

# Rosiglitazone ameliorates insulin resistance in brown adipocytes of Wistar rats by impairing TNF- $\alpha$ induction of p38 and p42/p44 mitogen-activated protein kinases

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## Abstract

**Aims/hypothesis.** TNF- $\alpha$  caused insulin resistance on glucose uptake and on insulin signalling in fetal brown adipocytes. Since treatment with TNF- $\alpha$  activates stress kinases, including c-jun NH2 terminal kinase (JNK), and p42/p44 and p38 mitogen-activated protein kinases (MAPK), we explored the contribution of these pathways to insulin resistance by TNF- $\alpha$ . Rosiglitazone is used to treat Type 2 diabetes as it improves insulin sensitivity in vivo. However, its ability to ameliorate TNF- $\alpha$ -induced insulin resistance in brown adipocytes remains to be explored.

**Methods.** We used fetal rat primary brown adipocytes cultured with TNF- $\alpha$ , with or without stress kinase inhibitors or rosiglitazone, and further stimulated with insulin. Then, we measured glucose uptake and GLUT4 translocation. To determine the insulin signalling cascade, we submitted cells to lysis, immunoprecipitation and immunoblotting.

**Results.** Exposure to TNF- $\alpha$  for 24 h impairs insulin stimulation of the phosphatidylinositol (PI) 3-kinase activity associated with IRS-2 and Akt activity. Pretreatment with PD98059 or PD169316, which inhibit p42/p44MAPK and p38MAPK respectively, restored insulin signalling and insulin-induced glucose uptake in the presence of TNF- $\alpha$ . However, in the presence of SP600125, an inhibitor of JNK, TNF- $\alpha$  still produced insulin resistance. Rosiglitazone ameliorated insulin resistance by TNF- $\alpha$  in brown adipocytes, restoring completely insulin-stimulated glucose uptake and insulin-induced GLUT4 translocation to plasma membrane in parallel to the insulin signalling cascade IRS-2/PI 3-kinase/Akt.

**Conclusions/interpretation.** Rosiglitazone treatment impaired TNF- $\alpha$  activation of p38 and p42/p44MAPK, restoring insulin signalling and leading to normalisation of glucose uptake.

**Keywords** Akt · Glucose uptake · GLUT4 translocation · Insulin · JNK · p38MAPK · p42/p44MAPK · PI 3-kinase · Rosiglitazone · TNF- $\alpha$

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**Abbreviations:** ECL, enhanced chemiluminescence · ERK, extracellular signal-regulated kinase · IKK, inhibitor kB kinase · JNK, c-jun NH2 terminal kinase · MAPK, mitogen-activated protein kinase · MBP, myelin basic protein · MEM, minimal essential medium · PI, phosphatidylinositol · PKC, protein kinase C · PMSF, phenylmethylsulfonyl fluoride · PPAR, peroxisome proliferator-activated receptor · TLC, thin layer chromatography

## Introduction

Insulin resistance is an important contributor to the pathogenesis of Type 2 diabetes mellitus. Tumour necrosis factor- $\alpha$  has been proposed as a link between adiposity and the development of insulin resistance, since most Type 2 diabetic patients are obese and have increased TNF- $\alpha$  expression in fat cells, and obese mice lacking TNF- $\alpha$  function are protected from developing insulin resistance [1, 2, 3]. Direct exposure of isolated cells to TNF- $\alpha$  inhibits insulin signalling and induces a state of insulin resistance in several systems including 3T3-L1 cells and human primary adipocytes [4, 5, 6]. The mechanism by which this

happens involves Ser phosphorylation of IRS-1, which converts IRS-1 into an inhibitor of the insulin receptor Tyr kinase activity [7, 8]. Moreover, Ser307 has been identified as a site for TNF- $\alpha$  phosphorylation of IRS-1, with activation of c-Jun N-terminal kinase (JNK) being involved in the phosphorylation of this residue [9, 10]. In this regard, ablation of *Jnk1* decreases the development of insulin resistance associated with dietary obesity [11]. Other studies suggested that p42/p44 and p38 mitogen-activated protein kinases (MAPKs) inhibit insulin signalling by diverse mechanisms in 3T3-L1 adipocytes [12, 13]. Recent work also implicated activation of inhibitor  $\kappa$ B kinases (IKK) by TNF- $\alpha$  on Ser phosphorylation of IRS-1 [14], while IKK inhibition with salicylate or targeted disruption of *Ikk $\beta$*  reversed obesity- and diet-induced insulin resistance [15].

Brown adipose tissue is a target tissue for insulin action, especially during late fetal development, when insulin supports survival, promotes adipogenic and thermogenic differentiation and regulates glucose uptake [16, 17, 18]. Acute insulin treatment stimulates glucose transport in brown adipocytes largely by mediating translocation of GLUT4 from an intracellular compartment to the plasma membrane, involving the activation of phosphatidylinositol (PI) 3-kinase, Akt 1 and 2 isoforms and the atypical protein kinase C (PKC) isoform  $\zeta$  [19, 20, 21]. We have previously described that TNF- $\alpha$  caused insulin resistance in brown adipocytes by decreasing IRS-2 Tyr phosphorylation and IRS-2-associated PI 3-kinase activity, while IRS-1 signalling was unaffected [22]. Furthermore, we had identified ceramide production as one of the mediators of insulin resistance by TNF- $\alpha$  and found that exogenously added C2-ceramide inhibited Akt activity through a ceramide-activated phosphatase [23]. However, other mediators activated in response to TNF- $\alpha$ , such as stress kinases and inflammatory pathways, could also contribute to insulin resistance in brown adipocytes.

An insulin-sensitising drug from the thiazolidinediones family, rosiglitazone improves insulin action in vivo and in vitro across a wide spectrum of insulin-resistant states, and has recently been introduced in the treatment of Type 2 diabetes [24, 25, 26]. Brown adipocytes are also a target for rosiglitazone action since they show high expression of peroxisome proliferator-activated receptor (PPAR) $\gamma$ , and rosiglitazone increased expression of the thermogenic uncoupling protein by binding to the PPAR $\gamma$  response element described in the enhancer of this gene [27]. We have also shown that rosiglitazone produces insulin sensitisation in cultured brown adipocytes [28]. Therefore, this cell model is an ideal system for investigating the effectiveness of rosiglitazone in the treatment of insulin resistance induced by TNF- $\alpha$ .

## Materials and methods

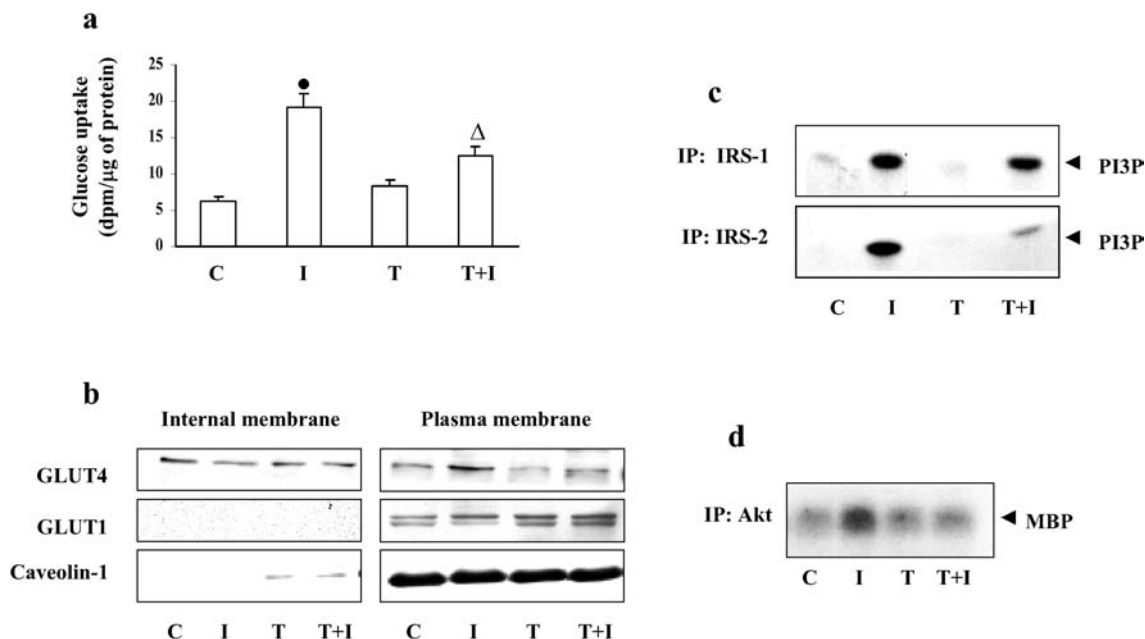
**Materials.** Insulin, BSA (fraction V, essentially fatty-acid-free) and myelin basic protein (MBP) were from Sigma (St. Louis, Mo., USA). TNF- $\alpha$  was purchased from Pharma Biotechnologie (Hanover, Germany). Rosiglitazone was kindly provided by Dr S. A. Smith (Glaxo SmithKline, Harlow, UK). PD169316 and PD98059 were purchased from Calbiochem (Calbiochem-Novabiochem Intl, La Jolla, Calif., USA). SP600125 was from Alexis (Lausen, Switzerland). Fetal calf serum, PBS and culture media were from Invitrogen (Paisley, UK). Autoradiographic films were Kodak X-O-MAT/AR (Eastman Kodak, Rochester, N.Y., USA). We purchased 2-deoxy-D[1- $^3$ H]-glucose (444 GBq/mmol), ( $\gamma$  $^{32}$ P)ATP and protein G-sepharose from Amersham Bioscience (Little Chalfont, UK). The anti-GLUT1 and anti-GLUT4 antibodies were supplied by Chemicon (Tamacula, Calif., USA). The anti-phospho and anti- (Akt, p42/p44MAPK, p38MAPK, JNK) antibodies were from Cell Signalling (Beverly, Mass., USA). We obtained antibodies against IRS-1 and IRS-2 from Upstate Biotechnology (Lake Placid, N.Y., USA), against caveolin-1(N-20) "sc-894" from Santa Cruz (Palo Alto, Calif., USA) and the mouse anti-FAS antibody from BD Biosciences Pharmingen (San Diego, Calif., USA). All other reagents used were of the purest grade available.

**Cell culture.** Fetal brown adipocytes were obtained from interscapular brown adipose tissue of 20-day Wistar rat fetuses and isolated by collagenase dispersion [29]. Isolated cells were plated in tissue culture dishes (1 $\times$ 10 $^6$  cells per 60-mm diameter dish or at 4 $\times$ 10 $^6$  cells per 100-mm diameter dish) in minimal essential medium (MEM) with Earle's salts supplemented with 10% FCS, the presence of serum being essential for the cells to attach to the plastic surface of the dishes. After 4 to 6 h of culture at 37  $^{\circ}$ C, cells were rinsed twice with PBS and a 70% confluent monolayer was observed under inverse light microscopy. Cells were maintained for 20 h in a serum-free MEM medium supplemented with 0.2% (w/v) BSA and then cultured for 24 h in the absence or presence of 1 nmol/l TNF- $\alpha$  without or with the following inhibitors: 800 nmol/l PD169316, 20  $\mu$ mol/l PD98059, 3  $\mu$ mol/l SP600125. They were then stimulated or not for 5 or 30 minutes with 10 nmol/l insulin. In another group of experiments cells were cultured for 24 h in the presence of TNF- $\alpha$  without or with 10  $\mu$ mol/l rosiglitazone, prior to insulin stimulation.

Wistar rats were obtained from the Complutense University Animal House. The principles of laboratory animal care were followed and the study was approved by the local ethics committee and carried out in accordance with the Declaration of Helsinki.

**Glucose transport determination.** Glucose transport was measured by incorporating 2-deoxy-D[1- $^3$ H]-glucose into cells during the last 10 min of culture as described previously [19]. Results are expressed as dpm/ $\mu$ g of protein or as fold increase over basal.

**Subcellular fractionation.** Cells were washed with ice-cold PBS and scraped into the homogenisation buffer containing: 20 mmol/l Tris-HCl, 2 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 mmol/l  $\beta$ -mercaptoethanol, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin (pH 7.4). After 10 min incubation cells were homogenised with 30 strokes of a Dounce homogeniser using a tight-fitting pestle. Nuclei were pelleted by centrifugation at 500 g for 5 min, and the low-speed supernatant was centrifuged at 100,000 g for 30 min. The high-speed supernatant constituted the internal



**Fig. 1.** TNF- $\alpha$  causes insulin resistance in cultured fetal brown adipocytes. Cells were serum-deprived for 20 h and further cultured for 24 h in the absence (C) or presence (T) of 1 nmol/l TNF- $\alpha$  and stimulated or not for 30 minutes with 10 nmol/l insulin (I). For panels (c) and (d), the time of insulin stimulation was 5 minutes. Glucose uptake (a) was measured during the last 10 minutes by incorporation of 2-deoxy-D[1- $^3$ H]-glucose into the cells. Results are means  $\pm$  SEM ( $n=10$ ). Statistical significance was tested and differences between values in the presence of I vs control (C) are represented by filled circles ( $p<0.001$ ), differences between values in the presence of T+I vs I are represented by open triangles ( $p<0.01$ ). **b.** Cells were harvested and submitted to subcellular fractionation. Western blotting was done on 10  $\mu$ g of internal and plasma membrane proteins from each condition, using anti-GLUT4, anti-GLUT1 and anti-Caveolin-1 antibodies, and developed with ECL. Cell lysates were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies (c) and assayed for PI 3-kinase activity or with anti-Akt (d) and assayed for MBP phosphorylation. Representative blots from four independent experiments are shown

**Western blotting.** Cells were lysed in lysis buffer I and cellular proteins (30  $\mu$ g) were submitted to SDS-PAGE, transferred to Immobilon membranes and blocked using 5% non-fat dried milk in 10 mmol/l Tris-HCl and 150 mmol/l NaCl pH 7.5. They were then incubated overnight with several antibodies as indicated in each case in 0.05% Tween-20, 1% non-fat dried milk in 10 mmol/l Tris-HCl and 150 mmol/l NaCl pH 7.5. Immunoreactive bands were visualised using the enhanced chemiluminescence (ECL-Plus) western blotting protocol (Amersham).

**JNK assay.** Cell lysates were obtained as described for immunoprecipitation. A pull-down of JNKs was performed with glutathione-S-transferase-c-Jun 79 protein, as described [31].

**Data analysis.** Results are means  $\pm$  SEM ( $n=4-10$ ) for duplicate dishes from four to ten independent experiments. Statistical significance was tested with ANOVA, followed by the protected least significant different test. A  $p$  value of less than 0.05 was considered significant. In experiments using X-ray films (Hyperfilm), different exposure times were used to ensure that bands were not saturated.

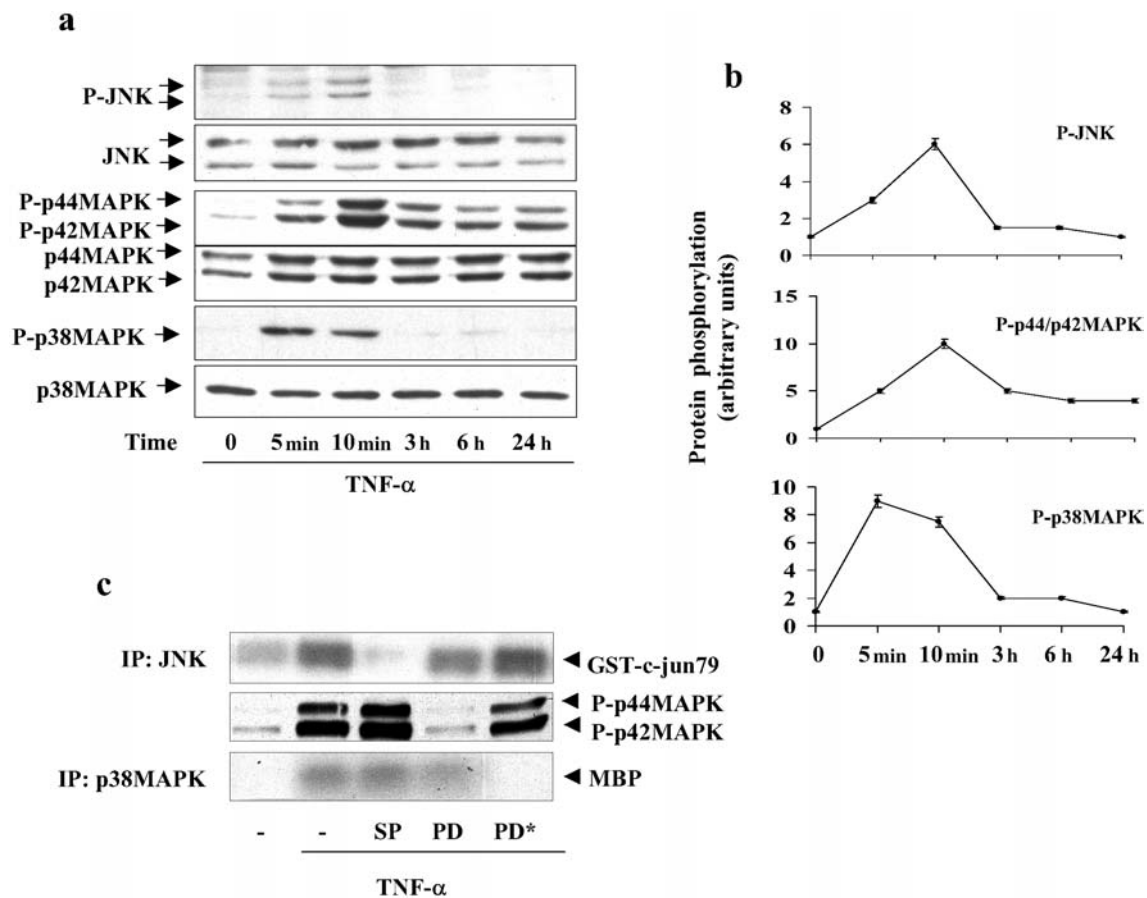
membrane fraction. The pellet was washed three times and extracted in ice-cold homogenisation buffer containing 1% Triton X-100 for 60 min. The Triton-soluble component (plasma membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100,000  $g$  for 15 min. Internal and plasma membrane fractions were kept at  $-70$   $^{\circ}$ C before protein quantification and western blotting with GLUT4, GLUT1 and caveolin-1 antibodies [23].

**Immunoprecipitations.** Cells were extracted with lysis buffer I containing: 10 mmol/l Tris, 50 mmol/l NaCl, 1% Triton X-100, 5 mmol/l EDTA, 20 mmol/l sodium pyrophosphate, 50 mmol/l NaF, 100  $\mu$ mol/l Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/l PMSF (pH 7.5), and then immunoprecipitated with different antibodies. PI 3-kinase activity was measured in anti-IRS-2 or anti-IRS-1 immunoprecipitates by in vitro phosphorylation of PI as previously described [23]. To determine Akt and p38MAPK activities, lysates were immunoprecipitated with anti-Akt or anti-p38MAPK and the immune complexes were used for in vitro phosphorylation of MBP as described [19, 30].

## Results

**Characterisation of TNF- $\alpha$  signalling pathways that contribute to insulin resistance in brown adipocytes.** TNF- $\alpha$  inhibited insulin-stimulated glucose uptake in several systems including primary fetal brown adipocytes. Cells pretreated with TNF- $\alpha$  for 24 h had a glucose uptake 30% higher than untreated cells. Insulin stimulation for 30 min increased basal glucose uptake 3-fold, but only produced a 1.5-fold increase in the glucose uptake of cells treated with TNF- $\alpha$ , 50% lower than that observed in the absence of TNF- $\alpha$  (Fig. 1a).

Insulin stimulation of glucose transport is mediated by the translocation of GLUT4 to the plasma membrane. To correlate the data on glucose uptake with



**Fig. 2.** TNF- $\alpha$  activates several kinases in fetal brown adipocyte primary cultures. **a.** Cells were serum-deprived for 20 h and further cultured for up to 24 h with or without 1 nmol/l TNF- $\alpha$ . Cells were then collected and protein extracts were analysed by western blotting with the corresponding antibodies against phosphorylated and total JNK, p42/p44MAPK and p38MAPK (representative blots are shown). Graphs (**b**) show densitometric analysis of phosphorylated proteins after standardisation using the total amount of protein. Values (means  $\pm$  SEM,  $n=4$ ) are expressed in arbitrary units, in which a value of 1 was assigned for kinase activity prior to stimulation with TNF- $\alpha$ . **c.** Cells were pretreated or not for 30 minutes with the inhibitors PD169316 (PD\*, 800 nmol/l), PD98059 (PD, 20  $\mu$ mol/l) and SP600125 (SP, 3  $\mu$ mol/l) and further incubated for 10 minutes with 1 nmol/l TNF- $\alpha$ . Then protein extracts were immunoprecipitated with anti-p38MAPK or pull-down with GST-c-Jun 79 for in vitro activity assays, or were analysed by western blotting for phosphorylated p42/p44MAPK. Representative experiments are shown

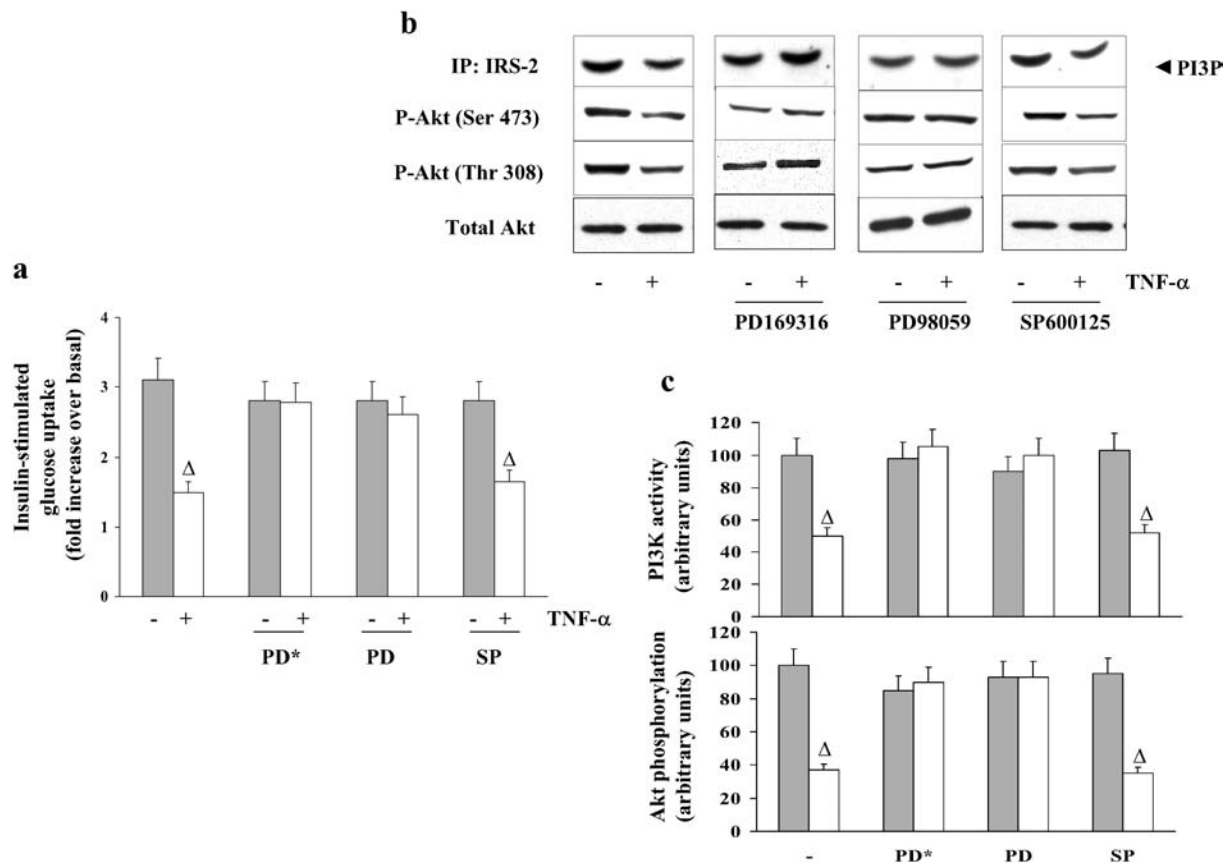
creased GLUT1 protein content at the plasma membrane, probably contributing to the increase observed in basal glucose uptake. Caveolin-1, an integral membrane protein from caveolae, was used as a marker protein of the plasma membrane and under the different treatments used the amount of caveolin-1 remained essentially unaltered.

The PI 3-kinase/Akt pathway has been broadly implicated in insulin stimulation of GLUT4 translocation. In brown adipocytes, insulin activated PI 3-kinase was associated either with IRS-1 or IRS-2 to a similar extent. TNF- $\alpha$  hardly modified insulin-induced PI 3-kinase activity associated with IRS-1, but it did inhibit PI 3-kinase activity associated with IRS-2 (Fig. 1c). Immunoprecipitation with the anti-Akt antibody and determination of the enzymatic activity revealed that TNF- $\alpha$  prevented insulin-stimulated Akt activation (Fig. 1d).

The molecular mechanism by which TNF- $\alpha$  induces insulin resistance is far from clear. This prompted us to investigate the signalling pathways elicited by TNF- $\alpha$  in brown adipocytes. Cells were deprived of serum for 20 h and further cultured for up to 24 h in the presence of 1 nmol/l TNF- $\alpha$ . At the end of the culture time cells were collected and protein extracts were analysed for activation of stress kinases (Fig. 2). TNF- $\alpha$  produced a transient phosphorylation of p38MAPK at 5 min and of JNK at 10 min. These activations were not detectable after 3 h of treatment. However, TNF- $\alpha$  in-

GLUT4 translocation, cells were pretreated or not with TNF- $\alpha$  for 24 h and stimulated with insulin for a further 30 min. Subcellular fractionation was then performed to obtain plasma membrane and internal membrane, and GLUT4 protein was detected by immunoblotting. Insulin increased GLUT4 translocation to the plasma membrane 3-fold with a concomitant decrease in the amount of GLUT4 in the internal membrane. This redistribution of GLUT4 was precluded after TNF- $\alpha$  treatment (Fig. 1b). However, TNF- $\alpha$  in-





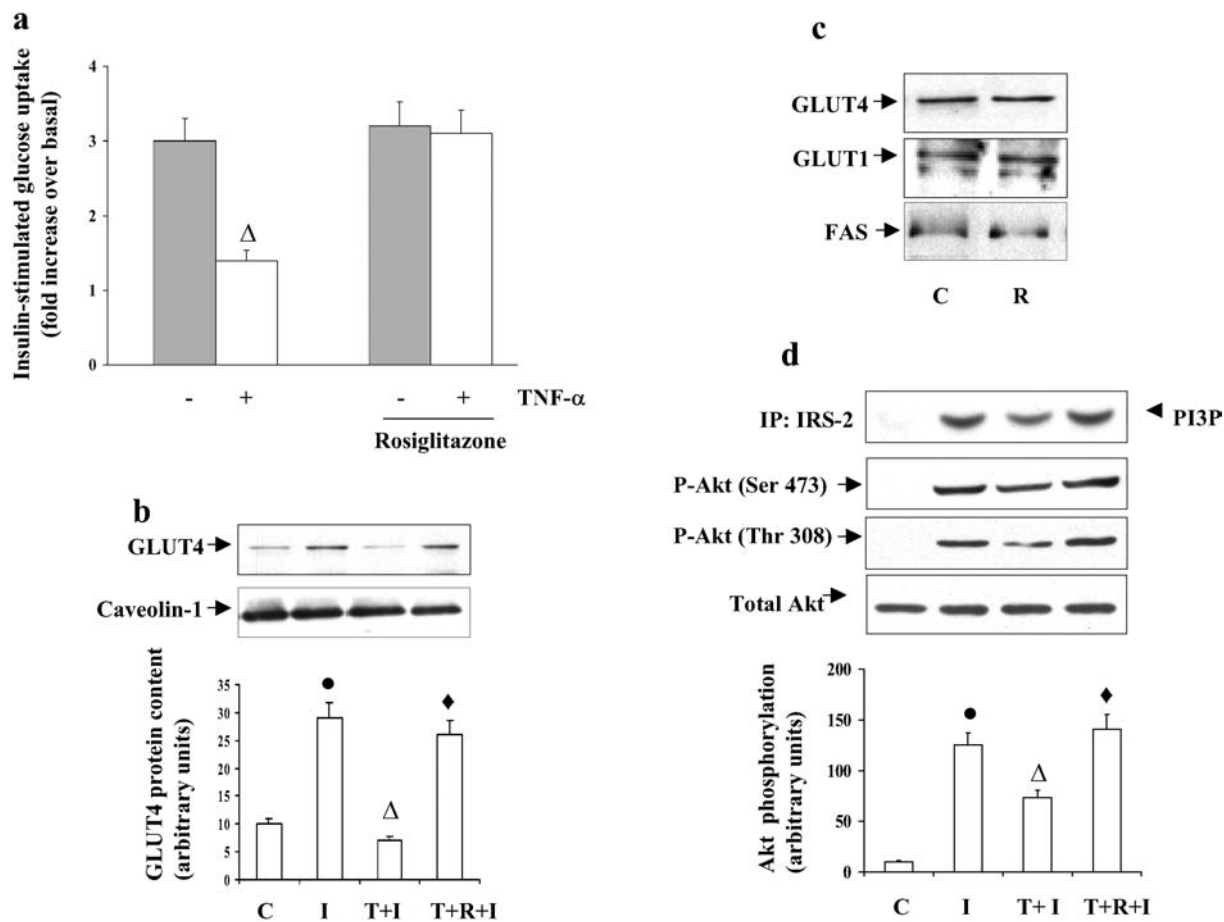
**Fig. 3.** Effect of inhibitors on TNF- $\alpha$ -induced insulin resistance in brown adipocytes. **a.** Cells were cultured for 24 h in the absence or presence of 1 nmol/l TNF- $\alpha$  and without or with the inhibitors PD169316 (PD\*, 800 nmol/l), PD98059 (PD, 20  $\mu$ mol/l) and SP600125 (SP, 3  $\mu$ mol/l). They were then stimulated or not for 30 minutes with 10 nmol/l insulin. Glucose uptake was measured during the last 10 minutes by incorporating 2-deoxy-D[1- $^3$ H]-glucose into the cells. Results are expressed as fold increase over basal glucose uptake without insulin and are means  $\pm$  SEM ( $n=10$ ). Cells (**b**) were cultured as above, but with only 5 min insulin stimulation. Cell lysates were immunoprecipitated with anti-IRS-2 antibody and assayed for PI 3-kinase activity. Cell lysates were also analysed by western blotting, using the corresponding antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308) and total Akt. Representative experiments are shown. **c.** The resulting densitometric analysis from four independent experiments. Filled bars: without TNF- $\alpha$ ; open bars: with TNF- $\alpha$ . Results (means  $\pm$  SEM) are expressed in arbitrary units, in which a value of 100 was assigned for kinase activity in the presence of insulin. Statistical significance was tested and differences between values in the presence of TNF- $\alpha$  vs absence of TNF- $\alpha$  are represented by the open triangle ( $p<0.01$ )

duced the maximal phosphorylation of p42/p44MAPK at 10 min, this kinase remaining activated throughout the treatment (24 h) (Fig. 2a, b).

Since TNF- $\alpha$  activated several kinases in brown adipocytes, we decided to impair these pathways with chemical inhibitors and determine if TNF- $\alpha$  produced insulin resistance under those circumstances. We used SP600125 (3  $\mu$ mol/l) to inhibit JNK, PD98059

(20  $\mu$ mol/l) to inhibit extracellular signal-regulated kinase (ERK) and PD169316 (800 nmol/l) as p38MAPK inhibitor, having previously tested the specificity and optimal inhibitory doses of these compounds in brown adipocytes (Fig. 2c). Cells were deprived of serum for 20 h and further cultured for 24 h in the absence or presence of 1 nmol/l TNF- $\alpha$  with or without inhibitors, prior to stimulation for 30 min with 10 nmol/l insulin. Glucose uptake was measured during the last 10 min of culture and results were expressed as fold increase over basal glucose uptake in the absence of insulin (Fig. 3a). As described above, insulin increased basal glucose uptake (3-fold) and TNF- $\alpha$  treatment inhibited insulin stimulation by 50%. When cells were cultured for 24 h in the presence of PD169316 or PD98059, insulin increased glucose uptake by 2.8-fold regardless of whether TNF- $\alpha$  was present or not. However, SP600125 at the optimal dose for JNK inhibition did not prevent insulin resistance by TNF- $\alpha$  (Fig. 3a). These data seem to indicate that although TNF- $\alpha$  activates several kinases in brown adipocytes, it is mostly p42/p44 and p38MAPK that contribute to the inhibitory effect of TNF- $\alpha$  on insulin-stimulated glucose uptake.

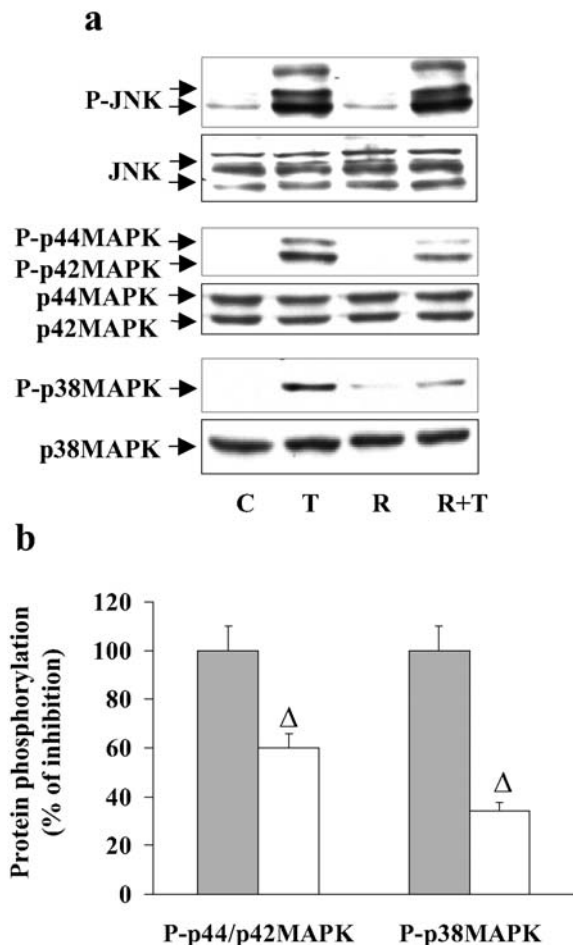
As TNF- $\alpha$  interferes with the insulin signalling cascade at the level of IRS-2, with the subsequent inhibition of PI 3-kinase and Akt, we next checked whether the inhibitors named above could block interference at this level. Cells were deprived of serum for 20 h and further cultured for 24 h in the absence or



**Fig. 4.** Rosiglitazone restored insulin-stimulated glucose uptake, GLUT4 translocation and the IRS-2/PI 3-kinase/Akt cascade in the presence of TNF- $\alpha$ . Cells were cultured for 24 h in the absence or presence of 1 nmol/l TNF- $\alpha$  (T) and/or 10  $\mu$ mol/l rosiglitazone (R) and stimulated or not for 30 minutes with 10 nmol/l insulin (I). **a.** Results for glucose uptake are expressed as in Figure 3a. Statistical significance was also tested as in Figure 3. **b.** Cells were harvested and subjected to subcellular fractionation. Western blotting was done on 10  $\mu$ g of plasma membrane proteins from each condition, using anti-GLUT4 and anti-Caveolin-1 antibodies (representative blot is shown). Corresponding autoradiograms for GLUT4 were quantitated by scanning densitometry. Results (means  $\pm$  SEM,  $n=4$ ) are expressed as arbitrary units, in which a value of 10 was assigned for control. Representative western blot analysis (**c**) is of GLUT4, GLUT1 and FAS from brown adipocytes, treated (R) or not (C) for 24 h with rosiglitazone. **d.** Cells were cultured as above, but insulin stimulation was for 5 min only. Cell lysates were immunoprecipitated with anti-IRS-2 antibody and assayed for PI 3-kinase activity. Cell lysates were also analysed by western blotting with the corresponding antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308) and total Akt (representative blots are shown). Values in graphs from densitometric analysis (means  $\pm$  SEM,  $n=4$ ) are expressed as arbitrary units, in which a value of 10 was assigned for control. Statistical significance was tested (**b**, **d**) and differences between values in the presence of I vs control (C) are represented by a closed circle ( $p<0.001$ ), between values in the presence of T+I vs I by an open triangle ( $p<0.01$ ), and between values in the presence of T+R+I vs T+I by a closed diamond ( $p<0.01$ )

presence of 1 nmol/l TNF- $\alpha$  with or without inhibitors, prior to stimulation for 5 min with 10 nmol/l insulin. PI 3-kinase activity was determined in the immune complexes after immunoprecipitation with the anti-IRS-2 antibody. In addition, cell lysates were analysed by western blotting with anti-phospho Akt antibodies for the regulatory residues Ser473 and Thr308, as well as with total Akt antibody (Fig. 3b). As expected, TNF- $\alpha$  inhibited insulin-activated PI 3-kinase that is associated with IRS-2. Moreover, upon insulin stimulation, Akt was highly Ser- and Thr-phosphorylated, both effects being impaired by pretreatment with TNF- $\alpha$ , which was consistent with our data on Akt activity (Fig. 1d). Under chronic treatment with SP600125 together with TNF- $\alpha$ , PI 3-kinase and Akt phosphorylation by insulin also decreased compared with the activation detected in the absence of TNF- $\alpha$ . However, when cells were cultured in the presence of PD169316 or PD98059, TNF- $\alpha$  did not produce insulin resistance at the level of PI 3-kinase associated to IRS-2 or phosphorylated Akt (Fig. 3b, c). This is consistent with the results for insulin-stimulated glucose uptake (Fig. 3a).

*Rosiglitazone ameliorates insulin resistance by impairing TNF- $\alpha$  activation of p38 and p42/p44 MAPKs.* Insulin resistance caused by TNF- $\alpha$  in brown adipocytes can be reduced by treatment with



**Fig. 5.** Rosiglitazone impairs the stimulation of p38 and p42/p44 MAPKs produced by TNF- $\alpha$ . Cells (**a**) were cultured for 24 h in the absence or presence 10  $\mu$ mol/l rosiglitazone (R) and stimulated or not for 10 minutes with 1 nmol/l TNF- $\alpha$  (T). Cell lysates were submitted to western blotting with the corresponding antibodies against phosphorylated and total JNK, p42/p44MAPK and p38MAPK (representative blots are shown). C, control. **b**. Values in graphs from densitometric analysis (means  $\pm$  SEM,  $n=4$ ) are expressed as arbitrary units in which a value of 100 was assigned for kinase activity in the presence of TNF- $\alpha$ . Grey bars: with TNF- $\alpha$ ; open bars: with rosiglitazone + TNF- $\alpha$ . Statistical significance was tested and differences between values in the presence of R vs its absence are represented by an open triangle ( $p<0.05$ )

PD169316 or PD98059. However, as these compounds are experimental tools, we tested rosiglitazone, a compound used in clinical studies to treat Type 2 diabetes, and checked the mechanism by which it restores insulin signalling and/or antagonises TNF- $\alpha$  signalling. Cells were deprived of serum for 20 h and further cultured for 24 h in the absence or presence of 1 nmol/l TNF- $\alpha$  with or without 10  $\mu$ mol/l rosiglitazone, prior to stimulation for 30 min with 10 nmol/l insulin. Under these conditions glucose uptake and GLUT4 translocation to the plasma membrane were determined (Fig. 4a, b). Rosiglitazone completely impaired TNF- $\alpha$ -induced insulin resis-

tance on glucose uptake (Fig. 4a) and restored GLUT4 translocation by insulin (Fig. 4b), without modifying levels of GLUT4, GLUT1 and FAS proteins (Fig. 4c). In parallel to glucose uptake, rosiglitazone completely restored insulin stimulation of IRS-2-associated PI 3-kinase and Akt phosphorylation in the presence of TNF- $\alpha$  (Fig. 4d).

Finally, we investigated whether rosiglitazone treatment antagonised the activation of kinases by TNF- $\alpha$ , some of which are involved in insulin resistance as indicated (Fig. 3). Cells were treated with rosiglitazone for 24 h prior to TNF- $\alpha$  treatment for 10 min, and the activation of stress kinases was determined (Fig. 5a). Rosiglitazone impaired TNF- $\alpha$  activation of p38MAPK and ERK, without affecting JNK activation (Fig. 5b).

## Discussion

TNF- $\alpha$  produced opposing physiological effects to insulin in the murine 3T3-L1 cell line, in human primary adipocytes and, as we previously showed, in rat primary brown adipocytes, inhibiting insulin-stimulated glucose uptake [4, 5, 22, 23, 32]. There are differences in the level at which TNF- $\alpha$  antagonisation of insulin signalling occurs: impaired insulin signalling at the IRS-1 level has been demonstrated for white adipocytes [7, 8], but chronic exposure to TNF- $\alpha$  caused insulin resistance in brown adipocytes by decreasing IRS-2 Tyr phosphorylation and IRS-2-associated PI 3-kinase activity, with IRS-1 signalling being unaffected [22]. Downstream PI 3-kinase Akt activation by insulin but not PKC $\zeta$  was also impaired under chronic TNF- $\alpha$  treatment in brown fat [23]. The molecular mechanism underlying TNF- $\alpha$ -mediated insulin resistance could involve activation of different Ser/Thr kinases by TNF- $\alpha$ , and in this regard MAPKs (JNK, ERK and p38), as well as IKK, were activated by TNF- $\alpha$  in fat cells [13, 33]. In this work, we show that TNF- $\alpha$  produced a transient phosphorylation of p38MAPK at 5 min and of JNK at 10 min, as well as a sustained activation of p42/p44MAPK throughout the 24-h treatment in brown adipocytes, the activation of these pathways seeming to be independent from each other.

To better understand the contribution of these kinases to insulin resistance, we used chemical inhibitors of these pathways in order to restore insulin signalling to normal levels despite the presence of TNF- $\alpha$ . The impairment of insulin stimulation of glucose uptake was abrogated by pretreatment with the inhibitor of p42/p44MAPK, PD98059, as well as with the inhibitor of p38MAPK, PD169316, with a complete restoration of IRS-2-associated PI 3-kinase activity and Akt phosphorylation by insulin. However, SP600125, an inhibitor of JNK, failed to prevent insulin resistance through TNF- $\alpha$ 's effect on glucose uptake and insulin signalling.



Both ERK and JNK have been said to mediate Ser/Thr phosphorylation of IRS-1 by TNF- $\alpha$ , weakening its Tyr phosphorylation by insulin and impairing the normal response to insulin on glucose uptake and lipolysis in white fat cells [9, 10, 12, 33]. In this regard, recent works have indicated that ERK, to a greater extent than p38MAPK and JNK, could inhibit insulin signalling at the level of IRS-1 and IRS-2 in 3T3-L1 adipocytes [13], while JNK mediated feedback inhibition of insulin signalling [34]. Moreover, increased phosphorylation of p38MAPK in human adipocytes and muscle from Type 2 diabetic subjects has recently been described [35, 36], suggesting a key role for this kinase in the development of insulin resistance and Type 2 diabetes. Our data indicate that ERK and p38MAPK could be major factors in TNF- $\alpha$ -induced insulin resistance in brown adipocytes, acting at the level of IRS-2, in agreement with our previous findings for a more prevalent implication of this docking protein in brown adipocytes as well as in other insulin-responsive cells [22, 23, 37]. Constitutive Ser/Thr phosphorylation is found in IRSs since more than a hundred of these residues are present in these proteins, as we recently detected in skeletal muscle cells [38]. However, additional Ser phosphorylation, as that induced when TNF- $\alpha$  activates ERK and p38MAPK, could inhibit IRS function. Based on sequence homology from data bases (online tool at <http://scansite.mit.edu>, last accessed 16/4/2004), Ser904 could be a potential ERK phosphorylation site in IRS-2, Ser652 could be a potential phosphorylation site for p38MAPK, and Ser367 is a phosphorylation site common to both kinases. Thus, it is possible that phosphorylation of these three residues in the IRS-2 protein will be necessary to produce insulin resistance in brown adipocytes, although we cannot rule out the involvement of other residues.

The ability of thiazolidinediones to ameliorate insulin resistance induced by TNF- $\alpha$  is well documented [39, 40, 41]. However, the mechanisms by which these compounds impair the activity of TNF- $\alpha$  are not understood. In this work we found that chronic exposure to rosiglitazone, as in human clinical use, completely restored insulin-induced glucose uptake and GLUT4 translocation in the presence of TNF- $\alpha$ , without modifying the expression of GLUT4 or GLUT1. Glucose uptake has been found to be increased by thiazolidinediones in isolated adipocytes and in 3T3-L1 adipocytes as a result of increased GLUT4 or GLUT1 expression [42, 43]. However, we demonstrate that this effect is a consequence of GLUT4 translocation in brown adipocytes. Our data also demonstrate a powerful effect of rosiglitazone in restoring insulin sensitivity upon activation of PI 3-kinase associated with IRS-2, and upon Akt phosphorylation, in TNF- $\alpha$ -treated brown adipocytes. This effect does not seem to be related to the differentiation-inducing properties of rosiglitazone, because levels of the adipogenic protein FAS remain unmodified.

Pioglitazone has also been reported to reduce TNF- $\alpha$ -induced insulin resistance in 3T3-L1 cells by a mechanism independent of the adipogenic activity of PPAR $\gamma$  [44]. In that model restoration of insulin response was mostly produced at the level of IRS-1/PI 3-kinase. Since our data indicated that TNF- $\alpha$ -induced insulin resistance could be mediated, at least in part, by activation of p38MAPK and ERK, we investigated whether rosiglitazone antagonises TNF- $\alpha$  signalling. In this regard, pretreatment with rosiglitazone impaired p38 and p42/p44MAPK activation by TNF- $\alpha$ , while JNK activation was not modified. These results are in agreement with those reported by Souza [45], who found that rosiglitazone blocked TNF- $\alpha$ -induced ERK but not JNK activation in 3T3-L1 adipocytes, resulting in reduced lipolysis. Our data indicate that rosiglitazone ameliorates TNF- $\alpha$ -induced insulin resistance by antagonising TNF- $\alpha$  activation of p38 and p42/p44 MAPKs, previously identified as the Ser/Thr kinases involved in insulin resistance in brown adipocytes. Very recent data supporting our findings report hyperphosphorylation of p38MAPK and increased IRS-1 Ser307 phosphorylation in adipose tissues of Zucker obese rats, both effects being reduced by treatment with rosiglitazone [46].

In conclusion, rosiglitazone treatment impaired TNF- $\alpha$  activation of p38MAPK and ERK, ameliorating TNF- $\alpha$  antagonism of the insulin signalling cascade IRS-2/PI 3-kinase and Akt, and completely restoring glucose uptake and GLUT4 translocation in cultured brown adipocytes.

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