytes,  $\alpha 2$ -adrenergic receptor ( $\alpha 2$ -AR) stimulation leads to a Gi/Go-dependent rearrangement of actin cytoskeleton. This is characterized by a rapid cell spreading, the formation of actin stress fibers and the increase in tyrosyl-phosphorylation of the Focal Adhesion Kinase (pp125FAK). These cellular events being tightly controlled by the small GTPase p21rhoA, the existence of a Gi/Go-dependent coupling of  $\alpha 2$ -AR to p21rhoA in preadipocytes was proposed.

In  $\alpha 2$ AF2-preadipocytes (a cell clone derived from the 3T3F442A preadipose cell line and which stably expresses the human  $\alpha 2$ C10-adrenergic receptor)  $\alpha 2$ -adrenergic-dependent induction of cell spreading, formation of actin stress fibers and increase in tyrosyl-phosphorylation of pp125FAK, were abolished by pretreatment of the preadipocytes with the C3 exoenzyme, a toxin which impairs p21rhoA activity by ADP-ribosylation. Conversely, C3 exoenzyme had no effect on the  $\alpha 2$ -adrenergic-dependent increase in tyrosyl-phosphorylation and shift of ERK2 Mitogen Activated Protein Kinase. Alpha2-adrenergic stimulation also led to an increase in GDP/GTP exchange on p21 rhoA, as well as to an increase in the amount of p21rhoA in the particulate fraction of  $\alpha 2$ AF2 preadipocytes. Stable transfection of  $\alpha 2$ AF2 preadipocytes with the C-terminal domain of  $\beta$ ARK1 ( $\beta$ ARK-CT) (a blocker of  $G\beta\gamma$ -action), strongly inhibited the  $\alpha 2$ -adrenergic-dependent increase in tyrosyl-phosphorylation and shift of ERK2, without modification of the tyrosyl-phosphorylation of pp125FAK and spreading of preadipocytes. These results show that  $\alpha 2$ -adrenergic-dependent reorganization of actin cytoskeleton requires the activation of p21rhoA in preadipocytes. Conversely to the activation of the p21ras/MAPK pathway, the  $\alpha 2$ -adrenergic activation of p21rhoA-dependent pathways are independent of the  $\beta\gamma$ -subunits of heterotrimeric G proteins.

## Introduction

Alpha2-adrenergic receptors ( $\alpha 2$ -AR) are G-protein-coupled receptors which mediate the action of catecholamines (epinephrine, norepinephrine) in a wide range of tissues (1). In white adipose tissue,  $\alpha 2$ -ARs belong to the  $\alpha 2A$ -subtype and are expressed in both adipocytes and preadipocytes. In adipocytes  $\alpha 2$ -ARs mediate inhibition of lipolysis through a Gi-dependent coupling to adenylyl cyclase (2). In preadipocytes, stimulation of  $\alpha 2$ -ARs generates a Gi/Go-dependent increase in cell proliferation. This effect was associated with rapid and transient tyrosine phosphorylation of the p44 and p42 Mitogen-Activated Protein Kinases (MAPK) ERK1 and ERK2 (3) reflecting the Gi/Go-mediated coupling of the  $\alpha 2$ -ARs to the small GTPase p21ras (4). Alpha2-adrenergic activation of the ras/MAPK pathway is mediated by the  $\beta\gamma$ -subunits of the heterotrimeric G proteins as demonstrated by the ability of the C-terminal domain of  $\beta$ ARK1 (a  $\beta\gamma$ -binding protein) to block this activation (5, 6).

In preadipocytes, stimulation of  $\alpha 2$ -ARs is also associated with striking Gi/Go-dependent rearrangement of actin cytoskeleton. This is characterized by a rapid spreading of the cells on their growing substratum, the formation of actin stress fibers and the increase in the tyrosyl-phosphorylation of the pp125 focal adhesion kinase (pp125FAK) (7). These cellular events are known to be tightly controlled by another GTPase belonging to the ras-superfamily, p21rhoA (8, 9). It has particularly been demonstrated that the C3 exoenzyme from *Clostridium botulinum* (a toxin which specifically block the action of p21rhoA by catalysing its ADP-ribosylation) is able to block the activation of cell motility, cell morphology and cell growth, generated by several agonists acting through G-protein coupled receptors such as lysophosphatidic acid, sphingosine 1-phosphate and thrombin (10, 11).

Considering the morphological changes generated by stimulation of the  $\alpha 2$ -ARs in preadipocytes, we analysed (i) the involvement of p21rhoA in  $\alpha 2$ -adrenergic-dependent reorganization of actin cytoskeleton and tyrosyl-phosphorylation of pp125FAK; (ii) the existence of a functional coupling between  $\alpha 2$ -ARs and p21rhoA; (iii) the putative involvement of  $\beta\gamma$ -subunits of G proteins in this coupling. In the present study we demonstrate that, in  $\alpha 2AF2$ -preadipocytes (a cell clone derived from the 3T3F442A preadipose cell line stably

expressing the human  $\alpha 2C10$ -adrenergic receptor),  $\alpha 2$ -adrenergic-dependent reorganization of actin cytoskeleton involves the activation of p21rhoA independently of a  $G\beta\gamma$ -mediated transduction pathway.

## Materials and methods

### Cell culture and transfection

The cells were grown at 37°C in DMEM supplemented with 10% donor calf serum (Gibco-Life technologies, NY) as previously described (7). Alpha2AF2 ( $\alpha$ 2AF2) preadipocytes were previously obtained by permanent transfection of the human  $\alpha$ 2C10-adrenergic receptor gene in the 3T3F442A preadipose cell line followed by G418-selection (3). As determined by radioligand binding analysis  $\alpha$ 2-adrenergic receptor density in  $\alpha$ 2AF2 preadipocytes was  $2050 \pm 90$  fmol/mg protein. Stable expression of the carboxyl-terminal domain of  $\beta$ ARK1 ( $\beta$ ARK-CT) was obtained by transfection of  $\alpha$ 2AF2 preadipocytes with a pZeo/ $\beta$ ARKCT vector, followed by the double selection G418/zeocin. pZeo/ $\beta$ ARKCT vector was obtained by subcloning an EcoRI-SalI fragment from pRK- $\beta$ ARK1 vector (6) (generous gift from Dr. Lohse) into pcDNA3.1/Zeo vector (Invitrogen) linearized by EcoRI-XhoI. The validity of the construct was verified by sequencing.

### Expression and purification of C3 exoenzyme

Recombinant C3 exoenzyme were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* (generous gift from Dr. Alan Hall) and purified on glutathione sepharose beads as previously described (12). C3 exoenzyme was released from the beads by thrombin (Calbiochem) cleavage and concentrated by centricon-3 (Amicon Inc, Beverly, USA).

### C3-exoenzyme-catalysed ADP-ribosylation of Rho proteins

Cells were pretreated or not with C3-exoenzyme for various period of time, washed twice in PBS and homogenized in extraction buffer (0.25M sucrose, 20mM Tris-HCL, 3mM MgCl<sub>2</sub>, 1mM EDTA, 1mM DTT, 1mM phenylmethylsulfonylfluoride, 2mM benzamidine, pH7.5). ADP-ribosylation was carried out in a total volume of 100  $\mu$ l containing 20mM Hepes pH8.0, 2mM MgCl<sub>2</sub>, 10mM thymidine, 0,1% deoxycholate, 10 mM NAD, 1 $\mu$ Ci [<sup>32</sup>P] NAD, 100  $\mu$ g of cell homogeneate and 0.5  $\mu$ g of C3 exoenzyme. The reaction was carried out at

30°C for 37 min and terminated by addition of 11 µl of 70% trichloroacetic acid for 15 min at 4°C. The proteins in the pellet were dissolved in 50µl of Laemmli buffer (200mM Tris-HCL pH6.8, 6%SDS, 2mM EDTA, 4% 2-mercaptoethanol and 10% glycerol) and separated on 12,5% SDS-polyacrylamide gel, stained with Coomassie brilliant blue R-250, dried and autoradiographed. The intensity of the spots was analyzed using an Image'quant software.

### **Morphological analysis**

Cell spreading activity was determined as previously described (7). Briefly, cells were previously retracted by serum starvation (which was characterised by an increased cell refringency) before addition of an alpha2-adrenergic agonist. The proportion of retracted cells was determined as the ratio between the number of refringent cells and the total number of cells present in a microscope field. Each value correspond to the mean of at least five separate fields.

Actin filaments were visualized as previously described (13). Cells grown on plastic culture plates were washed twice with PBS and fixed for 15 min in 3,7% (v/v) formaldehyde in PBS. Fixed cells were washed twice with PBS and permeabilized with 0,1% Triton X-100 in PBS for 2 min. Permeabilized cells were washed twice with PBS and incubated with 150nM of fluorescein isothiocyanate (FITC)-conjugated phalloidin PBS for 20 min. The cells were then extensively washed with PBS and examined using an epifluorescence microscope.

### **Detection of tyrosyl-phosphorylated proteins**

Immunoprecipitation of tyrosyl-phosphorylated FAK, ERK-2 and western-blot were carried out as described previously (7). Briefly, tyrosyl-phosphorylated proteins were immunoprecipitated with 15µl protein G-sepharose beads (Sigma Chemical Inc.) conjugated with an antiphosphorylated antibody (PY20, Transduction laboratories) and resuspended in 50µl Laemmli buffer. Tyrosyl-phosphorylated proteins were then separated on 8% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose and incubated with an anti-focal adhesion kinase antibody (FAK C-20; 0,5µg/ml, Santa Cruz Biotechnology, Inc.) followed by horseradish peroxydase-labeled secondary anti-rabbit

(Immunotech.). Immunoreactive bands were visualized by enhanced chemiluminescence detection (ECL, Amersham Life Science). After FAK-detection, the blot was stripped for 30' at 50°C in 100mM  $\beta$ -mercaptoethanol, 2% SDS, 62,5mM TrisHCl pH6.5 followed by extensive washes in TBST buffer (10mM Tris-HCL pH8.0, 150mM NaCl, 0,2% Tween-20). The blot was then incubated with anti-ERK2 (C-14, 1 $\mu$ g/ml, Santa Cruz Biotechnology, Inc.) to detect tyrosyl-phosphorylated ERK2. The intensity of the bands was measured using Image'quant software.

### **Detection of rhoA and $\alpha$ i2 proteins in particulate subcellular fraction**

Cells were lysed in extraction buffer (25mM Tris/HCL (pH7,5), 5mM EGTA (pH 7.5), 15mM NaCl, 1% n-octyl  $\beta$ -D-glucopyranoside, 1mM phenylmethylsulfonylfluoride, 20 $\mu$ g/ml leupeptin). Lysates were centrifuged at 120,000xg for 45 min to separate cytosolic and particulate fractions. Particulate fraction were resuspended in RIPA buffer (0,01M Tris-HCL (pH 7.0), 150mM NaCl, 2mM EDTA, 1mM sodium orthovanadate, 0,1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 2mM phenylmethylsulfonylfluoride) and centrifuged at 15,000g to eliminate non solubilized material. Protein concentrations were determined according to the method of Lowry (14) and separated on 12,5% SDS-polyacrylamide gel for western-blot analysis using anti-rhoA antibody (26C4, 1 $\mu$ g/ml, Santa Cruz Biotechnology, Inc.), and anti  $\alpha$ i2 antibody (gift from Dr Rouot).

### **Determination of GTP/GDP ratio**

The determination of the GTP/GDP ratio of p21rhoA was essentially as described (15). Cells were serum starved for 18 h and subsequently labeled with 0.25mCi/ml [ $^{32}$ P] orthophosphate for 3 h. Cells were stimulated with 1mM UK14304 for various times and lysed in 0.5% Nonidet P-40 buffer containing 20 mM Tris-HCL (pH7,5), 150 mM NaCl, 10mM MgCl<sub>2</sub>, 250 mM sucrose, 2 mM EGTA, 1mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM NaF, 1% triton X-100, 1 mM ATP, 0,1mM GTP, 0.1 mM GDP, 1 mM phenylmethylsulfonylfluoride. The soluble cell extract was incubated for 3 h at 4°C with 15  $\mu$ l protein G-sepharose beads (Sigma Chemical Co., St.Louis, MO) conjugated with a mouse anti-rhoA antibody (119, Santa-cruz Biotechnology,



Inc.). [ $^{32}\text{P}$ ] labeled GDP and GTP were eluted from the immunocomplexes with a buffer containing, 5mM EDTA, 2mM DTT, 0.2% SDS, 0.5mM GDP and 0.5mM GTP 20' at 68°C. After extensive washes GTP/GDP were separated by thin layer chromatography (Bakerflex PEI-F cellulose TLC plates, Bakerflex) in 1M  $\text{KH}_2\text{PO}_4$  (pH3.4) and autoradiographed. The intensity of the spots was analyzed using Image'quant software.

## Results

### Influence of the C3-exoenzyme on $\alpha$ 2-adrenergic-dependent regulation of preadipocyte morphology

In a previous study, we demonstrated that in preadipocytes,  $\alpha$ 2-adrenergic stimulation promotes rapid spreading, pp125FAK tyrosyl phosphorylation and actin stress fiber formation (7). These cellular events are known to be tightly controlled by the small GTPases of the rho family particularly p21 rhoA (8). In order to determine the involvement of rhoA in the  $\alpha$ 2-adrenergic-dependent regulation of preadipocyte morphology, the effect of C3 exoenzyme, a toxin which impairs the function of p21 rho (12), on  $\alpha$ 2-adrenergic-mediated spreading, stress fibers formation and pp125FAK phosphorylation in  $\alpha$ 2AF2 preadipocytes was tested.

In total  $\alpha$ 2AF2 preadipocyte lysate C3-exoenzyme catalysed the [ $^{32}$ P]ADP ribosylation of a unique band exhibiting a molecular weight of 21 KDa corresponding to p21 rho proteins (Figure 1.A) (12, (16). Seventy two hours pretreatment of intact  $\alpha$ 2AF2 preadipocytes with 10 $\mu$ g/ml C3-exoenzyme led to about 80% reduction in the amount of the 21KDa band (Figure 1.B) demonstrating the ability of C3-exoenzyme to penetrate into intact  $\alpha$ 2AF2 preadipocytes and ADP-ribosylate p21 rho proteins.

After 15 hours serum-starvation,  $\alpha$ 2AF2 preadipocytes exhibited a retracted morphology (Figure 2) characterized by the absence of actin stress fibers (visualized with FITC-labeled phalloidin) (Figure 3A). Fifteen minutes treatment with 1 $\mu$ M of the specific  $\alpha$ 2-adrenergic agonist UK14304 led the spreading of almost 80% of retracted  $\alpha$ 2AF2 (Figure 2) and by the formation of actin stress fibers (Figure 3B). UK14304-induced cell spreading and reorganization of actin cytoskeleton were abolished by 72 hours pretreatment of  $\alpha$ 2AF2 preadipocytes with 10  $\mu$ g/ml of C3 exoenzyme (Figures 2, 3C and 3D).

Five minutes treatment of 24 hours serum-starved  $\alpha$ 2AF2 preadipocytes with 1 $\mu$ M UK14304 led to an increase in the tyrosyl-phosphorylation of the focal adhesion kinase (pp125FAK) (Figure 4.A Control). The same stimulation also led to an increase in the tyrosyl-phosphorylation of the Mitogen Activated Protein Kinase ERK2 associated with a shift corresponding to the biphosphorylated (on tyrosine and threonine residues) and active form of

ERK2 (Figure 4.B Control). Seventy two hours pretreatment of  $\alpha$ 2AF2 preadipocytes with 10 $\mu$ g/ml of C3 exoenzyme led to a 75-80% reduction of UK14304-induced tyrosyl phosphorylation of pp125FAK (Figure 4A, C3) without alteration of UK14304-induced shift and tyrosyl phosphorylation of ERK2 (Figure 4B, C3).

These results revealed that, in  $\alpha$ 2AF2 preadipocytes, active p21rho proteins are required to mediate  $\alpha$ 2-adrenergic-dependent (i) spreading; (ii) actin stress fibers formation; and (iii) tyrosyl-phosphorylation of the focal adhesion kinase.

### **-Alpha2-adrenergic stimulation activates rhoA protein**

The above results suggested the existence of a functional coupling between  $\alpha$ 2-adrenergic receptors and p21rhoA. To test this hypothesis, we analysed the influence of the  $\alpha$ 2-adrenergic stimulation on the translocation of p21 rhoA from cytosol to plasma membrane and the capacity of GDP/GTP exchange on p21rhoA.

In control cells, the amount of p21 rhoA in the particulate fraction was lower as compared to the cytosoluble fraction (Figure 5.A). Stimulation of 24 h serum-starved  $\alpha$ 2AF2 preadipocytes with 1  $\mu$ M UK14304 led to a increase in the amount of rhoA in the particulate fraction. This effect was not significant (1.6 fold increase over control) before 15 treatment. (Figure 5B). Conversely, the amount of G $\alpha$ i2, which is exclusively localised in plasma membrane, was not modified by UK14304 treatment (Figure 5B).

The influence of  $\alpha$ 2-adrenergic stimulation on GDP/GTP exchange on p21rhoA was tested by GTP-loading assay. Stimulation of 24 h serum-starved  $\alpha$ 2AF2 preadipocytes with 1  $\mu$ M UK14304 led to a rapid and transient increase in the proportion of p21rhoA-GTP versus p21rhoA-GDP with a maximum increase of 6 fold after 2 minutes (Figure 6). These results revealed the existence of a functional coupling between  $\alpha$ 2-adrenoceptors and p21rhoA-proteins.

### **- G $\beta$ $\gamma$ -independent induction of spreading and tyrosyl-phosphorylation of the focal adhesion kinase**

We have previously shown that  $\alpha 2$ -adrenergic-dependent activation of actin cytoskeleton in preadipocytes was pertussis toxin-sensitive, a result demonstrating the involvement of heterotrimeric G proteins of the  $G_i/G_o$  family (7). In order to determine the putative influence of the  $G\beta\gamma$  subunits of activated G proteins in this regulation,  $\alpha 2AF2$  preadipocytes were stably transfected with the C-terminal domain of  $\beta ARK1$  ( $\beta ARK-CT$ ), a truncated protein known to block the action of the  $G\beta\gamma$  subunits of G proteins when overexpressed in a cell (6). The selection of the cell clones exhibiting a blockade of  $G\beta\gamma$  subunits, was based on the inhibition of the  $\alpha 2$ -adrenergic-dependent tyrosyl-phosphorylation and shift of ERK2. The activation of ERK proteins resulting from a  $G\beta\gamma$ -dependent activation of p21ras (5), it should be blocked by transfection of  $\beta ARK-CT$ . Conversely  $G\beta\gamma$ -independent activation of the p21ras/ERK pathway such as that induced by growth factors contained in the serum, should not be altered.

Based on this criteria two cell clones (clone 5 and clone 50) exhibiting similar behaviour were selected. Only results obtained with clone 50 are presented. As determined by radioligand binding assay  $\alpha 2$ -adrenergic receptor density was not significantly different between clone 50 and non-transfected  $\alpha 2AF2$  preadipocytes ( $1750 \pm 110$  vs  $2050 \pm 90$  fmol/mg protein). In clone 50 the tyrosyl-phosphorylation of ERK2 induced by 1  $\mu M$  UK14304 treatment was reduced about 80% as compared to non-transfected  $\alpha 2AF2$  preadipocytes (Figure 7). This was accompanied by an almost complete disappearance of the shifted form of ERK2 (Figure 7). Conversely, the tyrosyl-phosphorylation and shift of ERK2 promoted via treatment with 10% fetal calf serum was unaffected by  $\beta ARK-CT$  transfection (Figure 7).

We then tested the influence of  $G\beta\gamma$  blockade on the  $\alpha 2$ -adrenergic-dependent activation of p21rhoA-mediated pathways. For that we studied, in clone 50, the  $\alpha 2$ -adrenergic-dependent regulation of spreading and tyrosyl-phosphorylation of pp125FAK, cellular events demonstrated above to be dependent on p21rhoA activation. In clone 50, the induction of preadipocyte spreading generated by 15 min exposure to 1  $\mu M$  UK14304 was not significantly different as compared with non-transfected  $\alpha 2AF2$  preadipocytes (Figure 8). Similarly, in clone 50, the increase of pp125FAK tyrosyl-phosphorylation induced by treatment with 1  $\mu M$  UK14304 was not significantly altered as compared with  $\alpha 2AF2$  preadipocytes (Figure 9).

These results show that, in  $\alpha 2AF2$  preadipocytes,  $\beta$ ARK-CT-mediated blockade of  $G\beta\gamma$  subunits did not alter the  $\alpha 2$ -adrenergic-dependent regulation of cell spreading and focal adhesion kinase phosphorylation.

## Discussion

The present study demonstrates that (i) the coupling between  $\alpha 2$ -adrenergic receptors and the small GTPase p21rhoA is involved in the  $\alpha 2$ -adrenergic-dependent regulation of preadipocyte actin cytoskeleton and pp125FAK; and that (ii) conversely to the ras/MAPK pathway, the Gi/Go-dependent activation of p21rhoA/cytoskeleton pathway does not involve the  $\beta\gamma$  subunits of heterotrimeric G-proteins.

As described with other agents such as lysophosphatidic acid, shingosine-1-phosphate, or thrombin (8, 10, 11),  $\alpha 2$ -adrenergic-dependent activation of actin cytoskeleton was blocked by C3-exoenzyme pretreatment, a toxin known to specifically suppress the activity p21 rhoA (17, 18). Under its active form (bound to GTP), p21rhoA plays a key role in the reorganization of the actin network, particularly in the formation of actin stress fibers, but also in the formation of focal adhesion plaques and the tyrosyl-phosphorylation of the focal adhesion kinase (8, 9, 19). Therefore, our results obtained with the C3-exoenzyme demonstrated the implication of p21rhoA in the  $\alpha 2$ -adrenergic-dependent regulation of the actin cytoskeleton and in the control of adhesion of the preadipocyte. This observation suggested the existence of a possible coupling between  $\alpha 2$ -adrenergic receptors and p21rhoA.

This hypothesis was confirmed since we demonstrated that  $\alpha 2$ -adrenergic receptor stimulation leads to the activation of GDP/GTP exchange on p21rhoA characterized by a rapid and transient increase in the p21rhoA-GTP form as previously demonstrated for p21ras (4). This observation directly demonstrates the ability of the  $\alpha 2$ -adrenergic receptor to activate p21rhoA.  $\alpha 2$ -adrenergic stimulation also leads to an increase in the amount of p21rhoA in the particulate fraction of  $\alpha 2$ AF2 preadipocytes. This observation can be interpreted as the result of the translocation of a fraction of rhoA from the cytosol to the plasma membrane as classically described for the GTPases of the rho family (20, 21, 22). It is noticeable that whereas  $\alpha 2$ -adrenergic receptor-stimulated rhoA GTP-loading was maximal within 2 min of stimulation, no significant translocation was observed until 15 min. This suggests that, in our model, rhoA translocation is not required for rhoA activation. Knowing the close relationship between cytoskeleton activity and protein translocation, it is possible that rhoA

translocation is a consequence, rather than a cause, of rhoA-mediated reorganization of actin cytoskeleton. Another possibility is that rhoA translocation is independent of rhoA activation as previously proposed (33). Further investigations will be necessary to test these hypothesis.

In a previous study, we have demonstrated that the  $\alpha 2$ -adrenergic-dependent reorganization of actin cytoskeleton in preadipocytes was suppressed by pertussis toxin pretreatment of preadipocytes (7). Therefore, it is reasonable to propose that the coupling between  $\alpha 2$ -adrenergic receptors and p21rhoA involves an heterotrimeric G protein of the Gi/Go family. Gi proteins have been demonstrated to be involved in the  $\alpha 2$ -adrenergic-dependent activation of p21ras, via the G $\beta\gamma$  subunits of heterotrimeric G proteins (5, 23). Since p21rhoA and p21ras belong to the same superfamily they potentially exhibit very similar mode of regulation. Therefore, the involvement of the G $\beta\gamma$  subunits in the  $\alpha 2$ -adrenergic-dependent activation of p21rhoA could reasonably be suspected. Transient or permanent expression of  $\beta$ ARK-CT, a G $\beta\gamma$  binding protein, have been demonstrated as being a useful strategy to discriminate between  $\alpha$ - and  $\beta\gamma$ - mediated pathways (6). Our results demonstrates that permanent transfection of  $\beta$ ARK-CT in  $\alpha 2$ AF2 preadipocytes almost completely blocks the G $\beta\gamma$ -dependent activation of p21ras/MAPK pathway generated by  $\alpha 2$ -adrenergic-receptor stimulation. This blockade appears to specific of G $\beta\gamma$ , since the G $\beta\gamma$ -independent activation of p21ras/MAPK generated by growth-factor containing serum was not modified. Conversely to the ras/MAPK pathway, the  $\alpha 2$ -adrenergic-dependent regulation of spreading and increase of tyrosyl phosphorylation of pp125FAK were not altered by G $\beta\gamma$ -blockade. These data demonstrate that  $\beta\gamma$  subunits are not involved in the regulation of actin cytoskeleton nor in the activation of the Focal Adhesion kinase, two p21rhoA-dependent controled cellular events. Therefore, based on the action of pertussis toxin, it is likely that the G $\beta\gamma$ -independent coupling between  $\alpha 2$ -adrenergic-receptors and p21rhoA in preadipocytes, involves the  $\alpha i/\alpha o$ -subunits of the heterotrimeric G proteins. We previously showed that  $\alpha 2$ AF2 preadipocytes express the three pertussis toxin-sensitive  $\alpha$  subunits,  $\alpha i 2$ ,  $\alpha i 3$  and  $\alpha o$ , but not  $\alpha i 1$  (24). Further investigations will be necessary to determine which of these subunits are involved in  $\alpha 2$ -adrenergic-dependent activation of p21rhoA.

The existence of a  $G\beta\gamma$ -independent coupling between  $\alpha_2$ -adrenergic receptors and p21rhoA asks the question of its functional consequences in preadipocytes. Modifications of actin cytoskeleton are associated with numerous cellular events such as proliferation, differentiation, motility (25, 26). We have previously demonstrated that  $\alpha_2$ -adrenergic receptor stimulation increases preadipocyte proliferation, an effect which is associated with the tyrosyl-phosphorylation of ERK1, ERK2 MAPK (3), kinases which are dependent upon p21ras activation (27) and are implicated in cell cycle regulation (28). The results of the present study clearly demonstrate that p21rhoA is not involved in the  $\alpha_2$ -adrenergic-dependent activation of p21/MAPK pathway since C3-exoenzyme was without effect on ERK2 activation. However, even though the precise mechanism has not been completely elucidated, it has clearly been demonstrated by several groups that rho proteins are regulators of cell cycle regulation (29, 30). It has indeed been shown that C3-exoenzyme strongly inhibits the growth of several cell types (29) including  $\alpha_2AF_2$  preadipocytes (Bétuing, personal data). Therefore, it is reasonable to think that p21rhoA could be involved in the  $\alpha_2$ -adrenergic-dependent regulation of preadipocyte proliferation via an ERK-independent transduction pathway. Recently, Jinsi-Parimoo et al (31) proposed that p21rhoA is involved in the  $\alpha_2$ -adrenergic stimulation of phospholipase D in PC12 cells. Phospholipase D, is an enzyme involved in a broad spectrum of cellular events including mitogenesis (32). Therefore, it could be proposed that  $\alpha_2$ -adrenergic-dependent regulation of preadipocyte proliferation could involve a p21rhoA-dependent activation of phospholipase D.

In conclusion, this study emphasizes that in preadipocytes, in addition to their involvement in the activation of the  $G\beta\gamma$ -dependent p21ras/MAPK pathway,  $\alpha_2$ -adrenergic-receptors can also activate the p21 rhoA/cytoskeleton pathway in a  $G\beta\gamma$ -independent manner. Control of p21rhoA activity and actin cytoskeleton not only play an important role in cell morphological changes but are also crucial for cell cycle regulation. Therefore, depending on each kind of subunit ( $\beta\gamma$  or  $\alpha_i/\alpha_o$ ) the combined  $G_i/G_o$ -mediated stimulation of p21ras and p21rhoA can cooperate in the mediation of the  $\alpha_2$ -adrenergic-receptor- dependent regulation of preadipocyte proliferation and/or differentiation.



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**Abbreviations:** FAK, focal adhesion kinase; ADP, adenosine diphosphate; ERK, extracellular signal regulated kinase; GDP, guanosine diphosphate; GTP, guanosine triphosphate;  $\beta$ ARK-CT, C-terminal domain of  $\beta$ adrenergic receptor kinase; MAPK, mitogen activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; GST, glutathion S-transferase; PBS, phosphate buffer saline.

## LEGEND OF THE FIGURES.

**Figure 1: C3 exoenzyme catalysed ADP ribosylation in  $\alpha$ 2AF2 preadipocytes.**  $\alpha$ 2AF2 preadipocytes were exposed (C3) or not (control) to 10 $\mu$ g/ml C3 exoenzyme for 24, 48 or 72 hours. Cell lysates were prepared and subjected to *in vitro* C3 exoenzyme catalysed ADP-ribosylation as described in Materials and Methods. (A) Representative experiment. (B) Quantification from three separate experiments. Values correspond to the mean  $\pm$  SEM. Comparison with the control was performed using Student's t test : \*, P<0.05.

**Figure 2: Influence of C3 exoenzyme on  $\alpha$ 2-adrenergic-induced spreading in  $\alpha$ 2AF2 preadipocytes.**  $\alpha$ 2AF2 preadipocytes were treated (C3) or not (control) with C3 exoenzyme (72 hours, 10 $\mu$ g/ml). After serum starvation, control and C3 exoenzyme-treated  $\alpha$ 2AF2 preadipocytes were exposed (+) or not (-) to 1 $\mu$ M UK14304 for 15 minutes. Cell spreading was measured by quantifying the proportion of refringent cells present in a field (mean of five separate fields). Values represent the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*, P<0.05.

**Figure 3: Influence of C3 exoenzyme on  $\alpha$ 2-adrenergic-dependent stress fiber formation in  $\alpha$ 2AF2 preadipocytes.**  $\alpha$ 2AF2 preadipocytes were treated (C,D) or not (A,B) with C3 exoenzyme (72 hours, 10 $\mu$ g/ml). After serum starvation control and C3 exoenzyme-treated  $\alpha$ 2AF2 preadipocytes were exposed (B,D) or not (A,C) to 1 $\mu$ M UK14304 for 15 minutes. Actin filaments were visualized using FITC-labelled phalloidin as described in Materials and Methods. The pictures are representative of at least three separate experiments.

**Figure 4: Influence of C3 exoenzyme on  $\alpha$ 2-adrenergic-stimulated tyrosyl phosphorylations.**  $\alpha$ 2AF2 preadipocytes were treated (C3) or not (control) with C3 exoenzyme (72 hours, 10 $\mu$ g/ml). After serum starvation, control and C3 exoenzyme-treated  $\alpha$ 2AF2 preadipocytes were exposed (+) or not (-) to 1 $\mu$ M UK14304 for 2 minutes. The level of tyrosyl-phosphorylation of pp125FAK (A) and ERK2 (B) was determined and quantified as

described in Materials and Methods. Notice that ERK2 phosphorylation is also associated with a shift (ERK2\*). Values correspond to the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .

**Figure 5: Alpha2-adrenergic-dependent translocation of rhoA in  $\alpha 2AF2$  preadipocytes.**

(A) Cytosolic (CF) and particulate (PF) fractions of non stimulated  $\alpha 2AF2$  preadipocytes were separated and the amount of rhoA was measured by western-blot analysis as described in Materials and Methods. (B)  $\alpha 2AF2$  preadipocytes were exposed (UK) or not (control) to  $1\mu M$  UK14304 for various time. Particulate fraction of control and stimulated  $\alpha 2AF2$  preadipocytes were rapidly separated from the cytosolic fraction and the amount of RhoA as well as  $\alpha i2$  content (after stripping the blot) was measured by western blot analysis as described in Materials and Methods. Values correspond to the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .

**Figure 6: Alpha2-adrenergic stimulation stimulation of GDP/GTP exchange on rhoA.**

[ $^{32}P$ ] labelled  $\alpha 2AF2$  preadipocytes were exposed (UK) or not (control) to  $1\mu M$  UK14304 for various time. The relative proportion of [ $^{32}P$ ] GTP and GDP present in rhoA immunoprecipitate was determined as described in Materials and Methods. (A) Representative experiment. (B) Quantification from three separate experiments. Values correspond to the mean  $\pm$  SEM. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .

**Figure 7: Influence of stable transfection of the C-terminal domain of  $\beta ARK1$  ( $\beta ARK$ -CT) on  $\alpha 2$ -adrenergic-stimulated tyrosyl phosphorylation of ERK2 in  $\alpha 2AF2$  preadipocytes.**

$\alpha 2AF2$  preadipocytes (A) and clone 50 ( $\alpha 2AF2$  preadipocytes stably transfected with  $\beta ARK$ -CT polypeptide) (B) were serum starved and exposed to  $1\mu M$  UK14304 or FCS10% treatment for time indicated. The shift (ERK2\*) and the level of tyrosyl phosphorylation of ERK2 were determined as described in Materials and Methods. Values correspond to the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .

**Figure 8: Influence of the stable transfection of the C-terminal domain of  $\beta$ ARK1 ( $\beta$ ARK-CT) on  $\alpha$ 2-adrenergic-induced spreading in  $\alpha$ 2AF2 preadipocytes.**  $\alpha$ 2AF2 preadipocytes (white bars) and clone 50 ( $\alpha$ 2AF2 preadipocytes stably transfected with  $\beta$ ARK-CT polypeptide) (black bars) were serum-starved and exposed (UK) or not (control) to 1 $\mu$ M UK14304 for 15 minutes. Cell spreading was measured by quantification the proportion of retracted cells present in a field (mean of five separate fields). Values represent the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .

**Figure 9: Influence of the stable transfection of the C-terminal domain of  $\beta$ ARK1 ( $\beta$ ARK-CT) on  $\alpha$ 2-adrenergic-stimulated tyrosyl phosphorylation of pp125FAK in  $\alpha$ 2AF2 preadipocytes.**  $\alpha$ 2AF2 preadipocytes (A) and clone 50 ( $\alpha$ 2AF2 preadipocytes stably transfected with  $\beta$ ARK-CT polypeptide) (B) were serum starved and exposed to 1 $\mu$ M UK14304 or FCS10% treatment for time indicated. The level of tyrosyl phosphorylation of pp125 FAK were determined as described in Materials and Methods. Values correspond to the mean  $\pm$  SEM of three separate experiments. Comparison with the control was performed using Student's t test : \*,  $P < 0.05$ .