ADP-ribosylation factor 6 regulates endothelin-1-induced lipolysis in adipocytes

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ABSTRACT

Endothelin-1 (ET-1) induces lipolysis in adipocytes, where ET-1 chronic exposure results in insulin resistance (IR) through suppression of glucose transporter (GLUT)4 translocation to the plasma membrane and consequently glucose uptake. ARF6 small GTPase, which plays a vital role in cell surface receptors trafficking, has previously been shown to regulate GLUT4 recycling and thereby insulin signalling. ARF6 also plays a role in ET-1 promoted endothelial cell migration. However, ARF6 involvement in ET-1-induced lipolysis in adipocytes is unknown. Therefore, we investigated the role of ARF6 in ET-1-induced lipolysis in 3T3-L1 adipocytes. This was achieved by studying the effect of inhibitors for the activation of ARF6 and other signalling proteins on ET-1 induced lipolysis and ARF6 activation in the adipocytes. Our results indicate that ET-1 induces, through endothelin type A receptor (ET_A), lipolysis, the ARF6 activation and extracellular-signal regulated kinase (ERK) phosphorylation in adipocytes, further ET-1 stimulated lipolysis is inhibited by the inhibitors of ARF6 activation, ERK phosphorylation and dynamin, which is essential for endocytosis. Our studies also revealed that ARF6 acts upstream of ERK in ET-1-induced lipolysis. In summary, we determined that ET-1 activation of ET_A signalled through ARF6, which is crucial for lipolysis. © 2014 Elsevier Inc. All rights reserved.

1. Introduction

An increase in lipolysis, which leads to triglyceride (TG) breakdown and elevated plasma free fatty acids (FFAs), in adipocytes contribute to insulin resistance (IR) through suppressing insulin-stimulated glucose uptake [1]. The potent vasoconstrictor hormone endothelin-1 (ET-1) signals through G-protein coupled receptors (GPCRs) endothelin type A receptor (ET_A) and endothelin type B receptor (ET_B) [2,3]. Through the activation of ET_A, ET-1 can induce lipolysis in adipocytes [4]. ET-1 induces lipolysis through the activation/phosphorylation of ERK1/2, which then leads to the recruitment and activation of lipases and regulatory proteins required for TG hydrolysis [4]. Chronic ET-1 exposure impairs insulin-stimulated glucose uptake by increasing lipolysis, causes IR by depleting phosphatidylinositol

Abbreviations: ARF6, ADP-ribosylation factor 6; ET-1, endothelin-1; ET_A, endothelin type A receptor; ET_B, endothelin type B receptor; GPCR, G-protein coupled receptor; IR, insulin resistance; GLUT4, glucose transporter type 4; ERK, extracellular-signal regulated kinase; TG, triglyceride; FFA, free fatty acids; LCFA, long chain fatty acid; PI(3)P, phoshoinositol 3,4,5-phosphate; HSL, hormone sensitive lipase; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; GEF, guanine nucleotide exchange factor; GAP, GTPase activating factor; J2AR, J2-adrenergic receptor; IHR, luteinizing hormone receptor; PDK3, phosphatidylinositol 3-kinase; GSIS, glucose-stimulated insulin secretion; IBMX, methylisobutyl-xanthine; DMEM, Dulbecco’s modified Eagle’s medium; FCS, foetal calf serum; CS, calf serum; GGA3, Gogi-associated, gamma adaptin ear containing; ARF binding protein 3; PBD, protein binding domain; GST, glutathione S-transferase; PBS, phosphate buffered saline; IgG, immunoglobulin G; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidise; ECL, enhanced chemiluminescence; SD, standard deviation.

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4,5-bisphosphate (PIP2) and suppresses insulin-stimulated long chain fatty acid (LCFA) uptake in adipocytes [5–7]. Plasma ET-1 concentrations are raised in obese, IR and type 2 diabetic (T2D) patients [8,9]. The link between ET-1 and IR requires further attention to combat obesity and T2D.

ADP-ribosylation factors (ARFs) are Ras-related guanosine triphosphate (GTP)-binding proteins of about 21 kDa in size and regulated through activation by guanine nucleotide-exchange factors (GEFs) and inactivation by GTPase-activating proteins (GAPs) [10]. They are major regulators of intracellular membrane trafficking. There are six mammalian ARF proteins (ARFs 1–6) and among them ARF1 and ARF6 are the best characterised and least related [11]. ET-1 has been shown to promote endothelial cell migration through ARF6 activation [12]. ARF6 is also required for internalisation of the GPCRs such as β2-adrenergic receptor (β2AR) and Luteinizing hormone receptor (LHR) and isoproteolen-stimulated lipolysis in adipocytes [13–16]. ARF6 has also been associated with IR, where inactivation has been demonstrated to suppress glucose-stimulated insulin secretion (GSIS) [17,18], insulin signalling [19,20] and GLUT4 recycling to the plasma membrane [21]. Stimulation of β2AR by isoproteolen leads to activation of ARF6, protein kinase A (PKA) and perilipin on lipid droplets, which allows enhanced substrate accessibility and lipolysis by hormone sensitive lipase (HSL) [17]. Lipases such as adipose triacylglycerol lipase (ATGL), TAG hydrolase and adiponu- trin also participate in lipolysis, suggesting many pathways are involved in the regulation of the lipid metabolism.

Dynamin GTPase regulates β2AR and LHR internalisation by causing fission of clathrin coated vesicles [14,16,22]. It has been shown that ARF6 regulates dynamin GTPase activity through NM23–H1 during LHR internalisation [15]. The role of ARFs in the regulation of lipid metabolism through β2AR signalling was established recently [16]. However it is unknown whether ARF6 participate in ET-1 induced lipid metabolism or not. To investigate the role of ARFs in ET-1 induced lipolysis in adipocytes, we utilised glycerol release assays as read out for lipolysis. Chemical inhibitors and membrane permeable inhibitory peptides for ARFs and other signalling components were employed to assess their effect on lipolysis and ARF6 activation in ET-1 stimulated 3T3-L1 adipocytes. Our study here demonstrate the activation of ARF6 in ET-1 stimulated adipocytes and the involvement of activated ARF6 in ET-1 stimulated lipolysis in adipocytes through the ERK-phospho- phorylation and dynamin.

2. Materials and methods

2.1. Materials

Foetal calf serum (FCS), calf serum (CS) and Dulbecco’s modified Eagle’s medium (DMEM) were from Biosera (Uckfield, UK). 8-Br-cAMP, BQ–123, dynasore, ET–1, PD98059, brefeldinA (BFA) and secinH3 were from Abcam (Cambridge, UK). Penetratin, ARF1p-penetratin and ARF6p-penetratin were synthesised by Thermo Fisher Scientific (Cramlington, UK). LY294002 and Q511 were from R&D Systems Europe Ltd. (Abingdon, UK). Dexamethasone, forskolin, insulin, IBMX, rosiglitazone and free glycerol reagent were from Sigma-Aldrich (Poole, UK). Mouse anti-ARFβ antibody was from Santa Cruz biotechnology (Santa Cruz, CA). Anti-ERK1/2 and anti-total ERK1/2 antibodies were from New England Biolabs (Hitchin, UK). All other chemical, unless otherwise specified, were from Sigma-Aldrich (Poole, UK).

2.2. 3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes were grown to confluence in DMEM with high glucose containing 10% CS and PSG (2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin). Two days after reaching to confluence, cells were induced to differentiate by incubating them in the differentiation medium (0.5 mM IBMX, 0.25 μM dexamethasone, 10 μg/ml insulin, 2 μM rosiglitazone in FBM [DMEM with high glucose containing 10% FCS and PSG]) for 2 days and then in FSB containing 10 μg/ml insulin for 2 days [23]. Cells were grown in FSB for 6 further days with FSB medium replacing for every 2 days.

2.3. ARF6-GTP pulldown assay

ARF6 activation was assessed by using the GST-GGA3 protein binding domain (PBD) pulldown assay as described previously [14,24]. The GST–GGA3 PBD fusion protein was purified and coupled to glutathione-sepharose beads (GE Healthcare, Little Chalfont, UK) as described [25]. Differentiated adipocytes in a 10 cm plate were serum starved by washing twice with DMEM and incubating in DMEM containing 0.5% fatty acid free bovine serum albumin (BSA) for 16 h. The cells were then washed twice in ice- cold phosphate-buffered saline (PBS) and lysed at 4 °C for 15 min with 0.5 ml of ARF-GTP pulldown lysis buffer (25 mM Tris–HCl [pH 7.2], 150 mM NaCl, 5 mM MgCl2, 1% NP40, 5% glycerol) and 1% protease inhibitors. The cell lysates were centrifuged at 14,000 × g for 10 min at 4 °C to pellet cellular debris. A 0.4 ml fraction of each lysate was incubated with glutathione-beads coupled to 25 μg of purified GST-GGA3 PBD fusion protein at 4 °C for 2 h. The beads were washed three times with ARF–GTP pulldown wash buffer (50 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 150 mM NaCl and 1% Triton X-100) and boiled in 50 μl × SDS-PAGE loading buffer for 5 min to release the bound protein into buffer. The lysates that were not incubated with the beads (100 μl) were used to assess total ARF6 and ERK1/2, and ERK1/2 phosphorylation. ARF6-GTP bound to the beads and total ARF6, total ERK1/2 and ERK1/2-phospho in the inputs were determined by SDS-PAGE and immunoblotting using the anti-ARF6, anti-ERK1/2 and anti- phospho ERK1/2 antibodies.

2.4. Immunoblotting

Proteins were separated by 12% SDS-PAGE, transferred onto PVDF membrane and immunoblotted using appropriate primary and HRP-conjugated secondary antibodies as described previously [26].

2.5. Inhibition of ARF through penetratin-bound inhibitory peptides

The cell-permeating domain of Drosophila antennapedia protein (penetratin), and the N-myristoylated ARF1 (ARF1p; consists of 2–17 aa of ARF1) and ARF6 (ARF6p; consists of 2–13 aa of ARF6) peptides fused to penetratin, to give the property of membrane permeability, were used in this assay [14,27]. After serum starvation for 16 h, differentiated 3T3-L1 adipocytes were pre-incubated with 5 μM penetratin, ARF1p-penetratin or ARF6p-penetratin for 30 min. ET–1 (10 nM) was then added and incubated the cells for a further 4 h before glycerol content of the media was quantified.

2.6. Glycerol release assay

Stimulation of adipocytes and measurement of glycerol release were performed as described previously [28]. Before glycerol release assay, adipocytes were serum-starved as described above for 10 h. The cells were treated without or with chemical inhibitors before lipolytic stimulation with ET-1 or other stimulants. Lipolysis of triglycerides was quantified through the release of glycerol into the culture medium using the free glycerol reagent [29].
2.7. Statistical analyses

The statistical analysis software program GraphPad Prism was used to determine significance. The results are presented as means ± standard deviation (SD). Statistical significance was assessed by Student’s t-test or one-way ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

3.1. ET-1 stimulates lipolysis in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated with 10 nM ET-1 for various times and glycerol release was determined as the product of lipolysis. As shown in Fig. 1A, a time dependent increase in lipolysis was observed in cells treated with ET-1 when compared to the unstimulated control cells, with maximal lipolysis at 24 h of stimulation (1053.9 ± 40.3 vs 297.8 ± 49.7 nmol/ml in unstimulated cells, P < 0.001). 3T3-L1 adipocytes were then incubated for 4 h with varying concentrations of ET-1 (0–10−12 M) to study dose-dependent effect of ET-1 on lipolysis. The lipolysis increase in a dose dependent manner was observed (Fig. 1B). The maximal lipolysis seen with ET-1 at 10−8 M was six fold higher than that in the control unstimulated cells (491.5 ± 26.3 vs 749.8 ± 5.3 nmol/ml, P < 0.001). Unless otherwise indicated, a standard treatment protocol of 10 nM ET-1 for 4 h was used in further experiments.

To confirm ET-1 signals in adipocytes through ET, R [4], we analysed the effect of ET, R antagonist BQ-123 on ET-1 stimulated lipolysis in 3T3-L1 adipocytes. As shown in Fig. 1C, BQ-123 had no effect on basal lipolysis. However, BQ-123 completely inhibited ET-1 induced lipolysis, confirming that ET-1 induced lipolysis is mediated through ET, R. Since insulin has an anti-lipolytic effect on adipocytes (7), we assessed whether it inhibit ET-1-induced lipolysis (Fig. 1D). The presence of 10 nM insulin resulted in complete inhibition of ET-1 induced lipolysis. The glycerol released in adipocytes treated with 10 nM ET-1 and 10 nM insulin was close to that of basal.

3.2. ET-1 stimulation causes ARF6 and ERK1/2 activation in 3T3-L1 adipocytes

Active ARF6, the GTP-bound form, has been demonstrated to participate in adrenergic stimulated lipolysis in 3T3-L1 adipocytes [16]. The GST-GGA3 PBD specifically binds to the GTP-bound form of ARF, and therefore it has been used in an assay for detecting the ARF activation [14]. We used the GST-GGA3 PBD pulldown assay to assess if ET-1 stimulation of 3T3-L1 adipocytes causes ARF6 activation. 3T3-L1 adipocytes were treated with 10 nM ET-1 for various times and assessed the ARF6-GTP levels. As shown in Fig. 2A, ARF6 was activated at 1 min and maintained activation upto 1 h (5.4 ± 1.3-fold over basal, P < 0.01). The time-dependent assay revealed that the levels of ARF1-GTP were not increased in adipocytes upon stimulation with ET-1 (data not shown), indicating that ET-1 stimulation of 3T3-L1 adipocytes leads to the activation of ARF6 only. ET-1 has also been shown to activate ERK1/2, through the phosphorylation, in 3T3-L1 adipocytes [4]. The same time course assay revealed that ERK1/2 is phosphorylated at 1 min and activation peaked at 5 min (11.6 ± 3.1-fold over basal, P < 0.001), before returning to basal levels at 15 min (Fig. 2C).

Concentration-dependent activation of both ARF6 and ERK1/2 was assessed by incubating 3T3-L1 adipocytes with a variety of ET-1 concentrations (0–10−8 M) for 5 min. As shown in Fig. 2B, ARF6 was activated at 10−10 M, reaching the maximal activation at 10−8 M (6.4 ± 0.1-fold over basal, P < 0.001). ERK1/2 was phosphorylated at 10−10 M, increasing phosphorylation levels to maximal at 10−8 M (6.3 ± 1.2-fold over basal, P < 0.001) (Fig. 2D). These data demonstrate ET-1 induced activation of both ARF6 and ERK1/2 in a concentration-dependent manner. In ET-1 treated cells, ARF6 is
activated and maintained the active form throughout, whereas ERK1/2 is activated and peaked within 5 min.

3.3. ET-1 induced lipolysis is mediated by ARF6, ERK and dynamin

Previous studies suggested the involvement of ARF6, dynamin and ERK signalling proteins in agonist-induced lipolysis in differentiated 3T3-L1 adipocytes [4,16]. To analyse the role of ARF6, ERK1/2 and dynamin in ET-1 induced lipolysis, we made use of the chemical inhibitors specific for these signalling proteins. We first assessed the role of ARF6 in ET-1 induced lipolysis by using membrane permeable ARF6p-penetratin and ARF1p-penetratin peptides, which have been shown to inhibit ARF6 and ARF1 functions, respectively [30]. 3T3-L1 adipocytes were pre-treated with 5 μM penetratin (control), ARF1p-penetratin or ARF6p-penetratin, then stimulated with 10 nM ET-1 and measured the glycerol release. As shown in Fig. 3A, treatment of 3T3-L1 adipocytes with ARF6p-penetratin, but not ARF1p-penetratin, inhibited ET-1 induced lipolysis (294.4 ± 78.4 vs 420.7 ± 36.6 nmol/ml in control treated with no peptide, \( P < 0.01 \)). This indicated that ARF6 is essential for the lipolysis induced by ET-1.

ARF6 is active in the GTP-bound form and inactive in the GDP-bound form. ARF6 is activated by GEFs whereas it is inactivated by GAPs. ARF6 activation can be achieved with secinH3, an inhibitor of the cytohesin family of ARF GEFs but not with BFA, an ARF GEF inhibitor specific for the other ARFs activation [14]. Cells were stimulated with 10 nM ET-1 in the presence of different concentrations (0–100 μM) of secinH3 and measured the glycerol release. As shown in Fig. 3B, secinH3 inhibited ET-1 induced lipolysis by 2.2 fold at 6.25 μM concentration. The glycerol content released from adipocytes treated with 10 nM ET-1 and 6.25 μM secinH3 was lower when compared with that released by 10 nM ET-1 alone treated cells (387.9 ± 59.4 vs 536.7 ± 98.8 nmol/ml, \( P < 0.001 \)). The maximal inhibition of ET-1 induced lipolysis was observed in the presence of 100 μM secinH3 (204.9 ± 26.8 nmol/ml, \( P < 0.001 \)). Conversely, QS11, an inhibitor of ARF GAP, slightly increased glycerol release from 3T3-L1 adipocytes stimulated with 10 nM ET-1 in a dose dependent manner (Fig. 3C), reaching maximal lipolysis at 100 μM QS11 (575.0 ± 18.21 vs 492.4 ± 10.05 nmol/ml, \( P < 0.001 \)). However, the ARF GAP inhibitor BFA had no effect on ET-1 induced lipolysis (373.1 ± 38.45 nmol/ml vs 382.5 ± 15.32 nmol/ml in control, \( P < 0.001 \) [Fig. 3D]). Previous studies indicated that agonist (ET-1, EGF, hCG etc) activation of ARF6 is mediated by P13K [12,14,31], which is inhibitable by compounds such as LY290004. As shown in Fig. 3D, inhibition of P13K with LY294002 significantly reduced the lipolysis stimulated by ET-1 (160.8 ± 17.68 vs 382.5 ± 15.32 nmol/ml in control, \( P < 0.001 \)).

Next, cells were preincubated with different concentrations (0–100 μM) of the MEK-1 inhibitor, PD98059, for 1 h and measured 10 nM ET-1 stimulated glycoler release. As shown in Fig. 3E, the glycerol released from adipocytes stimulated with 10 nM ET-1 in the presence of 6.25 μM PD98059 was lower than that released by stimulation of the cells with ET-1 alone (457.8 ± 31.9 vs 597.8 ± 58.7 nmol/ml, \( P < 0.01 \)), the maximal inhibition of ET-1 induced lipolysis was observed in presence of 50 μM PD98059 (353.3 ± 38.4 nmol/ml, \( P < 0.001 \)). In presence of 6.25 μM PD98059, there was a 1.5 fold inhibition of ET-1 induced lipolysis. The role of dynamin in ET-1 induced lipolysis was assessed by measuring 10 nM ET-1 stimulated glycerol from 3T3-L1 adipocytes in the presence of different concentrations (0–100 μM) of dynasore, a dynamin chemical inhibitor. As shown in Fig. 3F, the lipolysis in adipocytes treated with 10 nM ET-1 and 6.25 μM dynasore showed a 2.2 fold inhibition compared to controls.

Fig. 2. ARF6 and ERK1/2 are activated in 3T3-L1 adipocytes by ET-1 stimulation. Differentiated 3T3-L1 adipocytes were serum starved and treated with ET-1 for various times (10 nM for 0–60 min (A and C) or at varying concentrations (0.1–1 μM) for 5 min (B and D). The cells were then lysed and the cell lysates were incubated with GST-GGA3 PBD resin. The protein bound to the resin was analysed by immunoblot using an anti-ARF6 antibody to analyse the levels of ARF6-GTP. The cell lysates that not incubated with the GST-beads were also immunoblotted with an anti-ARF6 antibody, an anti-ERK antibody and an anti-ERK phospho specific antibody for analysing total ARF6, total ERK1/2 and the phosphorylated ERK1/2 (ERK1/2-p). Blot images are representative of three separate experiments. Densitometric analysis of ARF6-GTP (A and B, lower panel) and ERK1/2-p (C and D, lower panel) is shown as a histogram, after normalizing to the expression of total ARF6 and total ERK1/2, respectively, present in the sample. *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \) compared with control.
dynasore was lower when compared with that in ET-1 alone treated cells (293.7 ± 43.7 vs 473.6 ± 73.9 nmol/ml, P < 0.01), where maximal inhibition was observed at 100 μM (144.0 ± 47.2 nmol/ml, P < 0.001). In the presence of 6.25 μM dynasore, there was a 2.6 fold inhibition of ET-1 induced lipolysis.

3.4. ARF6 acts upstream of ERK1/2 in ET-1 induced lipolysis

The activation of both ARF6 and ERK 1/2 was then assessed in ET-1 stimulated 3T3-adipocytes. Chemical inhibitors were again utilised to determine the sequence of the signalling pathway following ET,R activation. 3T3-L1 adipocytes were pre-incubated with secinH3 (50 μM) for 2 h, PD 98059, dynasore, BFA and LY429002 (50 μM) for 1 h. Cells were then stimulated with ET-1 (10 nM) for 5 min before lysing and subjecting to the GST C3A PBD pulldown assay. As shown in Fig. 4, secinH3, but not BFA, prevented the activation of ARF6 in ET-1 stimulated adipocytes (1.1 ± 0.2-fold vs 6.0 ± 0.6-fold, P < 0.001), and also ERK1/2 (1.4 ± 0.3-fold vs 6.4 ± 1.4-fold, P < 0.01). ERK1/2 phosphorylation was also prevented through treatment with PD98059 (1.3 ± 0.2-fold vs ET-1 alone, P < 0.01), but the MEK-1 inhibitor did not prevent ARF activation (6.0 ± 1.0-fold vs ET-1 alone). These observations imply that ARF6 could be upstream to ERK1/2 in ET-1 induced lipolysis signalling. Inhibition of PI3K also prevented the activation of ARF6 and ERK1/2, which is consistent with our previous observation that PI3K activates ARF6 in growth factor stimulated cells [31]. This would imply a role for PI3K in ET-1 induced lipolysis, acting upstream of ARF6 and ERK1/2. Inhibition of dynamin had no effect on either ARF6 or ERK1/2 activation.

3.5. Inhibition of ARF6 activation does not affect lipolysis induced by 8-Br-cAMP, IBMX or forskolin

We finally studied whether ARF6 play a role in ET-1-induced lipolysis by regulating ET,R downstream signalling pathway. For this purpose, the effect of inhibition of ARF6 activation on lipolysis stimulated by directly activating the signalling pathway downstream of ET,R was assessed. ET,R is a Gs coupled GPCR and therefore it stimulates cAMP production upon activation. A cell-membrane permeable cAMP analogue 8-Br-cAMP (1 mM), the phosphodiesterase (PDE) inhibitor IBMX (0.5 mM) and the adenyl cyclase activator forskolin (10 μM), which stimulate cAMP production independent of ET,R activation, were used to induce lipolysis [4]. 3T3-L1 adipocytes were treated with 8-Br-cAMP, IBMX and forskolin separately in the presence or absence of secinH3 (50 μM) and glycerol content was quantified. As shown in Fig. 5, the lipolysis induced by these three chemicals is not affected by ARF6 inactivation by secinH3, indicating that ARF6 regulates ET-1 induced lipolysis not through ET,R downstream Gs-coupled signalling pathway.
**4. Discussion**

This study was performed to investigate the role of ARF6 in ET-1 induced lipolysis of 3T3-L1 adipocytes and to determine the signalling pathways of this process. We have demonstrated the following in this study. First, ETA antagonist and insulin inhibit ET-1 induced lipolysis. Second, ARF6 and ERK1/2 activate upon ET-1 stimulation in 3T3-L1 adipocytes. Third, chemical inhibition of both ARF6 and ERK1/2 result in decreased ET-1 induced lipolysis. Fourth, ARF6 activation precedes the ERK1/2 activation in ET-1 signalling. Fifth, inactivation of dynamin inhibits ET-1 induced lipolysis and signals through different pathways to that of ARF6/ERK1/2. Sixth, chemical inhibition of ARF6 does not affect lipolysis induced by activators (8-Br-cAMP, IBMX and forskolin) of the pathway downstream of ETAR. Collectively, these data demonstrate that ARF6 is required for ET-1 induced lipolysis in adipocytes.

Inhibition of ARF1 and ARF6 through the use of membrane permeable inhibitory ARF1 and ARF6 peptides and chemical inhibitors (secinH3 and BFA) in our study revealed that ARF6, but not ARF1, was essential for ET-1 stimulated lipolysis. By using these peptides previously and chemical inhibitors, ARF6 has been demonstrated to participate in LHR receptor internalisation [14]. Furthermore, ARF6 has been shown to be required for isoprostane induced lipolysis [16] and ERK1/2 phosphorylation is involved in lipolysis stimulated by ET-1 [4]. Furthermore, secinH3, an inhibitor of ARF6 activation, treatment of adipocytes in this study lead to a significant decrease in ET-1 induced lipolysis and ERK1/2 phosphorylation. However PD98059, an ERK inhibitor, treatment of adipocytes reduced ET-1 stimulated lipolysis but had no effect on ET-1 stimulated ARF6 activation, indicating that ARF6 regulates ET-1 induced lipolysis through ERK1/2 phosphorylation. ET-1 stimulation of 3T3-L1 adipocytes led to increased lipolysis, transient ERK1/2 phosphorylation and sustained ARF6 activation. A previous study also reported transient phosphorylation of ERK1/2 in ET-1 stimulated preadipocytes [4]. Although exactly how transiently phosphorylated ERK1/2 regulate sustained lipolysis is unknown, it is possible that the phosphorylated ERK1/2 may be required only to initiate or recruit the signalling cascade that leads to lipolysis. In contrast, ARF6 may activate not only ERK1/2 but also several components within the signalling cascade and additional signalling components (for example dynamin) involved in lipolysis and therefore its sustained activation allow sustained lipolysis.

The involvement of dynamin in ET-1 stimulated lipolysis was also established in this study by using a dynamin chemical inhibitor dynasore, which inhibited ET-1 induced lipolysis but not the ARF6 activation or ERK1/2 phosphorylation. Dynamin is involved in cell surface receptor internalisation, in which its function is to regulate vesicle budding from coated pits [16]. It has been shown previously that dynamin is required for β2AR and LHR internalisation [14,22]. Dynamin also known to function down stream of ERK during preadipocyte migration [32]. If dynamin participates in ET-1 induced lipolysis by regulating ETAR internalisation, then its inhibition should effect not only ET-1 induced lipolysis but also the activity of ARF6 and ERK1/2, which function downstream of ETAR.

**Fig. 4.** ARF6 acts upstream of ERK 1/2 in ET-1 induced lipolysis. 3T3-L1 adipocytes were serum starved for 16 h and preincubated with SecinH3 (50 μM) for 2 h [14], and PD98059 (50 μM), Dynasore (50 μM), BFA (50 μM) or LY294002 (50 μM) for 1 h. Cells were then stimulated with ET-1 for 5 min. ARF6-GTP and ERK1/2-p in adipocytes neither pre-incubated nor stimulated with ET-1 were considered as basal activities. The cells were then lysed and a fraction of the cell lysate was incubated with GST-GGA3 PRD resin to analyse the levels of ARF6-GTP. The remaining cell lysate was used for analysis of total ARF, total ERK1/2 and the phosphorylated ERK1/2 (ERK1/2-p). (A) Total and the activated ARF6 (ARF6-GTP) were analysed by immunoblotting using an anti-ARF6 monoclonal antibody. (B) Total and ERK1/2-p were analysed by immunoblotting using an anti-ERK1/2 polyclonal antibody and an anti-ERK1/2-phospho polyclonal antibody, respectively. Blot images are representative of three separate experiments. Densitometric analysis of ARF6-GTP (A, lower panel) and ERK1/2-p (B, lower panel) is shown as a histogram after normalizing to the expression of total ARF6 and total ERK1/2, respectively, present in the sample. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control, **P < 0.01 and ***P < 0.001 compared with ET-1 alone.

**Fig. 5.** Inhibition of ARF6 activation does not affect lipolysis stimulated by 8-bromo-cAMP, IBMX or forskolin. 3T3-L1 adipocytes were serum starved for 16 h, preincubated for 2 h in the absence or presence of 50 μM secinH3 [14] and then treated with 8-bromo-cAMP, IBMX or forskolin. Glycerol concentration in the medium was measured. The results are means ± S.D of triplicate measurements and are representative of those for 3 separate experiments (**P < 0.001 compared with none).
Since, in this study, dynamin inhibition affects ET-1 stimulated lipolysis but had no effect on either ARF6 activation or ERK1/2 phosphorylation, it is possible that dynamin functions downstream of ERK in ET-1 induced lipolysis.

To determine the sequence of signalling following ET-1 stimulation that leads to lipolysis, chemical inhibitors of various signalling molecules were utilised. SecinH3, which affects ARF6 activation by inhibiting the cytohesin family of ARF GEFs, treatment resulted in inhibition of both ARF6 and ERK1/2 activation. Through the use of the MEK-1 inhibitor, ERK1/2 activation was understood to act downstream of ARF6 activation, which was unaffected by ERK1/2 inhibition. We have shown previously that ARF6 is activated through PI3K [14,31]. This is because the cytohesin family of ARF GEFs translocate from the cytosol to the plasma membrane in a PI3K-dependent manner, where they activate ARF6 [33,34]. Inhibition of PI3K by LY294002 treatment, prevented ARF6 and ERK1/2 activation in ET-1 stimulated cells. This is consistent with the role of PI3K in ARF6 activation. Ligand binding to GPCR initiates subsequent signalling, which is terminated by receptor internalisation and desensitisation [35]. Chronic ET-1 exposure induces IR through PIPLC depletion but suppresses LCFA uptake independent of the PIP2 levels [7]. However, the inhibition of ERK activation prevents ET-1 suppression of LCFA uptake, indicating that ET-1 suppresses LCFA through the ERK dependent pathway [7]. In conclusion, the findings here suggest ET-1 activation of ET\(_{\text{A}}\)R leads to activation of ARF6, which subsequently activates ERK1/2. The activated ET\(_{\text{A}}\)R then inhibits LCFA uptake and induces signalling upstream to TG hydrolysis. As shown in this study, dynamin inhibition with chemical inhibitor dynasore affected the ET-1 induced lipolysis. However, dynasore had no effect on the activation of ARF6 or ERK1/2 in ET-1 stimulated cells. Previous studies indicate that dynamin functions downstream of ARF6 and ERK [14,20,32]. There it is possible that dynamin is involved downstream ARF6 in ET-1 induced lipolysis.

Considering the results described in this study, we proposed a model including ARF6 activation and subsequent ERK1/2 activation within the ET-1 lipolysis pathway (Fig. 6). Following the activation of ET\(_{\text{A}}\)R by ET-1, cytohesins recruits to the plasma membrane in a PI3K dependent manner and activate ARF6, which in turn activates ERK1/2 through phosphorylation. The activated ERK1/2 then phosphorylates lipid droplet associated proteins and lipases, leading to dynamic changes in those proteins location and activity required for the TG hydrolysis [4]. In conclusion, the present study demonstrates the necessity of ARF6, dynamin and ERK1/2 activation within the ET-1 induced lipolysis. Further studies on the involvement of ARF6/ERK in regulation of lipid droplet associated proteins required for ET-1 induced lipolysis would further knowledge of the role of ARF6 in lipid metabolism and the close link between development of obesity, insulin resistance and T2DM.

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References


