Chronic insulin effects on insulin signalling and GLUT4 endocytosis are reversed by metformin

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Decreases in insulin-responsive glucose transport and associated levels of cell surface GLUT4 occur in rat adipocytes maintained in culture for 20 h under hyperinsulinaemic and hyperglycaemic conditions. We have investigated whether this defect is due to reduced signalling from the insulin receptor, GLUT4 expression or impaired GLUT4 trafficking. The effects of chronic insulin treatment on glucose transport and GLUT4 trafficking were ameliorated by inclusion of metformin in the culture medium. In comparison with the acute effects of insulin, chronic insulin treatment attenuated changes in signalling processes leading to glucose transport. These included insulin receptor tyrosine phosphorylation, phosphoinositide 3-kinase activity and Akt activity, which were all reduced by 60–70 %. Inclusion of metformin in the culture medium prevented the effects of the chronic insulin treatment on these signalling processes. In comparison with cells maintained in culture without insulin, the total expression of GLUT4 protein was not significantly altered by chronic insulin treatment, although the level of GLUT1 expression was increased. Trafficking rate constants for wortmannin-induced cell-surface loss of GLUT4 and GLUT1 were assessed by 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine (ATB-BMPA) photolabelling. In comparison with cells acutely treated with insulin, chronic insulin treatment resulted in a doubling of the rate constants for GLUT4 endocytosis. These results suggest that the GLUT4 endocytosis process is very sensitive to the perturbations in signalling that occur under hyperinsulinaemic and hyperglycaemic conditions, and that the resulting elevation of endocytosis accounts for the reduced levels of net GLUT4 translocation observed.

Key words: adipocytes, glucose transport.

INTRODUCTION

In adipose cells, GLUT4 is the primary glucose-transporter isoform mediating insulin-stimulated glucose transport [1,2]. In the basal state, the majority of the GLUT4 is sequestered into intracellular vesicle compartments which, following insulin stimulation, translocate to the plasma membrane, thereby allowing glucose entry. Hyperinsulinaemic and hyperglycaemic treatment produces insulin resistance *in vitro* [3] and *in vivo* [4–6]. We and others have shown that chronic insulin treatment of rat adipocytes in culture leads to a reduction in insulin-stimulated glucose transport. This defect is associated with an impaired net GLUT4 translocation to the cell surface of adipocytes [7,8]. Therefore we have investigated whether this impaired net translocation is associated with altered insulin signalling processes and/or a change in the kinetics of GLUT4 trafficking. In particular, we have examined the levels of insulin activation of receptor tyrosine kinase activity and phosphoinositide 3-kinase (PI-3K) activity as both of these steps are essential for insulin action on glucose transporter translocation [9–12]. Similarly, we have examined the activation of Akt (or protein kinase B) which has been implicated as an intermediate in this process [13,14]. However, there is some debate as to whether it is an essential step [15]. Normal insulin signalling produces a marked increase in the exocytosis of GLUT4, with a much smaller decrease in its endocytosis [16–18]. However, data presented here suggest that chronic insulin treatment leads to an increase in the GLUT4 endocytosis rate.

The oral biguanide metformin is used clinically to alleviate insulin resistance [19]. The concentrations of metformin required for *in vitro* responses are generally higher than the therapeutic doses used to treat non-insulin-dependent diabetes mellitus (NIDDM) patients [20]. *In vivo* metformin treatment has been found to ameliorate insulin resistance by inducing glucose transporter translocation in obese (fa/fa) Zucker rats [21] and streptozotocin diabetic rats [22]. We have previously shown that inclusion of metformin in the culture medium during the chronic insulin treatment of rat adipocytes can alleviate the down-regulation of glucose transport activity [8]. It is not known whether the metformin effects are due to reversal of a defect resulting from the chronic insulin treatment or whether it compensates for the defect by altering other processes. We therefore examined whether the metformin effects are transmitted throughout the cascade of known signalling intermediates and whether metformin leads to a reversal of perturbed GLUT4 and GLUT1 trafficking kinetics.

EXPERIMENTAL

Materials

Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. 3-*O*-Methyl-D-[U-^{14}C]glucose (3-OMG), [γ-^{32}P]ATP and ECL® reagents were from Amersham International. Protein A– and Protein G–Sepharose, PtdIns, mouse monoclonal anti-(phosphotyrosine-agarose) conjugate, protein kinase A-inhibitor fragment, protamine sulphate and anti-mouse and anti-rabbit horseradish peroxidase conjugated antibodies were from Sigma. Mouse monoclonal anti-phosphotyrosine

Abbreviations used: NIDDM, non-insulin-dependent diabetes mellitus; ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine; 3-OMG, 3-*O*-methyl-D-glucose; PI-3K, phosphoinositide 3-kinase; DTT, dithiothreitol.

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(4G10 clone) used for Western blotting was from Upstate Biotechnology, Lake Placid, NY, U.S.A. Anti-Akt (C20) and anti-insulin receptor β-subunit (C19) were from Santa Cruz Biotechnology. Rabbit polyclonal and monoclonal anti-p85 antibodies were from Transduction Laboratories, Lexington, KY, U.S.A. GLUT4 and GLUT1 antibodies were raised against C-terminal peptides as described in [23].

Adipocyte cell culture

Rat adipocytes were prepared from male Wistar rats (180–200 g) by collagenase-digestion of aseptically removed epididymal fat pads as described in [24]. The isolated adipocytes were maintained in a culture as described by Traxinger and Marshall [25] with some modifications. Briefly, 1 ml of a 40 % cell suspension was added to 20 ml of sterile Dulbecco's modified Eagle's medium (25 mM glucose), containing 25 mM Hepes (pH 7.4), 1 % (w/v) BSA, 100 units/ml penicillin, 100 mg/ml of streptomycin, and maintained at 37 °C for 20 h in a 10 % CO_2 atmosphere, either in the absence or the presence of 500 nM insulin, and with or without 1 mM metformin. After 20 h in culture, adipocytes were washed in an albumin/Hepes buffer at 37 °C, containing 500 nM insulin and 1 mM metformin as appropriate, and were re-suspended at 40 % cytocrit. A portion of cells cultured in the absence of any additions was acutely stimulated with 20 nM insulin at 37 °C.

Cell lysates and immunoprecipitation

For Western blotting of whole cell lysates, adipocytes were lysed directly (1:1, v/v) with electrophoresis sample buffer [230 mM Tris/HCl (pH 6.8), 2 mM EDTA, 20 % (v/v) glycerol, 4 % (w/v) SDS, 10 % (v/v) mercaptoethanol, 400 μM Na_3VO_4, 20 mM NaF, 2 mM sodium molybdate], and proteins were resolved by 8 % PAGE. Purified plasma membrane for Western blotting of insulin receptors was obtained as described in [26]. For immunoprecipitation, 1 ml of isolated adipocytes was briefly centrifuged in a microfuge, excess buffer removed and the cells lysed with 1 ml of lysis buffer [50 mM Hepes (pH 7.0), 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10 mM Na_2HPO_4, 10 % (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 % (v/v) Triton X-100, the protease inhibitors antipain, aprotonin, pepstatin A, leupeptin (each at 1 μg/ml), and 100 μM 4-(2-aminoethyl)benzenesulphonyl fluoride]. Lysates were vortexed, incubated on ice for 15 min and then centrifuged at 20000 ***g*** for 10 min at 4 °C. The infranatant was filtered through a 0.2 μm filter and the filtrate subjected to immunoprecipitation. Phosphotyrosine immunoprecipitation used 30 μl of a purified mouse monoclonal anti-phosphotyrosine-agarose conjugate. Immunoprecipitation of p85 used 4 μg of anti-p85 conjugated to 30 μl of a 50 % (v/v) slurry of Protein A–Sepharose. Akt immunoprecipitation used 10 μl of a 50 % (v/v) slurry of Protein G–Sepharose with 2 μg of anti-Akt1. Samples were rotated for 2 h at 4 °C and then washed twice with 12.5 mM Na_2HPO_4, 0.4 M NaCl, pH 7.2, and then twice with 12.5 mM Na_2HPO_4, 154 mM NaCl, pH 7.2 (both wash buffers contained 200 μM Na_3VO_4, 10 mM NaF, 1 mM sodium molybdate, and protease inhibitors).

PI-3K activity

Immune pellets for PI-3K assays were washed twice with 12.5 mM Na_2HPO_4 (pH 7.2), 154 mM NaCl, 1 % (w/v) Thesit, 1 mM dithiothreitol (DTT), twice with 100 mM Tris/HCl (pH 7.4), 0.5 M LiCl, 1 mM DTT, and then twice with 10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 1 mM DTT. PI-3K activity was measured directly in immunoprecipitates in a final volume of 50 μl containing 20 mM Hepes and 0.4 mM Na_2HPO_4 (pH 7.4), 0.4 mM EGTA, 10 mM $MgCl_2$ and 10 μg of PtdIns. The PtdIns was incubated with the immunoprecipitate for 5 min at approx. 18 °C before the addition of 40 μM ATP with 10 μCi of [γ-^{32}P]ATP for 20 min. The assay was stopped by the addition of 30 μl of 4 M HCl and 130 μl of chloroform/methanol (1:1, v/v). The tubes were vortex-mixed briefly, spun in a microfuge at 13400 ***g*** for 10 min, and then 10 μl of the lower phase was spotted on to a silica gel 60 plate that had been pre-treated with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid. Lipids were separated by TLC in the presence of boric acid as described [27]. Plates were dried and visualized by autoradiography. [^{32}P]PtdIns3*P* levels were estimated by cutting and scintillation counting appropriate areas of the plate.

Akt activity

Immune pellets for Akt assays were washed three times in an Akt wash buffer [50 mM Hepes (pH 7.6), 1 % (v/v) Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 30 mM Na_2HPO_4, 150 mM NaCl, 1 mM EDTA, 100 μM 4-(2-aminoethyl)benzenesulphonyl fluoride, 1 μg/ml of protease inhibitors antipain, aprotonin, leupeptin and pepstatin A], and then three times with 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$. Akt activity was measured directly in immunoprecipitates by adding 50 μl of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 ng of protein kinase A inhibitor peptide, 5 μM ATP and 2 μCi of [γ-^{32}P]ATP and 25 μg protamine sulphate (the substrate). Following reaction for 20 min at 30 °C, the reaction buffer was removed and 40 μl of 2 % (w/v) SDS was added to the pellets for 10 min. The reaction buffer components were combined with the SDS eluates and 20 μl of the mixture was spotted on to phosphocellulose paper and left to dry. The phosphocellulose paper was washed five times, each for five min, with a large excess of 75 mM phosphoric acid, once with distilled water and once with acetone. The dried paper was then added directly to scintillation fluid for counting of radioactivity.

3-OMG transport and cellular ATP levels

To examine glucose transport after culturing and washing, the uptake of 50 μM 3-OMG was determined as described for freshly isolated cells [24]. Cellular ATP levels were measured as described in [28].

Photolabelling of glucose transporters

To photolabel the glucose transporters, 1 ml of cells in albumin/Hepes buffer was added to 200 μCi of ATB[2-^{3}H]BMPA [2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoly-[2-^{3}H]1,3-bis(D-mannose-4-yloxy)-2-propylamine] in 0.1 ml of Hepes buffer without albumin in 35-mm polystyrene dishes and irradiated for 1 min in a Rayonet photochemical reactor as described previously [23]. Following irradiation, the cells were rapidly washed three times with albumin/Hepes buffer at 18 °C and solubilized in 1 ml of detergent buffer [2 % poly(oxyethylene-9-lauryl ether) in 5 mM phosphate buffer (pH 7.2), with 1 μg/ml each of the protease inhibitors antipain, aprotinin, leupeptin and pepstatin A]. GLUT4 and GLUT1 antisera were conjugated to 8 μl of Protein A–Sepharose [23]. The conjugates were washed with 5 mM phosphate buffer to remove excess antibody. The solubilized cells in detergent buffer were then mixed sequentially with anti-GLUT4 and then anti-GLUT1 antisera-Protein A-Sepharose conjugates. The immunoprecipitates were washed four times in detergent buffer. Labelled transporters were then released from

the conjugates with electrophoresis sample buffer containing 10% (w/v) SDS, 6 M urea and 10% (v/v) mercaptoethanol. The proteins were separated on 8% polyacrylamide gels in a discontinuous buffer system and gel lanes were then separated and sliced. The radioactvity in the gel slices was extracted and estimated as described previously [23].

Estimation of rate constants

Time course data for the net loss of transport activity and cell-surface glucose transporters following wortmannin treatment were fitted by non-linear regression to eqn. (1) [17]:

$$Tp = \frac{k_{ex}\{1-\exp[-t(k_{ex}+k_{en})]\}}{k_{ex}+k_{en}} + Tp_0 \cdot \exp[-t(k_{ex}+k_{en})] \qquad (1)$$

Tp and Tp_0 are the fractions of surface transporters at time t and zero, and k_{ex} and k_{en} are the exocytosis and endocytosis rate constants respectively. For the analysis of transport data, the rates of transport for all conditions were normalized to the unperturbed acute insulin treatment transport rate, and this ratio was multiplied by the Tp_0 value (0.45) for cell-surface GLUT4 in the acute insulin treatment [8,29]. The levels of labelling were normalized to $Tp_0 = 1.0$ as all the ATB-BMPA-tagged transporters were at the cell surface.

RESULTS

Protein levels of insulin receptor and PI-3K following chronic insulin treatment

To investigate whether adipose cells which were chronically treated with insulin had altered levels of insulin receptors, cell lysates were blotted for the β-subunit (Figure 1). To assess

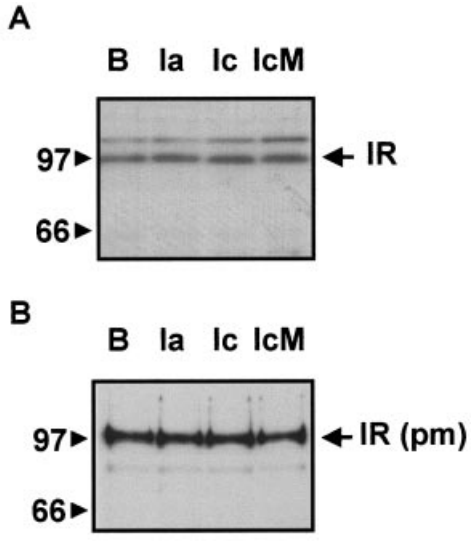

Figure 1 Western-blot analysis of the levels of insulin receptor following chronic insulin treatment

Adipocytes were cultured for 20 h either with no additions (B), or with 500 nM insulin (Ic), or with 500 nM insulin and 1 mM metformin (IcM). Cells were subsequently washed and adjusted to 40% cytocrit. A portion of basal cells was stimulated with 20 nM insulin (Ia). Portions of the cells (0.5 ml) from each condition were then directly lysed with sample buffer and 20 μg of lysate protein (**A**) were resolved by 8% PAGE. Proteins were transferred on to nitrocellulose and blotted for the β-subunit of the insulin receptor. (**B**) Insulin receptor (IR) was also analysed in purified plasma membrane (pm). The blots shown are representative of at least 3 separate experiments.

Table 1 Western-blot analysis of the levels of insulin receptor and PI-3K protein following chronic insulin treatment

Adipocytes were cultured with no additions (basal), in the presence of 500 nM insulin (insulin chronic), or with 500 nM insulin and 1 mM metformin (insulin chronic plus metformin). All cells were washed and adjusted to 40% cytocrit. A portion of basal cells was acutely stimulated with 20 nM insulin (insulin acute). Lysates or plasma membrane (PM) were Western blotted for insulin receptor (IR) and the p85 subunit of PI-3K and analysed by scanning densitometry. Values are in arbitrary absorbance units for the indicated number of experiments.

	IR lysate; $n = 3$	IR PM; $n = 4$	p85 lysate; $n = 3$
Basal	3.70 ± 0.51	10.0 ± 0.8	2.90 ± 0.46
Insulin acute	4.03 ± 0.40	9.9 ± 0.9	2.73 ± 0.13
Insulin chronic	4.37 ± 0.42	11.1 ± 1.5	2.83 ± 0.38
Insulin chronic plus metformin	4.27 ± 1.30	9.9 ± 1.9	2.91 ± 0.47

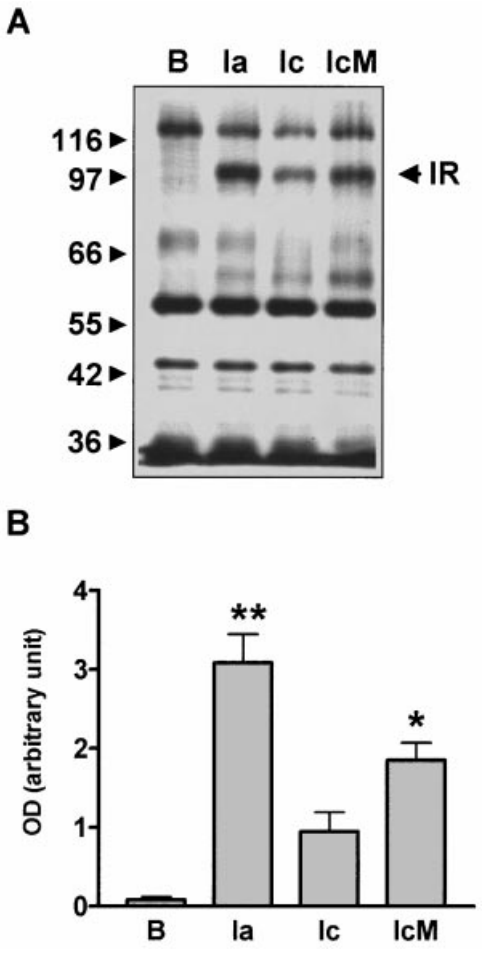

Figure 2 Effect of chronic insulin treatment on tyrosine phosphorylation of the insulin receptor β-subunit

Adipocytes were cultured for 20 h either with no additions (B), or with 500 nM insulin (Ic) or with 500 nM insulin and 1 mM metformin (IcM). Cells were subsequently washed and adjusted to 40% cytocrit. A portion of the cells cultured under basal conditions was acutely stimulated with 20 nM insulin for 2 min (Ia). Cells were lysed and phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine-agarose and then resolved by PAGE. The gels were then Western blotted using an anti-phosphotyrosine antibody. The autoradiogram shown is from a typical blot (**A**). The blots quantified in (**B**) are the mean ± S.E.M. from 5 separate experiments. ** $P < 0.01$ and * $P < 0.05$ versus Ic.

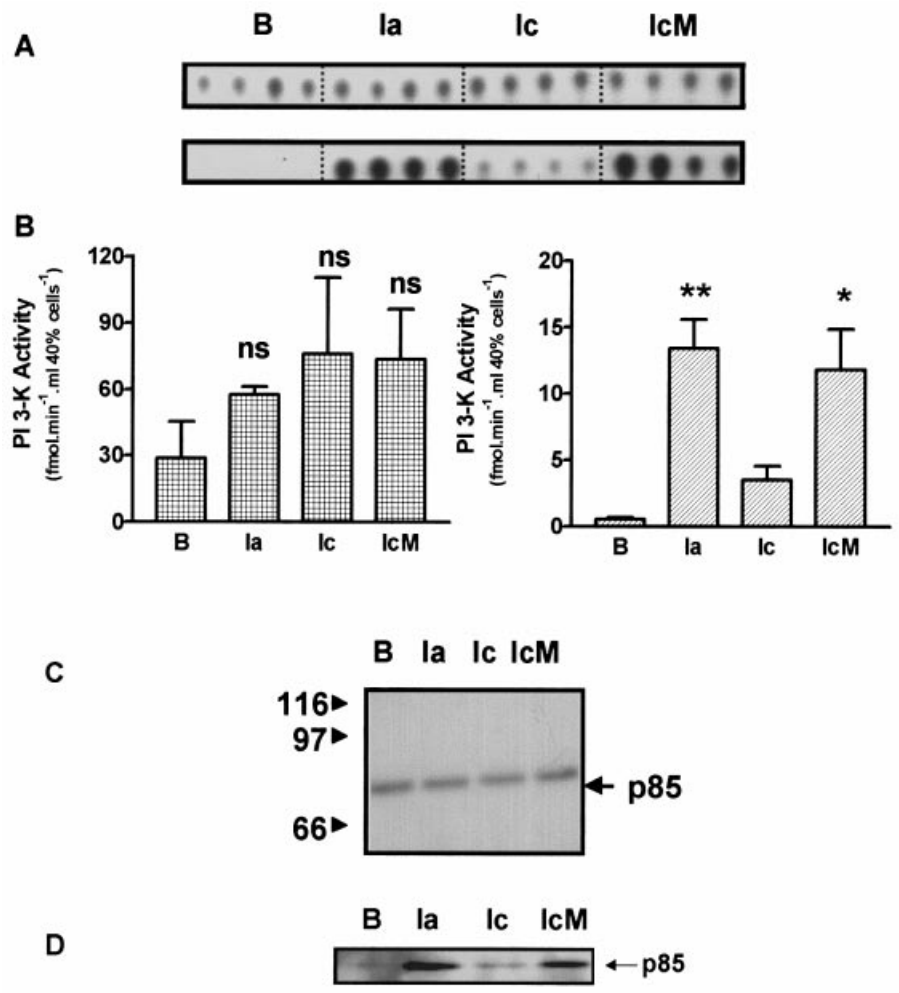

Figure 3 Effect of chronic insulin treatment on PI-3K activity associated with p85 and phosphotyrosine immunoprecipitates

Adipocytes were cultured for 20 h either with no additions (B), or with 500 nM insulin (Ic), or with 500 nM insulin and 1 mM metformin (IcM). Cells were subsequently washed and adjusted to 40% cytocrit. A portion of the cells cultured under basal conditions was stimulated with 20 nM insulin for 2 min (Ia). Aliquots (1 ml) of the cells were lysed and either p85 (**A**, top panel; **B**, left panel) or phosphotyrosine (**A**, lower panel; **B**, right panel) were immunoprecipitated from the detergent-soluble lysates. The immune pellets were then directly assayed for PI-3K activity. [^{32}P]PtdIns3*P* was resolved on TLC plates and visualized by autoradiography. The kinase reaction was performed in duplicate for each condition, with each reaction mix analysed by spotting twice on the TLC plate (**A**). The kinase activity measurements (**B**) are the mean $\pm$ SEM of 5 separate experiments. ** $P < 0.01$ and * $P < 0.05$ versus Ic; ns, not significant. The levels of the p85 subunit of PI-3K in lysates (**C**) and associated with the anti-phosphotyrosine immunoprecipitates were analysed by Western blotting (**D**)

whether these treatments led to a down-regulation of cell-surface-exposed insulin receptor, purified plasma membrane was also blotted for this protein (Figure 1). The levels of the p85 subunit of PI-3K in whole cell lysates were also determined (see Figure 3C). Scanning densitometry (Table 1) showed that the levels of these signalling molecules did not alter following chronic insulin treatment neither in the presence nor absence of metformin. The data on levels of insulin receptors (both in lysate and purified plasma membrane) are consistent with the studies of Lima et al. [30], in which insulin binding to intact rat adipose cells in culture was studied with tracer insulin. In this study only a slight (18%) decrease in tracer insulin binding to the cells following culture with high insulin and high glucose compared with high glucose alone was found. We cannot exclude an 18% decrease in receptors in the present study, as this would be within the resolution of the blotting procedure employed. We note that insulin receptor down-regulation appears to be greater in cell lines [31] than in the rat adipocyte culture system used in the present work. This variance probably reflects differences in the state of differentiation of the cells before the chronic insulin treatment.

Phosphorylation of the insulin receptor β-subunit following chronic insulin treatment

Since chronic insulin treatment did not correlate with reduced protein levels of either the insulin receptor or p85, the extent of activation of these intermediates were investigated. Tyrosine phosphorylation of the insulin receptor was analysed in phosphotyrosine immunoprecipitates obtained from detergent lysates. Immunoprecipitated proteins were blotted for phosphotyrosine (Figure 2A) and the autoradiograms were analysed using scanning densitometry (Figure 2B). Insulin treatment for 2 min produced extensive phosphorylation on tyrosine residues of the receptor β-subunit. However, in chronically insulin-treated cells there was approx. 70% reduction in the levels of tyrosine

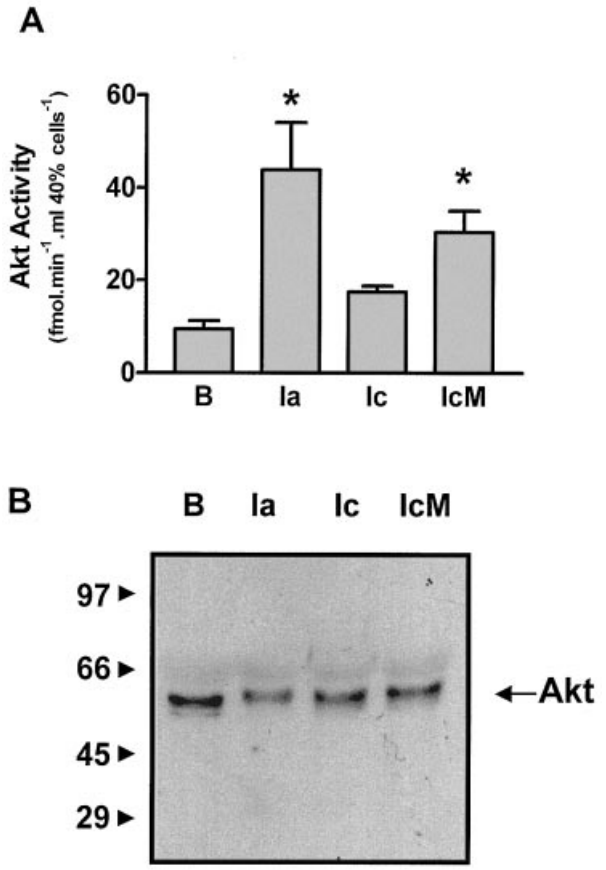

Figure 4 Akt activity following maintenance of adipocytes in culture

Adipocytes were cultured for 20 h either with no additions (B) or with 500 nM insulin (Ic) or with 500 nM insulin and 1 mM metformin (IcM). Cells were subsequently washed and adjusted to 40% cytocrit. A portion of the cells cultured under basal conditions was stimulated with 20 nM for 2 min (Ia). Cells were lysed and Akt immunoprecipitated from the detergent soluble lysates. Akt activity was directly measured in immune pellets. (**A**). The kinase activity measurements are means ± SEM from 4 separate experiments. P vs Ic, * < 0.05. Cell lysates were also subjected to Western-blot analysis for Akt (**B**). The Western blot is representative of 3 separate experiments.

phosphorylation of the receptor β-subunits compared with the acute insulin stimulation. This decrease was reversed when metformin was included in the chronic insulin culture (Figures 2A and 2B).

Levels of PI-3K activity following chronic insulin treatment

To determine whether chronic insulin treatment can alter total PI-3K activity, the activity in p85 immunoprecipitates was measured (Figure 3A, top panel). From the quantification of a series of these experiments (Figure 3B, left panel), it is evident that acute insulin treatment produced a relatively small, less than twofold increase above basal level. Neither chronic insulin nor metformin plus chronic insulin treatments produced a significant alteration in the total PI-3K activity in the p85 immunoprecipitates.

To determine the extent of PI-3K activity associated with tyrosine phosphorylated proteins, anti-phosphotyrosine-agarose immunoprecipitates were examined (Figure 3A, lower panel). After 20 h culture of the adipose cells without insulin, a subsequent acute stimulation with insulin increased the kinase activity in excess of 20-fold. In contrast, the PI-3K activity in cells cultured for 20 h in the presence of insulin was only 26 % of the acute insulin treatment level (Figure 3B, right panel). Treatment with metformin during the chronic insulin culture period prevented this decrease in PI-3K activity. These PI-3K activity measurements correspond closely with the levels of the p85 regulatory subunit of the enzyme that was found to be associated with phosphotyrosine immunoprecipitates (Figure 3D), but not the total amount of p85 (Figure 3C).

Table 2 Effect of chronic insulin treatment on glucose transport activity

Adipocytes were cultured with no additions (basal), in the presence of 500 nM insulin (insulin chronic), or with 500 nM insulin and 1 mM metformin (insulin chronic plus metformin) All cells were washed and adjusted to 40% cytocrit. A portion of basal cells was acutely stimulated with 20 nM insulin for 30 min (insulin acute). The rate constants of uptake of 3-OMG were then determined. Data are means ± SEM from 6–12 separate experiments. The effects of addition of 10 mM inosine to the culture medium were also studied. Data are from 2 separate experiments. *** $P < 0.001$ versus insulin.

Condition	Transport rate constant (sec^{-1})	*n*
Basal	0.019 ± 0.002	12
Insulin acute	0.121 ± 0.008 ***	12
Insulin chronic	0.060 ± 0.003	12
Insulin chronic plus metformin	0.136 ± 0.013 ***	6
Basal plus inosine	0.025, 0.029	2
Insulin chronic plus inosine	0.061, 0.065	2

Levels of Akt activity following chronic insulin treatment

To explore whether signalling changes are transmitted downstream of PI-3K, Akt was immunoprecipitated from cell lysates and the kinase activity determined. The Akt antibody used is known to immunoprecipitate Akt1 and Akt2. Acute insulin stimulation of the cells increased Akt activity by 4.7-fold (relative to basal cells), whereas chronic insulin treatment stimulated the activation of Akt by only 1.9-fold (Figure 4A). Inclusion of metformin in the chronic insulin culture medium alleviated this reduction in activity. Western-blot analysis of the levels of Akt in whole cell lysates (Figure 4B) indicated that the protein levels of the enzyme were not reduced by chronic insulin treatment.

Glucose transport following chronic insulin treatment

Cells cultured in the absence of insulin and then subsequently acutely insulin stimulated (20 nM) for 30 min produced a sixfold increase in 3-OMG transport (Table 2). Following 20 h culture in the presence of 500 nM insulin, the levels of glucose transport activity were only threefold above basal levels. The attenuation of glucose transport activity following chronic insulin treatment was alleviated when cells were cultured with 500 nM insulin in the presence of 1 mM metformin (Table 2). Culture of the adipocytes in the presence of metformin alone produced only a slight increase in glucose transport activity [8]. In cells cultured in either the absence or presence of 500 nM insulin, the ATP levels were 8.3 and 7.8 nmol/mg protein respectively. The inclusion of inosine (which reverses the effects of glucosamine-induced down-regulation of glucose transport activity in 3T3-L1 cells [28]), slightly increased the ATP levels (to 14.5 and 13.9 nmol/mg of protein respectively), but this did not reverse the effects of the chronic insulin and 25 mM glucose treatment in culture on the subsequently measured glucose transport activity (Table 2).

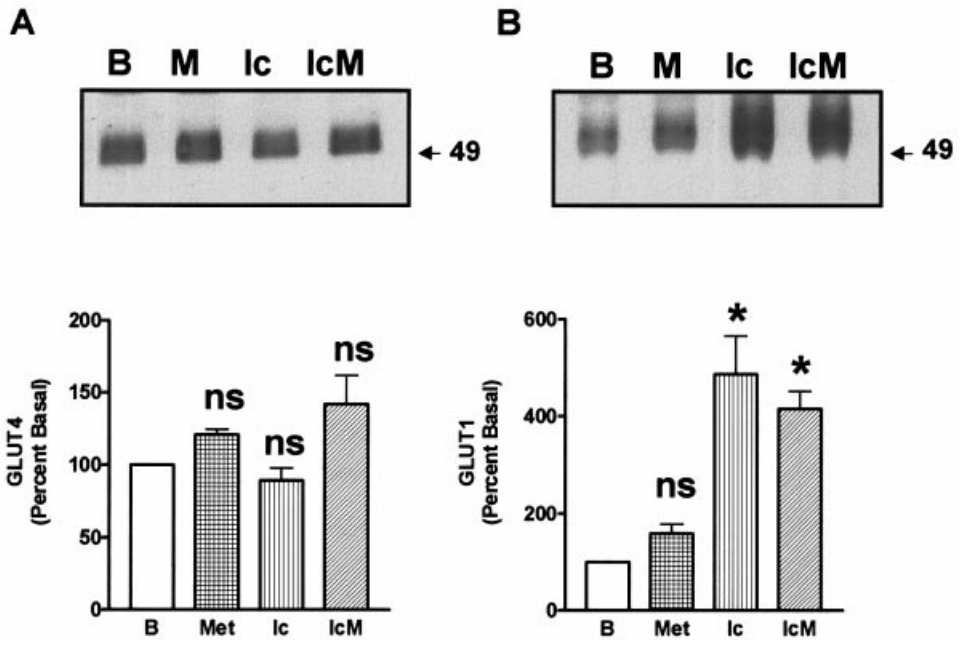

Figure 5 Western-blot analysis of total cellular GLUT4 and GLUT1 levels

Adipocytes were cultured for 20 h either with no additions (B), with 1 mM metformin (M), with 500 nM insulin (Ic), or with 500 nM insulin and 1 mM metformin (IcM). Cells were subsequently washed and adjusted to 40% cytocrit. Portions of the cells (0.5 ml) from each condition were then directly lysed with sample buffer and 20 μg of lysate protein were resolved by 10% PAGE. Proteins were transferred onto nitrocellulose and blotted for GLUT4 (**A**) or GLUT1 (**B**). The blots shown are representative of 3 separate experiments. The blots quantified (lower panels) are the means $\pm$ SEM from 3 separate experiments. * P (paired) < 0.05 versus basal; ns, not significant.

Cell levels of GLUT4 and GLUT1 following chronic insulin treatment

Prolonged maintenance of 3T3-L1 cells in the presence of insulin has been reported to produce large elevations of the total cellular GLUT1 [32,33], but variable effects on the levels of expression of GLUT4. These range from reports of no large changes [32,33] to reports of a loss of this isoform [34]. The variability probably occurs because of variable culture conditions or variable recovery of the GLUT4. In examining this issue for the rat adipocyte system described here, we found it necessary to determine GLUT4 levels in total cell lysates rather than in isolated membrane fractions. In particular, it was difficult to recover the GLUT4 in the low-density microsomes, possibly reflecting a relocalization of GLUT4 to a different subcellular membrane compartment following chronic insulin treatment [35]. In the total cell lysates, GLUT4 (Figure 5A) was found to be unaltered following chronic insulin treatment with or without metformin, but GLUT1 increased fourfold under these conditions (Figure 5B). Metformin treatment alone produced slight (but statistically insignificant) 1.2-fold increases in both GLUT4 and GLUT1. These data are consistent with our previous investigation of this issue in cultured rat adipocytes. We previously measured the total GLUT4 and GLUT1 in digitonin-permeabilized cells by photolabelling these transporters with ATB-BMPA [8]. Previous studies [8] have also indicated that GLUT1 levels are very low in rat adipocytes and, although increased following chronic insulin treatment, its contribution to transport activity is minimal.

Transporter trafficking kinetics following chronic insulin treatment

Cells were initially cultured and treated under the conditions described above (basal, acute insulin, chronic insulin and chronic insulin plus metformin), and were subsequently treated with 1 μM wortmannin. In each case there was a gradual reduction in glucose-transport activity, but this was more rapid following chronic insulin treatment (Figure 6). As the decrease in transport activity is related to a net reduction of transporters (mainly GLUT4) initially at the cell surface, the reduction in transport activity was analysed by curve-fitting to give single exponential decay half-times (Figure 6). Curve fitting to eqn. (1) provided an estimate of the endocytosis and exocytosis rate constants (Table 3). To determine the separate rates of loss of GLUT4 and GLUT1 from the cell surface following wortmannin treatment, we photolabelled these transporters with ATB-BMPA at the times indicated in Figure 6, and the separate isoforms were immunoprecipitated. This reagent is cell impermeable and only reacts with those glucose transporters remaining at the cell surface. The rates of decline in surface-available transporters were therefore curve-fitted to give decay half-times (Figure 6), and by using eqn. (1) to give endocytosis and exocytosis rate constants (Table 3).

The half-times for reduction in transport activity and cell-surface GLUT4 and GLUT1 were dependent on the conditions under which the cells were cultured. It is important to note that the half-times are model-independent parameters that are also independent of the total levels of the glucose transporters. Even if there were changes in the total amounts of these transporters (which in any case are small; Figure 5) these will not alter the half-times. From Figure 6 it is evident that the half-times for the reduction in transport activity correlate with the loss of GLUT4. There is a slight (approx. 1.3-fold) increase in the decay half-time comparing basal and acute-insulin pretreatments that is consistent with previous data [16–18]. However, the decay half-time in acutely insulin-stimulated cells is twice that of chronic

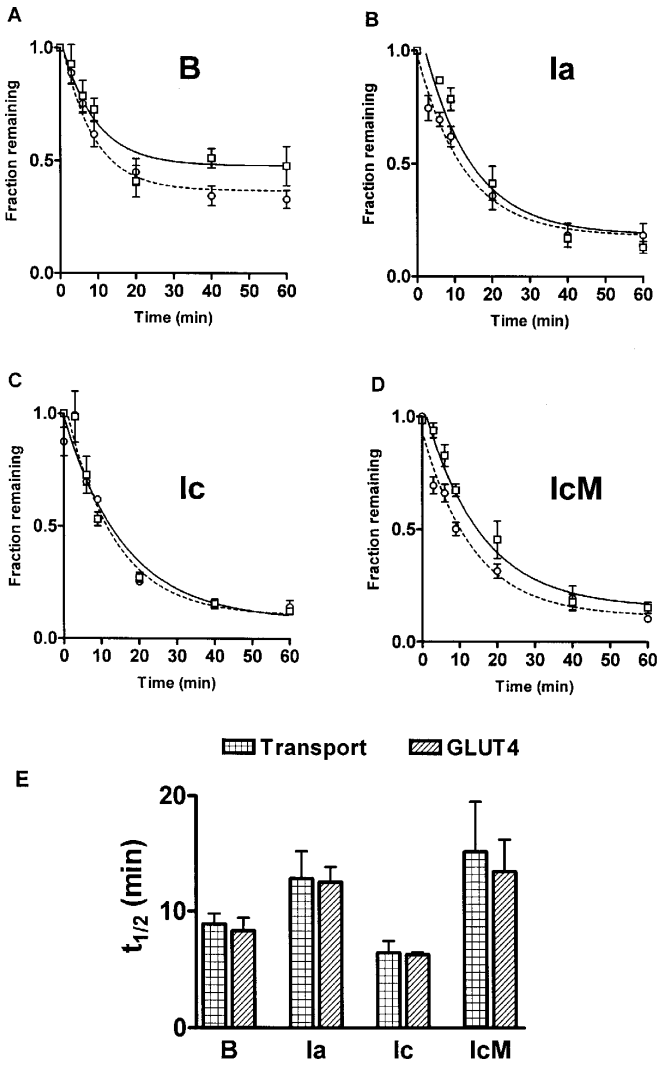

Figure 6 Rates of loss of glucose-transport activity and GLUT4 following wortmannin treatment

Adipocytes were cultured with either no additions (**A**), in the presence of 500 nM insulin (**C**), or with 500 nM insulin and 1 mM metformin (**D**). Cells were subsequently washed and adjusted to 40% cytocrit. A portion of basal cells was acutely stimulated with 20 nM insulin for 30 min (**B**). The rates of uptake of 3-OMG (□) and ATB-BMPA labelling of remaining cell surface GLUT4 (○) were then determined following treatment with 1 μM wortmannin for the indicated times. The data are plotted as the fractional reduction of the initial activity or level. The half-times (**E**) for the rate of reduction in glucose-transport activity (hatched bars) and GLUT4 (light bars) following treatment of the cells with 1 μM wortmannin were determined by curve fitting to an equation describing a single exponential decay. Results are the means ± S.E.M. from 3, (4 basal) experiments. Data are further analysed in Table 3.

insulin-treated cells, and this effect is completely ameliorated by culture with metformin.

The model-dependent rate constants for GLUT4 endocytosis following wortmannin treatment and derived directly from curve fitting to eqn. (1) are compared with the half-times in Figure 6. This analysis indicates that the effects of chronic insulin treatment on glucose-transport activity and the levels of cell-surface GLUT4 are accounted for by a doubling of the GLUT4 endocytosis rate constant (k_{en}). Metformin reverses this effect on endocytosis.

Wortmannin appears to have little or no effect on endocytosis as the values for k_{en} derived here are similar to those previously

Table 3 Effect of chronic insulin treatment on the rate constants for net loss of cell-surface GLUT4 and GLUT1

Adipocytes were cultured with no additions (B), in the presence of 500 nM insulin (Ic), or with 500 nM insulin and 1 mM metformin (IcM). A portion of basal cells was acutely stimulated with 20 nM insulin for 30 min (Ia). The endocytosis and exocytosis rate constants (k_{en} and k_{ex} respectively) for net reduction in glucose-transport activity, GLUT4 and GLUT1 following treatment of the cells with 1 μM wortmannin, were determined by curve fitting to eqn. 1. Results are the means ± S.E.M. from 3 experiments. ** $P < 0.01$, * $P < 0.05$ versus insulin.

		Transport ($min^{-1} \cdot 10^2$)	GLUT4 ($min^{-1} \cdot 10^2$)	GLUT1 ($min^{-1} \cdot 10^2$)
B	k_{en}	7.22 ± 0.63	5.86 ± 0.88	3.25 ± 0.86
	k_{ex}	0.27 ± 0.18	2.76 ± 0.55	5.67 ± 2.23
Ia	k_{en}	5.64 ± 1.15 *	4.93 ± 0.70 **	4.18 ± 0.37
	k_{ex}	0.20 ± 0.10	0.72 ± 0.26	2.31 ± 0.86
Ic	k_{en}	10.96 ± 1.51	9.61 ± 0.26	3.40 ± 0.20
	k_{ex}	0.33 ± 0.05	1.50 ± 0.17	3.17 ± 0.58
IcM	k_{en}	5.18 ± 1.24 *	5.27 ± 1.25 *	6.83 ± 2.76
	k_{ex}	0.10 ± 0.04	0.65 ± 0.12	3.23 ± 1.03

obtained in cells not treated with wortmannin [16,17,36]. As in 3T3-L1 cells [36], GLUT4 exocytosis is inhibited by wortmannin, since values for k_{ex} (Table 3) are much lower than those previously obtained in cells not treated with wortmannin [16,17,36]. These comparisons therefore suggest that it is the inhibition of exocytosis and not acceleration of endocytosis that leads to the perturbation of glucose transport and reduction of levels of cell-surface GLUT4 following wortmannin treatment. A rate constant for unperturbed GLUT4 exocytosis (k_{ex}) can be calculated if we assume (and as suggested above) k_{en} is unaltered by wortmannin. To do this the steady-state GLUT4 distributions (k_{ex}/k_{en}) are used. These ratios (k_{ex}/k_{en}) are approx. 0.81 and 0.33 for acute and chronic insulin treatments respectively [8,29]. For acute and chronic insulin treatments, the derived exocytosis rate constants are therefore approx. 0.039 and 0.032 min^{-1} respectively. From this analysis it is apparent that the calculated rate of GLUT4 exocytosis in the absence of wortmannin is not markedly altered by chronic insulin treatment.

The calculated GLUT1 endocytosis rate constants are generally lower than GLUT4, and are not markedly altered by chronic insulin treatment (Table 3). The calculated GLUT1 exocytosis rate constants following wortmannin treatment are generally higher than for GLUT4, possibly due to a lower wortmannin sensitivity.

DISCUSSION

Since the development of the chronic insulin treatment model for insulin resistance by Marshall and colleagues [7,25], considerable progress has been made in determining the locus of the insulin resistance in the pathway leading to glucose transport. In particular, the hexosamine pathway has been implicated by *in vitro* [37] and *in vivo* studies [22,38]. More recently, the glucosamine effect has been found to be associated with ATP depletion in 3T3-L1 cells [28]. In the study described here, we found that the chronic insulin effect can be reversed by metformin; we have therefore focused on determining whether the reversal effects of metformin are transmitted through all the known intermediates implicated in the signalling cascade. We have traced the phenomenon of reduced sensitivity to insulin, and the reversal effects of metformin, from receptor tyrosine phosphorylation through to the kinetics of glucose transporter trafficking. Interestingly, and unlike the studies in which insulin

insensitivity has been induced by glucosamine treatment, the effects we observe occur with high glucose concentrations in culture and where ATP levels are unaltered by the chronic insulin treatment.

Reduced insulin receptor kinase activity has been observed in insulin-resistant states [39–41]. The data presented here suggest that impaired signalling, at the level of receptor phosphorylation, is also associated with the chronic insulin treatment model of insulin resistance. Inclusion of metformin has been found to reverse the chronic insulin effects on receptor phosphorylation. There are reports that metformin treatment of obese (fa/fa) Zucker rats [21] and a 2 h metformin treatment of rat adipocytes [42] have no effect on the insulin receptor phosphorylation. However, there is contradictory evidence suggesting that metformin can produce increases in the receptor tyrosine phosphorylation in streptozotocin diabetic rats [22], erythrocytes [43], *Xenopus* oocytes [44], and basal insulin receptor tyrosine phosphorylation in vascular smooth muscle [45]. Recent evidence suggests that *in vivo* metformin directly activates receptor tyrosine kinase activity [46].

The reduced tyrosine phosphorylation of the insulin receptor following chronic insulin treatment and its reversal by metformin have effects on downstream processes that have been implicated in the signalling to glucose transporter translocation. The data suggest chronic insulin and metformin treatments do not alter the total amount or the catalytic activity of PI-3K directly, but do alter the coupling of PI-3K to upstream phosphotyrosine intermediates.

Reduced Akt and glucose-transport activities have been observed in human muscle from NIDDM patients [47]. Similarly, the activity of Akt following chronic insulin treatments *in vitro* (in the absence and presence of metformin) correlates with the changes occurring at the levels of insulin receptor phosphorylation and the phosphotyrosine-associated PI-3K. These data suggest that alterations in Akt activity are a consequence of the early processes and that there are probably no additional effects of these treatments (insulin or metformin) on enzyme activity. In turn, the changes in Akt also correlate with the changes observed in glucose transport activity but this may occur because both of these processes are related to the same early signalling changes. This correlation, therefore, does not provide evidence either for [48] or against [15] the possibility that changes in transport are mediated by Akt.

In dexamethasone-treated mice, metformin prevents insulin resistance in glucose transport, but this is not due to changes in GLUT4 or GLUT1 expression [49]. Our data also suggest that in rat adipocytes, metformin can reverse the effects of chronic insulin on glucose-transport activity, and that this effect is independent of the total cellular levels of these isoforms. Rather, we have found that the effects of chronic insulin treatment and its reversal by metformin are transmitted through the signalling intermediates to the GLUT4 endocytosis process.

There appears to be spare receptor and signalling capacity in the normal system leading to elevation of GLUT4 translocation. The concentration of insulin required to activate glucose transport maximally corresponds to only 14 % of the maximal receptor kinase activity [50]. On the other hand, only rather moderate deficiencies in these same signalling processes produce insulin resistance and down-regulation of cell surface glucose transporters. This appears to apply to both *in vitro* model systems and in human NIDDM [5]. We have shown here that only a low level of signalling (for example when phosphotyrosine-associated PI-3K is reduced by 60 %) is necessary for GLUT4 exocytosis, since this process apparently remains unaltered by chronic insulin treatment. However, the down-regulation of cell-surface GLUT4 is associated with a doubling of the rate of endocytosis of GLUT4. It is this effect which correlates with the reductions in insulin receptor phosphorylation and PI-3K activity. The effects on endocytosis persist under conditions in which exocytosis is blocked by wortmannin treatment. This suggests that, once elevated, the endocytosis rate is not dependent on further signalling via a wortmannin-sensitive PI-3K. Therefore, an explanation for the apparent poor correlation between high insulin sensitivity/signalling capacity and insulin-signalling resistance may be that the former is associated with GLUT4 exocytosis, while the latter is associated with GLUT4 endocytosis. Regulation of the endocytosis limb of the translocation pathway may involve a divergent signalling route or branch with a different insulin and PI-3K dependence.

Previous studies have shown that GLUT4 exocytosis is increased over ninefold by insulin [16,17], while endocytosis is slightly decreased by insulin under normal conditions [16–18]. It appears that the relatively small effect of acute insulin treatment to reduce endocytosis is lost following prolonged insulin treatment. However, as the endocytosis process appears to be more sensitive to perturbations in signalling, an acceleration of endocytosis may be an important component of the pathophysiological changes leading to insulin resistance.

We have found that metformin can reverse both signalling and the related GLUT4-trafficking defects associated with chronic insulin treatment. The metformin reversal appears to be primarily associated with altered insulin receptor tyrosine phosphorylation. Although its precise mechanism of action remains to be fully elucidated, the secondary effects on GLUT4 endocytosis provide a rationale for the therapeutic use of this compound in treatment of insulin resistance in human NIDDM.

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