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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_AR), 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-indcued lipolysis. In summary, we determined that ET-1 activation of ET_AR signalled through ARF6, which is crucial for lipolysis.

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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_AR) and endothelin type B receptor (ET_BR) [2,3]. Through the activation of ET_AR, 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_AR, endothelin type A receptor; ET_BR, 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; PIP2, phosphoinositol 4,5-phosphate; HSL, hormone sensitive lipase; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; GEF, guanine nucleotide exchange factor; GAP, GTPase activating factor; β2AR, β2-adrenergic receptor; LHR, luteinizing hormone receptor; PI3K, phosphatidylinositol 3-kinase; GSIS, glucose-stimulated insulin secretion; IBMX, methylisobutylxanthine; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; CS, calf serum; GGA3, Golgi-associated, gamma adaptin ear containing, ARF binding protein 3; PBD, protein binding domain; GST, glutathione S-transferase; PBS, phosphate buffered saline; IgG, immunoglubulin 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 (PIP_2) 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 (β_2AR) and Luteinizing hormone receptor (LHR) and isoproterenol-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 β_2AR by isoproterenol leads to activation of ARF6, protein kinase A (PKA) and perillipin 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 adiponutrin also participate in lipolysis, suggesting many pathways are involved in the regulation of the lipid metabolism.

Dynamin GTPase regulates β_2AR 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 ARF6 in the regulation of lipid metabolism through β_2AR 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 ARF6 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 analyse 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-phosphorylation 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 QS11 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-Arf6 antibody was from Santa Cruz biotechnology (Santa Cruz, CA). Anti-pERK1/2 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 FSM [DMEM with high glucose containing 10% FCS and PSG]) for 2 days and then in FSM containing 10 μg/ml insulin for 2 days [23]. Cells were grown in FSM for 6 further days with FSM 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 $MgCl_2$, 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 $MgCl_2$, 150 mM NaCl and 1% Triton X-100) and boiled in 50 μl 1× 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 Drosphila 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 16 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].

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