Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes

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Abstract Insulin acutely stimulated glucose uptake in rat primary brown adipocytes in a PI3-kinase-dependent but p70S6-kinase-independent manner. Since Akt represents an intermediate step between these kinases, this study investigated the contribution of Akt to insulin-induced glucose uptake by the use of a chemical compound, ML-9, as well as by transfection with a dominant-negative form of Akt (ΔAkt). Pretreatment with ML-9 for 10 min completely inhibited insulin stimulation of (1) Akt kinase activity, (2) Akt phosphorylation on the regulatory residue Ser473 but not on Thr308, and (3) mobility shift in Akt1 and Akt2. However, ML-9 did not affect insulin-stimulated PI3-kinase nor PKCζ activities. In consequence, ML-9 precluded insulin stimulation of glucose uptake and GLUT4 translocation to plasma membrane (determined by Western blot), without any effect on the basal glucose uptake. Moreover, ΔAkt impaired insulin stimulation of glucose uptake and GFP-tagged GLUT4 translocation to plasma membrane in transiently transfected immortalised brown adipocytes and HeLa cells, respectively. Furthermore, ML-9 treatment for 6 h down-regulated insulin-induced GLUT4 mRNA accumulation, without affecting GLUT1 expression, in a similar fashion as LY294002. Indeed, co-transfection of brown adipocytes with ΔAkt precluded the transactivation of GLUT4-CAT promoter by insulin in a similar fashion as a dominant-negative form of PI3-kinase. Our results indicate that activation of Akt may be an essential requirement for insulin regulation of glucose uptake and GLUT4 gene expression in brown adipocytes.

Key words: Glucose uptake; GLUT4 translocation; GLUT4 gene expression; Akt; Insulin

1. Introduction

Brown adipose tissue is a target tissue for insulin action, specially during late foetal development when insulin promotes both adipogenic and thermogenic differentiation of primary cells, as well as displays survival effects [1–3]. Glucose transport in foetal brown adipocytes is maintained mainly by the activity of the insulin-regulated glucose transporter GLUT4, although the ubiquitous GLUT1 glucose transporter is often expressed at appreciable levels together with GLUT4. During foetal life, GLUT1 is expressed at high level in rat brown adipose tissue, although GLUT4 mRNA expression increases greatly at day 22 of foetal development concomitant to the onset of differentiation of the tissue [1]. Acute insulin treatment stimulates glucose transport in adipocytes and myocytes largely by mediating translocation of GLUT4 from an intracellular compartment to the plasma membrane, as reviewed [4]. It is well established that activation of phosphatidylinositol (PI)3-kinase mediates GLUT4 redistribution to the plasma membrane; overexpression of the catalytic subunit of p110 produced GLUT4 translocation and increased glucose uptake [5,6], and inhibition of PI3-kinase (either by chemical inhibitors, or by microinjection of blocking p85 protein, or by transfection with a dominant-negative mutant of p85) precluded insulin-induced GLUT4 translocation [7,8]. The targets of PI3-kinase action are likewise controversial. Two classes of Ser/Thr kinases have been proposed to act downstream of PI3-kinase, Akt/PKB and the atypical protein kinase (PK) C isoforms ζ and λ (PKC ζ/λ). Expression of a constitutively active, membrane-bound form of Akt, using either stable or inducible expression systems, resulted in persistent localisation of GLUT4 at the plasma membrane and increased glucose uptake in 3T3-L1 adipocytes [9,10] but did not promote GLUT4 translocation or glucose transport in L6 myotubes [11]. Experiments involving expression of a dominant-negative Akt mutant are also controversial since both inhibition of insulin-stimulated GLUT4 translocation [12] or no effect have been described [13]. Very recently, ML-9 has been characterised as a chemical inhibitor of Akt activity, that prevents some of the metabolic effects of insulin in isolated rat fat cells [14]. Expression of PKCζ or λ are also reported to induce GLUT4 translocation, whereas expression of a dominant-interfering PKCζ inhibited GLUT4 translocation in 3T3L1 cells [15,16]. Our previous work showed that PI3-kinase and PKCζ could be involved in IGF-I/insulin-stimulation of glucose uptake in foetal brown adipocyte primary cultures [17,18]. Meanwhile Akt activation seems to be an absolute requirement for insulin rescue from apoptosis for these cells [19], the implication of Akt in insulin-induced glucose uptake in brown adipocytes has not been reported yet.

Although in vivo studies clearly indicate that GLUT4 expression in insulin-responsive tissues is under insulin and/or metabolic control [20,21], experiments using cultured fat cells

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Abbreviations: BSA, bovine serum albumin; MEM, minimal essential medium; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; FCS, foetal calf serum; PK, protein kinase; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; gal, galactosidase

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had failed to establish a stimulatory role of insulin in GLUT4 expression [22], and a positive effect of insulin on GLUT4 gene expression had only been observed when dexamethasone was also present [23,24]. However, in rat cardiomyocytes there are evidences of a direct effect of insulin on GLUT4 transcription [25]. In this regard, we have previously described that insulin up-regulated GLUT4 gene expression in brown adipocytes in a PI3-kinase-dependent manner [26]. Furthermore, Akt mediated the expression of certain genes, including GLUT3 and GLUT1 [27,28]. Whether Akt could be mediating chronic insulin effects on GLUT4 gene expression remains to be established. The studies described herein propose that insulin regulation on both glucose uptake and GLUT4 gene expression in foetal brown adipocytes is produced throughout the activation of Akt.

2. Materials and methods

2.1. Materials

1-(5-Chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine, also known as ML-9, was purchased from Alexis Co (Switzerland). LY294002 and rapamycin were from Calbiochem (Calbiochem-Novabiochem Intl, La Jolla, CA, USA). Insulin and bovine serum albumin (BSA) (fraction V, essentially fatty acid free) were from Sigma Chemical Co (St. Louis, MO, USA). Foetal calf serum (FCS), phosphate-buffered saline (PBS) and culture media were from Imperial Laboratories (Hampshire, UK). Trizol was from Gibco BRL (Life Technologies). Nylon membranes were GeneScreen™ (NEN Research Products, Boston, MA, USA). Autoradiographic films were Kodak X-O-MAT/AR (Eastman Kodak Co, Rochester, NY, USA). 2-Deoxy-D-[1-^{3}H]glucose (11.0 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{14}C]chloramphenicol and the multiprimer DNA-labelling system kit were purchased from Amersham (Aylesbury, UK). All other reagents used were of the purest grade available. The cDNAs used as probes were GLUT4/GLUT1 [29]. The MBS mammalian transfection kit and pCMVβ-galactosidase (β-gal) were supplied by Stratagene. The rabbit anti-GLUT1 and GLUT4 antibodies were supplied by Chemicon Inc (Tamacula, CA, USA). The anti-Caveolin-1 (N-20) antibody was from Santa Cruz Biotechnology (Palo Alto, CA, USA). The anti-phospho-specific Akt (Ser473) and phospho-Akt (Thr308) antibodies, the anti-phospho-specific p70S6-kinase (Thr421/Ser424) antibody and the anti-total Akt antibody were purchased by New England Biolabs (Beverly, MA, USA). The antibodies against Akt1 and Akt2 were from Upstate Biotechnology (Lake Placid, NY, USA). The anti-PKCζ antibody was purchased from Gibco BRL (Life Technologies). For anti-Tyr(P) immunoprecipitation, a monoclonal antibody (Py72) was the generous gift of Dr. E. Rozengurt (Imperial Cancer Research Foundation, London, UK).

2.2. Cell culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day Wistar rat foetuses and isolated by collagenase dispersion as described [1]. Cells were plated at 1×10^{6} cells/60 mm or at 4×10^{6} cells/100-mm diameter tissue culture dishes in minimal essential medium with Earle's salts (MEM) supplemented with 10% FCS. After 4 h of culture at 37°C, cells were rinsed twice with PBS and 70% of the initial cells attached to the dish forming a monolayer. Cells were maintained for 20 h in a serum-free medium supplemented with 0.2% (w/v) BSA before starting different treatments. For transfection experiments with primary brown adipocytes, cells were cultured for 24 h in the presence of 10% FCS prior to transfection. For transfection experiments we have also used a SV40 large T antigen-immortalised brown adipocyte cell line (MB 4.9.2) and HeLa cells, cultured as previously described [3].

2.3. Measurement of the glucose transport

Glucose transport was measured in duplicated dishes from four independent experiments as previously described [17].

2.4. Subcellular fractionation

Cells were washed with ice-cold PBS and scraped in homogenisation buffer containing: 20 mM Tris–HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 10 min incubation cells were homogenised with 30 strokes of a Dounce-homogenised using a tight-fitting pestle. Nuclei were pelleted by centrifugation at $500\times g$ for 5 min, and the low speed supernatant was centrifuged at $100\,000\times g$ for 30 min. The high speed supernatant constituted the internal membrane fraction. The pellet was washed three times and extracted in ice-cold homogenisation buffer containing 1% Triton X-100 for 60 min. The Triton-soluble component (plasma membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at $100\,000\times g$ for 15 min. Internal and plasma membrane fractions were kept at −70°C before protein quantification and Western blotting with GLUT4, GLUT1 and Caveolin-1 antibodies.

2.5. Western blotting

Cells were lysed as previously described [26] and cellular proteins (20 μg) were subjected to SDS–PAGE, transferred to Immobilon membranes, blocked and incubated overnight with the primary antibodies in 0.05% Tween-20, 1% non-fat dried milk in 10 mM Tris–HCl and 150 mM NaCl, pH 7.5. Immunoreactive bands were visualised using the enhanced chemiluminescence (ECL-plus) Western blotting protocol (Amersham Pharmacia Biotech). Protein determination was performed by the Bradford dye method, using Bio-Rad reagent and BSA as a standard [30].

2.6. PI3-kinase activity

PI3-kinase activity was measured in the anti-Tyr(P) immunoprecipitates by in vitro phosphorylation of PI as described [17].

2.7. PKCζ and PKB/Akt activities

Cells were extracted with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 1 μM PMSF, 25 μg/ml leupeptin and 25 μg/ml aprotinin) and immunoprecipitated with an anti-PKCζ or anti-total Akt antiserum [17]. Immune complexes were washed five times with ice-cold lysis buffer with 0.5 M NaCl and two times with kinase buffer (35 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 1 μM Na_3VO_4). The kinase reaction was performed in buffer containing 1 μCi of [γ^{32}P]ATP, 60 μM ATP, and 1 μg of MBP as a substrate for 30 min at 30°C, and was terminated by the addition of 4×SDS–PAGE sample buffer followed by boiling for 5 min at 95°C [31]. Samples were resolved in 12% SDS–PAGE and gels were dried out and subjected to autoradiography.

2.8. RNA extraction and analysis

For Northern blot analysis of RNA, at the end of the culture time, cells were washed twice with ice-cold PBS, and lysed directly with Trizol following the protocol supplied by the manufacturer for total RNA isolation. Total cellular RNA (10 μg) was subjected to Northern blot analysis, i.e. electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen™ and cross-linked to the membranes by UV light. Hybridisation was in 0.25 mM NaHP04, pH 7.2, 0.25 M NaCl, 100 μg/ml denatured salmon sperm DNA, 7% SDS and 50% deionised formamide, containing denatured ^{32}P-labelled cDNA (10^{6} cpm/ml) for 24 h, at 42°C, as previously described [26].

2.9. Transfection conditions

The following constructs were used for transfection experiments: pSG5-PKB-CAAX encodes an Akt protein with dominant-negative activity (ΔAkt). This construct is expressed under the control of the early SV40 promoter for eukaryotic expression and was a generous gift of B.M.Th. Burgering (Utrecht, The Netherlands) [31]. The dominant-negative p85 construct (Srα-Δp85), a protein that lacks the inter-SH2 region required for binding to p110, thereby blocking activation of p110 was kindly provided by M. Kasuga (Kobe, Japan) [32]. GLUT4-CAT(chloramphenicol acetyltransferase) (where the CAT reporter gene is under the control of a 2400 bp full-length 5′-flanking region of rat GLUT4 promoter, from positions −2212 to +164 relative to the transcription start site) was kindly provided by A. Zorzano (Barcelona, Spain) [33]. GFP (green fluorescent protein)-tagged GLUT4 was kindly provided by J. Tavaré (Bristol, UK) [34]. Primary brown adipocytes were cultured for 24 h in the presence of 10% FCS and then, transiently transfected according to the calcium phosphate-mediated protocol with 10 μg of GLUT4-CAT alone or combined

with 10 μg of ΔAkt or Δp85 together with 2 μg of pCMVβ-gal (to monitor transfection efficiency). After 4 h of incubation, cells were shocked with 3 ml of 15% glycerol–PBS for 2 min, washed, and then fed with serum-free medium (either in the absence or presence of insulin) for 24 h. Immortalised brown adipocytes (MB 4.9.2) or HeLa cells cultured in 10% FCS were transiently transfected with 10 μg of ΔAkt and/or 10 μg GFP-GLUT4 as described above, using a GFP vector to monitor transfection efficiency when required. Then cells were maintained for 24 h in 10% FCS, serum-starved overnight, and the stimulated or not with insulin for 30 min.

2.10. CAT determination

After GLUT4-CAT transfection as described above, cells were cultured for 24 h in a serum-free medium either in the absence or in the presence of insulin, and then cells were harvested, and lysates were prepared for CAT and β-gal activity assays, as previously described [26]. The amount of substrate acetylated was directly quantified with a radioimaging device (Fujifilm BAS-1000, Japan). CAT enzyme activity was expressed as a percentage of [^{14}C]chloramphenicol acetylated normalised to the internal control β-gal (assayed according to the Stratagene protocol). Transfections were performed from four independent experiments.

2.11. Translocation of GFP-GLUT4 to the plasma membrane

HeLa cells after GFP-tagged GLUT4 transfection as described above were overnight serum-deprived and incubated with 500 nM insulin for 30 min. Then, cells were analysed by fluorescence microscopy. Cells were scored as positive for GLUT4 translocation if they were observed to have a ring of fluorescence at the periphery. Different fields were read blind by two independent investigators.

3. Results

3.1. Akt mediates insulin stimulation of glucose uptake and GLUT4 translocation to the plasma membrane in brown adipocytes

The fact that glucose transport is induced in foetal brown adipocytes upon insulin stimulation [26], prompted us to investigate whether this effect could be blocked by chemical inhibitors of the insulin signalling pathway in those cells. Cells were serum-deprived for 20 h and further treated or not with 10 μM LY294002 or 25 ng/ml rapamycin for 30 min before being stimulated with insulin (10 nM) for further 30 min. Glucose uptake was measured during the last 10 min of culture by incorporation of 2-deoxy-D-[1-^{3}H]glucose into cells and results are expressed as disintegrations per min/1×10^6 cells (Fig. 1A). Cells treated with insulin showed an increase of 3-fold in glucose incorporation, compared with untreated cells. The compound LY294002, that inhibited insulin-stimulated PI3-kinase activity and consequently the phosphorylation of targets downstream PI3-kinase, such as Akt and p70S6-kinase (Fig. 1B), completely abolished insulin effects on glucose uptake. The protein p70S6-kinase is an effector of PI3-kinase and is also phosphorylated in response to insulin. However, blocking p70S6-kinase phosphorylation with rapamycin, did not modify insulin-stimulated glucose incorporation (Fig. 1). These results indicate that insulin increases glucose uptake through a pathway that involves PI3-kinase and downstream targets others than p70S6-kinase, in brown adipocytes.

Among the known targets downstream PI3-kinase, Akt and PKCζ have been reported to participate in insulin-induced glucose transport [7,12,15]. In order to determine the significance of both kinases in the insulin pathway leading to glucose incorporation in brown adipocytes we tested a compound, ML-9, that has been recently characterised as an inhibitor of Akt activity [14]. We first determined the effect

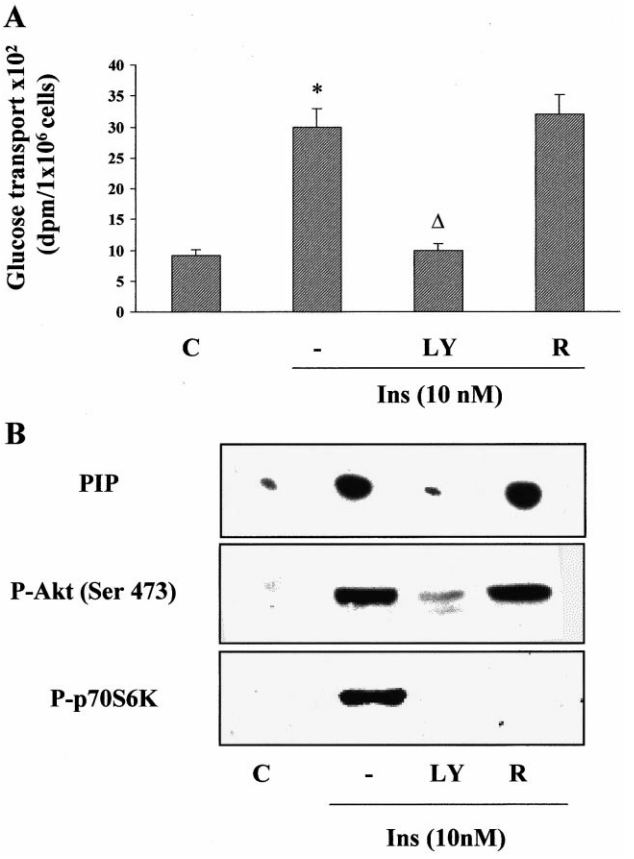

Fig. 1. LY294002 but not rapamycin inhibits insulin-stimulated glucose uptake. A: Rat primary brown adipocytes were treated or not with 10 μM LY294002 (LY) or 25 ng/ml rapamycin (R) for 30 min before being stimulated with insulin (10 nM) for a further 30 min. Glucose uptake was measured the last 10 min by incorporation of 2-deoxy-D-[1-^{3}H]glucose into the cells. Results are expressed as disintegration per min per 1×10^6 cells and are the mean ± S.E.M. ($n=8$) for duplicate samples from four independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test, where differences between values in the presence of insulin vs. control is represented by (*) and differences between values in the presence of insulin plus LY vs. insulin is represented by ($^\Delta$);*,$^\Delta P<0.01$. B: Brown adipocytes were treated as above except that insulin stimulation was for 5 min. Cells were then lysed and proteins assayed for phospho-Akt(Ser473) and phospho-p70S6 kinase levels by Western blot, or for PI3-kinase activity after immunoprecipitation with anti-Tyr(P) antibody.

of this inhibitor on insulin-stimulated PI3-kinase pathway in brown adipocytes. Serum-starved cells were preincubated for 10 min with different doses of ML-9 before being treated with insulin, and lysates were analysed for PI3-kinase, PKCζ and Akt activities as well as for phosphorylation of Akt and p70S6-kinase. Cells were immunoprecipitated with anti-Tyr(P), anti-PKCζ or anti-total Akt antibodies and determination of the enzymatic activities was performed. As shown in Fig. 2A, the stimulation of PI3-kinase and PKCζ activities in response to insulin was not affected by pretreatment with ML-9, however insulin-induced Akt activity was completely abolished in the presence of the inhibitor (Fig. 2B). In addition, cell lysates were analysed by Western blotting with the anti-phospho Akt antibodies for both regulatory residues Ser473 and Thr308, as well as with the total and isoform-specific Akt antibodies. Upon insulin stimulation, Akt/PKB was highly

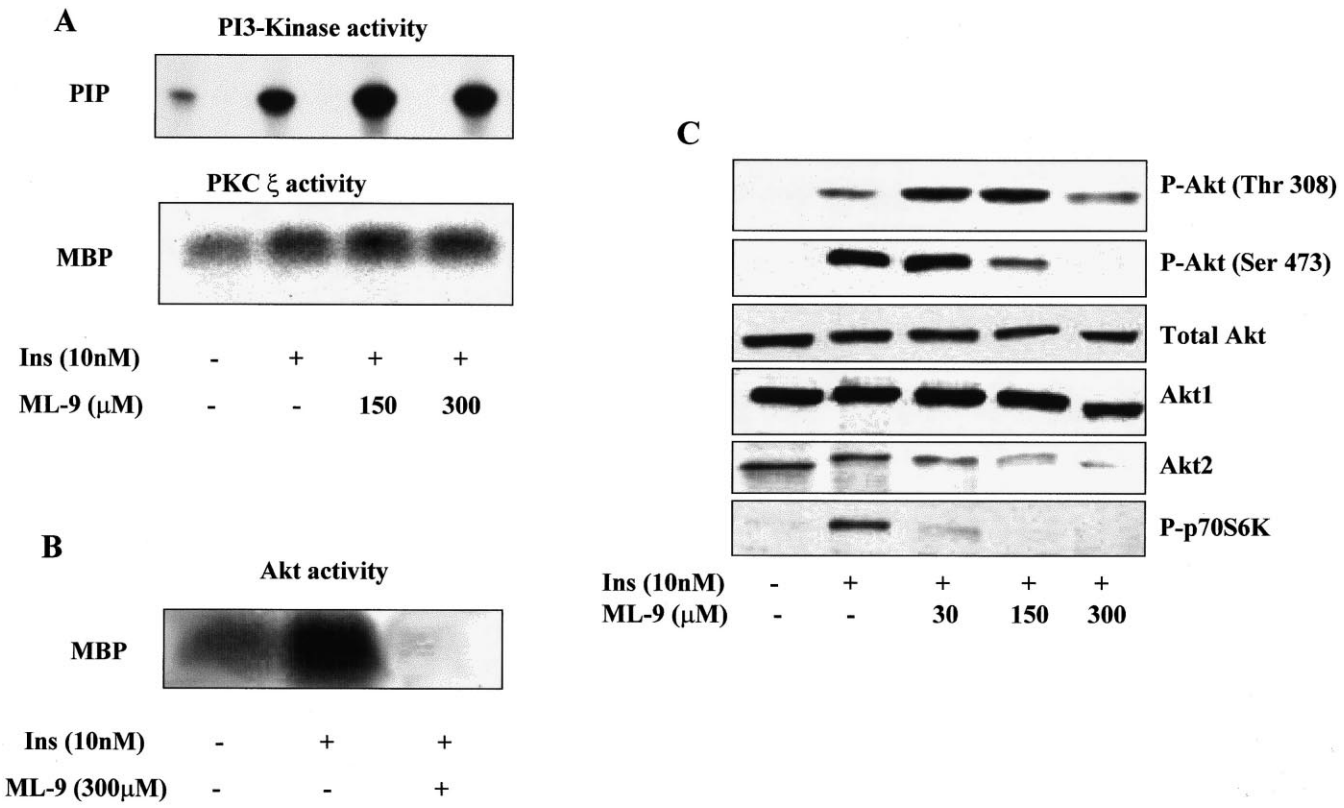

Fig. 2. Effect of ML-9 on insulin signalling pathway. A, B: Primary brown adipocytes were preincubated or not with ML-9 for 10 min at the doses indicated were further treated with insulin for 5 min. Cells were then lysed and either immunoprecipitated with anti-Akt or PKCζ antibodies and the resulting immune complexes assayed for MBP phosphorylation, or immunoprecipitated with anti-Tyr(P) antibody and assayed for PI3-kinase activity. Results shown are representative of at least four independent experiments. C: Cells were pretreated or not with ML-9 (30–300 μM) for 10 min before being stimulated with insulin for 5 min. Cells lysates were analysed by Western blotting with the corresponding antibodies against phospho-Akt(Ser473), phospho-Akt(Thr308), total Akt, Akt1 and Akt2 and phospho-p70S6K.

Ser-phosphorylated, and in a lower extent, Thr-phosphorylated. ML-9 inhibited Ser473 phosphorylation in a dose-dependent manner but had no effect on Thr308 phosphorylation (Fig. 2C). Moreover, antibodies raised against the total Akt, and the isoforms Akt1 and Akt2 revealed a mobility shift when cells were stimulated with insulin, this effect being not observed under pretreatment with ML-9 at 300 μM, when dephosphorylation at Ser473 occurred. The presence of ML-9 also precluded insulin-stimulated p70S6-kinase phosphorylation (Fig. 2C). Therefore, ML-9 blocks Akt and p70S6-kinase but neither PI3-kinase nor PKCζ stimulation by insulin, that allows us to differentiate between PI3-kinase-PKCζ pathway and Akt-dependent effects of insulin.

To evaluate the significance of Akt in the signalling pathway by which insulin induces glucose transport in brown adipocytes, we performed series of experiments pretreating cells with various doses (30–300 μM) of ML-9 for 10 min followed by stimulation with insulin (10 nM) for a further 30 min. Glucose uptake was measured during the last 10 min by incorporation of 2-deoxy-D-[1-^{3}H]glucose into cells. Basal glucose uptake either in the absence or presence of ML-9 was also determined. As depicted in Fig. 3A, ML-9 had not effect on the basal glucose uptake, even at the highest dose used (300 μM). However, insulin-induced glucose uptake was reduced about 50% by 30 μM ML-9. Higher concentrations of the Akt inhibitor totally precluded glucose transport stimulated by insulin, decreasing glucose uptake to basal levels. Insulin increases glucose incorporation mainly because its ability to recruit the insulin-responsive glucose transporter GLUT4 from intracellular compartments to the cell surface. We wanted to determine whether the inhibitory effects of ML-9 on insulin-induced glucose uptake were due to an impaired capacity of insulin for GLUT4 translocation. Brown adipocytes were treated as above and subjected to subcellular fractionation. Western blot protein analysis with anti-GLUT4 antibody indicated that insulin increased by 3-fold GLUT4 translocation to plasma membrane with a concomitant decrease of GLUT4 amount in the internal membrane. Both effects were prevented by ML-9 in a concentration-dependent manner (Fig. 3B), what indicates that the Akt inhibitor precludes insulin-induced glucose uptake by preventing GLUT4 translocation to the plasma membrane. However, neither ML-9 alone or in the presence of insulin modified GLUT1 protein content at the plasma membrane (data not shown). Caveolin-1, an integral protein from caveolae implicated in cellular transport processes [35] was used as a marker protein of plasma membrane and its expression remained essentially unaltered under the different treatments used (Fig. 3B).

To validate the data on insulin-induced glucose uptake obtained with the chemical inhibitor of Akt, ML-9, we decided to block Akt by transfection with a dominant-negative Akt construct (ΔAkt). Since immortalised brown adipocytes also express GLUT4 and have higher transfection efficiency than primary brown adipocytes, these cells were transiently trans-

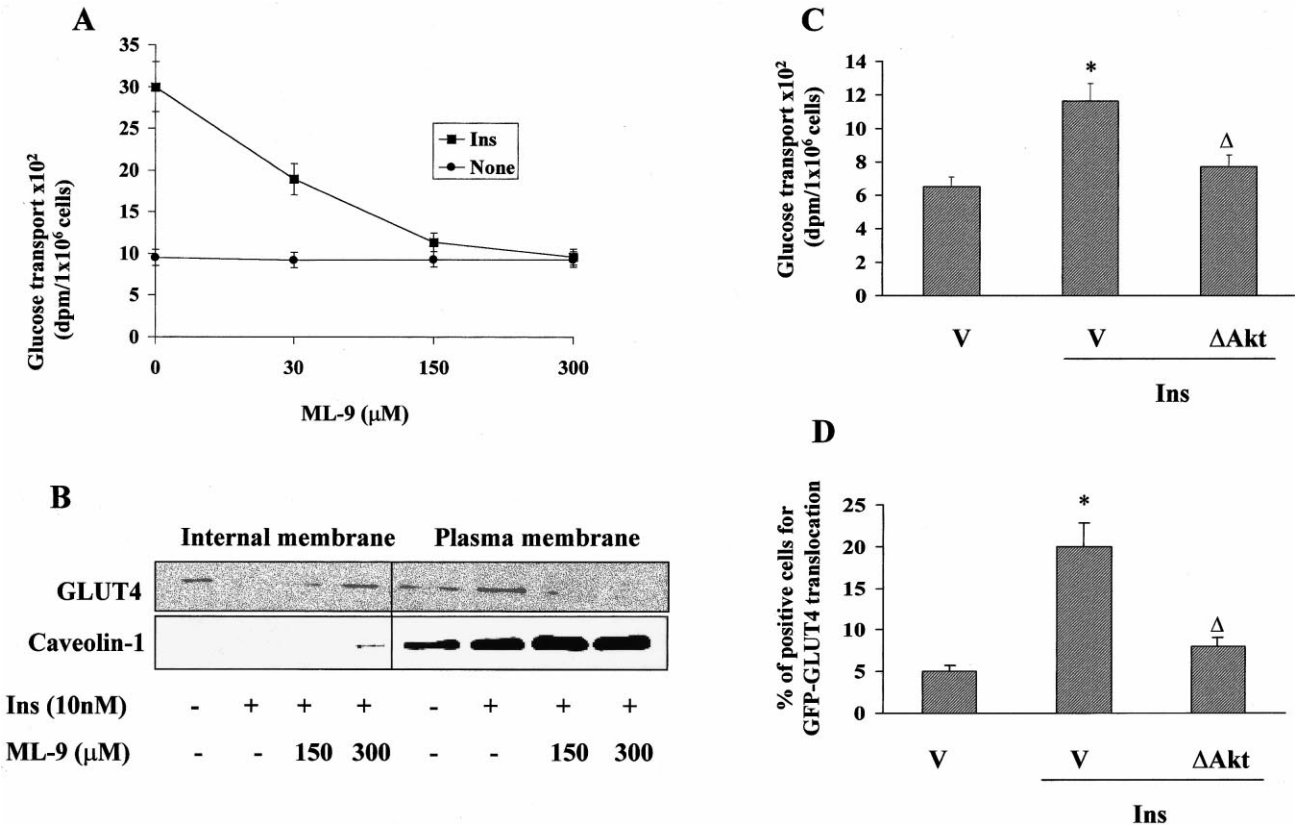

Fig. 3. ML-9 inhibits insulin-stimulated glucose uptake and GLUT4 translocation in a similar fashion that dominant-negative Akt. A: Primary brown adipocytes were preincubated for 10 min with increasing doses of ML-9 (0–300 μM) before 30 min of stimulation in the absence (none) or in the presence of 10 nM insulin (Ins). Glucose uptake was measured the last 10 min by incorporation of 2-deoxy-D-[1-^{3}H]glucose into the cells. Results are expressed as disintegration/min/1×10^6 cells and are the mean ± S.E.M. ($n=8$) for duplicate samples from four independent experiments. B: Cells were treated as described above and collected for subcellular fractionation. 10 μg of internal and plasma membrane proteins from each condition were subjected to SDS–PAGE, blotted on to nylon membrane, immunodetected with anti-GLUT4 and anti-Caveolin-1 antibodies and developed with ECL chemiluminescence. A representative experiment out of four is shown. C: Immortalised brown adipocytes were transiently transfected with 10 μg of ΔAkt (pSG5-PKB-CAAX, a dominant-negative form of Akt) or with the empty vector (V). 2 μg of GFP were added to each dish to monitor transfection efficiency. Cells were cultured for 24 h in 10% FCS medium, overnight serum-starved and stimulated or not with insulin (100 nM) for 30 min, with the glucose uptake determined during the last 10 min as described in A. Results are expressed as disintegration/min/1×10^6 cells and are the mean ± S.E.M. ($n=8$) for duplicate samples from four independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least significant difference test, where differences between values in the presence of insulin vs. control is represented by (*) and differences between values in the presence of insulin plus ΔAkt vs. insulin is represented by ($^\Delta$);*,$^\Delta P<0.01$. D: HeLa cells were transiently co-transfected with 10 μg of GFP-tagged GLUT4 together with 10 μg of ΔAkt or with the empty vector (V). Cells were cultured for 24 h in 10% FCS medium, overnight serum-starved and stimulated or not with insulin (500 nM) for 30 min, being GLUT4 translocation observed under fluorescence microscopy. Cells were scored as positive for GLUT4 translocation if they were observed to have a ring of fluorescence at the periphery. The percentage of positive cells are means ± S.E.M. for several fields for duplicate dishes from two independent experiments. Statistical significance was tested as in C.

fected with 10 μg of pSG5-PKB-CAAX (ΔAkt) or an empty vector together with 2 μg of GFP to monitor transfection efficiency. Cells were cultured for 24 h in 10% FCS medium, overnight serum-starved and stimulated or not with insulin (100 nM) for 30 min, with the glucose uptake determined during the last 10 min by incorporation of 2-deoxy-D-[1-^{3}H]-glucose as described above (Fig. 3C). In this cell line insulin increased glucose uptake by nearly 2-fold, this effect being almost completely abolished in the presence of an Akt protein with dominant-negative activity. Furthermore, we co-transfected HeLa cells, that do not constitutively express GLUT4 but are insulin-responsive cells [36], with 10 μg of a GFP-tagged GLUT4 construct together with 10 μg of the ΔAkt or an empty vector. Cells were cultured for 24 h in 10% FCS medium, overnight serum-starved and stimulated or not with insulin (500 nM) for 30 min, and the translocation of GFP-GLUT4 was determined with fluorescence microscopy (Fig. 3D). The characteristic ring of GLUT4 staining at the periphery of the cells in response to insulin stimulation was quantitated either in the absence (V) or in the presence of ΔAkt. Blocking Akt with a ΔAkt construct significantly precluded GFP-GLUT4 translocation to the plasma membrane. These data obtained with ΔAkt on impairment of insulin-induced glucose uptake and GLUT4 translocation in immortalised brown adipocytes and HeLa cells, respectively, are confirmatory of those obtained with ML-9 in primary brown adipocytes.

3.2. Akt is involved in insulin-induced GLUT4 gene expression

The results described above support the involvement of Akt in the pathway leading to glucose uptake under acute insulin stimulation. Insulin also plays a long-term role increasing GLUT4 levels of a PI3-kinase-dependent mechanism [26]. Because Akt is an effector downstream PI3-kinase we wanted to

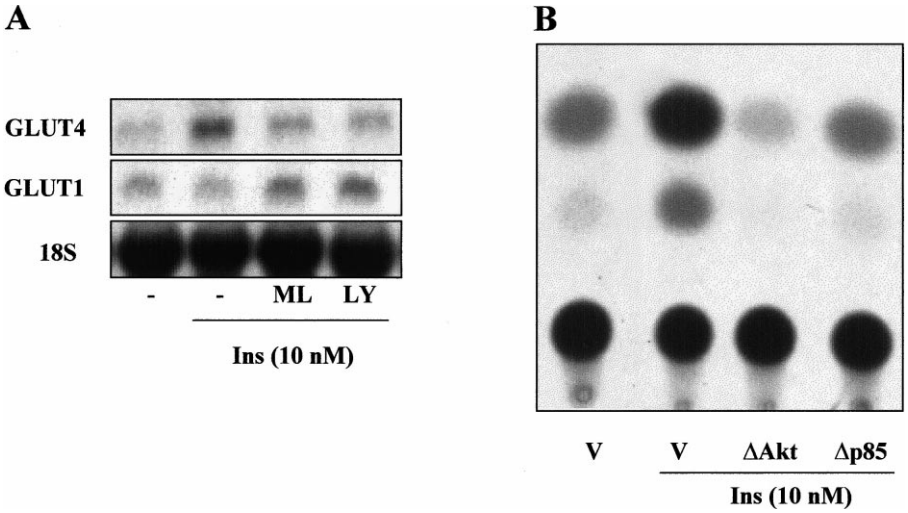

Fig. 4. Akt is involved in insulin-induced GLUT4 gene expression. A: Primary brown adipocytes (20 h serum-deprived) were treated for 6 h with 10 nM insulin, either in the absence or presence of 10 μM LY294002 (LY) or 300 μM ML-9, and untreated cells were used as control. Total RNA (10 μg) from each condition was subjected to Northern blot analysis and hybridised with GLUT4 and GLUT1 probes. An equal amount of loaded RNA was assessed by hybridisation with 18S RNA probe. Autoradiograms from a representative experiment out of four are shown. B: Primary brown adipocytes were transiently co-transfected with 10 μg of GLUT4-CAT together with 10 μg of Δp85 (a dominant-negative form of the regulatory subunit of PI3-kinase) or ΔAkt or with the empty vector (V). 2 μg of DNA-βgal were added to each dish to monitor transfection efficiency. After transfection cells were treated or not for 24 h with 10 nM insulin and at the end of the culture time collected and assayed for CAT activity. A representative experiment out of four is shown.

explore the possible implication of Akt in long-term effects of insulin on GLUT4 gene expression blocking Akt activity by two different ways: (1) using the chemical compound ML-9, and (2) transfecting cells with an Akt protein with dominant-negative activity. Brown adipocytes were treated for 6 h with insulin in the absence or presence of ML-9 or LY294002, and GLUT4 mRNA levels were analysed by Northern blot. As shown in Fig. 4A, insulin increased by 3-fold GLUT4 mRNA levels and this effect was abolished by the Akt inhibitor, in a similar fashion as the inhibitor of PI3-kinase did. Moreover, primary brown adipocytes were transiently co-transfected with 10 μg of GLUT4-CAT and 10 μg of ΔAkt or a dominant-negative p85 construct (Δp85) or an empty vector. After transfection, cells were fed with serum-free medium and cultured for 24 h in the absence or presence of insulin. As depicted in Fig. 4B, insulin stimulation of GLUT4-CAT fusion gene activity (4-fold increase) was almost precluded upon co-transfection with either the ΔAkt or the Δp85 construct. Both approaches suggest a role for Akt, downstream PI3-kinase, in insulin-induced GLUT4 gene expression.

4. Discussion

The insulin-stimulated PI3-kinase pathway seems to be the main route involved in glucose uptake in most insulin-responsive cells, including brown adipocytes. However, downstream or alternative pathways leading to this metabolic effect of insulin remain to be completely established. In this work we have investigated the contribution of Akt to insulin stimulation of glucose transport in brown adipocytes by two approaches, (1) using ML-9, proposed as a chemical inhibitor of Akt activity in a recent paper [14], and (2) blocking Akt with an Akt protein with dominant-negative activity. ML-9 treatment of primary brown adipocytes produced a complete inhibition of insulin-stimulated glucose uptake and an impairment of the redistribution of GLUT4 from internal membrane to plasma membrane in response to insulin. However, ML-9 was not affecting the glucose transport machinery per se since basal glucose uptake remained unchanged in its presence. Our results in primary brown adipocytes are in conformity with those reported in isolated fat cells [14]. All the results presented with the chemical inhibitor have been validated with the dominant-negative construct of Akt (ΔAkt). In this regard, ΔAkt transiently transfected precluded insulin-stimulation of glucose uptake in immortalised brown adipocytes and insulin-induced GFP-tagged GLUT4 translocation to the plasma membrane in HeLa cells.

Pretreatment with ML-9 did not affect insulin-stimulated PI3-kinase nor PKCζ activities, however, completely inhibited (1) insulin-stimulated Akt kinase activity immunoprecipitated with anti-total-Akt antibody, (2) Akt phosphorylation by insulin at the regulatory residue Ser473 but not at Thr308, (3) insulin-induced mobility shift in Akt1, Akt2 and total Akt separated in polyacrylamide gels and (4) phosphorylation of p70S6-kinase by insulin. We did not detect inhibition of PI3-kinase by ML-9, in agreement with Smith's data [14], but we found that ML-9 prohibited Akt activity and the phosphorylation of Akt at Ser473, in contrast with the lack of effect of this compound on Akt phosphorylation at the same dose found [14]. These results indicate that autophosphorylation of Akt on the hydrophobic site (Ser473) is not been produced when Akt is catalytically incompetent (by treatment with ML-9), as recently has been proposed [37]. Furthermore, our data agree with the inhibition produced by ceramide on Akt activation by dephosphorylation at Ser473, but not at the Thr308 residue, in TF-1 cells treated with the colony-stimulating factor [38]. Phosphorylation of p70S6-kinase by insulin also resulted in inhibition in the presence of ML-9, but this effect is probably the consequence of Akt inactivation, as recently proposed [11]. Insulin-induced glucose uptake in brown adipocytes seems to be independent of p70S6-kinase activation,

as demonstrated by the use of the specific inhibitor rapamycin. PKCζ could be another PI3-kinase downstream target involved in insulin-induced glucose uptake in adipocytes [16,17], but ML-9 did not inhibit this step although we have recently reported an inhibition of this enzyme with okadaic acid that precluded insulin-stimulated glucose transport. Although the contribution of Akt to insulin-induced glucose uptake is controversial, with both positive and negative evidences in the literature [9,11–13], this paper indicates that Akt activation seems to be an absolute requirement for insulin-induced glucose uptake in foetal brown adipocytes.

Furthermore, this work describes for the first time an inhibitory effect of long-term treatment with ML-9 in insulin induction of GLUT4 gene expression. Since long-term treatment with ML-9 produced down-regulation of the GLUT4 mRNA accumulation, inactivation of Akt, and possibly its translocation to the nucleus as recently proposed [39], would be producing this effect. This data suggest a role of Akt at the transcriptional level in brown adipocytes. To test this hypothesis we performed GLUT4-CAT transactivation experiments in the presence of the ΔAkt construct. Co-transfection with ΔAkt precluded insulin stimulation of GLUT4 promoter activity in a similar fashion as Δp85 did, indicating that PI3-kinase/Akt pathway is regulating GLUT4 gene transcription by insulin. Akt has been involved in the insulin-induced expression of other glucose transporters, such as GLUT1 in hepatoma cells and GLUT3 in skeletal muscle [27,28]. This work demonstrates for the first time that GLUT4 gene is transcriptionally regulated by insulin thought Akt activation, although the specific transcription factors involved remain to be established.

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