EXPERIMENTAL STUDY

Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes

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Abstract

Objective: Glucocorticoid excess leads to insulin resistance. This study explores the effects of glucocorticoids on the glucose transport system and insulin signalling in rat adipocytes. The interaction between glucocorticoids and high levels of insulin and glucose is also addressed.

Design and Methods: Isolated rat adipocytes were cultured for 24 h at different glucose concentrations (5 and 15 mmol/l) with or without the glucocorticoid analogue dexamethasone (0.3 μmol/l) and insulin (10^4 μU/ml). After the culture period, the cells were washed and then basal and insulin-stimulated glucose uptake, insulin binding and lipolysis as well as cellular content of insulin signalling proteins (insulin receptor substrate-1 (IRS-1), IRS-2, phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB)) and glucose transporter isoform GLUT4 were measured.

Results: Dexamethasone in the medium markedly decreased both basal and insulin-stimulated glucose uptake at both 5 and 15 mmol/l glucose (by ∼40–50%, $P < 0.001$ and $P < 0.05$ respectively). Combined long-term treatment with insulin and dexamethasone exerted additive effects in decreasing basal, and to a lesser extent insulin-stimulated, glucose uptake capacity ($P < 0.05$) compared with dexamethasone alone, but this was seen only at high glucose (15 mmol/l). Insulin binding was decreased (by ∼40%, $P < 0.05$) in dexamethasone-treated cells independently of surrounding glucose concentration. Following dexamethasone treatment a ∼75% decrease ($P < 0.001$) in IRS-1 expression and an increase in IRS-2 (by ∼150%, $P < 0.001$) was shown. Dexamethasone also induced a subtle decrease in PI3-K (by ∼20%, $P < 0.01$) and a substantial decrease in PKB content (by ∼45%, $P < 0.001$). Insulin-stimulated PKB phosphorylation was decreased (by ∼40%, $P < 0.01$) in dexamethasone-treated cells. Dexamethasone did not alter the amount of total cellular membrane-associated GLUT4 protein. The effects of dexamethasone *per se* on glucose transport and insulin signalling proteins were mainly unaffected by the surrounding glucose and insulin levels. Dexamethasone increased the basal lipolytic rate (∼4-fold, $P < 0.05$), but did not alter the antilipolytic effect of insulin.

Conclusions: These results suggest that glucocorticoids, independently of the surrounding glucose and insulin concentration, impair glucose transport capacity in fat cells. This is not due to alterations in GLUT4 abundance. Instead dexamethasone-induced insulin resistance may be mediated via reduced cellular content of IRS-1 and PKB accompanied by a parallel reduction in insulin-stimulated activation of PKB.

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Introduction

Type 2 diabetes is characterized by insulin resistance in the major target tissues coupled with insufficient insulin secretion and this leads to an impaired uptake and metabolism of glucose. The evolvement of type 2 diabetes is considered to be a gradual process, usually starting with insulin resistance. When there is a concomitant vulnerability in β-cell capacity, a relative insulin deficiency eventually occurs upon elevated demand, and diabetes develops.

The factors responsible for the early steps in the development of insulin resistance are not fully understood. One important factor could be insulin antagonistic

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hormones, e.g. glucocorticoids, growth hormone and catecholamines. Glucocorticoid hormones are produced in the adrenal cortex under the control of the hypothalamic–pituitary–adrenal axis. They have several important effects in different physiological systems, e.g. the regulation of salt and water balance, blood pressure, immune function and metabolism. Glucocorticoid excess results in insulin resistance (1) by blunting insulin's action to suppress hepatic glucose production and stimulate peripheral glucose utilization (2). Glucocorticoids also have a direct inhibitory effect on glucose-induced insulin release in the β-cells (3).

The mechanisms by which glucocorticoids inhibit insulin-stimulated glucose uptake in peripheral tissues are still largely unknown. Glucocorticoids could potentially inhibit glucose uptake at one or more steps along the signalling pathway through which insulin stimulates glucose transport. The uptake of glucose in insulin-sensitive cells like muscle and fat cells is to a great extent an insulin-regulated process, mediated primarily by the facilitative glucose transporter isoform GLUT4 (4–6). Most of GLUT4 (>90%) is in the basal state sequestered in an intracellular pool, and insulin stimulates glucose uptake mainly by recruiting this intracellular GLUT4 pool to the plasma membrane (5, 7, 8). The insulin signalling process involves a cascade of events initiated by insulin binding to the α-subunits of its cell-surface receptor. The receptor β-subunits becomes autophosphorylated and its intrinsic tyrosine kinase is activated eliciting tyrosine phosphorylation of insulin receptor substrates (IRSs) (9). Binding of the regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) to IRSs results in activation of PI3-K, which is necessary for insulin action on glucose transport (10–13), glycogen synthase (14) and anti-lipolysis (11). Recently, much interest has been focused on the serine/threonine protein kinase B (PKB) downstream of PI3-K (15) since PKB activation seems to be involved in GLUT4 translocation (16, 17).

Several situations of insulin resistance in man (18) as well as in animal models (19) have some degree of hypercortisolism, e.g. Cushing's syndrome and abdominal obesity. Pharmacological treatment with high doses of glucocorticoids leads to an impairment of insulin sensitivity. A previous study in 3T3-F442A adipocytes reported a dexamethasone-induced decrease in IRS-1 but increase in PI3-K protein levels, yielding a mild non-significant decrease in IRS-1-associated PI3-K activity (20). There are, however, still many unsolved questions about the mechanisms for dexamethasone-induced insulin resistance.

The aim of the present work was to investigate further the mechanisms involved in glucocorticoid-induced insulin resistance at the cellular level. This was studied in primary cultured rat adipocytes as an established model for insulin target tissues. In addition, adipocyte-specific defects can lead to abnormalities in other tissues, i.e. muscle and liver (21). The effects of long-term treatment with the glucocorticoid analogue dexamethasone on the glucose transport system as well as insulin signalling proteins were investigated. We also wished to elucidate the possible interaction of glucocorticoids vs hyperglycaemia and hyperinsulinaemia respectively.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 150–200 g were obtained from B&K, Sollentuna, Sweden. The study protocol was approved by the Umeå Ethical Committee for Animal Research.

Chemicals

D-[U-^{14}C]glucose (specific activity ~200–300 mCi/mmol), human monocomponent [^{125}I]Tyr-14-insulin (specific activity ~2000 Ci/mmol) and ECL Blotting reagents were purchased from Amersham Pharmacia Biotech, Freiburg, Germany. [γ-^{32}P]ATP (specific activity 5 Ci/μmol) was from Amersham Pharmacia Biotech, Amersham, Bucks, UK. Anti-IRS-1, IRS-2, p85α/β (PI3-K), Akt1/2 (PKB), GLUT1, GLUT4 polyclonal antibodies as well as secondary antibodies were obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). The anti-phospho-Akt1 (Ser^{473}) and anti-phospho-p70^{s6k} (Thr^{389}) antibodies as well as secondary antibodies were from New England Biolabs (Beverly, MA, USA). BSA (fraction V), N^6-(R-phenylisopropyl) adenosine (PIA), 8-bromo-cAMP and dexamethasone were from Sigma Chemical Co. (St Louis, MO, USA). Adenosine deaminase (ADA), glycerokinase and collagenase A were from Boehringer Mannheim (Mannheim, Germany). DMEM, medium 199, fetal calf serum (FCS), and penicillin/streptomycin were obtained from Gibco BRL, Life Technologies (Paisley, Strathclyde, UK). Human insulin (Actrapid, 100 U/ml) was from Novo Nordisk A/S (Copenhagen, Denmark).

Isolation and primary culture of rat adipocytes

Male Sprague–Dawley rats, freely fed, were killed by decapitation and epididymal fat pads were immediately excised and minced. Isolated fat cells were obtained by shaking the tissue in polypropylene containers at 37 °C for 1 h in medium 199 containing 5.6 mmol/l glucose with 40 mg/ml BSA and 0.6 mg/ml collagenase. The cells were then filtered through a nylon mesh and washed four times with fresh medium. Isolated adipocytes were placed in flasks containing DMEM with 5 or 15 mmol/l D-glucose as indicated, 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37 °C for 24 h unless otherwise specified in the absence or presence of dexamethasone at

0.3 µmol/l, a concentration known to produce a maximum inhibition of glucose transport (22), and insulin (10^4 µU/ml) under a gas phase of 95% O_2 and 5% CO_2. Glucose concentrations decreased after 24 h to ∼4 and ∼12 mmol/l, for cells cultured at initial glucose concentrations of 5 or 15 mmol/l respectively. Cell size was determined in isolated adipocytes as previously described (23) and it did not differ between dexamethasone-treated and control cells (diameter 59.2±1.3 vs 59.5±1.0 µm, means±S.E.M., NS).

Glucose uptake assay

Glucose uptake was assessed as previously described (24, 25). In brief, after washing four times, adipocytes (lipocrit 3–5%) were incubated at 37 °C in medium 199 without glucose for 15 min with 4% BSA, ADA (1 U/ml), PIA (1 µmol/l) and various insulin concentrations (0–1000 µU/ml). Subsequently D-[U-^{14}C]glucose (0.86 µmol/l) was added. After 1 h glucose uptake was terminated by transferring cells and medium to pre-chilled tubes on ice and adipocytes were immediately separated from the medium by centrifugation through silicone oil. Cell-associated radioactivity (as c.p.m.) was determined. To correct for extracellularly trapped isotope, cell-associated radioactivity obtained when D-[U-^{14}C]glucose was added immediately before separation of the medium was subtracted. Under these experimental conditions glucose uptake is mainly determined by the rate of transmembrane glucose transport, as previously reported (26). The cellular clearance of glucose from the medium was calculated according to the following formula and taken as an index of the rate of glucose uptake: cellular clearance of medium glucose = (c.p.m. cells × volume)/(c.p.m. medium × cell number × time).

Western analysis of total cellular lysates

After the culture period, cells were washed four times with PBS and lysed with 0.2–0.4 ml lysis buffer (25 mmol/l Tris–HCl, pH 7.4, 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamidine and 0.1 mmol/l 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride) and rocked for 2 h at 4 °C. The detergent-insoluble material was sedimented by centrifugation at 12 000 *g* at 4 °C and the supernatants collected. Protein determination was performed using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) with BSA as the standard. Forty micrograms of total cellular protein per lane were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and blocked overnight at 4 °C with 5% dry milk in 20 mmol/l Tris, 137 mmol/l NaCl, 0.5% Tween-20, pH 7.6. Immunological detection of IRS-1, IRS-2, p85α/β (PI3-K) and Akt1/2 (PKB) was carried out with specific polyclonal

antibodies, and immunoreactive bands were visualized using the ECL Western blotting protocol and quantified by densitometry (Molecular Analyst; Bio-Rad Laboratories, Hercules, CA, USA).

Western analysis of PKB and p70^{s6k} phosphorylation

After washing four times, adipocytes (lipocrit ∼15%) were incubated at 37 °C in medium 199 with 5.6 mmol/l glucose, 4% BSA, ADA (1 U/ml) and PIA (1 µmol/l) with and without a maximally stimulating concentration (1000 µU/ml) of insulin. After 10 min incubation, cells and medium were transferred to pre-chilled tubes on ice and adipocytes were immediately separated from the medium by centrifugation through silicone oil. Cell lysis, protein determination and Western blotting procedures were performed with appropriate antibodies as described above.

Western analysis of total cellular membrane GLUT4

After the culture period, cells were washed twice with PBS and homogenized for 30 s with a Polytron PT-MR 3000 (Kinematica AG, Littau, Switzerland) in TES homogenization buffer (20 mmol/l Tris–HCl, pH 7.4, 1 mmol/l EDTA, 255 mmol/l sucrose and 1 mmol/l phenylmethylsulphonyl fluoride). The homogenate was then centrifuged at 1000 *g* for 30 min at 4 °C and the supernatant collected and centrifuged at 212 000 *g* for 1 h at 4 °C. The resulting pelleted total membrane fraction was resuspended in TES and frozen at −70 °C. Protein determination, based on the method of Bradford (27), was performed using the Bio-Rad protein assay and bovine gamma globulin as the standard. Two micrograms of total cellular membrane protein per lane were separated by SDS-PAGE and transferred to Immobilon-P membranes and blocked overnight at 4 °C with 5% dry milk in 20 mmol/l Tris, 137 mmol/l NaCl, 0.5% Tween-20, pH 7.6. Immunological detection of GLUT4 was carried out with an anti-GLUT4 polyclonal antibody, and immunoreactive bands were visualized and quantified as above.

^{125}I-insulin binding to intact cells

After the culture period the fat cells were washed four times. Thereafter, the cells (lipocrit 5–10%) were incubated with ADA (1 U/ml), PIA (1 µmol/l) and 2 mmol/l KCN for 5 min at 37 °C to deplete the cells of ATP and stop receptor internalization and recycling (28). Subsequently, cell-surface binding of ^{125}I-insulin (0.2 ng/ml) was carried out for 60 min at 16 °C. After the incubation period, cells and medium were separated by centrifugation through dinonyl phthalate and ^{125}I-insulin binding to cells was measured. Non-specific

binding, defined as binding in the presence of $10^{5}\,\mu U/ml$ unlabelled insulin, was subtracted.

Lipolysis

After 24 h culture and washing four times, adipocytes (lipocrit 1–3%) were incubated in medium 199 containing 5.6 mmol/l glucose, 4% BSA, ADA (1 U/ml), PIA (1 μmol/l), 8-bromo-cAMP (5 mmol/l) and various insulin concentrations (0–100 μU/ml) for 60 min at 37 °C. After 1 h the reaction was stopped by transferring cells and medium to pre-chilled tubes on ice and adipocytes were immediately separated from the medium by centrifugation through silicone oil. Lipolysis was then assessed by determine the glycerol content in the medium (29). Glycerol was phosphorylated in the presence of glycerokinase and $[\gamma\text{-}^{32}P]$ATP for 30 min at 37 °C. Residual $[\gamma\text{-}^{32}P]$ATP was then hydrolysed in perchloric acid at 95 °C for 60 min. Free $[^{32}P]$phosphate was precipitated on ice in the presence of ammonium molybdate and triethylamine. After centrifugation, radioactivity of the supernatant reflecting phosphorylated glycerol was measured.

Statistical analysis

Statistical analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA). Results are given as means±S.E.M. and statistical significance was determined using one-way or two-way ANOVA when appropriate and the Bonferroni test as a post hoc test. The Kruskal–Wallis non-parametric test for unpaired values with the Mann–Whitney test as a post hoc test was used when comparing protein concentrations.

When appropriate, P-values were recalculated for post hoc analyses and $P < 0.05$ was considered statistically significant.

Results

Effect of dexamethasone and insulin on glucose uptake

Addition of dexamethasone to the culture medium during 24 h markedly decreased both basal and insulin-stimulated glucose uptake (by ~40–50%) compared with control cells, and this was true whether the glucose concentration was 5 or 15 mmol/l ($P < 0.001$ and $P < 0.05$ respectively) (Fig. 1A and B).

The combination of dexamethasone and insulin at 5 mmol/l glucose exerted no additional decrease in basal or insulin-stimulated glucose uptake compared with that produced by dexamethasone alone (Fig. 1A). In contrast, the concomitant presence of dexamethasone and high insulin during the 24 h cell culture at 15 mmol/l glucose exerted additive effects in decreasing basal glucose uptake (by ~70% compared with dexamethasone alone, $P < 0.05$) whereas maximally insulin-stimulated glucose uptake was not significantly reduced by the concomitant addition of insulin (Fig. 1B).

When calculating the acute insulin effect as per cent of basal non-stimulated glucose uptake, the combination of long-term dexamethasone and high insulin in the culture medium amplified the relative incremental effect of insulin, but this was partly a consequence of the impairment of basal glucose uptake (Fig. 1A and B).
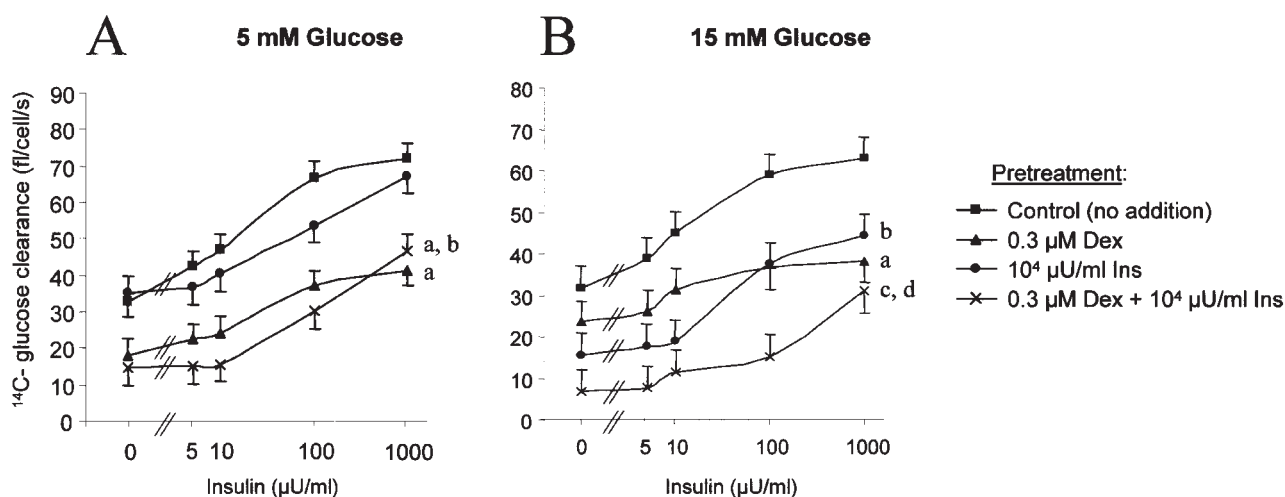

Figure 1 Effects of long-term dexamethasone and insulin pretreatment on glucose uptake in primary cultured rat adipocytes. Isolated cells were cultured for 24 h at different glucose concentrations ((A) 5 mmol/l), (B) 15 mmol/l) with or without dexamethasone (Dex, 0.3 μmol/l) and insulin (Ins, 10^{4} μU/ml). After washing, insulin (0–1000 μU/ml) was added for 15 min as indicated, and then D-[U-^{14}C]glucose clearance was measured during 60 min. Results are expressed as means±S.E.M. of six to nine separate experiments. In (A) a = $P < 0.001$ vs control, b = $P < 0.01$ vs Ins alone. In (B) a = $P < 0.05$ vs control, b = $P < 0.01$ vs control, c = $P < 0.001$ vs control alone, d = $P < 0.01$ vs dexamethasone alone. Differences between treatments were assessed with two-way ANOVA.

These results indicate a reduction in glucose uptake capacity when dexamethasone was present in the culture medium. Combined treatment with a high concentration of insulin exerted an additive down-regulating effect on glucose uptake, but only when cells were cultured in high glucose.

Time-course for the inhibitory effect of dexamethasone on glucose uptake

Cells were cultured with addition of dexamethasone for different time-periods. Both basal and maximally insulin-stimulated glucose uptake decreased upon dexamethasone treatment in a time-dependent manner (Fig. 2). However, the time-course for the inhibitory effect of dexamethasone on glucose uptake differed markedly for basal and insulin-stimulated glucose uptake. The maximal inhibition of basal glucose uptake (by ~70%) occurred already after 2 h of dexamethasone treatment. Control experiments revealed that shorter than 2 h exposure to dexamethasone did not exert a full impairment on basal uptake (not shown). In contrast, the maximally dexamethasone-induced impairment of insulin's ability to stimulate glucose uptake occurred after 24 h (Fig. 2).

Effect of dexamethasone and insulin on cellular content of insulin signalling proteins and GLUT4

Dexamethasone treatment for 24 h exerted a powerful decrease (by ~75%, $P < 0.001$) in total cellular
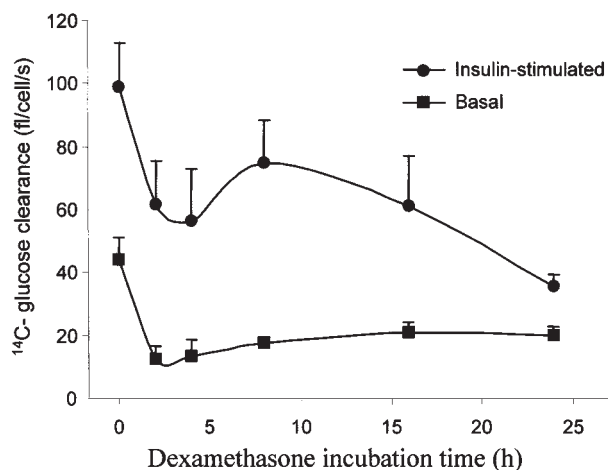

Figure 2 Time-course of the inhibitory effects of dexamethasone on basal and maximally insulin-stimulated glucose uptake in primary cultured rat adipocytes. Isolated cells were cultured for 24 h at 5 mmol/l glucose and 0.3 µmol/l dexamethasone was added during the final 2, 4, 8, 16 and 24 h of incubation (0 = no dexamethasone added). After washing, basal and maximally insulin-stimulated (1000 µU/ml) D-[U-^{14}C]glucose clearance was measured as in Fig. 1. Results are expressed as means±S.E.M. of four separate experiments.

IRS-1 content compared with control cells (Fig. 3A) at 5 mmol/l glucose. In contrast, an increase in IRS-2 (by ~150%, $P < 0.001$, Fig. 3B) was shown. Dexamethasone exerted a small but significant decrease (by ~20%, $P < 0.01$) in PI3-K (Fig. 3C). A consistent reduction in PKB was also seen following 24 h dexamethasone (by ~45%, $P < 0.001$, Fig. 3D). Shorter incubation times (2 or 8 h) with dexamethasone did not result in any changes in IRS-1, PI3-K or PKB content (not shown). Concomitant incubation with dexamethasone and insulin for 24 h at 5 mmol/l glucose decreased IRS-1 content further (by ~50%, $n = 4$, $P = 0.056$) compared with that produced by dexamethasone alone. However, in 15 mmol/l glucose, dexamethasone induced a reduction in IRS-1 (by ~85%, $n = 4$, $P < 0.05$) that was not further enhanced by insulin. The net effect of concomitant incubation with dexamethasone and insulin on IRS-2, PI3-K and PKB content, however, was not significantly different from that produced by dexamethasone alone, at both low and high glucose levels (not shown).

The total cellular membrane GLUT4 content in cells incubated in 5 mmol/l glucose was unchanged following treatment with dexamethasone and/or insulin in the culture medium (Fig. 4). Similarly, there was no effect of dexamethasone on GLUT4 expression when cells were cultured in 15 mmol/l glucose (not shown). Total cellular GLUT1 amount was not altered by dexamethasone treatment (95±9% of control, $n = 4$).

Support that there was no general toxic effect by dexamethasone or other added agents was provided by trypan blue exclusion tests verifying that cells were viable after culture.

Effect of dexamethasone on PKB and p70^{s6k} phosphorylation

After 24 h of culture in 5 mmol/l glucose, PKB phosphorylation was low in the absence of insulin in both normal and dexamethasone-treated cells (Fig. 5). Short-term (10 min) insulin treatment increased phosphorylation of PKB in both normal and dexamethasone-treated cells. The cellular content of phosphorylated PKB following insulin stimulation was, however, ~40% lower in dexamethasone-treated adipocytes than in control cells ($P < 0.01$, Fig. 5). Thus, it was reduced in parallel to the total cellular PKB amount. Pretreatment with dexamethasone for 24 h also reduced the ability of short-term insulin treatment to activate p70^{s6k} (~50% compared with the control situation, $P = 0.056$, $n = 4$).

Effect of dexamethasone on ^{125}I-insulin binding

A 24 h incubation with dexamethasone produced a reduction in cell-surface ^{125}I-insulin binding by ~40% compared with control cells at both 5 and
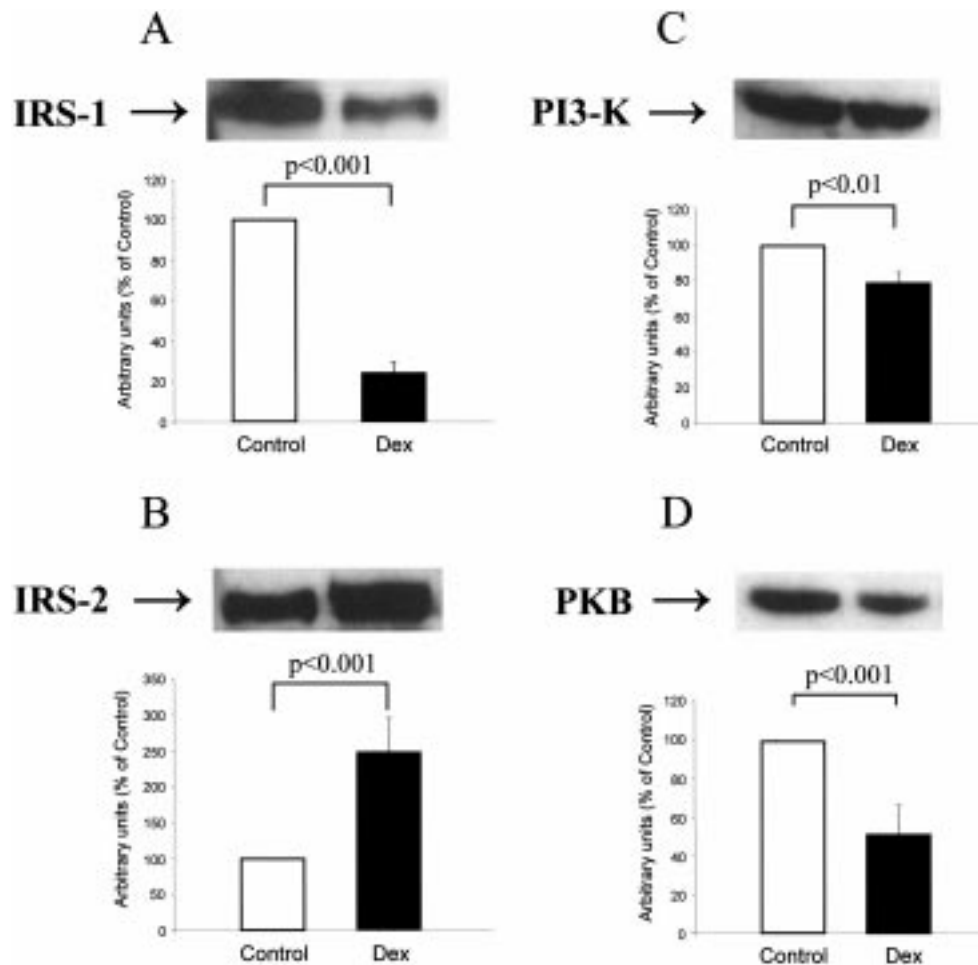
Figure 3 Content of IRS-1, IRS-2, PI3-K and PKB in total cellular lysates assessed by Western blotting. Isolated cells were cultured for 24 h at 5 mmol/l glucose with or without dexamethasone (Dex, 0.3 μmol/l). After washing, total cellular lysates were prepared as described in Materials and methods. Protein from total cellular lysates (40 μg) was separated on SDS-PAGE, followed by immunoblotting using anti-IRS-1 (A), IRS-2 (B), PI3-K (C) and Akt1/2 (PKB) (D) antibodies. One representative blot of eight separate experiments is shown. The results of video densitometry analysis are shown and are expressed as per cent of the amount of signalling protein in the control situation (i.e. without dexamethasone, 100%). Data are means±S.E.M. of eight separate experiments. Data are compared with the corresponding point with no dexamethasone.

15 mmol/l glucose ($P < 0.01$ and $P < 0.05$ respectively) (Fig. 6). Scatchard plots indicated a reduction in the number of available cell-surface binding sites after dexamethasone treatment but no apparent change in binding affinity (not shown).

Lipolysis

After cell culture in 5 mmol/l glucose, basal lipolysis differed significantly in control and dexamethasone-treated cells (glycerol release 2.0±0.8 vs 9.0± 2.4 nmol/10^5 cells respectively, $n = 5$, $P < 0.05$). In other experiments lipolysis was stimulated by a maximally effective concentration (5 mmol/l) of the cAMP analogue 8-bromo-cAMP. 8-bromo-cAMP-induced lipolysis was ∼30% lower in cells treated with dexamethasone compared with control cells (glycerol release

93±40 vs 130±68 nmol/10^5 cells, $n = 6$, $P < 0.05$) and the relative increase compared with non-stimulated cells was consequently smaller (not shown). Pretreatment with dexamethasone for 24 h did not affect the ability of insulin to counteract cAMP-stimulated lipolysis (Fig. 7). Similar results were seen when cells had been cultured in high glucose (not shown).

Discussion

In the present study we demonstrate effects of glucocorticoids on the glucose transport system, insulin signalling proteins, insulin binding, and lipolysis in rat adipocytes, and the interaction with insulin and glucose was elucidated. Independently of the surrounding glucose concentration, long-term incubation (24 h) with the glucocorticoid analogue dexamethasone
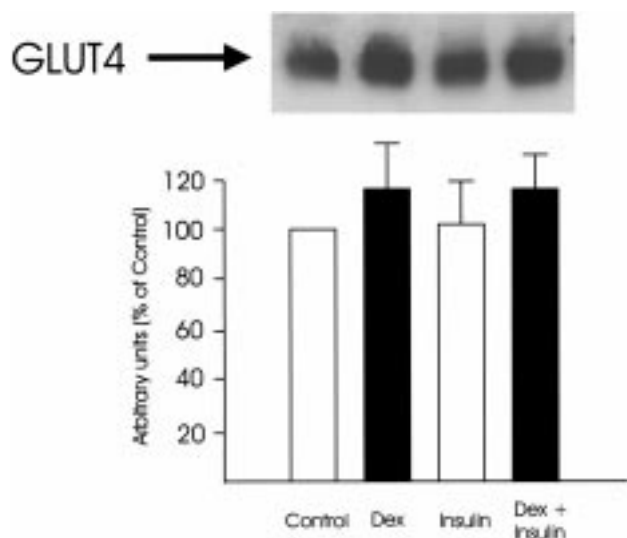
Figure 4 GLUT4 content in total cellular membranes assessed by Western blotting. Isolated cells were cultured for 24 h at 5 mmol/l glucose with or without dexamethasone (Dex, 0.3 μmol/l) and insulin (10^4 μU/ml). After washing, total cellular membranes were prepared as described in Materials and methods. Protein from total cellular membranes (2 μg) was separated on SDS-PAGE, followed by immunoblotting using anti-GLUT4 antibodies. One representative blot of 13 separate experiments is shown. The results of video densitometry analysis, in which a value of 100% was set to represent GLUT4 content in the absence of dexamethasone and insulin (control), are shown in the lower panel. Results are expressed as per cent of control, and means±S.E.M. of 13 separate experiments are shown. No significant differences were found between the different treatments.

Figure 5 Effect of dexamethasone on insulin-stimulated PKB phosphorylation. Isolated cells were cultured for 24 h at 5 mmol/l glucose with or without dexamethasone (Dex, 0.3 μmol/l). After washing, cells were incubated in the absence or presence of insulin (Ins, 1000 μU/ml) for 10 min and total cellular lysates were prepared as described in Materials and methods. Protein from total cellular lysates was separated on SDS-PAGE, followed by immunoblotting using an anti-phospho-Akt1 (Ser^{473}) antibody. One representative blot is shown. The results of video densitometry analysis are shown and are expressed as per cent of the cellular content of Ser^{473} phosphorylated PKB in the control situation in the presence of insulin (i.e. without dexamethasone, 100%). Data are means±S.E.M. of seven separate experiments.

decreased both basal and insulin-stimulated glucose uptake by ~40–50%. According to our results, an underlying mechanism for dexamethasone-induced insulin resistance could be a reduction in IRS-1 expression, and this is in accord with a few other studies. For example, down-regulation of IRS-1 following dexamethasone treatment has previously been observed in 3T3-L1 adipocytes (30). However, over-expression of IRS-1 in 3T3-L1 adipocytes did not improve the impaired insulin-stimulated 2-deoxy-D-glucose uptake induced by dexamethasone (31). These data suggest some other critical mechanism of dexamethasone in decreasing glucose uptake. In type 2 diabetics, on the other hand, a suppression of IRS-1 expression has been suggested to be a critical mechanism for insulin resistance (32, 33). In accord with data from experiments in the 3T3-L1 adipocyte cell-line (31), dexamethasone increases IRS-2 expression in rat adipocytes. The presently demonstrated effects of dexamethasone on IRS-1 and IRS-2 were, however, not previously shown in mature adipocytes.

The present study for the first time demonstrates a modest but consistent decrease in PI3-K and a substantial reduction in PKB content in rat adipocytes after long-term dexamethasone treatment. Our data on
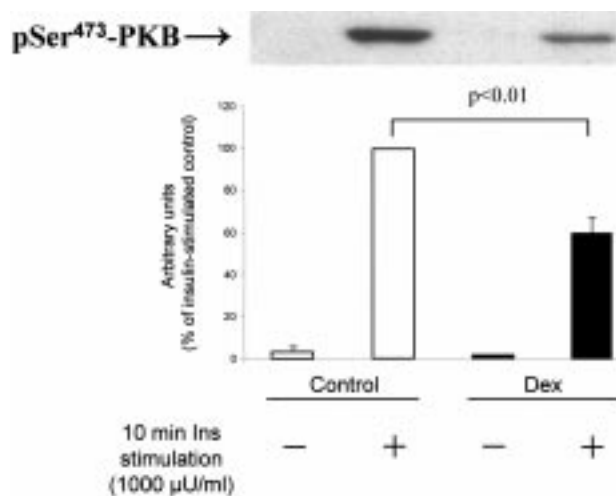
PKB differ from what is reported from 3T3-L1 adipocytes where dexamethasone did not induce any change in content or insulin-stimulated phosphorylation of PKB (31). This might be explained by differences in primary adipocytes and cell line-derived adipocytes. Insulin-stimulated PKB phosphorylation should mirror PKB activity (34) and the reduction
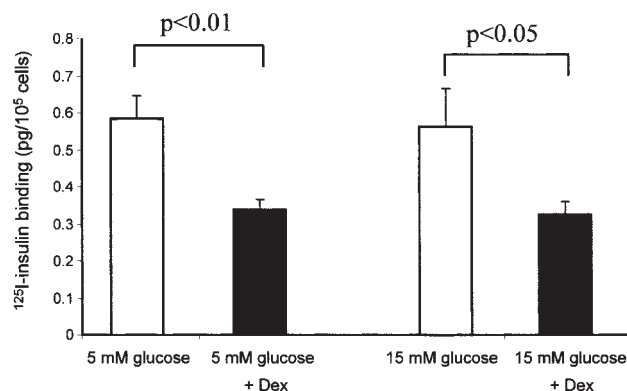

Figure 6 Effect of dexamethasone (Dex) on ^{125}I-insulin binding. Cells were cultured as in Fig. 1 at 5 or 15 mmol/l glucose as indicated. After washing, cells were treated with KCN for 5 min and subsequently cell-surface binding of ^{125}I-insulin was carried out for 60 min at 16 °C. Data are means±S.E.M. of 8 or 11 separate experiments.
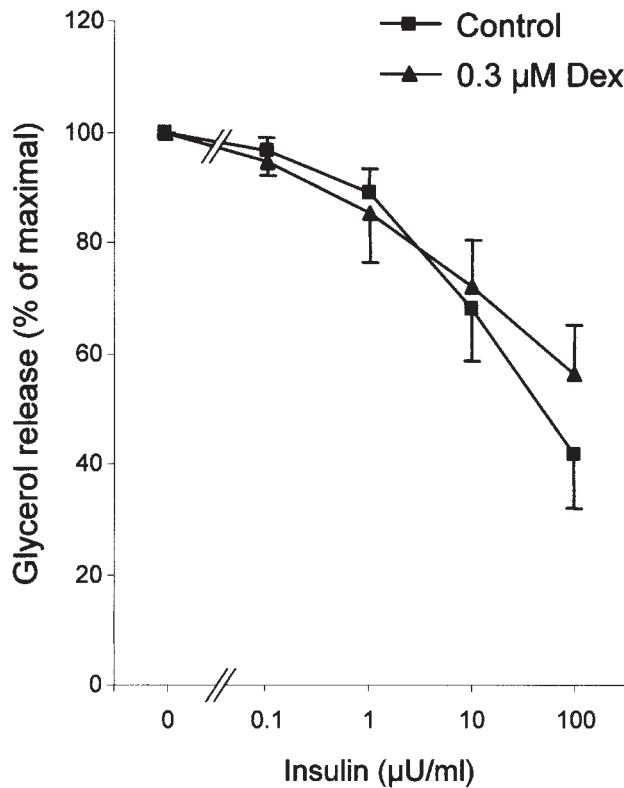
Figure 7 Dose−response curve for the antilipolytic effect of insulin on cAMP-stimulated lipolysis. Isolated cells were cultured for 24 h at 5 mmol/l glucose with or without dexamethasone (Dex, 0.3 μmol/l). After washing, the cells were further incubated for 60 min at 37 °C with 8-bromo-cAMP (5 mmol/l) and the indicated insulin concentrations. The glycerol release into the medium was subsequently measured and taken as an index of lipolysis rate. Results are expressed as per cent of maximal glycerol release (8-bromo-cAMP alone) and represent means±S.E.M. of six separate experiments. No significant differences were seen between dexamethasone-treated and control cells.

reported in the present study is probably of importance in the development of glucocorticoid-induced insulin resistance. The total amount of PKB is not reduced in fat cells from type 2 diabetic patients; however, the insulin-stimulated PKB activation is reduced (34, 35). A recent report showed that the phenotype of type 2 diabetes is achieved in mice deficient in PKBβ (36). These mice have elevated blood glucose accompanied by an increase in serum insulin levels. In addition, the effect of insulin to stimulate glucose uptake in muscle as well as suppressing hepatic glucose production was disturbed. Thus, reduced content or activation of PKB might be of importance in the development of insulin resistance and type 2 diabetes. Further support for this comes from a recent report showing that PKB inhibition prevents the stimulatory effect of insulin on glucose transport and GLUT4 translocation (37).

It is less likely that the small reduction in PI3-K should affect insulin action since present data have suggested a great redundancy for this protein (38). Since phosphorylation of IRS-1 and its associated PI3-K activity was not tested in our study, it cannot be excluded that the phosphorylation/activation of these proteins are normal despite reduced protein levels. However, the reduced PKB phosphorylation that we observed suggests that reduced signal protein content is accompanied by a parallel reduction in phosphorylation/activation of critical downstream signalling steps.

Other serine/threonine kinases have been shown to participate in insulin signalling pathways not primarily involved in glucose transport. p70[s6k] is a serine/threonine kinase that is stimulated by insulin and is important for protein synthesis (39). The activation of p70[s6k] is a complex process that involves many converging pathways (40). One of these pathways involved is the PI3-K/PKB pathway. We therefore studied the effect of dexamethasone on insulin's ability to phosphorylate/activate p70[s6k] and found that dexamethasone pretreatment decreased p70[s6k] phosphorylation by ∼50%, which is in agreement with a previous *in vivo* study (41). These data suggest that insulin-regulated protein synthesis may be altered in dexamethasone-treated cells, and also that other PKB actions beside glucose transport are affected.

A conceivable target of dexamethasone could be GLUT4 amount or function. A reduction in adipocyte GLUT4 content was previously reported in type 2 diabetes patients and could thus be linked to insulin resistance (42). We have recently shown that long-term insulin treatment increases both GLUT4 mRNA and protein expression at low glucose in 3T3-F442A adipocytes (43). In contrast, when the glucose concentration in the medium was high (25 mmol/l), insulin instead suppressed GLUT4 expression. However, in the present work on primary cultured adipocytes we found no stimulatory effect of insulin on GLUT4 content, emphasizing the possible discrepancies between animal or human cells and cell lines. Here we report that the negative effect of dexamethasone on glucose transport could not be explained by any depletion in cellular GLUT4 protein. Previous studies have shown diverging results with respect to dexamethasone effects on GLUT4 content. Following long-term incubations, dexamethasone has been reported to decrease (44) or not change (45) GLUT4 content in rat adipocytes. Our data support the concept that dexamethasone does not change the content of GLUT4 protein in fat cells but decreases the amount of some proteins that precede GLUT4 translocation in the insulin signalling pathway. It is also possible that dexamethasone somehow interacts with the intrinsic activity of GLUT4. In support of this, the translocation process of GLUT4 from intracellular compartments to the plasma membrane in response to insulin is reduced by dexamethasone (44). Our present data suggest that this effect could be mediated by reduced abundance and, in parallel,

an impaired activity of PKB. Another possibility would be a reduction in cellular GLUT1 content following glucocorticoid treatment. GLUT1 is considered to be responsible for a large part of the basal, non-stimulated glucose transport, and the finding of a similar impairment of basal and insulin-stimulated glucose uptake following dexamethasone may support this possibility. However, we found no effect of dexamethasone on cellular GLUT1 content.

Time-course experiments revealed that the inhibitory effect of dexamethasone differed with respect to basal and maximally insulin-stimulated glucose uptake, as the full inhibition was seen after 2 vs 24 h respectively. This suggests that dexamethasone down-regulates basal and maximally insulin-stimulated glucose uptake by partly different mechanisms. Support for this concept also come from the finding in high glucose medium, that the interaction between high insulin and dexamethasone differed between basal and maximally insulin-stimulated glucose transport respectively (Fig. 1B). It would be conceivable that the impairment of basal glucose uptake could be due to alterations in glucose transporter activity or function, whereas the inhibition of insulin's ability to fully activate glucose transport might be partly attributed to the demonstrated alterations in abundance and activity of insulin signalling molecules. This concept is supported by our present study, as cells cultured with dexamethasone for 8 h or less did not change IRS-1 or PKB content, while 24 h dexamethasone treatment suppressed IRS-1 and PKB expression considerably.

Dexamethasone reduced insulin binding in adipocytes cultured for 24 h. In rats treated with dexamethasone *in vivo*, insulin binding is also decreased (46), but it is difficult to establish whether this was caused by the glucocorticoid itself or by compensatory hyperinsulinaemia (47). Our data suggest that dexamethasone *per se* induces a decrease in insulin binding and that this was attributed to a reduction in the number of available cell-surface insulin receptors (28). This could contribute to insensitivity to insulin but probably not to the reduction in maximal insulin response in glucose uptake, since fat cells have a large proportion of spare receptors (48).

In man, elevated cortisol was reported to increase the rate of lipolysis (49) and data from rats suggested that the glucose–free fatty acid (FFA) cycle contributed to dexamethasone-induced insulin resistance and that glucose tolerance was normalized when the FFA level was lowered with nicotinic acid (50). Following dexamethasone treatment we also found an increase in basal lipolysis *in vitro*, but rather a decrease in cAMP-induced lipolysis and a normal antilipolytic effect of insulin. Taken together our data cannot rule out that an enhanced rate of lipolysis plays a role in dexamethasone-induced impairment in glucose uptake capacity.

In this study on cultured rat adipocytes we demonstrate that dexamethasone mainly exerts its suppression of glucose transport capacity independently of the prevailing glucose and insulin levels. If this is extrapolated to the *in vivo* setting, glucocorticoids may be detrimental for glucose turnover in subjects with normal glucose tolerance and impaired glucose tolerance as well as diabetic patients regardless of ambient glycaemic control and β-cell function. Dexamethasone may exert its diabetogenic effect partly by impairing insulin-stimulated glucose uptake. This may be caused by a reduction in IRS-1 and PKB expression, and the reduced insulin-stimulated activation of PKB may be the most important mechanism for reduced insulin action in dexamethasone-treated cells. Whether the suppression of signalling proteins is due to altered gene transcription, mRNA stability or translation, or altered post-translational processing remains to be established in future studies.

In conclusion, this work suggests that glucocorticoids impair glucose transport in fat cells both at physiological and at abnormally high glucose concentrations, and the effects were also mainly independent of the concomitant insulin level. Thus, *in vivo* glucocorticoids could potentially promote insulin resistance in non-diabetic as well as diabetic individuals. Reduced amounts of the signalling proteins IRS-1 and PKB following glucocorticoid exposure can be important mechanisms in this process. Further research is needed to clarify the interaction between glucocorticoids and other hormone systems that influence glucose turnover and this also includes the molecular mechanisms involved. It is also important to elucidate further the possible role of endogenous glucocorticoids in the pathogenesis of type 2 diabetes.

Acknowledgements

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