Dexamethasone Inhibits Insulin-Stimulated Recruitment of GLUT4 to the Cell Surface in Rat Skeletal Muscle

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To test the hypothesis that glucocorticoids reduce insulin-stimulated skeletal muscle glucose transport by inhibiting the recruitment of GLUT4 glucose transporters to the cell surface, we determined the effect of glucocorticoid treatment on cell-surface GLUT4 using the impermeant glucose transporter photolabel, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-[2-^{3}H]1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA), and GLUT4 immunoprecipitation. Male Sprague-Dawley rats were treated with dexamethasone ([Dex] 0.9 mg/kg for 2 days) and compared against pair-fed controls. 2-[^{3}H]deoxyglucose (2-[^{3}H]DG) uptake in isolated soleus muscles was measured under conditions in which uptake reflects glucose transport activity. In control muscles, 2-[^{3}H]DG uptake was stimulated eightfold by insulin (20 nmol/L). Dex treatment reduced maximal insulin-stimulated 2-[^{3}H]DG uptake by 48% ± 4% (mean ± SEM) and decreased cell-surface (ATB-[2-^{3}H]BMPA–photolabeled) GLUT4 by 48% ± 3%, despite an increase in total muscle GLUT4 content of 26% ± 7%. These findings indicate that glucocorticoid-induced inhibition of insulin-stimulated glucose transport in muscle is due to impaired recruitment of GLUT4 to the cell surface.

THE DIABETOGENIC EFFECT of exogenous or endogenous glucocorticoid excess results in part from the development of peripheral insulin resistance,[1] wherein insulin fails to normally stimulate glucose uptake into skeletal muscle, the main site of insulin-mediated glucose disposal.[2] This action of glucocorticoids is well known, but the precise mechanism of this common clinical problem remains uncertain. Recruitment of GLUT4 glucose transporters to the cell surface in response to insulin is thought to be the major event in the stimulation of glucose transport in skeletal muscle, as well as other insulin-sensitive peripheral tissues (adipose and heart).[3,4] Previous studies have demonstrated that glucocorticoid excess reduces insulin-stimulated, insulin-like growth factor-I (IGF-I)–stimulated, and hypoxia-stimulated hexose uptake in rat skeletal muscle, and also reduces hexose uptake even in the absence of insulin, suggesting that glucocorticoids may reduce muscle glucose transport, at least in part, by altering subcellular GLUT4 trafficking.[5]

To test the hypothesis that glucocorticoids impair recruitment of GLUT4 to the cell surface, we measured cell-surface–accessible GLUT4 content by photolabeling glucose transporters in isolated rat soleus muscles with the impermeant photoaffinity reagent, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-[2-^{3}H]1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA), with subsequent GLUT4 immunoprecipitation.[6-8] We report herein that the inhibition of insulin-stimulated glucose transport in muscle from glucocorticoid-treated rats can be accounted for by a reduction in cell-surface GLUT4.

MATERIALS AND METHODS

Materials

Bovine insulin (24.4 IU/mg), cytochalasin B, and radioimmunoassay-grade bovine serum albumin were obtained from Sigma (St Louis, MO). Dexamethasone sodium phosphate (Dex) was from American Regent Laboratories (Shirley, NY). Protein A–Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ). Thesit detergent was purchased from Boehringer Mannheim (Indianapolis, IN). 2-[1,2-^{3}H(*N*)]-deoxy-D-glucose (26 Ci/mmol), D-[1-^{14}C]-mannitol (49 mCi/mmol), and [^{125}I]protein A (9 μCi/μg) were purchased from Du Pont-NEN (Boston, MA). [^{125}I]-labeled sheep anti-mouse immunoglobulin G (IgG) antibody was from Amersham (Arlington Heights, IL). A rabbit polyclonal antiserum prepared against the 20–amino acid peptide corresponding to the COOH terminus of GLUT4 was kindly supplied by Hoffman-La Roche (Nutley, NJ), and anti-GLUT4 monoclonal antibody 1F8 was a gift from Dr Paul Pilch (Boston University School of Medicine, Boston, MA). ATB-[2-^{3}H]BMPA (10 Ci/mmol) was a gift from Dr G.D. Holman (University of Bath, Bath, UK).

Treatment of Animals

Male Sprague-Dawley rats (100 to 110 g; Charles River, Wilmington, MA) were fed standard rat chow. Treated rats received Dex (0.9 ± 0.1 mg/kg/d) in the drinking water (2.5 mg/L) for 2 days, whereas control rats received tap water alone and were pair-fed as previously described.[5] After 2 days of treatment, fasted (4 hours) rats were killed by carbon dioxide inhalation, and the soleus muscles were rapidly excised for hexose uptake measurement or photoaffinity labeling.

Hexose Uptake Measurement in Isolated Soleus Muscles

2-[^{3}H]deoxyglucose (2-[^{3}H]DG) uptake was measured under conditions in which uptake reflects glucose transport activity. Excised unsplit muscles were placed directly into stoppered 25-mL Erlenmeyer flasks and preincubated for 40 minutes at 29°C with orbital agitation at 180 rpm in gassed (95% O_2/5% CO_2) Krebs-Henseleit bicarbonate buffer containing 6.5 mmol/L glucose, 2 mmol/L sodium pyruvate, and 0.1% bovine serum albumin. After washing and further incubation for 30 minutes in fresh glucose-free buffer (with or without 20 nmol/L insulin), uptake was started by adding tracer concentrations of 2-[1,2-^{3}H(*N*)]-deoxy-D-glucose (1.5 μCi/mL, 57 nmol/L) and D-[1-^{14}C]-mannitol (0.3 μCi/mL) and was terminated at 30 minutes during the linear phase of 2-[^{3}H]DG uptake. ^{3}H and ^{14}C radioactivity in 10% trichloroacetic acid extracts of the muscles and in the uptake medium was determined by

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dual-channel liquid scintillation counting, and mannitol uptake was used to correct for 2-[^{3}H]DG uptake into the interstitial space.

Photoaffinity Labeling of Isolated Rat Soleus Muscles

The methods of Wilson and Cushman[6] and Lund et al[7] were used with modifications. Muscles were isolated as already described, and after the 30-minute incubation in glucose-free buffer, muscles (up to four at a time) were transferred to 1 mL of the same gassed buffer (with or without insulin) containing 1 mCi/mL (100 μmol/L) ATB-[2-^{3}H]BMPA. Muscles were incubated in the dark with agitation for 10 minutes (during which time the medium equilibrated completely with the interstitial space [data not shown]) at 17°C, a temperature at which photolabel internalization is markedly reduced.[6,7] Duplicate 3-μL medium aliquots were removed after each incubation for determination of radioactivity. Muscles were then irradiated at 17°C for 4 minutes (two 2-minute intervals) 5 cm perpendicular from the center of a planar array of four 15-W 300-nm fluorescent tubes (catalog no. R-15-3000; Southern New England Ultraviolet, Branford, CT) housed in an inverted UV transilluminator with the gelatin filter removed and the line voltage boosted to 135 V. Muscles were manually turned over between radiation intervals. Preliminary studies showed that 4 minutes of irradiation was sufficient to achieve maximal labeling of GLUT4 with ATB-[2-^{3}H]BMPA under these conditions. The muscles were then blotted, trimmed, and frozen.

Preparation of Detergent Extracts of Total Crude Muscle Membranes

Crude muscle membranes were prepared and extracted with Thesit detergent as previously described.[6] The extract protein concentration was determined in duplicate 5-μL aliquots using the BCA Protein Assay Reagent from Pierce (Rockford, IL) with bovine serum albumin as a standard.

GLUT4 Immunoprecipitation

Anti-GLUT4-COOH-terminus antibody coupled to protein A–Sepharose was used as previously described,[6] except that the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)/bead-elution buffer contained 4% SDS and 150 mmol/L dithiothreitol. Preliminary immunoprecipitation experiments demonstrated virtually complete clearing of GLUT4 from Thesit extracts and elution of GLUT4 from protein A–Sepharose. GLUT4 was immunoprecipitated with equal efficiency from control and Dex-treated rat muscles.

Immunoblot Analysis of GLUT4

For quantitation of GLUT4 in Thesit extracts, Thesit was first removed by chloroform/methanol extraction, and immunoblot analysis was performed as previously described[9] using the polyclonal anti-GLUT4-COOH-terminus antiserum and [^{125}I]protein A. For quantitation of immunoprecipitated GLUT4 eluted from protein A–Sepharose beads, monoclonal antibody 1F8 (5 μg IgG/mL) and [^{125}I]-labeled sheep anti-mouse IgG antibody (0.3 μCi/mL) were used.

Quantitation of ATB-[2-^{3}H]BMPA–Labeled GLUT4

Immunoprecipitated GLUT4 was subjected to SDS-PAGE, and ^{3}H was determined in gel slices by liquid scintillation counting as previously described.[6] The data were expressed as disintegrations per minute per milligram total crude membrane extract protein used in immunoprecipitation, and were normalized to 1 mCi ATB-[2-^{3}H]BMPA/mL incubation medium to correct for differences in medium radioactivity among experiments.

Statistical Analysis

The data are expressed as the mean ± SEM. For single comparisons, P values were calculated by Student's unpaired t test (two-tailed).

RESULTS

Effect of Dex Treatment on Body Weight and Soleus Muscle Characteristics

As expected, pair-fed control animals (n = 23) gained weight (+12 ± 1 g) and Dex-treated animals (n = 21) lost weight (−7 ± 1 g) over the 2-day treatment period.[5,10] The final weight of soleus muscles from control and Dex-treated rats was comparable (49 ± 1 mg [n = 46] and 48 ± 1 mg [n = 42], respectively), and the Thesit-extractable protein yield in total crude soleus membranes from control and Dex-treated rats was similar (20 control muscles, 10.2 ± 0.3 mg/g tissue; 16 Dex-treated muscles, 10.7 ± 0.5 mg/g tissue; $P > .2$). GLUT4 content per gram of extract protein, expressed as a percentage of the mean control value in each of five experiments, increased in soleus muscles from Dex-treated rats (126% ± 7%, n = 20 muscles) compared with controls (100% ± 3%, n = 15 muscles; $P < .005$), as previously reported.[5,10]

Effect of Dex Treatment on Basal and Insulin-Stimulated 2-[^{3}H]DG Uptake in Isolated Soleus Muscles

In soleus muscles from control animals, a maximally effective concentration of insulin (20 nmol/L) stimulated 2-[^{3}H]DG uptake by 8.2 times the basal level (mean stimulation in three experiments). In the same experiments, both basal and insulin-stimulated 2-[^{3}H]DG uptake were decreased (42% and 39%, respectively) in muscles from Dex-treated animals (uptake in pmol/g wet weight/30 min, n = 9 muscles per group: basal, control 12 ± 1 and Dex-treated 7 ± 1, $P < .01$; insulin-stimulated, control 93 ± 4 and Dex-treated 57 ± 5, $P < .001$), as previously shown.[5]

Effect of Dex Treatment on Cell-Surface GLUT4 in Insulin-Stimulated Isolated Soleus Muscles

The impermeant glucose transporter photolabel ATB-[2-^{3}H]BMPA, in combination with GLUT4 immunoprecipitation, was used to identify and quantify cell-surface GLUT4 in isolated soleus muscles. Figure 1 (inset) illustrates typical SDS-PAGE profiles of ATB-[2-^{3}H]BMPA–photolabeled GLUT4 immunoprecipitated from Thesit extracts of crude soleus membranes. In insulin-stimulated muscle, a distinct peak of ^{3}H counts is observed in the molecular mass range corresponding to that of GLUT4 in immunoblots. Cytochalasin B, a specific glucose transporter competitive inhibitor, effectively blocked labeling of GLUT4 by ATB-[2-^{3}H]BMPA in insulin-stimulated muscles, as previously demonstrated.[6,7] In the basal state, ATB-[2-^{3}H]BMPA–photolabeled (cell-surface) GLUT4 was low, and ^{3}H counts from photolabeled GLUT4 were not sufficiently above background (Fig 1, inset) to allow meaningful comparison of unstimulated muscles from control and Dex-treated animals. In insulin (20 nmol/L)-stimulated muscles, Dex treatment decreased ATB-[2-^{3}H]BMPA–photolabeled GLUT4 by 48% ± 3% (Fig 1; by comparison, Dex treatment reduced 2-[^{3}H]DG uptake by 48% ± 4% in parallel muscles from the

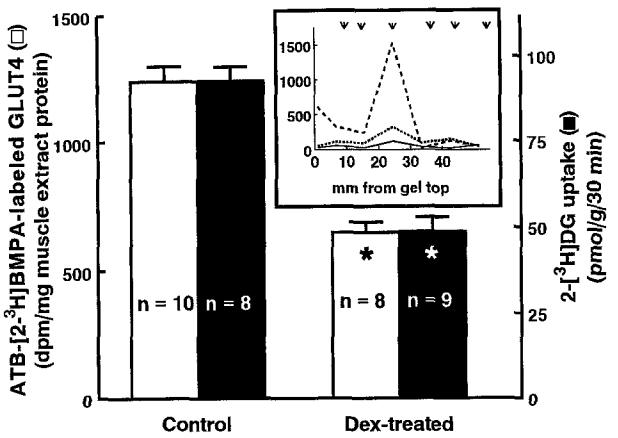

Fig 1. ATB-[2-^{3}H]BMPA–labeled (cell-surface) GLUT4 and 2-[^{3}H]DG uptake in rat soleus muscle. Isolated muscles were incubated in the presence of 20 nmol/L insulin and then used for measuring either ATB-[2-^{3}H]BMPA–labeled GLUT4 (□) or 2-[^{3}H]DG (■) uptake (n = no. of muscles per group). *$P < .001$ *v* control. Error bars are 1 SE. Inset: SDS-PAGE profiles of GLUT4 immunoprecipitates from 3 ATB-[2-^{3}H]BMPA–photolabeled control rat soleus muscles incubated in the absence of insulin (—), in the presence of insulin 20 nmol/L (- - - -), or in the presence of insulin 20 nmol/L and cytochalasin B 50 μmol/L (dotted line). Radioactivity (y-axis in dpm/mg crude muscle membrane extract protein) in each gel slice is plotted at the position of the middle of the slice. The first slice represents the gel origin (thin ≈ 1-mm slice). Positions of molecular weight markers are indicated by arrows (from left to right: 143, 97, 50, 35, 30, and 22 kd).

same experiments). The Dex-induced reduction of photolabeled GLUT4 is the same whether expressed per milligram of protein in detergent extracts of muscle membranes or per gram wet weight of muscle, since Dex treatment did not alter the Thesit-extractable protein yield in crude muscle membrane preparations.

DISCUSSION

We have previously shown that glucocorticoid-induced insulin resistance is characterized by a reduction of basal, insulin-stimulated, IGF-I–stimulated, and hypoxia-stimulated muscle glucose transport, despite an increase in muscle GLUT4 content.[5] These findings suggested that glucocorticoids may act, in part, by inhibiting the recruitment of GLUT4 to the cell surface. In the present study using the impermeant glucose transporter photoaffinity reagent ATB-[2-^{3}H]BMPA in combination with GLUT4 immunoprecipitation, we show that the glucocorticoid-induced impairment of insulin-stimulated glucose transport in skeletal muscle can be accounted for by a reduction in cell-surface–accessible GLUT4. In intact cells, the combination of these photolabeling and immunoprecipitation techniques can be used to identify only the glucose transporters that are capable of transporting glucose across the plasma membrane, ie, transporters at the cell surface that are physically accessible to glucose.[11,12] Indeed, studies have demonstrated that the stimulation of glucose transport by insulin in rat and mouse soleus muscles can be completely accounted for by the increase in cell-surface GLUT4 assessed by photoaffinity labeling with ATB-[2-^{3}H]BMPA.[6-8]

Venkatesan et al[13] recently reported that in vivo Dex treatment did not affect subcellular distribution of GLUT4, suggesting that the mechanism by which GLUT4 is translocated to the plasma membrane remained intact. However, in their study, the methods used to identify and quantify glucose transporters were based on subcellular fractionation, a method that cannot distinguish between transporters in the plasma membrane that are accessible to and capable of transporting glucose, ie, functional, and those that are not. The apparent discrepancy between the observations of the present study and those of Venkatesan et al[13] may be related to differences in the techniques used to assess plasma membrane GLUT4 content. Indeed, in studying the effect of high-fat feeding on GLUT4 recruitment to the plasma membrane, an analogous discrepancy between the results obtained using these two decidedly disparate techniques has also been noted.[8,14]

Since studies in rat adipocytes have shown that Dex-induced inhibition of glucose transport results from a decrease in the maximal rate rather than an increase in K_t,[15,16] the present results are most consistent with the hypothesis that glucocorticoid-induced muscle insulin resistance is due, at least in part, to impaired recruitment of GLUT4 to the cell surface rather than to a reduction in cell-surface GLUT4 intrinsic activity. Such an effect could result from alterations in GLUT4 trafficking steps that allow interaction of plasma membrane-associated GLUT4 with extracellular glucose,[12,17] including fusion of plasma membrane–docked GLUT4-containing vesicles. Further deciphering of the mechanisms underlying glucocorticoid-induced and other forms of skeletal muscle insulin resistance will require a more complete understanding of the molecular events involved in subcellular GLUT4 trafficking.

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