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Alterations in insulin signalling pathway induced by prolonged insulin treatment of 3T3-L1 adipocytes

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Summary Insulin-induced glucose transport stimulation, which results from the translocation of glucose transporter 4 (GLUT 4)-containing vesicles, is completely blocked after prolonged insulin treatment of 3T3-L1 adipocytes. Since GLUT 4 expression was reduced by only 30 %, we looked at the insulin signalling pathway in this insulin-resistant model. Insulin-induced tyrosine phosphorylation of the major insulin receptor substrate IRS 1 was reduced by 50 ± 7 %, while its expression was decreased by 70 ± 4 %. When cells were treated with wortmannin (a PI3-kinase inhibitor) together with insulin, the expression of IRS 1 diminished to a much lower extent. Associated with the decrease in IRS 1 expression and phosphorylation, the activation by insulin of anti-phosphotyrosine immunoprecipitable PI3-kinase activity and of $p44^{mapk}$ and $p42^{mapk}$ activities was altered. However, the expression of these proteins was normal and $p44^{mapk}$ activity remained responsive to the tumour promoter TPA. Those results indicate that prolonged insulin treatment of 3T3-L1 adipocytes induces an insulin-resistant state with a reduced ability of insulin to stimulate the PI3-kinase and the MAP-kinases and a blockade of glucose transporter translocation. [Diabetologia (1995) 38: 1148–1156]

Key words Insulin signalling, MAP-kinase, PI3-kinase, IRS 1, GLUT 4 translocation, insulin resistance, wortmannin.

In adipose tissue and skeletal muscle, insulin stimulation of glucose transport results from the translocation of vesicles containing the glucose transporters GLUT 4 from an intracellular compartment to the plasma membrane [1–3]. However, the molecular events between the activation of the insulin receptor tyrosine kinase and the translocation of GLUT 4 remain ill-defined. Some insight into the understanding of insulin signalling has been obtained with the cloning of the major insulin receptor substrate IRS 1 (insulin receptor substrate 1) [4]. In most cells, this 160–185 kDa protein is rapidly tyrosine-phosphorylated after insulin exposure, and then docks several proteins containing src-homology 2 (SH2) domains such as the phosphotyrosine phosphatase Syp, Nck, the p85 subunit of the phosphatidylinositol 3′-kinase (PI3-kinase) and GRB 2 [5]. GRB 2 couples the Ras guanine-nucleotide releasing factor Sos to IRS 1 and then links the insulin receptor to the Ras/mitogen-activated protein kinases (MAPK) signalling pathway [6, 7]. A whole set of data suggests that IRS 1 is essential for some, if not all, of insulin's biological responses [5, 8]. Indeed, when IRS 1 levels are reduced by overexpression of an antisense cDNA, insulin mitogenic response is blocked [9]. In a similar fashion, serine/threonine phosphorylation of IRS 1 induces a decrease in its insulin-induced tyrosine phosphoryla-

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Abbreviations: GLUT, Glucose transporter; TPA, tumour promoter; MAPK, mitogen-activated protein kinase; IRS, insulin receptor substrate; SH2, src homology 2; GRB, GRB: Growth factor Receptor bound protein; PVDF, polyvinyliden difluoride; HDM/LDM, high density/low density microsomes; MBP, myelin basic protein; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethanesulphonyl fluoride; PI3-kinase: phosphatidylinositol 3′-kinase

tion and a blockade in the stimulation of glucose transport and PI3-kinase activity [10]. However, the possible implication of the different molecules of the cascade following IRS 1 phosphorylation in the regulation of insulin-induced glucose transport remains unknown.

Insulin resistance for glucose transport can result from various defects including: 1) alterations in insulin receptor functioning, 2) depletion of the GLUT 4 transporter pool and 3) alterations in the post-receptor signalling pathway [11]. An insulin-resistant state due to defects in signal transmission has been obtained following a prolonged insulin treatment of 3T3-L1 adipocytes [12]. Indeed, this treatment, which did not modify the insulin receptor and the GLUT 4 amounts, totally prevented the GLUT 4 translocation in response to acute insulin stimulation [12], and induced a decrease in IRS 1 expression due to proteolysis [13, 14].

In the present work, we have taken advantage of this model to investigate whether the decrease in IRS 1 amount was associated with defects in PI3-kinase and MAPK activation, and whether such alterations could be correlated with the lack of insulin-induced GLUT 4 translocation.

Materials and methods

Antibodies. Antibodies to GLUT 4 and GLUT 1 were obtained from rabbits injected with peptides corresponding to the 12 or 14 amino acids of the C-terminus of GLUT 4 and GLUT 1, respectively [15], and were used at 1/500 and 1/200 dilution for immunoblotting experiments. Antibodies to IRS 1, kindly provided by Dr. C. R. Kahn (Boston, Mass., USA) were raised in a rabbit using a synthetic peptide derived from the sequence 489–507 of IRS 1 [16]. Antibodies to $p44^{mapk}$ and $p42^{mapk}$ were obtained by immunizing rabbits against a peptide corresponding to the C-terminus of each protein [17]. Antibodies to p85 PI3-kinase and antibodies to phosphotyrosine were from UBI (Lake Placid, N. Y., USA) or obtained in our laboratory after injection of a rabbit with phosphotyrosine coupled to bovine immunoglobulins.

Cell culture. The 3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) 10 % fetal calf serum and induced to differentiate into adipocytes as described [18]. These 3T3-L1 adipocytes were used between 8 and 12 days after initiation of differentiation, when more than 95 % of the cells exhibited the adipocyte phenotype. Prolonged insulin treatment of 3T3-L1 adipocytes was performed as described [12] with minor modifications. Cells were incubated for different periods of time in DMEM/10 % fetal calf serum with 100 nmol/l insulin. Cells were then washed three times at 37 °C with Krebs-Ringer-Mes buffer (in mmol/l: 136 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.25 $MgSO_4$, 10 Mes, pH 6.0). Cells were then incubated for 1 h in the same buffer supplemented with 25 mmol/l D-glucose and 0.2 % (w/v) bovine serum albumin (BSA) and washed three times with the same buffer, then three times with phosphate buffered saline (PBS)/BSA. Cells were incubated in PBS/BSA for 20–30 min before being stimulated. Control cells were submitted to the same washing protocol as the chronically insulin-treated cells.

Deoxyglucose uptake measurement. The 3T3-L1 adipocytes were incubated for 10 min in PBS/BSA with or without 100 nmol/l insulin. Then, 2-deoxy-[1-^{3}H] D-glucose (100 μmol/l, 0.1 μCi) was added for 5 min. Cells were washed twice with ice-cold PBS, solubilized in 0.1 normal NaOH and their associated radioactivity was determined.

Subcellular fractionation of 3T3-L1 adipocytes and GLUT 4 immunodetection. The 3T3-L1 adipocytes (cells from two 150-mm dishes) were incubated with or without 100 nmol/l insulin for 15 min at 37 °C in 10 ml of PBS/BSA. Cells were washed twice with PBS, scraped in 10 ml of TES buffer (in mmol/l: 20 Tris pH 7.4, 1 EDTA, 250 sucrose, pH 7.4, aprotinin 100 U/ml and 1 mmol/l PMSF) and homogenized with 15 strokes in a Thomas Potter (Type C Philadelphia, PA, USA). Homogenates were centrifuged at 16,000 *g* for 20 min. The supernatant was centrifuged for 90 min at 210,000 *g* to obtain a pellet referred to as high-density/low-density microsomes (HDM/LDM). In parallel, the 16,000 *g* pellet was rehomogenized in 4 ml of TES buffer, overlaid on a sucrose cushion (38 % w/v, in Tris/EDTA buffer) and centrifuged for 60 min at 100,000 *g*. The plasma membranes between the two layers were collected, diluted in Tris buffer, and pelleted by centrifugation for 75 min at 210,000 *g*. Pellets of each fraction were resuspended in 50–100 μl TES and protein content determined by the Bio-Rad assay (Richmond, CA, USA) with BSA as standard.

Proteins from the different fractions (50–100 μg) were solubilized in Laemmli buffer (3 % SDS, 70 mmol/l Tris, 11 % glycerol) containing bromophenol blue (0.05 %) and 2-mercaptoethanol (700 mmol/l) and separated by SDS/PAGE with a 10 % resolving gel [19]. Proteins were transferred to a polyvinyliden difluoride (PVDF) sheet. The efficiency of the transfer and the similarity in the protein amounts in the different lanes were controlled by coloration with Ponceau Red. The sheet was incubated with blocking buffer (PBS/5 % fat skimmed dry milk) for 2 h at room temperature. The sheet was then incubated overnight at 4 °C with the antibodies to GLUT 4. After three washes (10 min each) in PBS containing 1 % Nonidet P40 (Fluko, Bucks, Switzerland), the sheet was incubated for 1 h at room temperature with [^{125}I] protein A (5×10^5 cpm/ml blocking buffer) and washed as above. Blots were submitted to autoradiography. Quantification was performed by counting the radioactivity associated with the bands and/or by scanning (Hoefer Scientific Instrument San Francisco, CA, USA).

Immunodetection of phosphotyrosine-containing proteins and other proteins (IRS 1, p85 PI3-kinase, $p44^{mapk}$). Following prolonged treatment with insulin, 3T3-L1 adipocytes were stimulated for 7 min with or without 100 nmol/l insulin. Cells were then washed twice with PBS and solubilized for 40 min at 4 °C in 1 ml of buffer A (in mmol/l: 20 Tris pH 7.4, 137 NaCl, 100 NaF, 10 EDTA, 2 Na_3VO_4, 10 pyrophosphate, 1 PMSF, 1 % Nonidet P40, 100 U/ml aprotinin). Then, cell lysates (50–100 μg proteins) were treated with Laemmli buffer and separated on SDS/PAGE with a 7.5 % polyacrylamide resolving gel. Proteins were transferred to PVDF membranes. Membranes were saturated with PBS/5 % BSA for 2 h at room temperature and incubated overnight with antibodies to phosphotyrosine (1 μg/ml saturation buffer), IRS 1 (1/200 in saturation buffer), $p44^{mapk}$ (1/100 in saturation buffer), p85 PI3-kinase (1/1000 in saturation buffer). Membranes were washed as described above. When antibodies to phosphotyrosine were used, a further incubation was performed for 1 h at room temperature with rabbit anti-mouse immunoglobulins. Finally, sheets were incubated with [^{125}I] protein A and washed as described above.

Determination of PI3-kinase activity. Control or chronically insulin-treated 3T3-L1 adipocytes were stimulated or not with 100 nmol/l insulin and solubilized in 1 ml buffer A for 40 min at 4 °C. Lysates were centrifuged for 10 min at 13,000 *g*. Supernatants were incubated for 90 min at 4 °C with antibodies to phosphotyrosine or to the PI3-kinase 85 kDa subunit coupled to protein A sepharose beads. Thereafter, immune pellets were washed twice with each of the three following buffers: (a) PBS containing 1 % Nonidet p40 and 200 μmol/l Na_3VO_4; (b) 100 mmol/l Tris pH 7.4, 0.5 mol/l LiCl, 200 μmol/l Na_3VO_4, and (c) 10 mmol/l Tris pH 7.4, 100 mmol/l NaCl, 1 mmol/l EDTA, 200 μmol/l Na_3VO_4. PI3-kinase activity was measured on the immune pellets as previously described [20, 21].

Measurement of $p42^{mapk}$ and $p44^{mapk}$ activities. Lysates obtained from cells treated as described above were used to measure MAPK activities. Phosphorylated $p42^{mapk}$ was visualized by its characteristic retardation in electrophoretic mobility [22]. The $p44^{mapk}$ activity was measured by incubating cell lysates with antibodies to $p44^{mapk}$ coupled to protein A sepharose beads for 90 min at 4 °C. Immune pellets were washed three times with homogenization buffer and twice with HNT buffer (30 mmol/l Hepes pH 7.4, 30 mmol/l NaCl, 0.1 % Triton X-100) containing 200 μmol/l Na_3VO_4. Pellets were resuspended in 50 μl of HNT buffer and $p44^{mapk}$ activity was measured by addition of 10 μl of a solution containing 30 mmol/l Hepes, 30 mmol/l NaCl, 0.1 % Triton X-100, 30 μmol/l [γ-^{32}P] ATP (10 μCi), 6 mmol/l DTT, 60 mmol/l $(CH_3COO)_2Mg$, 1 mg/ml myelin basic protein (MBP). After 15 min, samples were spotted on phosphocellulose paper and immersed in a bath of 1 % orthophosphoric acid. Papers were washed three times for 10–15 min each and dried before quantification by Cerenkov counting [17].

Results

Glucose transport and glucose transporter translocation in chronically insulin-treated 3T3-L1 adipocytes. Previous studies have shown that prolonged insulin-treatment of 3T3-L1 adipocytes induces an inability of the cells to recruit GLUT 4 to the cell surface following an acute insulin stimulation [12]. Since in our 3T3-L1 adipocytes, the previously published conditions [12] profoundly decreased GLUT 4 expression, insulin treatment was slightly modified. We first verified that the cells became insulin-resistant for glucose transport. As shown in Table 1, in control cells, acute insulin treatment increased deoxyglucose transport eightfold. By contrast, after 10 h of chronic-insulin treatment, basal transport was five times higher than in control cells but did not increase further upon an acute insulin stimulation.

The total amount of GLUT 1 and GLUT 4 proteins in cell homogenates was quantified by immunoblotting. It should be noted that insulin treatment did not modify the total cellular protein content (data not shown) and thus identical protein amounts were analysed, which corresponded to identical cell numbers. As shown in Table 1, GLUT 1 expression was increased by two fold while GLUT 4 amount decreased by 30 % following prolonged insulin treatment. To investigate whether the alteration in glucose transport stimulation was due to an alteration in GLUT 4 translocation, control and chronically insulin-treated cells were stimulated or not for 15 min with 100 nmol/l insulin and submitted to subcellular fractionation. Following electrophoresis, proteins were transferred to PVDF membrane and GLUT 4 molecules were identified using specific antibodies and [^{125}I]-labelled protein A. A typical autoradiogram is shown in Figure 1 and the quantification of three experiments is presented in Table 2. In control and treated cells, most of the GLUT 4 transporters were located in HDM/LDM in the absence of insulin. After insulin stimulation of control cells, the

Table 1. Effect of prolonged insulin treatment on insulin-induced glucose transport and glucose transporter expression

	Control cells	Treated cells
Deoxyglucose uptake		
Basal	0.28 ± 0.02	1.51 ± 0.29
Insulin	2.30 ± 0.70	1.81 ± 0.35
Glucose transporters		
GLUT 1	100	190 ± 28
GLUT 4	100	67 ± 7

Control or chronically insulin-treated 3T3-L1 adipocytes, were stimulated or not for 15 min with 100 nmol/l insulin before measuring 2-deoxyglucose (DOG) uptake (0.1 mmol/l, 0.1 μCi/assay) during a 5-min period as described in Methods. Values are expressed as nmol of DOG incorporated/assay and are the means ± SEM of three-four experiments. Glucose transporters were quantified as follows: cellular homogenates (100 μg of proteins) were subjected to SDS/PAGE; proteins were transferred to PVDF sheets and immunodetected with antibodies to GLUT 4 or GLUT 1 as described in Methods. Results are the means ± SEM of three-four different experiments and are expressed as a percentage of the values obtained in control cells

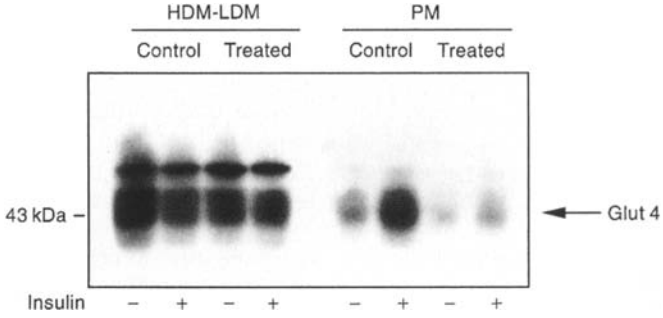

Fig. 1. Effect of prolonged insulin-treatment on insulin-induced GLUT 4 translocation. Control and chronically insulin-treated 3T3-L1 adipocytes were stimulated for 15 min with or without 100 nmol/l insulin. Cells were fractionated in high density/low density microsomes (HDM/LDM) and plasma membranes (PM) as described in Methods. Proteins from each fraction were separated by SDS-PAGE, transferred to PVDF sheets and incubated with antibody to GLUT 4 and [^{125}I]-protein A. Sheets were then submitted to autoradiography. A typical autoradiogram is shown

Table 2. Effect of prolonged insulin treatment on insulin-induced GLUT 4 translocation

	Control cells	Treated cells
GLUT 4 in PM fraction		
Basal	1	0.68 ± 0.15
Insulin	2.39 ± 0.49[a]	0.98 ± 0.17
GLUT 4 in HDM/LDM fraction		
Basal	1	0.67 ± 0.06
Insulin	0.60 ± 0.01[a]	0.54 ± 0.05

Control or chronically insulin-treated 3T3-L1 adipocytes were stimulated or not for 15 min with 100 nmol/l insulin before subcellular fractionation into plasma membranes (PM) and high/low density microsomes (HDM/LDM) as described in Methods. Glucose transporters were quantified in each fraction by immunodetection. Results are the means ± SEM of three different experiments. To allow comparison amongst experiments, the amounts of GLUT 4 were expressed relative to the amount of transporters present in control basal conditions.
[a] Insulin effect was significant with $p < 0.05$

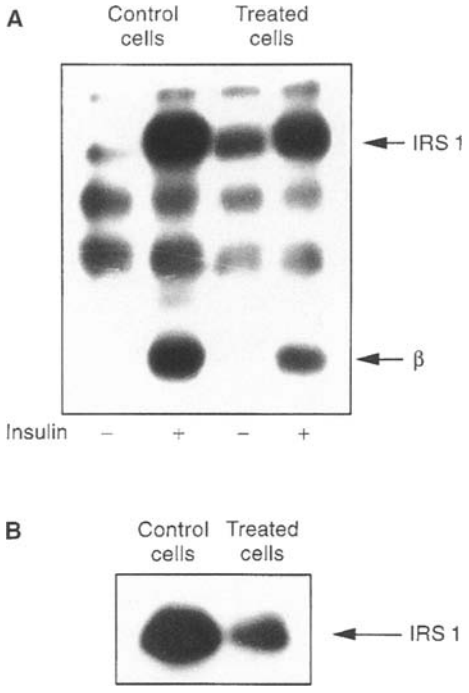

Fig. 2 A, B. Effect of prolonged insulin-treatment on insulin-induced tyrosine phosphorylation of proteins. Control and chronically insulin-treated 3T3-L1 adipocytes were treated with or without 100 nmol/l insulin for 7 min. Proteins were solubilized in buffer containing phosphatase inhibitors and subjected to SDS/PAGE. The proteins were transferred to PVDF sheets, then incubated with antibodies to phosphotyrosine (panel **A**) or to IRS 1 (panel **B**) as described in Methods. The sheets were then submitted to autoradiography. Typical autoradiograms are presented. Quantification of five different experiments indicated that, after prolonged insulin treatment, insulin-induced tyrosine phosphorylation of its receptor and IRS 1 was decreased by 40 %, while the IRS 1 amount was reduced by 70 ± 5 %. β refers to the insulin receptor beta-subunit

amount of GLUT 4 decreased by 40 % in the HDM/LDM and increased 2.4-fold in plasma membranes. In contrast, no significant change in the subcellular distribution of GLUT 4 occurred in treated cells. Thus, as previously reported [12], after prolonged insulin treatment, 3T3-L1 adipocytes became insulin-resistant for glucose transport and GLUT 4 translocation indicating that the signalling mechanism leading to translocation was altered.

Insulin receptor autophosphorylation and IRS 1 phosphorylation. To investigate which steps in insulin signalling could be altered after prolonged exposure to the hormone, we looked for the ability of insulin to stimulate protein tyrosine phosphorylation. Control or insulin-treated 3T3-L1 adipocytes were acutely exposed or not to insulin, homogenized and cellular proteins were separated on SDS/PAGE, transferred to PVDF membranes and immunoblotted with antibodies to phosphotyrosine (Fig. 2 A) or IRS 1 (Fig. 2 B). As shown in Figure 2 A, in control cells, insulin stimulated the tyrosine phosphorylation of two proteins, one with a M_r of 95 k corresponding to the insulin receptor β-subunit, and another one with a M_r of 160 k, identified as IRS 1 with a specific antibody (Fig. 2 B). In chronically insulin-treated cells, the phosphorylation of IRS 1 was decreased by 50 ± 7 % (mean ± SEM of six experiments) in response to an acute insulin stimulation. IRS 1 expression, measured using an antibody to IRS 1, (Fig. 2 B), was decreased by 70 ± 4 % (mean ± SEM of six determinations) in chronically insulin-treated cells compared to control cells.

Effect of prolonged insulin treatment on PI3-kinase activity. Since activation of PI3-kinase results from its binding to phosphorylated IRS 1, we have investigated whether the decreased IRS 1 amount and phosphorylation modified PI3-kinase activation. Control and 3T3-L1 adipocytes treated with insulin for 10 h were acutely stimulated with insulin for different periods of time before measuring PI3-kinase activity in immunoprecipitates obtained with antibodies to phosphotyrosine. As shown in Figure 3, in control cells, the insulin stimulation of PI3-kinase activity was rapid, with maximal effect (60-fold over basal) obtained after 2 min of stimulation, then this activity decreased rapidly and reached a plateau within 15 min. In cells chronically treated with insulin for 10 h, basal PI3-kinase activity was slightly increased (two fold over untreated cells) and the time course of PI3-kinase activation after an acute insulin stimulation was similar to control cells, but maximal activity was reduced by 70 %. This inhibition was not due to a change in the total amount of the enzyme detected by immunoblotting (Fig. 3, inset).

Effect of prolonged insulin treatment on $p44^{mapk}$ and $p42^{mapk}$ activities. Insulin-induced IRS 1-GRB2/Sos complex formation is thought to be partly involved

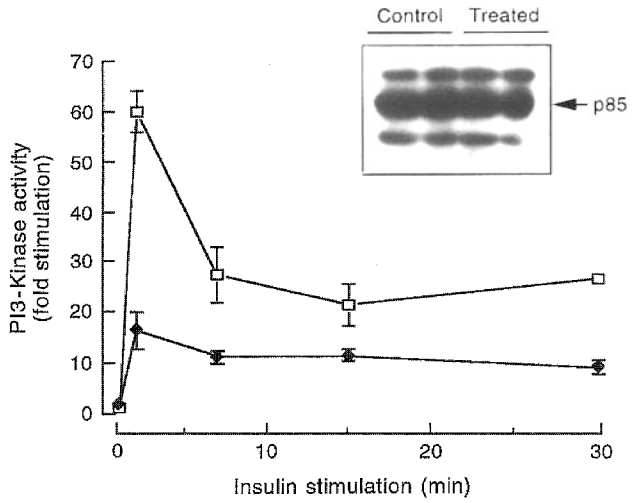

Fig. 3. Insulin stimulation of PI3-kinase activity in control and chronically insulin-treated 3T3-L1 adipocytes. Control cells (□) and chronically insulin-treated cells for 10 h (◆) were incubated in absence or presence of 100 nmol/l insulin for 2–30 min before homogenization and immunoprecipitation with antibodies to phosphotyrosine. PI3-kinase assay was performed on the immune pellet as described in Methods. Quantification of the PI-3-P product was performed by scintillation counting. To allow for comparison amongst experiments, the activities were expressed relative to the activity measured in control cells incubated in the basal condition. In treated cells, the basal activity was 1.98 ± 0.45, mean ± SEM of three experiments. In the inset is shown the immunodetection of p85 PI3-kinase subunit. Identical amounts of proteins from control and treated cells were analysed as described in Methods

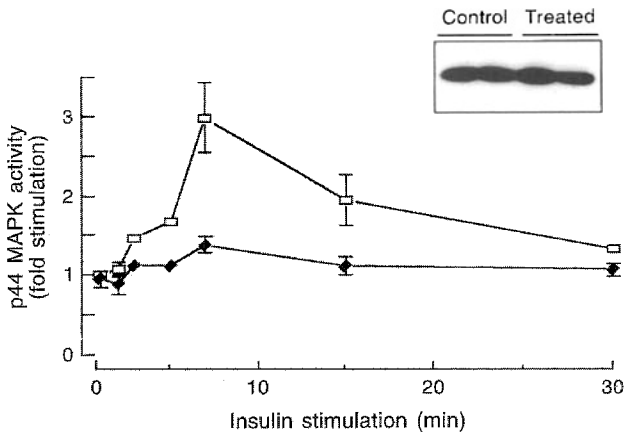

Fig. 4. Insulin stimulation of $p44^{mapk}$ activity in control and chronically insulin-treated 3T3-L1 adipocytes. Control cells (□) and chronically insulin-treated cells (◆) were incubated for 10 h in absence or in presence of 100 nmol/l insulin for 2–30 min before homogenization and immunoprecipitation with antibodies to $p44^{mapk}$. $p44^{mapk}$ activity was measured by phosphorylation of MBP as described in Methods. To allow for comparison amongst experiments, the activities were expressed relative to the activity measured in control cells incubated in the basal condition. In treated cells, the basal activity was 0.95 ± 0.11, mean ± SEM of three experiments. In the inset is shown the immunodetection of $p44^{mapk}$. Identical amounts of proteins from control and treated cells were analysed as described in Methods

in coupling the insulin receptor kinase to the Ras signalling pathway leading to the MAPK activation. Thus, we have investigated whether changes in IRS 1 phosphorylation and/or amount, induced by prolonged insulin treatment, could modify MAPK activation. Cells were stimulated with insulin and solubilized as described above. Following immunoprecipitation with a specific antibody, $p44^{mapk}$ activity was determined with MBP as substrate. As shown in Figure 4, in control cells, $p44^{mapk}$ activity was increased by threefold within 7 min of insulin stimulation and returned to basal values within 30 min. After 10 h of chronic-insulin treatment, basal activity was unchanged but the insulin-induced $p44^{mapk}$ activity was markedly blunted with only a slight stimulation remaining within 7 min. This lack of activation was not due to a change in $p44^{mapk}$ expression as determined by immunoblotting experiment (Fig. 4, inset). Further, tumour promotor (TPA) (1 μmol/l) was as efficient in activating $p44^{mapk}$ activity in control cells (2.92 ± 0.05-fold) as in chronically insulin-treated 3T3-L1 adipocytes (2.57 ± 0.22-fold).

To detect activation of $p42^{mapk}$, a gel shift assay was used since the phosphorylated form of the kinase has a slower electrophoretic mobility than the non-phosphorylated form [22]. Cell lysates were submitted to SDS/PAGE and $p42^{mapk}$ detected by immunoblotting. Acute insulin stimulation induced a retardation in the electrophoretic mobility of the kinase due to its phosphorylation in control but not in chronically insulin-treated cells (Fig. 5).

To determine whether the defect in the activation of PI3-kinase and $p44^{mapk}$ occurred with a similar time course following insulin treatment, 3T3-L1 adipocytes were chronically treated or not with 100 nmol/l insulin for different periods of time. The cells were washed and PI3-kinase or $p44^{mapk}$ activities were measured after 2 or 7 min of insulin stimulation, the time corresponding to their respective peak activation. Both PI3-kinase and $p44^{mapk}$ activities decreased in parallel when the time of insulin treatment increased from 1 h to 10 h (data not shown).

Wortmannin inhibits the insulin-induced decrease in IRS 1 expression. To search for the mechanism of the decrease in IRS 1 expression induced during long-term insulin treatment, we studied the effect of the PI3-kinase inhibitor, wortmannin [23, 24]. In this series of experiments a high concentration of insulin (500 nmol/l) was used to provoke a profound diminution of IRS 1 and GLUT 4. Indeed, when cells were treated for 5 h with insulin (500 nmol/l) (Fig. 6), a marked degradation of IRS 1, accompanied by a shift in its molecular weight was observed while the decrease in GLUT 4 was observed only after 24 h. Wortmannin (1 μmol/l) prevented these insulin ef-

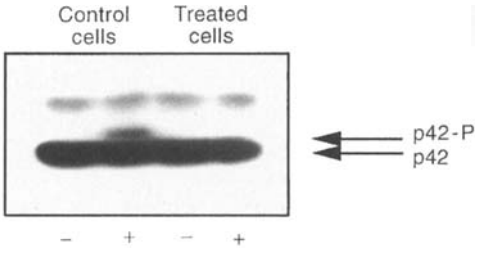

Fig. 5. Effect of prolonged insulin-treatment on $p42^{mapk}$ activity. Cells were incubated with or without insulin (100 nmol/l) for 7 min and then solubilized. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibody to $p42^{mapk}$ and revealed with [^{125}I] protein A. The positions of the phosphorylated form (p42-P) and the unphosphorylated form (p42) of the MAPK are indicated

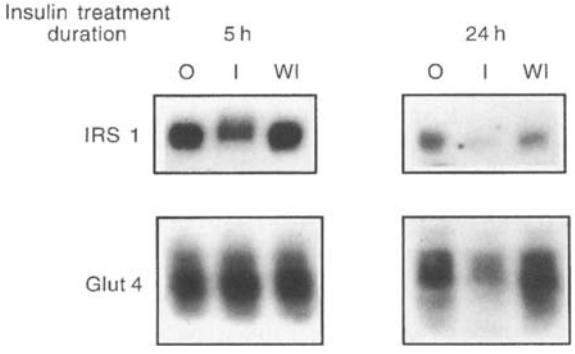

Fig. 6. Wortmannin prevents the insulin-induced decrease in IRS 1 and GLUT 4 expression. 3T3-L1 adipocytes were treated for 5 or 24 h in absence (O) or in presence of 500 nmol/l insulin (I, WI) and 1 μmol/l wortmannin (WI) and then washed as described in Methods. Cellular proteins were separated by SDS/PAGE and immunoblotted with antibodies to IRS 1 (top panel) or to GLUT 4 (bottom panel). A typical autoradiogram is shown

fects on IRS 1 and GLUT 4 expression, suggesting that PI3-kinase was involved in these insulin effects.

Discussion

Previous studies have shown that prolonged treatment of 3T3-L1 adipocytes with insulin leads to an increased basal glucose transport [12, 25, 26] and to an insulin-resistant state for this transport [12, 26]. In the previously published studies [12, 25, 26], the total amount of GLUT 4 was not changed but the GLUT 4 translocation from the internal compartment to the cell surface in response to an acute insulin stimulation was completely blocked [12]. For unknown reasons, the same experimental conditions, i.e. 500 nmol/l insulin for 24 h, dramatically decreased the total GLUT 4 amount in our 3T3-L1 cell line (Fig. 6) and in other studies [27, 28]. As previously described [13], chronic insulin treatment also decreased the IRS 1 content, a decrease which was attributed to an enhancement of IRS 1 degradation [14]. These effects of insulin on IRS 1 and GLUT 4 expression were blocked by wortmannin, a PI3-kinase inhibitor [23, 24], indicating that PI3-kinase is likely to play a role in this process. PI3-kinase is a dual specificity enzyme, with a lipid kinase activity and a serine kinase activity [29]. This serine kinase activity which is able to phosphorylate IRS 1 is also blocked by wortmannin [30, 31]. From our results it is not possible to decipher whether the insulin-induced downregulation of IRS 1 results from its serine/threonine phosphorylation or from the increase in lipid kinase activity, but the blockade of IRS 1 and GLUT 4 downregulation by wortmannin indicates that PI3 kinase is involved in this long-term insulin effect.

In the following series of experiments, in which biological insulin responses were measured, we reduced both the length of insulin treatment to 10 h and the hormone concentration to 100 nmol/l, conditions which induced insulin resistance for glucose uptake and glucose transporter translocation with only a moderate decrease in GLUT 4 expression (Fig. 1). We then looked for the defects in the insulin signalling pathway leading to glucose transporter translocation. The amount of IRS 1 was reduced by 40 %. It should be noted that chronic-insulin treatment affected protein expression differently, although total protein concentrations were similar in control and treated cells. While it markedly depressed IRS 1 and moderately decreased GLUT 4, it increased GLUT 1 but did not change the two p42 and $p44^{mapk}$ isoforms or PI3-kinase levels. Tyrosine phosphorylation of the insulin receptor and IRS 1 was reduced by 50 % while the IRS 1 amount was decreased by 70 % suggesting that tyrosine phosphorylation per IRS 1 molecule was increased in this insulin-resistant model. Tyrosine phosphorylated IRS 1 binds and activates PI3-kinase [32, 33] and also binds the GRB2-Sos complex which is involved in the activation of the Ras/MAPK pathways [5–7]. Thus, a reduction in the tyrosine phosphorylation of IRS 1 might result in alteration in the activation of PI3-kinase and MAPK. Our results indicate that in chronically insulin-treated cells, the activation of both PI3-kinase and MAPKinases was reduced by 70–80 % without a change in the expression of these enzymes. This reduction in activity was more closely correlated to the decrease in IRS 1 amount (70 %) than to its tyrosine phosphorylation (50 %). This could be due to the fact that hyperphosphorylation of IRS 1 occurs on tyrosine residues which are not in canonical sequences involved in the binding of PI3-kinase or GRB2-Sos complex. However, the MAPK cascade can also be initiated by the binding of the GRB2-Sos complex to Shc [34] and Shc has even been suggested to play a more important role than IRS-1 in insulin stimulation of $p21^{ras}$-activation in cells overexpressing insulin receptors [35, 36]. A reduction in the tyrosine

phosphorylation and/or in the amount of Shc could also contribute to the defect in MAPK activation.

A key role of IRS 1 in the transmission of insulin effect on glucose transport is supported by a series of observations. First, the IRS 1 amount increases dramatically when 3T3-L1 fibroblasts differentiate into adipocytes. This enhancement in IRS 1 expression parallels the increase in the amount of insulin receptors, the appearance of GLUT 4 expression and the occurrence of an insulin-induced glucose transport [13]. Second, following a treatment of 3T3-L1 adipocytes with okadaic acid, there was a close correlation between the reductions of IRS 1 tyrosine phosphorylation and of the stimulation of glucose transport in response to insulin [10]. Third, in many physiopathologic states linked to insulin resistance, the tyrosine phosphorylation of IRS 1 is reduced [37, 38]. If all those observations argue for a role of IRS 1 in the stimulation of glucose transport, the pathway downstream of IRS 1 remains ill-defined. In this study, we have shown that both the activation of PI3-kinase and MAPK was markedly altered. However, the potential role of these two enzymes in the stimulation of glucose transport is controversial. Indeed, okadaic acid and TPA, which activate MAPK but not PI3-kinase induce glucose transporter translocation [39–42]. Further, the microinjection of a constitutively active form of Ras activates glucose transport in cardiac myocytes [43] and the overexpression of activated Ras mimicks insulin action in 3T3-L1 adipocytes [44]. By contrast, in another study, the same last approach gave opposite results [45], and thrombin, epidermal growth factor and serum which also activate MAPK do not stimulate glucose transport [46–48]. A role for PI3-kinase in glucose transport is also strongly supported by the use of inhibitors such as wortmannin [23, 24, 49] or LY294002 [50] which inhibit insulin-induced glucose transport stimulation. However, wortmannin would not only inhibit PI3-kinase as claimed previously but also MAPK [51, 52]. To add to the confusion, it has been recently demonstrated that Ras could also regulate the activity of PI3-kinase by interacting with the catalytic subunit of the enzyme [53]. Overexpression of active Ras could thus activate PI3-kinase as well as MAPK. Finally, alterations in PI3-kinase activation and glucose transport stimulation have been observed in various insulin-resistant states [20, 54, 55], but MAPK activation has not been measured in the same models. Further, the reduction in PI3-kinase activation seems to be more profound [20] and appears earlier [37] than the defect in glucose transport in insulin-sensitive tissue of obese animals.

In conclusion, it appears that insulin resistance induced by chronic insulin treatment of 3T3-L1 adipocytes is associated with multiple defects, at the level of IRS 1, PI3-kinase and MAPK, and GLUT 4 translocation. Those defects are very similar to the alterations observed in hyperinsulinaemic states found in obese humans or rodents. This model will thus be very useful for a better understanding of the mechanisms of the alterations in the pathways leading from insulin receptor to GLUT 4 translocation.

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