Insulin and insulin-like growth factor I up-regulate *GLUT4* gene expression in fetal brown adipocytes, in a phosphoinositide 3-kinase-dependent manner

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Fetal brown adipocytes cultured in a serum-free medium, containing 5 mM glucose, expressed both *GLUT4* and *GLUT1* glucose transporters at the mRNA and protein level. Treatment with either insulin or insulin-like growth factor (IGF)-I at physiological concentrations up-regulates the expression of the *GLUT4* gene, producing a time-dependent mRNA accumulation (7-fold increase at 24 h) and a 2.5-fold increase in the amount of protein in the total membrane fraction. However, insulin treatment down-regulates *GLUT1* mRNA and protein expression. Moreover, either insulin or IGF-I transactivates a full-promoter *GLUT4*-chloramphenicol acetyltransferase gene (*CAT*) construct transiently transfected to the cells, without affecting *GLUT1*-*CAT* activity. In consequence, insulin treatment for 24 h increased by 3-fold the basal glucose uptake. Inhibition of phosphoinositide (PI) 3-kinase activity with chemical agents such as wortmannin or LY294002 partially blocked insulin-induced *GLUT4* mRNA accumulation, insulin-induced *GLUT4* protein

content, *GLUT4*-*CAT* transactivation and glucose uptake. Furthermore, co-transfection of brown adipocytes with a dominant-negative form of PI 3-kinase precluded the transactivation of the *GLUT4* promoter by insulin. However, inhibition of p70S6 kinase (p70^{s6k}) with rapamycin or of mitogen-activated protein kinase (MAPK) with PD098059 does not preclude insulin effects on *GLUT4* gene expression or glucose uptake. Our results show for the first time a positive effect of insulin on *GLUT4* gene expression in fetal brown adipocytes, suggesting the existence of insulin response element(s) in its promoter. Moreover, PI 3-kinase, but not p70^{s6k} or MAPK, is an essential requirement for insulin regulation of *GLUT4* gene expression.

Key words: glucose uptake, *GLUT4*/*GLUT1* mRNA, *GLUT4*/*GLUT1* promoter activity, *GLUT4*/*GLUT1* protein content, insulin signalling.

INTRODUCTION

Brown adipose tissue is specialized for non-shivering thermogenesis in neonates, responsible for heat production associated with the expression of the mitochondrial uncoupling protein 1 [1]. Differentiation of this tissue also encompasses an adipogenic programme related to lipid synthesis, and its accumulation results in a multilocular-fat-droplets phenotype [2], with glucose being the main lipogenic substrate [3,4]. Glucose transport in 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* [5]. During fetal life, *GLUT1* is expressed at a high level in brown adipose tissue, although *GLUT4* mRNA expression has been shown to increase greatly at day 22 of fetal development concomitantly with the onset of adipogenic and thermogenic differentiation of the tissue [2,6]. In previous work, we demonstrated that fetal brown adipocytes display binding sites of high affinity for both insulin and insulin-like growth factor (IGF)-I. Moreover, insulin and IGF-I induced adipogenesis-related gene expression and cytosolic lipid content in 20-day-old fetal brown adipocytes in primary culture [4]. These observations prompted us to examine the roles of IGF-I and insulin in the regulation of *GLUT4* and *GLUT1* gene expression in fetal brown adipocytes by studying the accumulation of mRNA and protein, as well as the transcriptional activity of *GLUT4* and *GLUT1* promoters.

A complex network of intracellular signalling pathways mediates the pleiotropic effects of insulin/IGF-I on metabolic processes as well as on gene expression. Effects of insulin and IGF-I are produced by the activation of its cell-surface receptors, which possess tyrosine kinase activity [7], leading, upon ligand binding, to receptor autophosphorylation and tyrosine phosphorylation of insulin receptor substrates (IRS-1 and IRS-2) [8]. Indeed, phosphorylated IRSs interact with numerous SH2 (Src-homology domain 2) domain-containing proteins, including the regulatory subunit of the phosphoinositide (PI) 3-kinase, and the guanine-nucleotide exchange factor Grb2/SOS. PI 3-kinase is a heterodimer composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110), capable of phosphorylating phosphoinositides at the 3' position of the inositol ring, which initiates phospholipid turnover [9]. Downstream of PI 3-kinase several targets have been identified, including the serine/threonine kinase p70S6 kinase (p70^{s6k}), which seems to be involved in regulating the phosphorylation of initiation factors controlling protein synthesis [10,11]. Grb2/SOS activation results in the sequential activation of Ras, Raf and the mitogen-activated protein kinase (MAPK) cascade [12]. Acute insulin treatment stimulates glucose transport in insulin-sensitive cells, such as adipocytes and myocytes, largely by mediating translocation of *GLUT4* and, to a lesser extent, *GLUT1* from an intracellular compartment to the plasma membrane [13,14]. Activation of PI 3-kinase seems to mediate *GLUT4* redistribution to the plasma membrane; overexpression the catalytic subunit of p110

Abbreviations used: IGF, insulin-like growth factor; CAT, chloramphenicol acetyltransferase; PI, phosphoinositide; MAPK, mitogen-activated protein kinase; p70^{s6k}, p70S6 kinase; IRS, insulin receptor substrate; MEM, minimal essential medium with Earle's salts; β -gal, β -galactosidase; FCS, fetal-calf serum.

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produced GLUT4 translocation and increased glucose uptake in 3T3L1 cells and in isolated rat adipocytes [15–17]. Inhibition of PI 3-kinase, either by chemical inhibitors, by microinjection of blocking p85 protein or a dominant-negative mutant of p85, precluded insulin-induced GLUT4 translocation [18,19]. In this regard, inhibition of IGF-I-induced IRS-1-associated PI 3-kinase activity with wortmannin or LY294002 leads to an inhibition of IGF-I-induced glucose uptake in fetal brown adipocytes [20]. Although the mechanism by which insulin increases short-term glucose uptake seems to involve signalling complexes containing the p85/p110-type PI 3-kinase, the mechanism by which insulin produces chronic effects on glucose uptake throughout induction of glucose-transporter gene expression remains largely unexplored.

Accordingly, we have investigated the signalling network leading chronic insulin treatment (24 or 48 h) to increase glucose uptake in brown adipocytes, which involved stimulation of the GLUT4 promoter transcriptional activity and up-regulation of GLUT4 mRNA and protein expression. In this regard, the requirement of PI 3-kinase has been studied using either chemical inhibitors, such as LY294002 or wortmannin [21,22], or with the dominant-negative p85 α regulatory subunit of PI 3-kinase construct (Δ p85), transiently transfected into brown adipocytes. The importance of p70^{s6k}, a downstream target of PI 3-kinase, has been tested using the specific inhibitor rapamycin. Moreover, the requirement of the Ras/MAPK pathway has been tested using the inhibitor of MEK-1 (MAPK kinase-1), PD098059 [23]. Our results show that PI 3-kinase, but not p70^{s6k} or Ras/MAPK, is an essential requirement for insulin-induced up-regulation of GLUT4 mRNA and protein, and for transactivation of the GLUT4 promoter. However, *GLUT1* gene expression is not regulated by insulin in fetal brown adipocytes.

MATERIALS AND METHODS

Materials

Insulin, BSA (fraction V, essentially fatty acid free) and wortmannin were from Sigma Chemical Co (St. Louis, MO, U.S.A.). IGF-I, LY294002 and rapamycin were purchased from Calbiochem (Calbiochem-Novabiochem, La Jolla, CA, U.S.A.). PD098059 was from New England Biolabs (Beverly, MA, U.S.A.). Fetal-calf serum (FCS), PBS and culture media were from Imperial Laboratories (Andover, Hants, U.K.). RNazol B was from Biotecx Lab (Dallas, TX, U.S.A.). Nylon membranes were GeneScreen™ (NEN Research Products, Boston, MA, U.S.A.). Autoradiographic films were Kodak X-OMAT/AR (Eastman Kodak Co, Rochester, NY, U.S.A.). 2-Deoxy-D-[1-³H]glucose (11.0 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol), [¹⁴C]-chloramphenicol and the multiprimer DNA-labelling system kit were purchased from Amersham (Amersham, Bucks., U.K.). All other reagents used were of the purest grade available. The cDNAs used as probes were *GLUT4* and *GLUT1* [24]. The transfection MBS mammalian transfection kit was from Stratagene (La Jolla, CA, U.S.A.). The plasmid constructs used for transfection experiments were *GLUT4*-(chloramphenicol acetyltransferase) *CAT* and *GLUT1-CAT*, kindly provided by Dr. A. Zorzano (Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain) [25], dominant-negative p85 α construct (Δ p85) was kindly provided by Dr. M. Kasuga (Kobe University, Kobe, Japan) [26], and pCMV β -galactosidase (β -gal) was supplied by Stratagene. The rabbit anti-GLUT1 and anti-GLUT4 antibodies were supplied by Chemicon Inc. (Tamaulaca, CA, U.S.A.). The anti-phospho-MAPK and anti-phospho-p70^{s6k} antibodies were from New England Biolabs. For anti-Tyr(P) immunoprecipitation, a mono-

clonal antibody (Py72) was the generous gift of Dr. E. Rozengurt (Imperial Cancer Research Fund, London, U.K.).

Cell culture

Fetal brown adipocytes were obtained from interscapular brown adipose tissue of 20-day-old Wistar rat fetuses and isolated by collagenase dispersion as described in [4]. Cells were plated at 1.5×10^6 cells/60 mm tissue culture dish in 2.5 ml of minimal essential medium with Earle's salts (MEM) supplemented with 10 % FCS. After 4–6 h of culture at 37 °C, cells were rinsed twice with PBS, and 70 % of the initial cells were 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 and, for the experiments described, this time defines the starting point for IGF-I or insulin stimulation at the doses indicated in each case. Cells were cultured in the presence of insulin for 24 or 48 h, as indicated in each case, either in the absence or in the presence of specific inhibitors of its signalling pathways, such as wortmannin (20 nM), LY294002 (10 μ M), rapamycin (25 ng/ml) or PD098059 (30 μ M). In other experiments, cells were cultured for 24 h in the presence of inhibitors and then stimulated for 10 min with insulin.

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 RNazol B following the protocol supplied by the manufacturer for total RNA isolation [27]. Total cellular RNA (20 μ g) was submitted to Northern-blot analysis, i.e. electrophoresed on 0.9 % agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen™ membranes using a VacuGene blotting apparatus (LKB-Pharmacia, Uppsala, Sweden) and cross-linked to the membranes by UV light. Hybridization was in 0.25 mM NaHPO₄ (pH 7.2)/0.25 M NaCl/100 μ g/ml denatured salmon sperm DNA/7 % SDS/50 % deionized formamide, containing denatured ³²P-labelled cDNA (10⁶ c.p.m./ml) for 40 h, at 42 °C, as described in [28]. cDNA labelling was carried out with [α -³²P]dCTP to a specific radioactivity of 10⁹ c.p.m./ μ g of DNA by using a multiprimer DNA-labelling-system kit. For serial hybridization with different probes, the blots were stripped and rehybridized subsequently as needed in each case. Membranes were subjected to autoradiography and relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Each Northern-blot analysis was performed from four independent experiments.

PI 3-kinase activity

PI 3-kinase activity was measured by phosphorylation *in vitro* phosphorylation of PI as previously described previously [20]. At the end of the culture time cells were washed with ice-cold PBS, solubilized in lysis buffer [10 mM Tris/HCl/5 mM EDTA/50 mM NaCl/30 mM sodium pyrophosphate/50 mM NaF/100 μ M Na₃VO₄/1 % Triton X-100 (pH 7.6)] containing leupeptin (10 μ g/ml), aprotinin (10 μ g/ml) and 1 mM PMSF. Lysates were clarified by centrifugation at 15000 g for 10 min at 4 °C, and proteins were immunoprecipitated with a monoclonal antibody anti-Tyr(P) (Py72). The immunoprecipitates were washed successively in PBS containing 1 % Triton X-100 and 100 μ M Na₃VO₄ (twice), 100 mM Tris (pH 7.5), in PBS containing 0.5 M LiCl, 1 mM EDTA and 100 μ M Na₃VO₄ (twice), and in 25 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EDTA (twice). To each pellet were added 25 μ l of 1 mg/ml L- α -

phosphatidylinositol/L- α -phosphatidyl-L-serine sonicated in 25 mM Hepes (pH 7.5) and 1 mM EDTA.

The PI 3-kinase reaction was started by the addition of 100 nM [γ - ^{32} P]ATP (10 μ Ci) and 300 μ M ATP in 25 μ l of 25 mM Hepes (pH 7.4)/10 mM $MgCl_2$ /0.5 mM EGTA. After 15 min at room temperature the reaction was stopped by the addition of 500 μ l of chloroform/methanol (1:2, v/v) in a 1% concentration of HCl plus 125 μ l of chloroform/125 μ l of HCl (10 mM). The samples were centrifuged, and the lower organic phase was removed and washed once with 480 μ l of methanol/(100 mM HCl plus 2 mM EDTA) (1:1, v/v). The organic phase was extracted, dried under vacuum, and resuspended in chloroform. Samples were applied to a silica gel TLC plate (Whatman, Clifton, NJ, U.S.A.). TLC plates were developed in propan-1-ol/acetic acid (2 M; 65:35, v/v), dried, visualized by autoradiography and quantified by scanning laser densitometry.

Subcellular fractionation

For preparation of total membrane fraction, cells from five dishes of 100-mm diameter were pooled for each experimental condition after harvesting with ice-cold PBS. Cells were then homogenized in an ice-cold buffer containing 20 mM Hepes, 250 mM sucrose, 2 mM EGTA, 0.2 mM PMSF, 1 μ M leupeptin (pH 7.4). Nuclei and unbroken cells were removed by centrifugation at 2000 g for 10 min. Total membrane fraction was prepared by centrifugation of the supernatant in a Superspeed centrifuge at 190 000 g for 1 h and 4 °C. The membrane pellets were resuspended in homogenization buffer before storage at -20 °C, and proteins were determined.

Western blotting

For Western-blot analysis of GLUT4 and GLUT1 after SDS/PAGE, plasma-membrane proteins (100 μ g) were transferred to Immobilon membranes, blocked using 5% non-fat dried milk in 10 mM Tris/HCl/150 mM NaCl (pH 7.5), and incubated overnight with anti-GLUT1 antibody (1:1000) or anti-GLUT4 antibody (1:500) in 0.05% Tween-20/1% non-fat dried milk in the buffer indicated above. For Western blotting of MAPK and p70^{S6k} proteins, cells were lysed in lysis buffer as described in the PI 3-kinase protocol, and cellular proteins (50 μ g) were submitted to SDS/PAGE, transferred to membranes, blocked and incubated overnight with anti-phospho-MAPK antibody (1:1000) or with anti-phospho-p70^{S6k} antibody (1:1000) as indicated above. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL-Plus) Western blotting protocol (Amersham). Western-blot analysis was performed in four independent experiments.

Measurement of glucose uptake

For glucose uptake determination, cells that had been serum-deprived for 20 h were cultured for a further 24 h in the presence or absence of insulin alone or together with the inhibitors, and incubated with 2-deoxy-D-[1- ^{3} H]glucose (500 nCi/ml) to a final concentration of 0.1 mM in KRP buffer [135 mM NaCl/5.4 mM KCl/1.4 mM $CaCl_2$ /1.4 mM $MgSO_4$ /10 mM sodium pyrophosphate (pH 7.4)] during the last 60 min of culture. Cells were then washed three times with ice-cold KRP buffer and solubilized in 1 ml of 1% SDS, as described in [29]. The radioactivity of a 200 μ l aliquot was determined in a liquid-scintillation counter. Glucose transport was determined in triplicate dishes from four independent experiments. In parallel dishes, proteins were determined by the Bradford dye method [30], using the Bio-Rad reagent (Bio-Rad, Richmond, CA, U.S.A.) and BSA as the standard.

Transfection conditions

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. The plasmid constructs used were *GLUT4-CAT* (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) [25]; *GLUT1-CAT* (containing the *CAT* reporter gene downstream of the GLUT1 promoter, from positions -2106 to +134, relative to the transcription start site) [25]; the promoterless pCAT-basic vector and pCMV β -gal (a viral-promoter-driven expression of the reporter gene β -gal). DNA-*CAT* (15 μ g) together with 2 μ g of DNA- β -gal (to monitor transfection efficiency) were added to each dish and, after 4 h of incubation, cells were shocked with 3 ml of 15% glycerol for 2 min, washed, and then fed with serum-free medium. After 24 h (either in the absence or presence of insulin with or without inhibitors) cells were harvested and lysates were prepared for *CAT* and β -gal activity assays. In some experiments, a dominant-negative p85 α construct (SR α - Δ p85) [26] was also used in co-transfection with *CAT* constructs. DNA-*CAT* (10 μ g) in the presence of 10 μ g of dominant-negative construct or empty vector to keep constant the amount of DNA transfected, together with 2 μ g of DNA- β -gal, were added to each dish, and after the transfection cells were cultured for 24 h in the presence of insulin. *CAT* activity was determined by incubating 50 μ l of cell extracts with 0.25 μ Ci of [^{14} C]chloramphenicol/0.5 mM acetyl-CoA in 0.25 M Tris (pH 7.8) at 37 °C for 12 h, and then samples were submitted to TLC. The amount of substrate acetylated was directly quantified with a radioimaging device (Fujifilm BAS-1000, Fuji, Japan). *CAT* enzyme activity was expressed as a percentage of [^{14} C]chloramphenicol acetylated normalized to the internal control β -gal (assayed according to the Stratagene protocol). Transfections were performed from four independent experiments.

RESULTS

Insulin and IGF-I up-regulated the expression of *GLUT4* gene in a time-dependent manner and transactivated the *GLUT4-CAT* fusion gene, without affecting *GLUT1* gene expression

We first characterized the expression of *GLUT4* and *GLUT1* genes in fetal brown adipocytes in primary culture. Cells (after 4 h of attachment and culture for 20 h in a serum-free medium) were cultured for a further 24 h in a serum-free medium either in the absence or in the presence of increasing doses of insulin, the expression of *GLUT4* and *GLUT1* genes being studied by Northern blotting as depicted in Figure 1(A). Both GLUT4 2.8 kb and GLUT1 2.8 kb mRNA species were expressed in 20-day-old fetal brown adipocytes cultured in a serum-free medium. Moreover, a second mRNA isoform of approximately 2.3 kb for GLUT4 was weakly expressed in these experimental conditions (Figure 1A). However, both GLUT4 mRNA isoforms increased dramatically throughout 24 h of culture in the presence of insulin, this effect being dose-dependent; there was a 5-fold increase with 1 nM insulin, and a 7-fold increase at 10 or 100 nM insulin, resulting from the densitometric analysis after normalization with the amount of hybridized 18S rRNA. In contrast, GLUT1 mRNA accumulation decreased after 24 h of culture in the presence of insulin, regardless of the dose used (Figure 1A).

Since fetal brown adipocytes also bear a high number of high-affinity binding sites for IGF-I, we tested in a time-course experiment the effect of both IGF-I and insulin on GLUT4 mRNA expression. Fetal brown adipocytes (24 h serum-starved)

Figure 1 Insulin and IGF-I up-regulate GLUT4 mRNA expression in fetal brown adipocytes: time-dependency

(A) Cells were serum-deprived for 20 h prior to culture for 24 h either in the absence or presence of insulin (ins) in the doses indicated. Total RNA (10 µg) was submitted to Northern-blot analysis and hybridized with labelled *GLUT4* and *GLUT1* cDNAs. A final hybridization with the 18 S rRNA cDNA was performed for normalization. (B) A time-course experiment of GLUT4 mRNA accumulation was performed after treatment of brown adipocytes with either 10 nM insulin (ins) or 10 nM IGF-I (I). Autoradiograms from representative experiments out of four are shown.

were cultured for up to 24 h in the absence or presence of either 10 nM insulin or 10 nM IGF-I, the accumulation of GLUT4 mRNA species being analysed by Northern blotting (Figure 1B). Accumulation of GLUT4 mRNA was observed as early as 3 h after stimulation with either IGF-I or insulin. Both factors were equally potent in inducing GLUT4 mRNA accumulation; this accumulation was time-dependent, reaching maximal values after 24 h of treatment (Figure 1B).

The results shown above on GLUT4 mRNA accumulation seem to indicate a transcriptional regulation of this gene by insulin and IGF-I. Consequently, we decided to study the effect of these factors on the transactivation of the GLUT4 promoter driving the expression of the *CAT* reporter gene. Upon culture with 10% FCS medium for 24 h, primary brown adipocytes were transiently co-transfected with 15 µg of *GLUT4-CAT* fusion gene DNA [rat GLUT4 full-length promoter (2212 bp)-*CAT*] or with 15 µg of *GLUT1-CAT* fusion gene DNA [rat GLUT1 full-length promoter (2106 bp)-*CAT*], together with 2 µg of pCMVβ-gal for internal control of transfection. Upon transfection, cells were cultured for 24 h in serum-free medium either in the absence (control) or presence of increasing doses of insulin (1 and 10 nM)

Figure 2 Insulin and IGF-I transactivate the *GLUT4-CAT* fusion gene transiently transfected in fetal brown adipocytes

(A) Fetal brown adipocytes upon culture in 10% FCS-MEM medium were transiently transfected with 15 µg of rat full-promoter *GLUT4-CAT* fusion gene together with 2 µg of pCMVβ-gal for internal control of transfection. After transfection, cells were cultured for 24 h in a serum-free medium either in the absence (C) or in the presence of either IGF-I (10 nM) or insulin (ins; 1–10 nM). At the end of the culture time, cells were collected and assayed for CAT activity as described under Materials and methods. (B) Cells were transfected with *GLUT1-CAT* fusion gene and cultured in the absence or presence of 10 nM IGF-I or 10 nM insulin as indicated in (A). Representative experiments out of four are shown.

or IGF-I (10 nM), and at the end of the culture period cells were collected and assayed for CAT activity. Representative GLUT4-CAT and GLUT1-CAT TLC experiments in primary brown adipocytes are shown in Figures 2(A) and 2(B), respectively. The *GLUT4-CAT* fusion gene was weakly transactivated in control primary brown adipocytes, whereas treatment with 10 nM IGF-I or 1 nM insulin resulted in a 6-fold increase in CAT activity, and 10 nM insulin caused an 8-fold increase in CAT activity (Figure 2A), resulting from TLC of CAT directly counted in a phosphorimager, and values were normalized to β-gal activity.

Figure 3 Effect of wortmannin, LY294002, rapamycin and PD098058 on insulin-induced PI 3-kinase, p70^{S6K} and MAPK signalling pathways in fetal brown adipocytes

Cells were serum-deprived for 20 h prior to culture for 24 h in the presence of 10 nM wortmannin (W), 10 μM LY294002 (LY), 30 μM PD098058 (PD) or 25 ng/ml rapamycin (R) and subsequently stimulated for 10 min with 10 nM insulin; some dishes remaining untreated and used as control (C). **(A)** Cells were lysed and immunoprecipitated with a monoclonal antibody anti-Tyr(P). Conversion of phosphatidylinositol into phosphatidylinositol 3-phosphate (PIP) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was analysed by TLC. **(B)** Cells were lysed and total protein (50 μg) was submitted to SDS/PAGE and immunodetected with the anti-phospho-p70^{S6K} antibody. Arrow indicates the position of phosphorylated p70^{S6K} protein. **(C)** Cells were treated as indicated in **(B)**, and immunodetected with the anti-phospho-MAPK antibody. The positions of phosphorylated p42 and p44 proteins are indicated by arrows. Representative experiments out of four are shown.

The *GLUT1-CAT* fusion gene was transactivated to a slightly higher extent than *GLUT4-CAT* in untreated cells, however, this activity remained essentially unmodified in the presence of either insulin or IGF-I (Figure 2B).

Inhibition of PI 3-kinase, but not Ras/MAPK or p70^{S6K} signalling pathways, precluded insulin effects on *GLUT4* gene expression and *GLUT4* promoter activity

In order to define the signalling pathways by which insulin and IGF-I exert their biological effects on *GLUT4* gene expression we used specific inhibitors of PI 3-kinase and Ras/MAPK pathways,

Figure 4 Inhibition of PI 3-kinase pathway, but not p70^{S6K} or MAPK pathways, precludes insulin-induced *GLUT4* mRNA accumulation and *GLUT4* protein content

(A) Cells were serum deprived for 20 h prior to culture for 24 h either in the absence (C) or in the presence of 10 nM insulin (ins) alone or together with 10 nM wortmannin (W), 10 μM LY294002 (LY), 30 μM PD098058 (PD) or 25 ng/ml rapamycin (R). Total RNA (10 μg) was submitted to Northern-blot analysis and hybridized with labelled *GLUT4* and *GLUT1* cDNAs. A final hybridization with the 18 S rRNA cDNA was performed for normalization. Autoradiograms from representative experiments out of four are shown. **(B)** Cells were serum deprived for 20 h prior to culture for 48 h either in the absence (C) or in the presence of 10 nM insulin (ins) alone or together with LY294002, PD098058 or rapamycin. Cells from five dishes of 100-mm diameter were pooled for each experimental condition, and total membrane fraction was obtained as described in Materials and methods. Total membrane proteins (100 μg) were submitted to SDS/PAGE, blotted on to nylon membrane, immunodetected with the anti-*GLUT4* and anti-*GLUT1* antibodies and developed with ECL chemiluminescence. Representative experiments out of four are shown.

and studied *GLUT4* mRNA and protein expression. PI 3-kinase activity was inhibited either with wortmannin, which directly binds and inhibits the catalytic p110 subunit of PI 3-kinase, or with a structurally unrelated inhibitor, LY294002 [20–22]. A downstream target of PI 3-kinase, p70^{S6K}, was inhibited by treatment with the immunosuppressant rapamycin [31]. The Ras/MAPK pathway was inhibited with the inhibitor of MEK-1, PD098059 [23]. We first tested the doses at which the inhibitors were effectively inhibiting its target pathways, and also checked any possible cross-talk in the insulin pathways in fetal brown adipocytes (Figure 3). Cells (after 4 h of attachment and culture for 20 h in a serum-free medium) were cultured for a further 24 h in a serum-free medium in the presence of the inhibitors and stimulated for 10 min with 10 nM insulin; some dishes remaining untreated and used as control. The whole-cell lysates were subjected to immunoprecipitation with the anti-Tyr(P) antibody,

Table 1 Effect of the insulin-signalling inhibitors on insulin induction of GLUT4/GLUT1 mRNA, protein content and promoter activity

GLUT4 and GLUT1 mRNA levels are represented as arbitrary units from densitometric analysis of Figure 4(A) after standardization using the 18 S rRNA signal. GLUT4 and GLUT1 protein content is represented as arbitrary units from densitometric analysis of Figure 4(B). *GLUT4-CAT* activity is shown as arbitrary units from densitometric analysis of Figure 5 and *GLUT1-CAT* activity from Figure 2(B), after normalization with β -gal activity. All the results are means $\pm$ S.E.M ($n = 4$) 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 versus control are represented by * and differences between values in the presence of insulin plus inhibitors versus insulin are represented by †; * and †, $P < 0.01$.

Treatment	GLUT4 mRNA	GLUT4 protein	<i>GLUT4-CAT</i>	GLUT1 mRNA	GLUT1 protein	<i>GLUT1-CAT</i>
None	11 $\pm$ 2	17 $\pm$ 3	10 $\pm$ 2	48 $\pm$ 6	24 $\pm$ 3	16 $\pm$ 3
Insulin (10 nM)	75 $\pm$ 8*	78 $\pm$ 9*	70 $\pm$ 8*	26 $\pm$ 4*	13 $\pm$ 2*	15 $\pm$ 2
Insulin + wortmannin (20 nM)	48 $\pm$ 6†	—	39 $\pm$ 5†	24 $\pm$ 3	—	—
Insulin + LY (10 μ M)	38 $\pm$ 4†	47 $\pm$ 6†	23 $\pm$ 4†	25 $\pm$ 2	12 $\pm$ 2	—
Insulin + rapamycin (25 ng/ml)	78 $\pm$ 8	75 $\pm$ 6	76 $\pm$ 9	18 $\pm$ 4	13 $\pm$ 3	—
Insulin + PD (30 μ M)	84 $\pm$ 9	77 $\pm$ 7	84 $\pm$ 10	28 $\pm$ 3	11 $\pm$ 2	—

and the resulting immune complexes were assayed for PI 3-kinase activity as described under Materials and methods. Wortmannin inhibited insulin stimulation of PI 3-kinase at 20 nM, whereas LY294002 was effective at 10 μ M (Figure 3A). No effect on insulin-stimulated PI 3-kinase activity was observed in the presence of rapamycin or PD098059. For the determination of p70^{s6k} and MAPK activities, whole-cell lysates were subjected to Western-blot analysis with anti-phospho-p70^{s6k} and anti-phospho-MAPK antibodies, as described under Materials and methods (Figure 3). As shown in Figure 3(B), insulin stimulation of serine/threonine phosphorylation of p70^{s6k} was completely blocked by rapamycin at the dose of 25 ng/ml. The inhibitors of PI 3-kinase, wortmannin and LY294002, at the doses tested previously, also completely inhibited insulin stimulation of p70^{s6k}; this result confirms that this pathway is downstream of PI-3 kinase. However, PD098059 did not affect insulin-stimulated p70^{s6k} (Figure 3B). Insulin stimulation of p42 and p44 phosphorylation was completely abolished in the presence of 30 μ M PD098059, the amount of phospho-MAPK under insulin treatment remaining unaffected by incubation of cells with either wortmannin, LY294002 or rapamycin (Figure 3C). These data clearly indicate that insulin signals throughout two independent pathways, MAPK and PI 3-kinase/p70^{s6k}, in fetal brown adipocytes.

Once the optimal doses for inhibition of insulin pathways were established, we tested the effect of these inhibitors on *GLUT4* gene expression (Figure 4). Cells (after 4 h of attachment and culture for 20 h in a serum-free medium) were cultured for a further 24 h in a serum-free medium with 10 nM insulin either in the absence or presence of wortmannin, LY294002, rapamycin or PD098059: the expression of *GLUT4* and *GLUT1* genes was studied by Northern blotting, as depicted in Figure 4(A). The resulting densitometric analysis after normalization with the amount of hybridized 18 S rRNA is shown in Table 1. The presence of wortmannin in the culture medium together with insulin partially precludes (35% inhibition) insulin-induced *GLUT4* mRNA accumulation. A higher inhibition (50%) in the expression of *GLUT4* mRNA induced by insulin was observed when the PI 3-kinase inhibitor LY294002 was present in the culture medium, probably due to the better stability in aqueous solution of LY294002 compared with wortmannin [32]. However, no changes in the induction of *GLUT4* mRNA expression by insulin were observed when the inhibitors of either p70^{s6k} or MAPK, rapamycin or PD098059 (Figure 4A, Table 1), were added. Although insulin does not have a positive effect on *GLUT1* mRNA expression, as described in Figure 1 [in fact *GLUT1* mRNA accumulation significantly decreased by 50% in

the presence of insulin for 24 h (Table 1)], we reblotted the membranes from cells treated with the different inhibitors of insulin signalling to check if those inhibitors could have any effect on *GLUT1* expression. *GLUT1* mRNA accumulation in the presence of insulin and inhibitors remained essentially unaltered as compared with insulin alone; only a slight decrease in *GLUT1* mRNA was observed in the presence of rapamycin (Figure 4A, Table 1).

In addition to the mRNA accumulation, we studied the effect of insulin treatment (either in the absence or presence of inhibitors) on *GLUT4* and *GLUT1* protein distribution in total membrane, by Western-blot analysis. Cells (after 4 h of attachment and culture for 20 h in a serum-free medium) were cultured for a further 48 h in a serum-free medium with 10 nM insulin either in the absence or presence of LY294002, PD098059 or rapamycin. Then cells were harvested, homogenized and total membrane fraction was obtained as described in Materials and methods. Membrane proteins (100 μ g) were submitted to Western-blot analysis and immunoblotted for *GLUT4* and *GLUT1* detection (Figure 4B). Densitometric analysis from four independent experiments is shown in Table 1. Both *GLUT4* and *GLUT1* proteins were immunodetectable in total membrane in fetal brown adipocytes cultured for 72 h in a serum-free medium, in agreement with the data from mRNA. Treatment of cells with insulin for 48 h increased the amount of *GLUT4* protein in the total membrane by 2.5-fold compared with untreated cells; meanwhile *GLUT4* protein content was down-regulated by 40% under PI 3-kinase inhibition (Figure 4B, Table 1). No changes on *GLUT4* protein content were observed under PD098059 or rapamycin treatment as compared with insulin-treated cells. The amount of *GLUT1* in plasma membrane decreased by nearly a 50% under treatment with insulin, remaining very low in the presence of inhibitors and insulin (Figure 4B).

Moreover, we have performed *GLUT4-CAT* transactivation experiments in the presence of inhibitors of both PI 3-kinase and Ras-MAPK pathways to confirm the data reported above. In these experiments PI 3-kinase activity was inhibited by either chemical inhibitors of p110, such as wortmannin or LY294002, or by co-transfection with the dominant-negative p85 α construct (Δ p85), a protein that lacks the inter-SH2 region required for binding to p110, thereby blocking activation of p110 [26]. Primary brown adipocytes, upon culture in 10% FCS-MEM medium for 24 h, were transiently co-transfected with 15 μ g of *GLUT4-CAT* fusion gene DNA together with 2 μ g of pCMV β -gal for internal control of transfection. Upon transfection, cells were cultured for 24 h in a serum-free medium in the presence of 10 nM insulin, either in the absence or presence of 10 μ M LY294002, 25 ng/ml

Figure 5 Inhibition of PI 3-kinase pathway, but not p70^{S6K} or MAPK pathways, precludes insulin-induced GLUT4 promoter activity

(A) Fetal brown adipocytes, upon culture in 10% FCS-MEM medium, were transiently transfected with 15 μ g of rat full-promoter *GLUT4*-CAT fusion gene together with 2 μ g of pCMV β -gal for internal control of transfection. After transfection, cells were cultured for 24 h in a serum-free medium either in the absence (C) or in the presence of 10 nM insulin (ins) alone or together 10 nM wortmannin (W), 10 μ M LY294002 (LY), 30 μ M PD098058 (PD) or 25 ng/ml rapamycin (R). (B) Cells were transiently co-transfected with 10 μ g of *GLUT4*-CAT together with 10 μ g of Δ p85 (a dominant-negative form of the regulatory subunit of PI 3-kinase) or with the empty vector (V), and after transfection treated for 24 h with 10 nM insulin. At the end of the culture time, cells were collected and assayed for CAT activity as described under Materials and methods. Representative experiments out of four are shown.

Table 2 Effect of the insulin-signalling inhibitors on insulin induction of glucose uptake

Cells were serum deprived for 20 h prior to culture for 24 h either in the absence (None) or in the presence of insulin alone or together with inhibitors. Glucose uptake was determined over the last hour of the incubation, as described in Materials and methods, and is expressed as disintegration per min per μ g of protein. The results are means $\pm$ S.E.M. ($n = 4$) 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 versus control are represented by * and differences between values in the presence of insulin plus inhibitors versus insulin are represented by †; * and †, $P < 0.01$.

Treatment	Glucose uptake (d.p.m./ μ g of protein)
None	153 $\pm$ 13
Insulin (10 nM)	423 $\pm$ 41*
Insulin + wortmannin (20 nM)	278 $\pm$ 40†
Insulin + LY (10 μ M)	224 $\pm$ 26†
Insulin + rapamycin (25 ng/ml)	550 $\pm$ 71
Insulin + PD (30 μ M)	569 $\pm$ 78

rapamycin or 30 μ M PD098059 (Figure 5A). In another set of experiments, cells were transiently co-transfected with 10 μ g of rat full-promoter *GLUT4*-CAT fusion gene in the presence 10 μ g of Δ p85 or empty vector, together with 2 μ g of pCMV β -gal for internal control of transfection. Upon transfection, cells were cultured for 24 h in serum-free medium in the presence of 10 nM insulin (Figure 5B). At the end of the culture period, cells were collected and assayed for CAT activity, CAT TLC was directly counted in a phosphorimager, and values normalized to β -gal activity from four experiments are shown in Table 1. Insulin stimulation of *GLUT4*-CAT fusion gene activity was partially precluded upon treatment with wortmannin (46% inhibition) or LY294002 (more than 70% inhibition). However, rapamycin did

not modify the insulin-stimulated GLUT4-CAT activity, in a similar fashion to PD098059 (Figure 5A, Table 1). Insulin stimulation of *GLUT4*-CAT fusion gene activity was almost precluded upon co-transfection with the Δ p85 construct, with an effect similar to treatment with LY294002 (Figure 5B).

Chronic insulin treatment induced glucose uptake in brown adipocytes and this effect is dependent on PI 3-kinase activity

PI 3-kinase is required for the movement of the glucose transporters to the cell membrane on acute insulin stimulation in both white and brown adipose tissues and in muscle cells [33–35]. Moreover, inhibition of PI 3-kinase activity resulted in an impaired insulin-induced *GLUT4* gene expression and promoter activity in fetal brown adipocytes. So, we decided to study glucose uptake after 24 h of treatment with insulin either in the absence or presence of the inhibitors of insulin signalling, wortmannin, LY294002, PD098059 or rapamycin. Control cells were cultured in a serum-free medium for 24 h in the absence of insulin. Cells were in the presence of 2-deoxy-D-[1-³H]glucose during the last 60 min of culture. Experiments using parallel dishes were carried out for the determination of proteins, since the use of inhibitors and/or insulin for 24 h can affect the protein content. Results are expressed as disintegration per min per μ g of protein throughout 1 h of labelling (Table 2). Insulin (10 nM) treatment for 24 h induced glucose uptake by 3-fold compared with levels observed in cells maintained in a serum-free medium. The presence of wortmannin or LY294002 significantly decreased insulin stimulation of glucose uptake, producing 35–50% inhibition. However, neither rapamycin nor PD098059 significantly modified the insulin-induced glucose uptake.

DISCUSSION

Although numerous studies *in vivo* clearly indicate that GLUT4 expression in adipocytes is under insulin and/or metabolic control [36,37], studies using cultured fat cells failed to establish

a stimulatory role of insulin in GLUT4 expression [38–40]. In fact, either in adipocyte cell lines or in adult primary white or brown adipocytes, insulin by itself increased GLUT1 but did not change or either decreased GLUT4 expression [38–40]. In this paper we have found that insulin or IGF-I at physiological concentrations up-regulate the expression of the *GLUT4* gene, producing a time-dependent mRNA accumulation and significantly increasing the amount of GLUT4 protein located in total membrane fraction in fetal brown adipocytes. In addition, insulin and IGF-I induce *GLUT4* gene expression at the transcriptional level, producing the transactivation of the *GLUT4-CAT* fusion gene (transiently transfected in primary cells). In consequence, insulin treatment for 24 h resulted in a significant increase in glucose uptake determined by incubating cells for the last hour in the presence of labelled deoxyglucose. In contrast, the *GLUT1-CAT* fusion gene is not transactivated by treatment with insulin or IGF-I, and in fact insulin treatment resulted in a significant down-regulation of GLUT1 mRNA and protein expression, probably due to mRNA and protein degradation.

Furthermore, GLUT4 mRNA expression under insulin/IGF-I stimulation resulted in the appearance of a new 2.3 kb isoform additional to the canonical 2.8 kb isoform. Although the existence of 5' heterogeneity in the rat GLUT4 mRNA has been reported, describing the appearance of an isoform containing an incomplete spliced intron [41], we found for the first time a shorter mRNA isoform. Whether this isoform is the result of the existence of alternative polyadenylation sites, and its contribution to GLUT4 protein synthesis, remains to be established.

Our results show for the first time a positive effect of insulin and IGF-I on the GLUT4 promoter, indicating the existence of an insulin response element within 2.2 kb of the 5' flanking region to govern the transcription of *GLUT4* gene. These results agree with those obtained in rat cardiomyocytes where there is evidence of a direct effect of insulin on *GLUT4* transcription [42]. However, either in adult white or brown adipocytes a positive effect of insulin on *GLUT4* gene expression has only been observed when it has been combined with dexamethasone [39,40,43]. Although only the occurrence of response elements for cAMP, tri-iodothyronine and glucocorticoids on the *GLUT4* gene promoter have yet been proposed [25,39,44], our results seem to suggest the likely existence of IGF-I and/or insulin response elements on the GLUT4 promoter. Whether these insulin regulatory elements could be located at the brown adipose tissue GLUT4 enhancer [45] remains to be established. Furthermore, since fetal brown adipocytes showed increased expression of c/EBP β in the presence of the PPAR γ transcription factor under insulin stimulation [46], we cannot rule out an indirect effect throughout these transcription factors to induce *GLUT4* expression, as has recently been described [47].

To address the point of the underlying molecular mechanisms involved in IGF-I/insulin effects on *GLUT4* gene expression, we have effectively blocked PI 3-kinase, p70^{s6k} and Ras/MAPK pathways with specific inhibitors under insulin stimulation. Our results show that inhibition of PI 3-kinase with either LY294002 or wortmannin down-regulated insulin-induced GLUT4 mRNA accumulation and GLUT4 protein content, without affecting *GLUT1* gene expression. Moreover, both inhibitors precluded insulin transactivation of the GLUT4 promoter. In a similar fashion, Δ p85 transfection blocked insulin-induced transactivation of the GLUT4 promoter. However, rapamycin has no effect on either insulin-induced up-regulation of GLUT4 mRNA or protein, or on transactivation of the GLUT4 promoter, indicating that p70^{s6k} activity is not involved in the insulin signalling leading to *GLUT4* gene expression. In this regard, p70^{s6k} seems to be more directly involved in regulating translation initiation

by insulin in 3T3L1 adipocytes [10]. Other downstream targets of PI 3-kinase, such as the serine/threonine kinase Akt or the atypical protein kinase C ζ , recently shown to be involved in acute insulin effects on GLUT4 translocation [48,49], should be further investigated in the insulin signalling leading to *GLUT4* gene expression. Furthermore, the insulin signalling involved in *GLUT4* gene expression in fetal brown adipocytes is independent of MAPK activation. Determination of glucose uptake in the presence of insulin together with insulin signalling inhibitors revealed changes on glucose uptake in parallel with those observed for *GLUT4* gene expression, indicating that GLUT4 rather than GLUT1 is contributing to glucose uptake under chronic insulin treatment. We have recently reported that the activation of PI 3-kinase by IGF-I is a requirement for brown adipocyte adipogenic differentiation [20] and that both PI 3-kinase and MAPK activation are required to increase activator protein-1 DNA-binding activity upon insulin treatment, leading to UCP1 promoter transactivation [50]. However, insulin up-regulation of *GLUT4* gene expression seems to be mainly dependent on PI 3-kinase activity. In this regard, PI 3-kinase seems to be necessary not only for acute effects of insulin on GLUT4 translocation to the plasma membrane in both white and brown adipose tissues but also for the insulin signalling leading to *GLUT4* gene expression in fetal brown adipocytes.

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