Tumor Necrosis Factor-alpha produces Insulin Resistance in Skeletal Muscle by activation of inhibitor kB kinase in a p38 Mitogen-activated Protein Kinase-dependent manner*

Cristina de Alvaro¶, Teresa Teruel‡, Rosario Hernandez¶, and Margarita Lorenzo**
Departamento de Bioquimica y Biologia Molecular II, Facultad de Farmacia, Universidad Complutense, 28040-Madrid (Spain).

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Abbreviations: TNF, tumor necrosis factor; PI, phosphatidylinositol; IR, insulin receptor; IRS, IR substrate; MAPK, mitogen-activated protein kinase; JNK, c-jun NH2 terminal kinase; IkB, inhibitor kB; IKK, IkB kinase; BSA, bovine serum albumin; DMEM, Dulbecco’s minimal essential medium; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence; TLC, thin layer chromatography; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride.

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‡ Recipient of a postdoctoral fellowship from the Comunidad Autonoma de Madrid.

** To whom correspondence and reprints requests should be addressed at Departamento de Bioquimica y Biologia Molecular II, Facultad de Farmacia, Universidad Complutense, 28040-Madrid (Spain). FAX 34-91-3941779, PHONE 34-91-3941858
E-mail: mlorenzo@farm.ucm.es
SUMMARY

Insulin stimulation produced a reliable 3-fold increase in glucose uptake in primary neonatal rat myotubes, which was accompanied by a similar effect on GLUT4 translocation to plasma membrane. Tumor necrosis factor (TNF)-alpha caused insulin resistance on glucose uptake and GLUT4 translocation by impairing insulin stimulation of insulin receptor (IR) and insulin receptor substrate (IRS)-1 and IRS-2 Tyr phosphorylation, IRSs-associated phosphatidylinositol (PI) 3-kinase activation and Akt phosphorylation. Since this cytokine produced the sustained activation of stress and proinflammatory kinases, we have explored the hypothesis that insulin resistance by TNF-alpha could be mediated by these pathways. In this study we demonstrate that pretreatment with PD169316 or SB203580, inhibitors of p38 mitogen-activated protein kinase (MAPK), restored insulin signaling and normalized insulin-induced glucose uptake in the presence of TNF-alpha. However, in the presence of PD98059 or SP600125, inhibitors of p42/p44MAPK or c-jun NH2 terminal kinase (JNK), respectively, insulin resistance by TNF-alpha was still produced. Moreover, TNF-alpha produced inhibitor kB kinase (IKK-beta) activation and IkB-beta and alpha degradation in a p38MAPK-dependent manner, and treatment with salicylate (an inhibitor of IKK), completely restored insulin signaling. Furthermore, TNF-alpha produced Ser phosphorylation of IR and IRS-1 (total and on Ser307 residue), these effects being completely precluded by pretreatment with either PD169316 or salicylate. Consequently, TNF-alpha through activation of p38MAPK and IKK produces the serine phosphorylation of IR and IRS-1, impairing its tyrosine phosphorylation by insulin, and the corresponding activation of PI3-kinase and Akt, leading to insulin resistance on glucose uptake and GLUT4 translocation.
INTRODUCTION

Insulin increases glucose transport into cells of target tissues, primarily muscle (skeletal and cardiac) and fat (white and brown). Acute insulin treatment stimulates glucose transport in adipocytes and myocytes largely by mediating translocation of GLUT4 from an intracellular compartment to the plasma membrane, as reviewed (1) (2) (3). This effect is accomplished by activation of phosphatidylinositol (PI)3-kinase, Akt and the atypical protein kinase C (PKC) isoforms ζ and λ in most cellular models (4) (5) (6) although other PKC isoforms seem to play a role in skeletal muscle (7) (8). Recent discoveries have shown the presence of a second insulin signaling pathway leading to GLUT4 translocation in a PI3-kinase independent manner, involving the adaptor protein Cbl and the activation of a small GTP binding protein TC10 (9) (3). Furthermore, insulin can activate glucose uptake without producing GLUT4 translocation, this effect involves activation of p38 mitogen activated protein kinase (MAPK), as it has been specifically reported for muscle cells (10).

Insulin resistance, defined as a smaller than normal response to a given amount of insulin, is an important contributor to the pathogenesis of type 2 diabetes mellitus. Both genetic and environmental factors can contribute to develop insulin resistance. Targeted disruption of the IGF-I and insulin receptor (IR), or of GLUT4, selectively in skeletal muscle, causes insulin resistance and insulin intolerance (11) (12) (13). Tumor necrosis factor (TNF) -α has been proposed as a link between adiposity and the development of insulin resistance, since I) the majority of type 2 diabetics are obese, II) TNF-α is highly expressed in adipose tissues of obese animals and humans (14) (15), and III) obese mice lacking either TNF-α or its receptors showed protection for developing insulin resistance (16) (17). Infusion of TNF-α to adult rats reduce systemic insulin sensitivity associated with major changes in adipocyte gene expression favoring free fatty acids release, without changes on muscle gene expression (18). These data suggest that impaired activity of the insulin signaling transduction pathways rather than changes in gene expression may be contributing to the development of insulin resistance in muscle of TNF-α-treated animals.
Direct exposure of fat cells to TNF-α inhibits insulin-stimulated glucose uptake in several systems including 3T3-L1 cells, human primary adipocytes and primary brown adipocytes (19) (20). The mechanism proposed involves Ser phosphorylation of insulin receptor substrate (IRS)-1 that converts IRS-1 in an inhibitor of the insulin receptor Tyr kinase activity (21). Furthermore, Ser307 has been identified as a site for TNF-α-induced phosphorylation of IRS-1, being activation of c-Jun N-terminal kinase (JNK) involved in the phosphorylation of this residue (22). Other studies suggest that p42/p44 and p38MAPKs could inhibit insulin signaling by diverse mechanisms in 3T3-L1 adipocytes (23). In this regard, an enhanced basal activation of MAPKs in adipocytes from type 2 diabetic patients has been recently reported (24). Moreover, other works also implicated inhibitor kB kinase (IKK) activation by TNF-α on Ser phosphorylation of IRS-1 (25), meanwhile IKK inhibition with salicylates or targeted disruption of Ikk-β produced reversal of obesity and diet-induced insulin resistance (26).

Ceramide and fatty acids had been reported to induce insulin resistance in skeletal muscle (27) (28) (29), and its production could be consequence of sphingomyelinase or lipolytic activation by TNF-α (30) (31). However, a direct effect of TNF-α on insulin resistance in muscle, which is responsible for 80% of the glucose disposal of the body, is controversial. Several reports did not detect TNF-α inhibitory action on insulin-induced glucose uptake although TNF-α per se highly increased basal glucose uptake (32) (33) (34). But others observed TNF-α inhibitory effect on insulin action without modifying basal glucose uptake in muscle cells (35) (8). On the other hand, in most of these studies insulin stimulation of glucose uptake was very poor since virtually all cultured skeletal muscle cell lines have been found to be deficient in GLUT4 expression. Accordingly, in this work we have prepared primary cultures of neonatal rat myotubes that under physiological conditions, respond to insulin by increasing 3-fold glucose uptake and GLUT4 translocation to the plasma membrane. Both effects were impaired by chronic treatment with TNF-α. This cytokine, through activation of p38MAPK and IKK produces the phosphorylation of the residue Ser307 in IRS-1, impairing the insulin signaling cascade at the level of IR and IRS-1Tyr phosphorylation, IRS-1-associated PI3-kinase and Akt activities.
**EXPERIMENTAL PROCEDURES**

**Materials**- Insulin, bovine serum albumin (BSA) (fraction V, essentially fatty acid free), myelin basic protein (MBP) and antibody anti-β-actin were from Sigma Chemical Co (St. Louis, MO). TNF-α was purchased from Pharma Biotechnologie (Hannover, Germany). PD169316 and PD98059 were purchased from Calbiochem (Calbiochem-Novabiochem Intl, La Jolla, CA). SP600125 and SB203580 were from Alexis (Switzerland). Horse serum, phosphate buffered saline (PBS), trypsin-EDTA, culture media and Trizol were from Invitrogen (Paisley, UK). Nylon membranes were GeneScreen™ (NEN Research Products, Boston, MA). Autoradiographic films were Kodak X-O-MAT/AR (Eastman Kodak Co, Rochester, NY). 2-Deoxy-D[1-3H]-glucose (11.0 Ci/mmol), (γ32P)ATP and protein G-sepharose were purchased from Amersham Bioscience (Little Chalfont, UK). The rabbit anti-GLUT1, anti-GLUT4 and anti-P-Ser antibodies were supplied by Chemicon (Tamacula, CA). The anti-phospho and anti-(Akt, p42/p44MAPK, p38MAPK, JNK) antibodies were from Cell Signaling (Beverly, MA). Antibodies against IRS-1, IRS-2, P-Tyr (4G10) and P-IRS-1(Ser307) were from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against P-Tyr (PY20) “sc-508”, IR β-chain “sc-09”, caveolin-1(N-20) “sc-894”, IKK-β “sc-7607” and IκB-β “sc-945” were from Santa Cruz (Palo Alto, CA). The anti-IR β-chain antibody was from Oncogene Science (Uniondale, NY). All other reagents used were of the purest grade available.

**Cell culture**- Skeletal muscle cells were prepared from thigh muscles obtained from 3- to 5-days neonatal rats according to the method described in (7) with some modifications. Neonatal rats were killed by cervical dislocation. The muscles from the fore- and hind limbs were removed (carefully dissected to discard fat and connective tissues), washed in phosphate buffered saline (PBS) pH 7.5 to remove excess blood cells, triturated and homogenized with scissors. Then the minced muscle was transferred to a Ca2+-free 0.25% trypsin solution containing EDTA (1mM) for incubation with continuous stirring at 37°C. Cells were collected after successive 20- to 30-min trypsinization periods of incubation until all tissue was dispersed, and then centrifuged for 5 min at 1000xg. The successive supernatants were filtered and reserved in growth medium. For the last incubation, the resting tissue was digested with a solution of collagenase II
(Worthington) in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS (100 UI/ml), 20-30 minutes at 37°C with stirring. Finally, all the supernatants were centrifuged for 5 minutes at 2000xg. The last pellet was washed with PBS, resuspended in growth medium and preplated for 20 to 30 min in 100 mm-diameter tissue culture dishes to reduce the number of fibroblasts. The supernatant was collected and the remaining myoblasts were diluted with growth medium to a concentration of 1x10^6 cells/ml. To facilitate adhesion of muscular cells to the plastic surface, and to avoid attachment of fibroblasts, cells were seeded in collagen-coated dishes. Optimal plating densities were 1x10^6 cells in six-well dishes and 5x10^6 cells in 100 mm-diameter dishes. Cells were cultured in growth medium (Dulbecco’s Minimal Essential Medium DMEM plus 10% horse serum) in a water-saturated atmosphere of 95% air-5% CO\(_2\) at 37°C during the first 3-4 days, when cells proliferate until reaching confluence, as observed under inverse light microscopy. Next, cells were cultured for further 4-5 days in differentiation medium (2% horse serum-DMEM) when cells fuse and differentiate into multi-nucleated myotubes. Finally, myotubes were cultured overnight in serum-free, low glucose (1000 mg/L glucose) DMEM supplemented with 1% (w/v) BSA before starting different treatments.

**Glucose transport determination**-Glucose uptake was measured during the last 10 min of culture by incorporation of labeled 2-deoxy-glucose and parallel dishes were used for protein determination, individual values were expressed as disintegrations per min/µg of protein, as previously described for brown adipocytes (36). Results were expressed as percentage of stimulation over basal (100).

**Subcellular fractionation**-Cells were washed with ice-cold PBS and scrapped in homogenization buffer containing: 20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (pH 7.4). After a 10 min incubation cells were homogenized with 30 strokes of a Dounce homogenizer using a tight-fitting pestle. Nuclei were pelleted by centrifugation at 500xg for 5 min, and the low speed supernatant was centrifuged at 100,000xg for 30 min. The high speed supernatant constituted the internal membrane fraction. The pellet was washed three times and extracted in ice-cold homogenization 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 100,000xg for 15
min. Internal and plasma membrane fractions were kept at −70°C before protein quantification and Western blotting with GLUT4 and Caveolin-1 antibodies (20).

**Immunoprecipitations**- Cells were extracted with lysis buffer I containing: 10 mM Tris, 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 20 mM Sodium pyrophosphate, 50 mM NaF, 100 μM Na3VO4, 1 μM PMSF (pH 7.5), and immunoprecipitated with different antibodies against IRSs or IR or p38MAPK or IKK-β. PI3-kinase activity was measured in anti-IRSs immunoprecipitates by *in vitro* phosphorylation of PI as previously described (20).

**p38 and IKK in vitro kinase assays**- These assays were performed in anti-p38MAPK α and β and IKK-β immunoprecipitates as described (37). Immune complexes were washed five times with ice-cold lysis buffer containing 0.5 M NaCl and two times with kinase buffer (35 mM Tris, pH 7.5, 10 mM MgCl2, 0.5 mM EGTA, 1 μM Na3VO4). The kinase reaction was performed in a kinase buffer containing 1 μCi of (γ32P)ATP, 60 μM ATP, and 1 μg of MBP as a substrate for 30 min at 30°C, and was terminated by the addition of 2 X SDS-PAGE sample buffer followed by boiling for 5 min at 95°C. Samples were resolved in 12% SDS-PAGE and gels were dried out and subjected to autoradiography.

**Western blotting**- Cells were lysed in lysis buffer I and cellular proteins (30 μg) were submitted to SDS-PAGE, transferred to Immobilon membranes and were blocked using 5% nonfat dried milk in 10 mM Tris-HCl and 150 mM NaCl pH 7.5, and incubated overnight with several antibodies as indicated in each case in 0.05% Tween-20, 1% non-fat dried milk in 10 mM Tris-HCl and 150 mM NaCl pH 7.5. Immunoreactive bands were visualized using the enhanced chemiluminiscence (ECL-Plus) Western blotting protocol (Amersham).
Data analysis- Results are means ± S.E. (n=4 or 10) for duplicate dishes from four to ten independent experiments. Statistical significance was tested with a one-way analysis of variance followed by the protected least-significant different test. P values less than 0.05 were considered significant. In experiments using X-ray films (Hyperfim), different exposure times were used to ensure that bands were not saturated.
RESULTS

TNF-α inhibits insulin-induced glucose transport in a p38MAPK-dependent manner in skeletal muscle.

Isolated neonatal muscle cells were differentiated in low serum until the formation of myotubes. Then, cells were shifted overnight to serum-free, low glucose medium, and further cultured for 24 h in the absence or presence of 1nM TNF-α prior to the stimulation for 30 min with 50 nM insulin. Glucose uptake was measured during the last 10 min of culture by incorporation of labeled 2-deoxy-glucose and results were expressed as percentage of stimulation over basal (control = 100) (Fig. 1A). Insulin stimulation for 30 min significantly increased (3-fold) basal glucose uptake. Cells pretreated with TNF-α for 24 h showed a 3-fold higher glucose uptake than untreated cells, and under this circumstance insulin stimulation did not further stimulate glucose uptake. As insulin stimulation of glucose transport is mediated by the translocation of GLUT4 to the plasma membrane, we decided to confirm the above data on glucose uptake by examining GLUT4 translocation. After subcellular fractionation, GLUT4 protein in plasma membrane fractions was detected by immunoblotting (Fig. 1B). Insulin increased by 3-fold GLUT4 translocation to plasma membrane fraction but this effect was not produced when cells were pretreated with TNF-α prior to insulin stimulation. In parallel, the disappearance of GLUT4 localization in internal membrane produced by insulin was not observed when TNF-α was present together with insulin. Caveolin-1, an integral membrane protein from caveolae, was used as a marker protein of the plasma membrane and its amount remained essentially unaltered under the different treatments used (Fig. 1B). Moreover, the increase in basal glucose uptake observed under TNF-α treatment was not the result of GLUT4 translocation, as shown in Fig. 1B. However, the levels of GLUT1 protein and mRNA detected under TNF-α treatment were increased compared to controls, without being affected the expression of GLUT4 protein (Fig. 1C). Moreover, since TNF-α treatment was performed in differentiated myotubes, no changes in the state of differentiation of the cells were observed (data not shown).

The mechanism by which TNF-α produced insulin resistance on glucose uptake is far to be clear, and prompted us to investigate the signaling pathways elicited by chronic
TNF-\(\alpha\) treatment in skeletal muscle. Cells were overnight serum deprived and further cultured for up to 24 h in the presence of 1 nM TNF-\(\alpha\). At the end of the culture time cells were collected and protein extracts were analyzed for activation of stress kinases by Western blotting using the corresponding antibodies against phosphorylated and total p38 and p42/p44MAPK and JNK (Fig. 2A). TNF-\(\alpha\) produced a peak of phosphorylation of p38MAPK at 5 min, this activation being sustained for at least 6 h of treatment. Furthermore, TNF-\(\alpha\)-induced phosphorylation of p42/p44MAPK was detectable along 6 h of treatment; these activations on MAPKs were no detectable after 24 h. JNK phosphorylation by TNF-\(\alpha\) was detected after 30 min of stimulation, this kinase remained active for 6 h. The changes observed in the state of phosphorylation of these kinases seem to reflect changes in their activities, because the protein levels are similar in all the conditions. Next, we investigated which p38MAPK isoform resulted activated by TNF-\(\alpha\), using \textit{in vitro} kinase assays of MBP phosphorylation in immunoprecipitates against the p38MAPK isoforms \(\alpha\) and \(\beta\) (Fig. 2B). Stimulation for 5 min with TNF-\(\alpha\) (1 nM) activated mainly the p38MAPK activity associated to the \(\beta\) isoform, meanwhile 50 nM insulin, that has been shown to stimulate phosphorylation of p38MAPK in C2C12 cells (38), activated mainly the p38\(\alpha\) isoform. Both isoforms resulted activated in a similar extent after stimulation with 500 mM sorbitol.

In order to investigate if the sustained activation of p38 and p42/p44 MAPK and JNK by TNF-\(\alpha\) could be contributing to insulin resistance we decided to impair these pathways by the use of chemical inhibitors and determine if TNF-\(\alpha\) was able to produce insulin resistance under those circumstances. We used SP600125 (SP) (3 \(\mu\)M) to inhibit JNK, PD98059 (PD) (20 \(\mu\)M) to inhibit ERK and two different compounds PD169316 (PD*) (800 nM) or SB203580 (SB) (10 \(\mu\)M) as p38MAPK inhibitors, after testing the specificity and optimal inhibitory doses of these chemicals in neonatal muscle cells (Fig. 2C). Glucose uptake was determined in cells cultured for 24 h in the absence or presence of 1 nM TNF-\(\alpha\) with or without inhibitors, prior to the stimulation for 30 min with 50 nM insulin (Fig. 1A). When cells were cultured for 24 h in the presence of PD or SP or PD*/SB, no significant changes on insulin or TNF-\(\alpha\)-stimulated glucose uptake were detected. However, treatment with either PD* or SB completely restored insulin-stimulation of glucose uptake in the presence of TNF-\(\alpha\), leading to a 5-fold stimulation over basal and to a 2-fold increase over TNF-\(\alpha\) (Fig. 1A). This effect was not observed
in the presence of PD or SP. A similar restoration on insulin-induced translocation of GLUT4 from internal membrane to the plasma membrane was detected in the presence of TNF and PD* for 24 h, meanwhile this effect was not observed in the presence of PD (Fig. 1B). Furthermore, the presence of the inhibitors for 24 h did not modify the expression of GLUT4 or GLUT1 proteins (Fig. 1C). The above data seem to indicate that although TNF-α activates several MAPKs in skeletal muscle, mostly p38β MAPK could be contributing to TNF-α inhibitory effect on insulin-stimulated glucose uptake.

**TNF-α impairs insulin signaling cascade in a p38MAPK-dependent manner.**

The next step was to identify at which level TNF-α was interfering insulin signaling cascade, and if that interference could be avoided in the presence of the inhibitor of p38 or p42/p44 MAPKs. Overnight serum deprived myotubes were cultured for 24 h in the absence or presence of 1 nM TNF-α with or without PD or PD*, prior to the stimulation for 5 min with 50 nM insulin. Insulin induced Tyr phosphorylation of IR observed in IR immunoprecipitates, an effect that was impaired by pretreatment with TNF-α (Fig. 3A). However, treatment with PD* but not with PD, completely restored IR Tyr phosphorylation by insulin in the presence of TNF-α. Treatment with TNF-α for 24 h not only decreased Tyr phosphorylation of IR by insulin but also produced Ser phosphorylation of the IR, as demonstrated in immunoprecipitates with P-Ser antibodies and Western blotting with IR β-chain antibody. However, Ser phosphorylation of IR was decreased in myotubes pretreated with PD*, contributing to the restoration on Tyr phosphorylation (Fig. 3B).

Insulin-induced Tyr phosphorylation of IRS-1 and IRS-2 was significantly impaired under pretreatment with TNF-α, without significant changes on the expression of IRSs (Fig. 4A and B). However, treatment with PD* in the presence of TNF-α completely restored Tyr phosphorylation by insulin at the level of IRS-1, being the effect partial at the level of IRS-2. Treatment with TNF-α for 24 h produced Ser phosphorylation of IRS-1, being this phosphorylation precluded by pretreatment with PD* but not with PD, as demonstrated in immunoprecipitates with P-Ser antibodies and Western blotting with IRS-1 antibody (Fig. 4C). Moreover, TNF-α produced phosphorylation on the Ser307 residue of IRS-1 in a p38MAPK dependent manner, as demonstrated by direct Western blotting with anti-P-IRS-1(Ser307) specific antibody.
PI3-kinase activity associated to IRSs was determined in the immune complexes after immunoprecipitation with either the anti-IRS-1 or anti-IRS-2 antibodies (Fig. 5A). TNF-α impaired insulin-induced PI3-kinase activity in IRS-2 and more evident, in IRS-1 immunoprecipitates, but this inhibitory effect was not produced in the presence of PD*. In addition, cell lysates were analyzed by Western blotting with anti-phospho Akt antibodies for both regulatory residues Ser473 and Thr308 (Fig. 5B). Upon insulin stimulation Akt was highly Ser and Thr phosphorylated, both effects being impaired by the pretreatment with TNF-α in a p38MAPK-dependent manner (Fig. 5B). All these data indicated that TNF-α impaired insulin activation of IR/IRS-1/Akt signaling cascade in a p38MAPK-dependent p42/p44-independent manner, in a similar fashion as detected for glucose uptake.

**Inhibition of IKKs by salicylate or PD* precludes insulin resistance by TNF-α in skeletal muscle.**

Insulin resistance by TNF-α in skeletal muscle can be impaired by inhibition of p38MAPK. However, TNF-α, besides activating stress kinases, also activates proinflammatory kinases such as IKKs that recently have been implicated in the development of insulin resistance. Accordingly, we decided to explore the potential implication of IKKs in insulin resistance by TNF-α, and if there was any connection with the activation of p38MAPK. Overnight serum deprived myotubes were cultured for up to 24 h in the absence or presence of 1 nM TNF-α, and IKKs activation was detected indirectly by degradation of IkB-α and β, monitored by Western blotting (Fig. 6A). As expected, TNF-α induced IkB-α and β degradation after 5 min of treatment, this effect was essentially maintained along 24 h of treatment. Insulin stimulation for 5 min had no effect on IkB-α and β degradation by TNF-α. However, this effect was avoided in the presence of PD*, but not in the presence of PD, indicating that TNF-α activation of IKKs was dependent on p38MAPK, as determined by *in vitro* kinase assay in IKK-β immunoprecipitates as well as by Western blotting (Fig. 6B). However, p38MAPK activation by TNFα was independent on IKKs activation, since in the presence of an IKK inhibitor, salicylate, p38MAPK phosphorylation was still detected (Fig. 6C). Accordingly, we decided to block IKKs activation by TNF-α by incubating myotubes with salicylate, to check if it was able to restore insulin signaling (Fig. 7).
Cells cultured for 24 h in the presence of 1 nM TNF-α with 5 mM salicylate showed a complete restoration of insulin signaling at the level of IR and IRS-1 Tyr phosphorylation, PI3-kinase activation associated to IRS-1 and Akt phosphorylation on both Ser473 and Thr308 residues, as shown in Fig. 7. Furthermore, impairment of TNF-α-induced IKKs activation by salicylate completely prohibited Ser307 phosphorylation of IRS-1 by TNF-α, indicating that the phosphorylation of this residue by TNF-α play a major role in the development of insulin resistance in neonatal myotubes (Fig. 7B and D).
DISCUSSION

TNF-α has been reported to produce insulin resistance in several cellular systems, such as white and brown adipocytes, and in some skeletal muscle cell lines (19) (20) (35) (8). However, insulin stimulation of glucose uptake in these cells lines, including L6 and C2C12 myotubes, was very poor. Accordingly, we have developed primary cultures of neonatal skeletal muscle that represent a suitable system for investigating the molecular basis of TNF-α-induced insulin resistance. Isolated muscle cells were differentiated in low serum until the formation of myotubes and then, maintained in low glucose medium mimicking physiological environment. Under these experimental conditions, acute insulin stimulation produced a reliable 3-fold increase in glucose uptake, that was accompanied by a similar effect on GLUT4 disappearance from internal membrane and translocation to plasma membrane. Thus, primary neonatal rat myotubes respond to insulin in a similar extent than primary fetal rat brown adipocytes (36) (20). Chronic exposure to TNF-α impaired both insulin-stimulated glucose uptake and GLUT4 translocation, without being affected the expression of GLUT4 or the state of differentiation of the myotubes. Our results in primary neonatal myotubes are in agreement with those obtained in muscle in vivo (18), and indicated that insulin resistance by TNF-α seems to be the consequence of an antagonism in the activation of the complete insulin signaling cascade from IR to Akt rather than changes on muscle gene expression. Furthermore, the significant increase in basal glucose uptake produced by TNF-α treatment in primary skeletal muscle cells, was not due to GLUT4 translocation to the plasma membrane, and seemed to be associated to increased GLUT1 expression, as has been described before in several muscle cells (39) (29).

The molecular mechanism underlying TNF-α-mediated insulin resistance could involve activation of different Ser/Thr kinases by TNF-α, and in this regard MAPKs (JNK, ERK and p38) as well as IKK resulted activated by TNF-α in fat cells (40). In this work we show that TNF-α produced a sustained phosphorylation of JNK, p38 and p42/p44MAPK along the first 6 h of treatment. Acute insulin stimulation (5 min) produces a transient phosphorylation of MAPKs, as we previously reported (38) (41), but insulin activates p38αMAPK meanwhile TNF-α activates the β isoform, as we demonstrate in this work. To evaluate the contribution of sustained activation of these
kinases to insulin resistance, we used the chemical inhibitors SP, PD*/SB and PD, that specifically prohibited activation of these pathways by TNF-α in primary muscle cells. Inhibition of p38MAPK with either PD* or SB completely restored insulin-stimulated glucose uptake and insulin signaling, meanwhile inhibition of p42/p44MAPK or JNK did not abrogate TNF-α-induced insulin resistance. Although activation of both isoforms of p38MAPK by insulin has been proposed to activate glucose uptake without producing GLUT4 translocation (42) (10), other reports indicate that p38MAPK could play a role in oxidant-induced inhibition of insulin-regulated glucose transport in L6 muscle cells (43). Furthermore, adenovirus-mediated transfections of constitutively active MKK6/3 mutants in L6 myotubes has been reported to diminish glucose transport induced by insulin via down-regulation of GLUT4 gene expression (44).

Once identified p38MAPK as the potential kinase by which TNF-α was producing insulin resistance, we deeply explored how interfering insulin signaling cascade was. Chronic treatment with TNF-α was producing Ser phosphorylation of IRS-1 and IR in a p38MAPK-dependent manner, this Ser phosphorylation was weaken the Tyr phosphorylation induced by insulin and impairing the normal response to insulin on glucose uptake. Both p42/p44MAPK and JNK have been proposed to mediate TNF-α Ser/Thr phosphorylation of IRS-1 in white fat cells (22). Very recent works indicated that p42/p44MAPK, in a greater extent than p38MAPK and JNK, could inhibit insulin signaling at the level of IRS-1 and IRS-2 in 3T3-L1 adipocytes (40), meanwhile JNK could mediate feedback inhibitory effect of insulin (45). Our data indicate that p38MAPK but not p42/p44MAPK or JNK could be the major player in TNF-α-induced insulin resistance in neonatal skeletal muscle. Furthermore, Ser307 of IRS-1 seems to be one of the residues phosphorylated by TNF-α via p38MAPK, although we can not discard that other residues either in IRS-1 or IRS-2 or IR could be also target for TNF-α-induced insulin resistance in skeletal muscle.

Several reports also implicated IKK activation by TNF-α on Ser phosphorylation of IRS-1 (25) (26), and a recent work indicated that aspirin rescued insulin-induced glucose uptake in 3T3-L1 adipocytes treated with TNF-α (46). In this regard, activation of IKK detected indirectly by IκB-α or β degradation or directly by in vitro kinase assay in IKK-β immunoprecipitates was observed along the chronic treatment with TNF-α.
And more important, this activation was dependent on the functionality of p38MAPK. Inhibition of IKK activation with salicylate completely restores insulin signaling to normal levels despite the presence of TNF-α. Furthermore, salicylate did not affect p38MAPK activation by TNF-α. These data seem to indicate that IKK could act downstream of p38MAPK, and mediate TNF-α-induced insulin resistance on skeletal muscle. Our results are consistent with the requirement of p38MAPK in the activation of NF-kB transcriptional factor in response to IL-1β (47).

Accordingly, chronic TNF-α treatment impaired insulin stimulation of glucose uptake and GLUT4 translocation to the plasma membrane. This cytokine, through activation of IKK in a p38MAPK-dependent manner produces the phosphorylation of the residue Ser307 in IRS-1, impairing the insulin signaling cascade at the level of IR and IRS-1Tyr phosphorylation, IRS-1 associated PI3-kinase and Akt activity.

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FIGURE LEGENDS

FIG. 1. TNF-α inhibits insulin-induced glucose transport in a p38MAPK-dependent manner in skeletal muscle.

A) Primary neonatal rat myotubes were cultured for 24 h in the absence (C) or presence of 1 nM TNF-α (T) without or with 800 nM PD169316 (PD*) or 10 µM SB203580 (SB) or 20 µM PD98059 (PD) or 3 µM SP600125 (SP), and stimulated or not for 30 min with 50 nM insulin (I). Glucose uptake was measured during the last 10 min by incorporation of 2-Deoxy-D[1-3H]-glucose into the cells. Results are expressed as percentage of stimulation over control (C = 100) and are means ± S.E. (n=10). Statistical significance was tested and differences between values in the presence of I vs. C are represented by (●), between values in the presence of T vs. C are represented by (♦), between T+I vs. T are represented by (∆) and between values in the presence of T+I+PD* vs. T+I are represented by (∆). B) Cells cultured as in A were harvested and subcellular fractionated. Plasma and internal membrane proteins were submitted to SDS/PAGE, blotted onto nylon membrane, immunodetected with anti-GLUT4 and anti-Caveolin-1 (Cav-1) antibodies and developed with ECL chemiluminiscence. Representative experiments are shown. Histograms from densitometric analysis expressed in arbitrary units are means ± S.E. (n=4). Statistical significance was tested and differences between values in the presence of I vs. control (C) are represented by (●), between values in the presence of T+I vs. I are represented by (∆) and between T+I+PD* vs. T+I are represented by (∆). C) Cells were cultured for 24 h in the absence or presence of TNF-α and inhibitors, at the doses used in A, and after harvested, extracts were analyzed by Western blotting with the corresponding antibodies against GLUT4 and GLUT1 proteins or analyzed by Northern blotting with a GLUT1 cDNA. Representative experiments are shown.

FIG. 2. TNF-α activates JNK and p38 and p42/p44 MAPK in skeletal muscle.

A) Primary neonatal rat myotubes were cultured for up to 24 h in the absence or presence of 1 nM TNF-α. At the end of the culture time cells were collected and protein extracts were analyzed by Western blotting with the corresponding antibodies against phosphorylated and total p38 and p42/p44MAPK, and JNK. Representative experiments are shown as well as the densitometric analysis of phosphorylated proteins after
standardization using the total amount of protein, expressed in arbitrary units (a.u.). B) Myotubes (C) were stimulated for 5 min with 1 nM TNF-α, or 50 nM insulin (Ins), or 500 mM sorbitol (Sorb), lysed and immunoprecipitated with either anti-p38MAPK α or β antibodies. p38MAPK activity was assayed in the resulting immune complexes for MBP phosphorylation, and autoradiography. Histograms resulted from the densitometric analysis of the autoradiograms, represented phosphorylated MBP levels in arbitrary units (a.u.) and are means ± S.E. (n=4). C) Cells were cultured for 6 h either in the absence (C) or presence of 1 nM TNF-α without or with 800 nM PD169316 (PD*), or 10 μM SB203580 (SB), or 20 μM PD98059 (PD), or 3 μM SP600125 (SP). Protein extracts were analyzed by Western blotting with the corresponding antibodies against phosphorylated JNK, p38 and p42/p44MAPK, and total p38MAPK. Representative experiments are shown. Inhibition of p38MAPK β activity by PD* or SB is shown in a representative experiment performed for MBP phosphorylation in anti-p38MAPK β immunoprecipitates (IP), as described in B.

FIG. 3. TNF-α impairs insulin-induced Tyr phosphorylation of IR in a p38MAPK-dependent manner.

Cells were cultured for 24 h in the absence (C) or presence of 1 nM TNF-α (T) without or with 800 nM PD169316 (PD*) or 20 μM PD98059 (PD) and stimulated or not for 5 min with 50 nM insulin (I). A) Cell lysates were immunoprecipitated with antibodies against IR and immunoblotted with anti-P-Tyr (4G10) antibody (PY) or with the antibody against the β-chain of IR. (B) Cell lysates were immunoprecipitated with antibodies against P-Ser and immunoblotted with the antibody against the β-chain of IR. Representative experiments are shown. Histograms from densitometric analysis expressed in arbitrary units (a.u.) are means ± S.E. (n=4). Statistical significance was tested and differences between values in the presence of I vs. control C are represented by (●), between values in the presence of T or T+I+PD vs. C are represented by (●), between values in the presence of T+I vs. I are represented by (△) and between T+I+PD* vs. T+I are represented by (▲).
FIG. 4. TNF-α increases Ser phosphorylation and impairs insulin-induced Tyr phosphorylation of IRS-1 and IRS-2.

Cells were cultured as indicated in Fig.3 and lysates were immunoprecipitated with anti-IRS-1 (A) or anti-IRS-2 (B) antibodies and immunoblotted with anti-P-Tyr (4G10) antibody (PY) or with the antibodies against IRSs. (C) Cell lysates were immunoprecipitated with antibodies against P-Ser and immunoblotted with anti-IRS-1. A direct Western blot with the anti-P-IRS-1(Ser307) specific antibody is also shown. Representative experiments are shown. Histograms from densitometric analysis of phosphorylated IRSs expressed in arbitrary units (a.u.) are means ± S.E. (n=4). Statistical significance was tested as in Fig.3.

FIG. 5. PD169316 restores insulin stimulation of PI3-kinase and Akt activities in the presence of TNF-α.

Cells were cultured as indicated in Fig.3 and lysates were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and assayed for PI3-kinase activity (A). Cell lysates were also analyzed by Western blotting with the corresponding antibodies against phospho-Akt(Ser473), phospho-Akt(Thr308) and Akt (B). Representative experiments are shown. Histograms from densitometric analysis of PI3K activity and P-Akt expressed in arbitrary units (a.u.) are means ± S.E. (n=4). Statistical significance was tested as in Fig.3.

FIG. 6. TNF-α activates IκB-α and β degradation and IKK-β in a p38MAPK-dependent manner in skeletal muscle.

A) Primary neonatal rat myotubes were cultured for up to 24 h in the absence or presence of 1 nM TNF-α. At the end of the culture time cells were collected and protein extracts were analyzed by Western blotting with the corresponding antibodies against IκB-α, IκB-β and β-actin. Representative experiments are shown. B) Cells were cultured as indicated in Fig. 3, and protein extracts were analyzed by Western blotting as in A, or by in vitro kinase assay in immunoprecipitates with anti-IKK-β antibody. Representative experiments are shown. Histograms from densitometric analysis expressed in arbitrary units (a.u.) are means ± S.E. (n=4). Statistical significance was tested and differences between values in the presence of T or T+I or T+I+PD vs. C are represented by (♦) and between T+I+PD* vs. T+I are represented by (Δ). C) Cells were...
treated for 6 h in the absence or presence of TNF-α without or with 5 mM salicylate (Sal), and proteins were analyzed by Western blotting with the corresponding antibodies against phosphorylated and total p38 MAPK. Representative experiments are shown.

**FIG. 7. Salicylate precludes insulin resistance by TNF-α in skeletal muscle.**

Cells were cultured for 24 h in the absence (C) or presence of 1 nM TNF-α (T) without or with 5 mM salicylate (Sal) and stimulated or not for 5 minutes with 50 nM insulin (I). **A)** Cell lysates were immunoprecipitated with antibodies against IR and immunoblotted with anti-P-Tyr (4G10) antibody (PY) or with the antibody against the β-chain of IR. **B)** Lysates were immunoprecipitated with anti-IRS-1 or anti-P-Ser antibodies and immunoblotted with anti-P-Tyr (4G10) antibody or with the antibody against IRS-1. A direct Western blot with the anti-P-IRS-1(Ser307) specific antibody is also shown. **(C)** PI3-kinase activity was assayed in anti-IRS-1 immunoprecipitates. **D)** Cell lysates were also analyzed by Western blotting with the corresponding antibodies against phospho-Akt(Ser473), phospho-Akt(Thr308), Akt, IkB-α and IkB-β. Representative experiments out of four are shown. Histograms from densitometric analysis from B and C are expressed in arbitrary units (a.u.) and are means ± S.E. (n=4). Statistical significance was tested and differences between values in the presence of I vs. control (C) are represented by (●), between values in the presence of T+I vs. I are represented by (Δ) and between T+I+Sal vs. T+I are represented by (▲).
Fig. 1
Fig. 2

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A

P-p38MAPK

p38MAPK total

P-p38 (a.u.)

P-p44MAPK

P-p42MAPK

p44MAPK

p42MAPK

P-p42/p44 (a.u.)

P-JNK

JNK total

P-JNK (a.u.)

TNF-α (1 nM) 0 5' 30' 1h 6h 24h

B

p38α activity (a.u.)

p38β activity (a.u.)

C

p38MAPK total

P-p38MAPK

P-p44MAPK

P-p42MAPK

JNK total

P-JNK

IP: p38β

MBP

C  TNF-α PD* PD  SP

TNF-α

PD*  PD*  PD  SP

---

Fig. 2
A

IP: IR

WB: PY

PY-IR

WB: IR

IR-β

P-Tyr-IR

(arbitrary units)

C  I  T  -  PD*  PD

T+I

B

IP: P-Ser

WB: IR

P-Ser-IR

P-Ser-IR

(arbitrary units)

C  I  T  -  PD*  PD

T+I

Fig. 3
Fig. 4

A

IP: IRS-1

WB: PY

WB: IRS-1

P-Tyr-IRS-1

(a. u.)

C  I  T  -  PD*  PD  T+I

B

IP: IRS-2

WB: PY

WB: IRS-2

P-Tyr-IRS-2

(a. u.)

C  I  T  -  PD*  PD  T+I

C

IP: P-Ser

WB: IRS-1

P-Ser-IRS-1

P-Ser (307) IRS-1

P-Ser-IRS-1

(a. u.)

C  I  T  -  PD*  PD  T+I
Fig. 5

A

IP:IRS-1

PI3K activity (a.u.)

IP:IRS-2

PI3K activity (a.u.)

B

P-Akt (S473)

P-Akt (T308)

Akt total

PI3K activity (a.u.)

PI3P

PI3P

C       I       T        - PD*   PD

T+I

Fig. 5
Fig. 6
**Fig. 7**
Tumor necrosis factor-alpha produces insulin resistance in skeletal muscle by activation of inhibitor kB kinase in a p38 mitogen-activated protein kinase-dependent manner

Cristina De Alvaro, Teresa Teruel, Rosario Hernandez and Margarita Lorenzo

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