Insulin and dexamethasone induce *GLUT4* gene expression in foetal brown adipocytes: synergistic effect through CCAAT/enhancer-binding protein α

Rosario HERNANDEZ¹, Teresa TERUEL¹ and Margarita LORENZO²

Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad Complutense, 28040-Madrid, Spain

Treatment of foetal brown adipocytes in primary culture with either dexamethasone or insulin, at physiological concentrations, for 24 h up-regulates the expression of the *GLUT4* gene, producing a synergistic effect on mRNA accumulation (20-fold increase), in the amount of protein in the total membrane fraction (8-fold increase) and in the transactivation of a full-promoter *GLUT4*-chloramphenicol acetyltransferase gene (*CAT*) construct (7-fold increase). However, *GLUT1* expression remains essentially unmodified regardless of the presence of the hormones. As a consequence, exposure of brown adipocytes to dexamethasone and insulin results in a dramatic increase of glucose uptake (12-fold). Dexamethasone induces the expression of CCAAT/enhancer-binding protein (C/EBP) α , insulin promotes

myocyte enhancer factor-2 DNA-binding activity and both combined produces a significant increase in C/EBP α DNA-binding activity. Moreover, co-transfection with a wild-type C/EBP α construct transactivates a full-promoter *GLUT4*-*CAT* fusion gene, whereas a dominant-negative C/EBP α expression vector impairs the hormonal effects. Our results show that the synergism between insulin and glucocorticoids on glucose uptake is a consequence of the activation of the *GLUT4* promoter by the transcription factor C/EBP α .

Key words: CCAAT/enhancer-binding protein α (C/EBP α), glucose uptake, *GLUT4*, myocyte enhancer factor-2 (MEF-2).

INTRODUCTION

Brown adipose tissue is specialized in non-shivering thermogenesis in small mammals and is responsible for heat production associated with the expression of the mitochondrial uncoupling protein 1 ('UCP-1'). Since fatty acids are thermogenic fuels, this tissue also performs lipid synthesis. It is well known that insulin regulates lipogenesis and glucose is an essential lipogenic substrate. Moreover, glucose transport is maintained mainly by the activity of the insulin-regulated glucose transporter *GLUT4* in this tissue [1]. Foetal brown adipocytes display high-affinity binding sites for both insulin and insulin-like growth factor I ('IGF-I'), and both signals induce *GLUT4* mRNA expression concomitant with the onset of adipogenic- and thermogenic-differentiation programs [2–4].

In principle, there are at least three mechanisms by which insulin could modulate *GLUT4* function contributing to increased glucose uptake in peripheral tissues, including brown adipocytes. First, insulin could promote translocation of pre-existing intracellular sequestered populations of *GLUT4* proteins to the cell surface. Secondly, insulin could alter the intrinsic transport activity of *GLUT4* proteins at the cell surface by direct modifications of *GLUT4* itself or by interaction with other regulatory molecules. Thirdly, insulin could up-regulate the amount of *GLUT4* protein by increased biosynthesis, decreased degradation or both. Although these mechanisms are not mutually exclusive, the first model has been the most intensively studied; the participation of phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B ('PKB') and several protein kinase C isoforms in insulin-induced *GLUT4* redistribution to the plasma membrane in adipocytes and myocytes is well established [5–11]. Regarding the second model, a potential role for p38 mitogen-activated protein kinase ('MAP kinase') in the activation of

GLUT4 transporter by insulin has been proposed in some cellular systems [12]. However, the mechanism by which insulin could contribute to glucose uptake through regulation of *GLUT4* gene expression remains largely unexplored.

The expression of *GLUT4* is subject to tissue-specific, hormonal and metabolic regulation [5,13]. Studies *in vivo* clearly indicate that *GLUT4* gene expression is down-regulated in states of relative insulin deficiency such as streptozotocin-induced diabetes and chronic fasting, a situation that is reversed by insulin treatment and refeeding [14–16]. However, *in vitro* studies failed to establish a stimulatory role of insulin on *GLUT4* expression in white adipocytes and skeletal muscle cells [17], suggesting that additional factors could be involved *in vivo*. Dexamethasone, a synthetic glucocorticoid, has been shown to increase *GLUT4* expression in myocytes [18] as well as to interact with insulin in *GLUT4* expression in white adipocytes [19]. This prompted us to evaluate the potential role of dexamethasone as a co-regulator with insulin of glucose transporter expression using foetal rat brown adipocytes in primary culture. Our results indicate that insulin [activating myocyte enhancer factor (MEF)-2 DNA-binding activity] or dexamethasone [increasing CCAAT/enhancer-binding protein (C/EBP)- α] alone up-regulated *GLUT4* mRNA and protein expression through activation of the *GLUT4* promoter. A synergistic effect was observed on *GLUT4* gene expression leading to a dramatic increase in glucose uptake.

MATERIALS AND METHODS

Materials

Insulin, dexamethasone and BSA (fraction V, essentially fatty acid-free) were from Sigma. Foetal calf serum (FCS), PBS, minimal essential medium with Earle's salts (MEM) and Trizol

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; MEF, myocyte enhancer factor; FAS, fatty acid synthase; FCS, foetal calf serum; MEM, minimal essential medium with Earle's salts; β -gal, β -galactosidase; SREBP1, sterol-regulatory-element-binding protein 1.

¹ Both authors contributed equally to the experimental work.

² To whom correspondence should be addressed (e-mail: mlorenzo@farm.ucm.es).

were from Invitrogen. Nylon membranes were GeneScreen™ (PerkinElmer Life Science). Autoradiographic films were Kodak X-O-MAT/AR (Eastman Kodak). 2-Deoxy-D-[1-³H]glucose, [α -³²P]dCTP, [¹⁴C]chloramphenicol and the multiprimer DNA-labelling system kit were purchased from Amersham Biosciences. The mammalian transfection kit was from Stratagene. All other reagents used were of the purest grade available. The cDNAs used as probes were GLUT4, GLUT1, C/EBP α and fatty acid synthase (FAS) [20–22]. Rabbit anti-GLUT1 and GLUT4 antibodies were supplied by Chemicon. Anti-C/EBP α (sc-61) and anti-(sterol-regulatory-element-binding protein 1) (SREBP1; sc-8984) antibodies were from Santa Cruz Biotechnology.

Cell culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day Wistar rat fetuses and isolated by collagenase dispersion as described previously [2]. Cells were plated at 1.5×10^6 cells/60 mm-diameter or 4×10^6 cells/100 mm-diameter tissue-culture dishes in MEM supplemented with 10 % (v/v) 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 then maintained for 20 h in a serum-free MEM supplemented with 0.2 % (w/v) BSA, before culture for a further 24 h in the absence or presence of various doses of dexamethasone, insulin or both.

Measurement of glucose uptake

Cells were cultured as described above and 2-deoxy-D-[1-³H]glucose (1 μ Ci/ml) was added to the culture medium (MEM containing 0.2 % BSA) to a final concentration of 5 mM during the last 2 h of the culture time. Cells were then washed three times with ice-cold PBS and solubilized in 1 ml of 1 % (w/v) SDS, as described previously [23]. The radioactivity of a 200 μ l aliquot was determined in a scintillation counter. In parallel dishes, proteins were determined by the Bradford method [24], using Bio-Rad reagent and BSA as the standard.

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 [25]. Total cellular RNA (15 μ g) was subjected to Northern-blot analysis, i.e. electrophoresed on 0.9 % agarose gels containing 0.66 M formaldehyde, transferred on to GeneScreen™ membranes using a VacuGene blotting apparatus (LKB-Pharmacia) and cross-linked to the membranes by UV light. Hybridization was in 0.25 M NaHPO₄ (pH 7.2), 0.25 M NaCl, 100 μ g/ml denatured salmon sperm DNA, 7 % (w/v) SDS and 50 % deionized formamide containing denatured ³²P-labelled cDNA (10⁶ c.p.m./ml) for 24 h at 42 °C, as described previously [1]. cDNA labelling was carried out with [α -³²P]dCTP to a specific activity 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 (Amersham Biosciences).

Western blotting

Cells were lysed as described previously [4] and cellular proteins or total membrane proteins (30 μ g) were subjected to SDS/PAGE, transferred on to Immobilon membranes and were blocked using 5 % (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris/HCl (pH 7.5) and 150 mM NaCl], and incubated overnight with various antibodies, as indicated, in each case in Tris-buffered saline containing 0.05 % Tween-20 and 1 % (w/v) non-fat dried milk. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL-Plus) Western blotting protocol (Amersham Biosciences).

Transfection conditions and chloramphenicol acetyltransferase (CAT) determination

The following plasmid constructs were used for transfection experiments: full-promoter *GLUT4-CAT* fusion gene (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 +147, relative to the transcription start site); deleted-promoter *GLUT4-CAT* fusion gene (a promoter with 257 bp deleted from positions –110 to +147), both were kindly provided by Dr A. Zorzano (Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain) [26]; wild-type C/EBP α (a eukaryotic expression vector for wild-type C/EBP α) [21]; Δ C/EBP α (a eukaryotic expression vector for a dominant-negative mutant of C/EBP α), kindly provided by Dr A. F. Gombart (Division of Hematology, University of California, Los Angeles, CA, U.S.A.); and pCMV β -galactosidase (β -gal) (a viral promoter-driven expression of the reporter β -gal gene). Δ C/EBP α harbours a point mutation at nucleotide 1063 of the C/EBP α gene (C $\rightarrow$ G substitution) that results in the amino acid substitution Arg³⁰⁵ $\rightarrow$ Pro in the fork region between DNA-binding basic domain and the leucine zipper dimerization domain of the C/EBP α protein, conferring a dominant-negative property whereby the mutant polypeptide heterodimerizes with the wild-type polypeptide and prevents it from binding to DNA [27]. Primary brown adipocytes were cultured for 24 h in MEM containing 10 % (v/v) FCS and then transiently transfected using the calcium phosphate-mediated protocol with 10 μ g of *GLUT4-CAT* fusion gene alone or combined with 10 μ g of wild-type or dominant-negative C/EBP α together with 2 μ g of pCMV β -gal (to monitor transfection efficiency). After incubation for 4 h, cells were shocked with 3 ml of PBS containing 15 % (v/v) glycerol for 2 min, washed and fed with serum-free medium (either in the absence or presence of insulin and/or dexamethasone) for 24 h. Cells were then harvested and lysates were prepared for CAT and β -gal activity assays. CAT activity was determined by incubating 50 μ l of cell extracts with 0.25 μ Ci of [¹⁴C]chloramphenicol and 0.5 mM acetyl CoA in 0.25 M Tris (pH 7.8) at 37 °C for 12 h. After incubation, samples were subjected to TLC. The amount of substrate acetylated was quantified directly with a radioimaging device (Fujifilm BAS-1000). CAT enzyme activity was expressed as a percentage of [¹⁴C]chloramphenicol acetylated and normalized to the internal control β -gal (assayed according to the Stratagene protocol).

Gel-shift assay

Nuclear extracts were prepared from 4×10^6 cells. Cells were resuspended at 4 °C in buffer A [10 mM Hepes/KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol and 0.2 mM PMSF], allowed to swell on ice for 10 min, and then vortex-mixed

for 10 s. Samples were centrifuged, and the pellet was resuspended in ice-cold buffer C [20 mM Hepes/KOH (pH 7.9), 25 % (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF and 0.75 μ g/ml each of leupeptin and aprotinin] and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction was stored at -70 °C. The gel mobility-shift assay was performed essentially as described previously [28]. The double-stranded oligonucleotide used as the MEF-2 probe, corresponding to the MEF-2 site in the -522/-420 enhancer from the *GLUT4* promoter, was composed of the sequence 5'-CGTGGGAGCTAAAAATAGC-3'; the double-stranded oligonucleotide used as the C/EBP α probe, corresponding to the nuclear factor-interleukin 6 ('NF-IL6') site of the cyclo-oxygenase 2 ('COX2') promoter, was 5'-GGGTATTATGCAATTGGAAG-3'. The MEF-2 and C/EBP α double-stranded oligonucleotides were chemically synthesized by Sigma (Genosys). Oligonucleotides were labelled by using Klenow polymerase and [α -³²P]dCTP. The binding reaction was performed at 4 °C for 15 min in a buffer containing 0.5 ng of double-stranded oligonucleotide probe, 2 μ g of poly(dIdC) and 10 μ g of protein in buffer C and supplemented with 35 mM MgCl₂. For supershift and competition assays, nuclear extracts were previously incubated for 15 min at 4 °C with 1 μ l of the corresponding antibodies or 100-fold excess of unlabelled probe. DNA-protein complexes were resolved on a 6 % (w/v) polyacrylamide gel. The gel was dried and exposed to film at -70 °C.

Data analysis

Results are presented as means $\pm$ S.E.M. ($n = 8$ or 12) for duplicate or triplicate dishes from four independent experiments. Statistical significance was tested with a one-way ANOVA, followed by the protected least-significant different test. $P < 0.01$ was considered significant. In experiments using X-ray films (Hyperfilm), different exposure times were used to ensure that bands were not saturated.

RESULTS

Synergistic effect of insulin and dexamethasone on *GLUT4* gene expression, *GLUT4* promoter activity and glucose uptake

Foetal rat brown adipocytes when isolated and maintained for 20 h in culture expressed adipogenic and thermogenic genes, such as FAS and uncoupling protein-1 ('UCP1'), whereas the expression of the insulin-regulated glucose transporter *GLUT4* was barely detectable [2]. Under these conditions, treatment for 24 h with insulin up-regulated *GLUT4* gene expression in a PI 3-kinase/Akt-dependent manner [4]. We have explored the effect of dexamethasone and/or insulin on *GLUT4* gene expression in this cell model. Foetal rat brown adipocytes (after 4 h of attachment and cultured for 20 h in a serum-free medium) were cultured for a further 24 h in MEM containing 0.2 % BSA (with 5 mM glucose) either in the absence or presence of various doses of dexamethasone and/or insulin. The expression of *GLUT4* and *GLUT1* genes was studied by Northern blot, and the resulting densitometric analysis, after normalization with the amount of hybridized 18 S rRNA, are shown in Figure 1(A). A representative experiment is depicted in Figure 1(B). Insulin induced *GLUT4* mRNA in a dose-dependent manner, producing the maximal effect (8-fold) at 10 nM, whereas dexamethasone increased basal expression of *GLUT4* mRNA to the same level as insulin did (8-fold) at 100 nM. In the presence of both hormones, a synergistic effect was observed even at the lower dose of insulin

(1 nM) or dexamethasone (10 nM) used, producing a maximal induction (20-fold increase) at 10 nM insulin and 100 nM dexamethasone (Figures 1A and 1B). These doses were used in further experiments in the present study. Regarding the expression of the ubiquitous glucose transporter *GLUT1*, its mRNA level remained unmodified regardless of the presence of insulin or dexamethasone (Figure 1B). In order to validate if the effect of dexamethasone on *GLUT4* mRNA accumulation was dependent on the differentiation-inducing properties of the hormone, the expression of the adipogenic marker FAS was simultaneously analysed (Figure 1B). Dexamethasone did not modify the basal expression of FAS mRNA, but increased by 5-fold insulin-induced FAS gene expression. In addition to the mRNA accumulation, we studied the effect of insulin and dexamethasone treatment on *GLUT4* and *GLUT1* protein expression by Western-blot analysis (Figure 1C). Both insulin and dexamethasone alone increased *GLUT4* protein content in total membrane fraction 2-fold, and treatment with the two hormones together increased the *GLUT4* protein level 8-fold. No changes in *GLUT1* protein content were observed under any of the treatments.

These results seem to indicate that dexamethasone and insulin are inducing transcriptional activation of *GLUT4*. Consequently, we decided to study the effect of both factors on the transactivation of the *GLUT4* promoter driving the expression of the *CAT* reporter gene. After culture with MEM containing 10 % (v/v) FCS for 24 h, primary brown adipocytes were transiently co-transfected with 10 μ g of full-promoter *GLUT4-CAT* fusion gene [rat *GLUT4* full-length promoter (2400 bp)-*CAT*] 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 in the absence or presence of 100 nM dexamethasone and/or 10 nM insulin. At the end of the culture period, cells were collected and assayed for *CAT* activity. A representative *GLUT4-CAT* TLC experiment in primary brown adipocytes is shown in Figure 2(A). Densitometric analysis from the *CAT* TLC were directly counted in a phosphorimager, and values were normalized to β -gal activity. The *GLUT4-CAT* fusion gene was weakly transactivated in control primary brown adipocytes, whereas treatment with either 10 nM insulin or 100 nM dexamethasone resulted in a 3-fold increase, and when the two hormones were added together, the *CAT* activity was increased 7-fold (Figure 2A). These results provide evidence of a synergistic action of these hormones on *GLUT4* promoter activity in a similar manner to that observed for *GLUT4* mRNA and protein content.

One of the primary metabolic responses mediated by insulin is the stimulation of the facilitated glucose transport in insulin-responsive tissues such as brown adipose tissue. Since insulin and dexamethasone treatment for 24 h produced a dramatic increase in the expression of *GLUT4*, we decided to study glucose uptake under these experimental conditions. Glucose uptake was determined by incubation of cells in the culture medium in the presence of 2-deoxy-D-[1-³H]glucose during the last 2 h of culture. Parallel dishes were used for determination of protein content, since long-term treatments can affect the protein content. Results are expressed as d.p.m./ μ g of protein throughout 2 h of labelling (Figure 2B). Treatment with either insulin (10 nM) or dexamethasone (100 nM) alone for 24 h induced glucose uptake 3-fold compared with levels observed in cells maintained in the absence of hormones. The combined presence of insulin and dexamethasone dramatically increased glucose uptake 12-fold. The synergistic effect of dexamethasone and insulin on the induction of glucose uptake was found in a similar manner to the effect observed for *GLUT4* promoter transactivation, mRNA and protein content.

Figure 1 Insulin and dexamethasone up-regulate GLUT4 mRNA expression and GLUT4 protein accumulation in foetal brown adipocytes

(A) Cells were serum-deprived for 20 h prior to culture for 24 h in the absence or presence of various doses of insulin (without or with 100 nM dexamethasone; upper panel) or dexamethasone (without or with 10 nM insulin; lower panel). Total RNA (10 μ g) was subjected to Northern-blot analysis and hybridized with labelled GLUT4 cDNA. A final hybridization with the 18 S rRNA cDNA was performed for normalization. Densitometric analysis of GLUT4 mRNA levels are shown. Results are means $\pm$ S.E.M. ($n=8$). Statistical significance was tested as described in the Materials and methods section. * $P < 0.01$ between values in the presence of insulin and/or dexamethasone compared with control. ♦ $P < 0.01$ between values in the presence of dexamethasone and insulin compared with insulin or dexamethasone alone. (B) Cells were cultured for 24 h in the absence (C) or the presence of 10 nM insulin (Ins), 100 nM dexamethasone (Dex) or both (Dex + Ins). Total RNA (10 μ g) was subjected to Northern-blot analysis and hybridized with labelled GLUT4, GLUT1, FAS and 18 S rRNA cDNAs. Representative autoradiograms are shown. (C) Cells were cultured as in (B), and total membrane protein (30 μ g) was subjected to Western blotting with anti-GLUT4 or anti-GLUT1 antibodies. Representative autoradiograms are shown. Histograms represent densitometric analysis of GLUT4 protein levels. Results are means $\pm$ S.E.M. ($n=8$). Statistical significance was tested as described in (A).

Transcription factors involved in up-regulation of GLUT4 gene expression by insulin and dexamethasone

The specific transcription factors involved in the transactivation of GLUT4 promoter subjected to tissue-specific, hormonal and metabolic regulation have not been totally identified yet. However, the existence of binding domains to C/EBPs has been demonstrated in the GLUT4 promoter [26]. First, we decided to study the effect of treatment of brown adipocytes with dexamethasone and/or insulin for 24 h on C/EBP α mRNA and protein expression by Northern and Western blotting as shown in Figures 3(A) and 3(B) respectively. Insulin alone slightly decreased C/EBP α expression, dexamethasone increased C/EBP α mRNA and protein accumulation 2-fold, and no further increase was observed in the presence of the two hormones. This positive effect of dexamethasone on C/EBP α protein expression, regardless of the presence of insulin, was not observed for other transcription factors determined, such as SREBP1. As a consequence, we decided to study the ability of nuclear proteins from cells cultured for 24 h in the absence or presence of dexamethasone and/or insulin to bind to a C/EBP α double-stranded oligonucleotide in gel mobility-shift experiments (Figure 4A). Protein binding to DNA was observed in control brown adipocytes and this binding was increased approx. 2-fold by dexamethasone treatment and 4-fold in the combined presence of dexamethasone and insulin. This band appears to be specific,

since it was supershifted with the antibody against C/EBP α and disappeared in competition experiments with 100-fold molar excess of unlabelled probe. These results indicate that dexamethasone increases C/EBP α protein content and its binding to DNA and, although insulin does not induce C/EBP α expression, it is able to potentiate the activation of C/EBP α DNA-binding activity induced by dexamethasone, producing a dramatic up-regulation of GLUT4 gene expression.

The promoter region of the GLUT4 gene also contains a conserved binding site for the MEF-2 family of transcription factors that seems to be necessary for full promoter activity [29]. Accordingly, gel mobility-shift experiments were performed with a probe containing the MEF-2 site in the -522/-420 enhancer from the GLUT4 promoter, and nuclear extracts were obtained from cells treated as described above (Figure 4B). Two specific bands were detected in all the conditions, which disappeared in competition experiments with unlabelled probe. Insulin treatment doubled nuclear protein binding to DNA, regardless of the absence or presence of dexamethasone, when compared with untreated cells. These data suggest that the activation of MEF-2 could be responsible for insulin induction of GLUT4 gene expression.

Another approach to confirm the involvement of C/EBP α and MEF-2 in dexamethasone and insulin effects on GLUT4 gene expression was to perform transfection experiments with a deleted-promoter GLUT4-CAT fusion gene, which lacks

Figure 2 Synergistic effect of insulin and dexamethasone on *GLUT4* promoter activity and glucose uptake

(A) Foetal brown adipocytes cultured in MEM containing 10% (v/v) FCS were transiently transfected with 10 μ g of 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 in the absence (C) or the presence of 10 nM insulin (Ins), 100 nM dexamethasone (Dex) or both (Dex+Ins). At the end of the culture time, cells were collected and assayed for CAT activity, and the relative CAT activity normalized to β -gal activity is represented in the histogram. Results are means $\pm$ S.E.M. ($n = 8$). Statistical significance was tested as described in the legend for Figure 1(A). (B) Cells were serum-deprived for 20 h prior to culture for 24 h in the absence or the presence of 10 nM insulin (Ins), 100 nM dexamethasone (Dex) or both (Dex+Ins). Glucose uptake was determined over the last 2 h of culture by addition of 2-deoxy-D-[1- 3 H]glucose to the culture medium, as described in the Materials and methods section, and was expressed as d.p.m./ μ g of protein. Results are means $\pm$ S.E.M. ($n = 12$). Statistical significance was tested as described in the legend for Figure 1(A).

the region containing these response elements (rat *GLUT4* promoter of 257 bp) [13] (Figure 5A). This *GLUT4-CAT* fusion gene was not transactivated in the presence of dexamethasone and/or insulin. Finally, for a more direct demonstration of the involvement of C/EBP α in the induction of *GLUT4* by dexamethasone and its activation by the combined presence of insulin, we decided to use a genetic approach by using wild-type and dominant-negative C/EBP α expression vectors (Figures 5B and 5C). Co-transfection of a wild-type C/EBP α and full-promoter *GLUT4-CAT* produced a 3-fold stimulation of *GLUT4-CAT* fusion gene activity. Furthermore, insulin was also able to potentiate further (5-fold increase) *GLUT4-CAT* transactivation induced by co-transfection with C/EBP α

expression vector (Figure 5B). Moreover, co-transfection of a dominant-negative form of C/EBP α , which prevents wild-type polypeptide binding to DNA [27], and full-promoter *GLUT4-CAT* totally impaired the transactivation produced by dexamethasone and insulin treatment (Figure 5C).

DISCUSSION

GLUT4 expression is decreased in adipose tissue in human obesity and Type II diabetes [30]. Furthermore, adipose-selective targeting of the *GLUT4* gene impaired insulin action in muscle and liver [31], whereas a glycaemic improvement was observed in diabetic mice overexpressing *GLUT4* [32]. Most studies for regulating glucose uptake in peripheral tissues have been focused on the identification of factors and pathways that improve insulin induction of *GLUT4* translocation to the plasma membrane. However, insulin and/or other factors can contribute to elevation of glucose uptake by inducing the expression of *GLUT4*. In this regard, we have shown previously [4] that the expression of *GLUT4* protein could be induced by chronic treatment with insulin. The present study demonstrates that insulin and dexamethasone produce a synergistic effect at the transcriptional level, which leads to the accumulation of *GLUT4* mRNA and protein and, as a consequence, dramatically increases glucose uptake.

Dexamethasone was shown to be a potent inducer of *GLUT4* gene expression in foetal brown adipocytes and this effect was accompanied by parallel stimulation of glucose uptake. The increase in *GLUT4* expression produced by dexamethasone was independent of the differentiation-inducing properties of the hormone, since the expression of the adipogenic marker *FAS* remains unmodified. These results agreed with the positive effect of dexamethasone on *GLUT4* expression observed in neonatal primary brown adipocytes [33] as well as in C2C12 muscle cells, although, in this cell line, the effect did not contribute to an increased insulin-stimulation of glucose uptake [18]. In contrast, dexamethasone failed to induce *GLUT4* gene expression in 3T3-F442A adipocytes [19]. Furthermore, dexamethasone, when combined with insulin, produced a synergistic effect on both *GLUT4* protein content and glucose uptake in foetal brown adipocytes in a similar fashion as described in 3T3-F442A adipocytes [19]. Moreover, dexamethasone potentiated insulin-induced *FAS* gene expression in brown adipocytes, as shown in hepatocytes [34].

In addition, insulin or dexamethasone induce *GLUT4* gene expression at the transcriptional level, producing the transactivation of the *GLUT4-CAT* fusion gene transiently transfected into primary foetal brown adipocytes. Since the 2400 bp of the 5'-flanking region of the rat *GLUT4* gene is devoid of sequences similar to known insulin- or glucocorticoid-responsive elements [13], these hormones could be acting indirectly by modulating the expression of transcription factors to govern the transcription of *GLUT4* gene. In this regard, the existence of C/EBP α - and MEF-2-binding domains has been described in mouse *GLUT4* promoter [13] and, in fact, the hormonal effects were lost in experiments using a 257 bp *GLUT4* promoter with the C/EBP α - and MEF-2-binding domains deleted. Insulin treatment resulted in the induction of MEF-2-binding activity in electrophoretic mobility gel-shift assays regardless of the absence or presence of dexamethasone. These data agree with the presence of a *GLUT4*-MEF-2 binding complex reported previously [29] in brown adipose tissue and indicates for the first time a positive effect of insulin on MEF-2 transcription factor in foetal brown adipocytes, a finding in agreement with the effect found in muscle from diabetic mice treated with insulin [29].

Figure 3 Dexamethasone induces C/EBPα mRNA expression and protein accumulation

Serum-deprived brown adipocytes cultured for 24 h in the absence (C) or the presence of 10 nM insulin (Ins), 100 nM dexamethasone (Dex) or both (Dex + Ins) were subjected to Northern blotting with labelled C/EBPα and 18S rRNA cDNAs (**A**) and Western blotting with anti-C/EBPα or anti-SREBP1 antibodies (**B**). Representative autoradiograms are shown. Histograms represent densitometric analysis of C/EBPα mRNA and protein levels. Results are means $\pm$ S.E.M. ($n = 8$). Statistical significance was tested as described in the legend for Figure 1(A).

Figure 4 Regulation of C/EBPα and MEF-2 DNA-binding activities by insulin and dexamethasone

Brown adipocytes (20 h serum-starved) were cultured for a further 24 h in a serum-free medium in the absence (C) or the presence of 10 nM insulin (Ins), 100 nM dexamethasone (Dex) or both (Dex + Ins). At the end of the culture period, cells were collected and nuclei isolated. Nuclear extracts (10 μ g) were subjected to electrophoretic mobility-shift assay using a C/EBPα (**A**) or MEF-2 (**B**) consensus site radiolabelled probe. Representative autoradiograms and densitometric analysis are shown. Supershift experiments with the antibody against C/EBPα and competition experiments with 100-fold molar excess of unlabeled probe are also shown. Results are means $\pm$ S.E.M. ($n = 8$). Statistical significance was tested as described in the legend for Figure 1(A).

Figure 5 Wild-type C/EBP α construct transactivates GLUT4 promoter and a dominant-negative form impairs the hormonal effects

(A) Foetal brown adipocytes cultured in MEM containing 10% (v/v) FCS were transiently transfected with 10 μ g of deleted-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 in the absence (C) or the presence of insulin 10 nM (Ins), dexamethasone 100 nM (Dex) or both (Dex+Ins). At the end of the culture period, cells were collected and assayed for CAT activity as shown in a representative experiment. (B) Cells were co-transfected with 10 μ g of wild-type C/EBP α expression vector (wt C/EBP α) or an empty vector (V) together with 10 μ g of full-promoter *GLUT4*-CAT fusion gene, incubated for 24 h in a serum-free medium in the absence (–) or presence (+) of 10 nM insulin (Ins), and assayed for CAT activity. The relative CAT activity is represented in the histogram. Results are means $\pm$ S.E.M. ($n=8$). Statistical significance was tested as described in the Materials and methods section. * $P < 0.01$ between values in the presence of C/EBP α compared with control. ♦ $P < 0.01$ between values in the presence of insulin compared with its absence in C/EBP α -transfected cells. (C) Cells were co-transfected with 10 μ g of a dominant-negative C/EBP α expression vector (Δ C/EBP α) or an empty vector (V) together with 10 μ g of full-promoter *GLUT4*-CAT fusion gene, incubated for 24 h in a serum-free medium in the presence of 100 nM dexamethasone and 10 nM insulin (Dex+Ins), and assayed for CAT activity.

Several pieces of evidence suggest that C/EBP α could be a regulator of *GLUT4* gene expression [35], and our data demonstrate that co-transfection of primary brown adipocytes

with a wild-type C/EBP α expression vector transactivates the *GLUT4*-CAT fusion gene in a similar fashion as dexamethasone. These results are in agreement with the reconstitution of *GLUT4* gene expression observed in NIH-3T3 cells by ectopic expression of C/EBP α [36]. Furthermore, chronic dexamethasone treatment resulted in the induction of C/EBP α mRNA and protein levels in foetal brown adipocytes, in agreement with the effect observed in human preadipocytes [37]. Consequently, dexamethasone increased C/EBP α DNA-binding activity in nuclear extracts, and this effect was potentiated by the combined presence of insulin. Since insulin did not modify C/EBP α expression, the synergistic effect with dexamethasone on C/EBP α DNA-binding activity could be due to a phosphorylation event at the level of this transcription factor. In fact, insulin was able to increase further the transactivation of GLUT4 promoter produced by the C/EBP α expression vector transiently co-transfected into brown adipocytes. Furthermore, the stimulatory effect of dexamethasone and insulin on GLUT4 promoter activity was impaired when cells were co-transfected with a dominant-negative C/EBP α expression vector. In conclusion, we found individual and synergistic up-regulatory effects on GLUT4 mRNA and protein content by insulin and dexamethasone, mediated by the transcription factor C/EBP α , leading to a dramatic increase in glucose uptake.

T.T. was the recipient of a postdoctoral fellowship from the Comunidad Autonoma de Madrid. R.H. was the recipient of a postgraduate fellowship from the Ministerio de Educacion. This work was supported by Grant BMC2002-01322 from Direccion General de Investigacion Cientifica, Ministerio de Ciencia y Tecnologia, Spain. We thank COST/B17 Action on 'Insulin resistance, obesity and diabetes mellitus in the elderly' from the European Commission.

REFERENCES

- 1 Teruel, T., Valverde, A. M., Alvarez, A., Benito, M. and Lorenzo, M. (1995) Differentiation of rat brown adipocytes during late foetal development: role of insulin-like growth factor I. *Biochem. J.* **310**, 771–776
- 2 Teruel, T., Valverde, A. M., Benito, M. and Lorenzo, M. (1996) Insulin-like growth factor I and insulin induce adipogenic-related gene expression in fetal brown adipocyte primary cultures. *Biochem. J.* **319**, 627–632
- 3 Teruel, T., Valverde, A. M., Navarro, P., Benito, M. and Lorenzo, M. (1998) Inhibition of PI 3-kinase and RAS blocks IGF-I and insulin-induced uncoupling protein 1 gene expression in brown adipocytes. *J. Cell Physiol.* **176**, 99–109
- 4 Valverde, A. M., Navarro, P., Teruel, T., Conejo, R., Benito, M. and Lorenzo, M. (1999) Insulin and insulin-like growth factor I up-regulate *GLUT4* gene expression in fetal brown adipocytes, in a phosphoinositide 3-kinase-dependent manner. *Biochem. J.* **337**, 397–405
- 5 Watson, R. T. and Pessin, J. E. (2001) Subcellular compartmentalization and trafficking of the insulin-responsive glucose transporter, GLUT4. *Exp. Cell Res.* **271**, 75–83
- 6 Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T. and Olefsky, J. M. (1996) Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. *J. Biol. Chem.* **271**, 17605–17608
- 7 Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scott, P. H., Lawrence, J. C. J. and Roth, R. A. (1998) Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J. Biol. Chem.* **273**, 11937–11943
- 8 Bandyopadhyay, G., Standaert, M. L., Sajan, M. P., Karnitz, L. M., Cong, L., Quon, M. J. and Farese, R. V. (1999) Dependence of insulin-stimulated glucose transporter 4 translocation on 3-phosphoinositide-dependent protein kinase-1 and its target threonine-410 in the activation loop of protein kinase C- ζ . *Mol. Endocrinol.* **13**, 1766–1772
- 9 Hernandez, R., Teruel, T. and Lorenzo, M. (2001) Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes. *FEBS Lett.* **494**, 225–231
- 10 Valverde, A. M., Lorenzo, M., Navarro, P., Mur, C. and Benito, M. (2000) Okadaic acid inhibits insulin-induced glucose transport in fetal brown adipocytes in an Akt-independent and protein kinase C ζ -dependent manner. *FEBS Lett.* **472**, 153–158
- 11 Lorenzo, M., Teruel, T., Hernandez, R., Kayali, A. G. and Webster, N. J. (2002) PLC γ participates in insulin stimulation of glucose uptake through activation of PKC ζ in brown adipocytes. *Exp. Cell Res.* **278**, 146–157

- 12 Sweeney, G., Somwar, R., Ramlal, T., Volchuk, A., Ueyama, A. and Klip, A. (1999) An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. *J. Biol. Chem.* **274**, 10071–10078
- 13 Ezaki, O. (1997) Regulatory elements in the insulin-responsive glucose transporter (GLUT4) gene. *Biochem. Biophys. Res. Commun.* **241**, 1–6
- 14 Berger, J., Biswas, C., Vicario, P. P., Strout, H. V., Saperstein, R. and Pilch, P. F. (1989) Decreased expression of the insulin-responsive glucose transporter in diabetes and fasting. *Nature (London)* **340**, 70–72
- 15 Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I. and Pessin, J. E. (1989) Regulation of glucose transporter messenger RNA in insulin-deficient states. *Nature (London)* **340**, 72–74
- 16 Charron, M. J., Katz, E. B. and Olson, A. L. (1999) *GLUT4* gene regulation and manipulation. *J. Biol. Chem.* **274**, 3253–3256
- 17 Flores-Riveros, J. R., McLenithan, J. C., Ezaki, O. and Lane, M. D. (1993) Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: effects on transcription and mRNA turnover. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 512–516
- 18 Tortorella, L. L. and Pilch, P. F. (2002) C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression. *Am. J. Physiol. Endocrinol. Metab.* **283**, E514–E524
- 19 Hajdуч, E., Hainault, I., Meunier, C., Jardel, C., Hainque, B., Guerre-Millo, M. and Lavau, M. (1995) Regulation of glucose transporters in cultured rat adipocytes: synergistic effect of insulin and dexamethasone on GLUT4 gene expression through promoter activation. *Endocrinology* **136**, 4782–4789
- 20 Birnbaum, M. J. (1989) Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell (Cambridge, Mass.)* **57**, 305–315
- 21 Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. and McKnight, S. L. (1988) Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* **2**, 786–800
- 22 Amy, C. M., Witkowski, A., Naggert, J., Williams, B., Randhawa, Z. and Smith, S. (1989) Molecular cloning and sequencing of cDNAs encoding the entire rat fatty acid synthase. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3114–3118
- 23 Valverde, A. M., Lorenzo, M., Navarro, P. and Benito, M. (1997) Phosphatidylinositol 3-kinase is a requirement for insulin-like growth factor I-induced differentiation, but not for mitogenesis, in fetal brown adipocytes. *Mol. Endocrinol.* **11**, 595–607
- 24 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 25 Teruel, T., Clapham, J. C. and Smith, S. A. (1999) PPAR α activation by Wy 14643 induces transactivation of the rat UCP-1 promoter without increasing UCP-1 mRNA levels and attenuates PPAR γ -mediated increases in UCP-1 mRNA levels induced by rosiglitazone in fetal rat brown adipocytes. *Biochem. Biophys. Res. Commun.* **264**, 311–315
- 26 Vinals, F., Ferre, J., Fandos, C., Santalucia, T., Testar, X., Palacin, M. and Zorzano, A. (1997) Cyclic adenosine 3',5'-monophosphate regulates GLUT4 and GLUT1 glucose transporter expression and stimulates transcriptional activity of the GLUT1 promoter in muscle cells. *Endocrinology* **138**, 2521–2529
- 27 Asou, H., Gombart, A. F., Takeuchi, S., Tanaka, H., Tanioka, M., Matsui, H., Kimura, A., Inaba, T. and Koeffler, H. P. (2003) Establishment of the acute myeloid leukemia cell line Kasumi-6 from a patient with a dominant-negative mutation in the DNA-binding region of the C/EBP α gene. *Genes, Chromosomes Cancer* **36**, 167–174
- 28 Conejo, R., de Alvaro, C., Benito, M., Cuadrado, A. and Lorenzo, M. (2002) Insulin restores differentiation of Ras-transformed C2C12 myoblasts by inducing NF- κ B through an AKT/P70S6K/p38-MAPK pathway. *Oncogene* **21**, 3739–3753
- 29 Thai, M. V., Guruswamy, S., Cao, K. T., Pessin, J. E. and Olson, A. L. (1998) Myocyte enhancer factor 2 (MEF2)-binding site is required for *GLUT4* gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *J. Biol. Chem.* **273**, 14285–14292
- 30 Garvey, W. T., Maianu, L., Huecksteadt, T. P., Birnbaum, M. J., Molina, J. M. and Ciaraldi, T. P. (1991) Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *J. Clin. Invest.* **87**, 1072–1081
- 31 Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I. and Kahn, B. B. (2001) Adipose-selective targeting of the *GLUT4* gene impairs insulin action in muscle and liver. *Nature (London)* **409**, 729–733
- 32 Gibbs, E. M., Stock, J. L., McCoid, S. C., Stukenbrok, H. A., Pessin, J. E., Stevenson, R. W., Milici, A. J. and McNeish, J. D. (1995) Glycemic improvement in diabetic db/db mice by overexpression of the human insulin-regulatable glucose transporter (GLUT4). *J. Clin. Invest.* **95**, 1512–1518
- 33 Shimizu, Y., Kielar, D., Masuno, H., Minokoshi, Y. and Shimazu, T. (1994) Dexamethasone induces the GLUT4 glucose transporter, and responses of glucose transport to norepinephrine and insulin in primary cultures of brown adipocytes. *J. Biochem. (Tokyo)* **115**, 1069–1074
- 34 Rufo, C., Gasperikova, D., Clarke, S. D., Teran-Garcia, M. and Nakamura, M. T. (1999) Identification of a novel enhancer sequence in the distal promoter of the rat fatty acid synthase gene. *Biochem. Biophys. Res. Commun.* **261**, 400–405
- 35 Cooke, D. W. and Lane, M. D. (1999) The transcription factor nuclear factor I mediates repression of the GLUT4 promoter by insulin. *J. Biol. Chem.* **274**, 12917–12924
- 36 El-Jack, A. K., Hamm, J. K., Pilch, P. F. and Farmer, S. R. (1999) Reconstitution of insulin-sensitive glucose transport in fibroblasts requires expression of both PPAR γ and C/EBP α . *J. Biol. Chem.* **274**, 7946–7951
- 37 Zilberfarb, V., Siquier, K., Strosberg, A. D. and Issad, T. (2001) Effect of dexamethasone on adipocyte differentiation markers and tumour necrosis factor- α expression in human PAZ6 cells. *Diabetologia* **44**, 377–386

Received 27 February 2003/11 March 2003; accepted 17 March 2003

Published as BJ Immediate Publication 17 March 2003, DOI10.1042/BJ20030325