

# TNF- $\alpha$ impairs insulin signaling and insulin stimulation of glucose uptake in C<sub>2</sub>C<sub>12</sub> muscle cells

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**Del Aguila, Luis F., Kevin P. Claffey, and John P. Kirwan.** TNF- $\alpha$  impairs insulin signaling and insulin stimulation of glucose uptake in C<sub>2</sub>C<sub>12</sub> muscle cells. *Am. J. Physiol.* 276 (*Endocrinol. Metab.* 39): E849–E855, 1999.—Physiological stressors such as sepsis and tissue damage initiate an acute immune response and cause transient systemic insulin resistance. This study was conducted to determine whether tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a cytokine produced by immune cells during skeletal muscle damage, decreases insulin responsiveness at the cellular level. To examine the molecular mechanisms associated with TNF- $\alpha$  and insulin action, we measured insulin receptor substrate (IRS)-1- and IRS-2-mediated phosphatidylinositol 3-kinase (PI 3-kinase) activation, IRS-1-PI 3-kinase binding, IRS-1 tyrosine phosphorylation, and the phosphorylation of two mitogen-activated protein kinases (MAPK, known as p42<sup>MAPK</sup> and p44<sup>MAPK</sup>) in cultured C<sub>2</sub>C<sub>12</sub> myotubes. Furthermore, we determined the effects of TNF- $\alpha$  on insulin-stimulated 2-deoxyglucose (2-DG) uptake. We observed that TNF- $\alpha$  impaired insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activation by 54 and 55% ( $P < 0.05$ ), respectively. In addition, TNF- $\alpha$  decreased insulin-stimulated IRS-1 tyrosine phosphorylation by 40% ( $P < 0.05$ ). Furthermore, TNF- $\alpha$  repressed insulin-induced p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation by 81% ( $P < 0.01$ ). TNF- $\alpha$  impairment of insulin signaling activation was accompanied by a decrease ( $P < 0.05$ ) in 2-DG uptake in the muscle cells ( $60 \pm 4$  vs.  $44 \pm 6$  pmol·min<sup>-1</sup>·mg<sup>-1</sup>). These data suggest that increases in TNF- $\alpha$  may cause insulin resistance in skeletal muscle by inhibiting IRS-1- and IRS-2-mediated PI 3-kinase activation as well as p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation, leading to impaired insulin-stimulated glucose uptake.

tumor necrosis factor- $\alpha$ ; phosphatidylinositol 3-kinase; mitogen-activated protein kinase

THE STRESS of tissue damage is characterized by an alteration in body homeostasis, an acute immune response, and mononuclear cell release of cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, and IL-6 (4, 34). Furthermore, physiological stress resulting from muscle damage is accompanied by the development of whole body transient insulin resistance (1, 2, 7, 20). Recent studies performed in our laboratory (19, 21) have confirmed an increase in insulin secretion after the physiological stress induced by eccentric exercise in young subjects. Therefore, an increase in pancreatic  $\beta$ -cell secretion may be a mecha-

nism to compensate for the transient systemic insulin resistance frequently associated with eccentric exercise-induced muscle damage (1, 20). However, the molecular mechanisms associated with stress-induced insulin resistance are not known. Recent publications suggest that TNF- $\alpha$  may impair the insulin signaling pathway in cultured adipocytes and hepatocytes (10, 15). However, the effects of TNF- $\alpha$  on the insulin signaling pathway in skeletal muscle are less clear. In addition, TNF- $\alpha$  infusion in rodents has been shown to induce whole body insulin resistance during euglycemic-hyperinsulinemic clamps (22). Thus increased production of TNF- $\alpha$  by mononuclear cells could provide a link between muscle tissue damage and transient insulin resistance, possibly through a mechanism associated with altered regulation of the insulin signaling pathway.

The insulin signaling pathway is comprised of a complex array of protein kinases and protein phosphatases that regulate insulin action (5, 33). Insulin receptor substrate (IRS)-1 and IRS-2 are docking proteins that are pivotal in initiating the pleiotropic effects of insulin through phosphatidylinositol 3-kinase (PI 3-kinase; see Refs. 5 and 29) and mitogen-activated protein kinases (MAPK) p42<sup>MAPK</sup> and p44<sup>MAPK</sup> (5). Both PI 3-kinase and MAPK are activated by insulin via tyrosine phosphorylation and have been shown to be involved in the regulation of glucose uptake and glycogen synthesis, respectively (3, 5, 8). Thus the purpose of this study was to determine the effects of TNF- $\alpha$  on insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activation, p42<sup>MAPK</sup>/p44<sup>MAPK</sup> tyrosine phosphorylation, and 2-deoxyglucose (2-DG) uptake in skeletal muscle cells.

## METHODS

**Materials.** C<sub>2</sub>C<sub>12</sub> myoblasts were purchased from the American Type Culture Collection (Rockville, MD). Recombinant mouse TNF- $\alpha$  was purchased from Genzyme Diagnosis (Cambridge, MA). Anti-active MAPK polyclonal antibody was purchased from Promega (Madison, WI). Monoclonal antiphosphotyrosine antibody PY20 and monoclonal antiserum to the p85 regulatory subunit of the PI 3-kinase were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antiserum to rabbit IRS-1 and IRS-2 were obtained from Upstate Biotechnology (Lake Placid, NY). Okadaic acid was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Phosphatidylserine and phosphatidylinositol were purchased from Avanti Polar Lipids. All biochemicals, 2-DG, cell culture reagents, and FBS were from Sigma (St. Louis, MO), and radiochemicals (2-[<sup>3</sup>H]DG and <sup>32</sup>P labeled) were from Du Pont-New England Nuclear. Protein assay reagents

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were from Bio-Rad Laboratories, and chemiluminescence reagents were from Amersham.

**Cell culture.**  $C_2C_{12}$  myoblasts were cultured in 100-mm dishes in an atmosphere of 5%  $CO_2$  at 37°C in DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin (100 U/ml) to reach 100% confluence. Myoblast differentiation was induced with DMEM supplemented with 5% horse serum, L-glutamine, and penicillin/streptomycin for 72 h. Differentiated myotubes were then starved for 5 h in serum-free DMEM before treatment.

**Insulin and TNF- $\alpha$  treatment.** Serum-free myotubes were treated with insulin (100 nM) for 3 or 15 min, with TNF- $\alpha$  (10 ng/ml) alone for 1 h, or with TNF- $\alpha$  for 1 h followed by insulin for 3 or 15 min. All of the treatments were performed in duplicate in three independent experiments. Medium was then removed, and cells were washed two times with ice-cold PBS. Cell lysates were obtained by scraping the cells in lysisate buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 2 mM  $NaVO_4$ , 1 mM benzamidine, 0.2 M 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 10  $\mu$ g/ml of antipain, pepstatin, aprotinin] or in PI 3-kinase immunoprecipitation buffer [50 mM HEPES, 137 mM NaCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM  $NaVO_4$ , 1% Nonidet P-40 (NP-40), 10% glycerol, 2  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, 1.5 mg/ml benzamidine, 0.2 MAEBSF, 10  $\mu$ g/ml antipain, and 0.5  $\mu$ g/ml pepstatin]. Cell lysates were then spun down, and the cell pellet/debris was discarded. Supernatant protein concentration was determined by the Bio-Rad DC protein assay.

**Determination of IRS-1- and IRS-2-associated PI 3-kinase activity.** A 1-mg sample of cell lysate was immunoprecipitated with either 4  $\mu$ g of IRS-1 or IRS-2 polyclonal antibodies, rocking overnight at 4°C. A 40- $\mu$ l sample of slurry protein A-Sepharose was added to the immunoprecipitate for 2 h, and immunocomplexes were obtained by brief centrifugation at 9,000 rpm and washed three times in PBS-1% NP-40, two times in 500 mM LiCl-100 mM Tris, pH 7.6, and one time in 10 mM Tris·HCl, pH 7.4, 100 mM NaCl, and 1 mM trans-1,2-diaminoacyclohexane-*N,N,N,N'*-tetraacetic acid. The pellets were spun down one more time and washed in PI 3-kinase adenosine assay buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10 mM  $MgCl_2$ , 0.5 mM EGTA, and 120  $\mu$ M adenosine). The final pellet was resuspended in 40  $\mu$ l of PI 3-kinase adenosine assay buffer. A 50- $\mu$ l sample of phosphatidylinositol and phosphatidylserine was dried down in a nitrogen stream and sonicated in 100  $\mu$ l of 20 mM HEPES-1 mM EDTA, pH 7.4. The lipid mixture was kept on ice, and 5  $\mu$ l of this mixture (2  $\mu$ g/ $\mu$ l of phosphatidylinositol) were added to each sample. The solution was mixed by sonication and incubated for 10 min at 30°C on a heat block. A mixture consisting of 170  $\mu$ Ci of [ $\gamma$ - $^{32}P$ ]ATP and 280  $\mu$ M unlabeled ATP was prepared, and the reaction was started by adding 5  $\mu$ l of this mixture to each sample. After 10 min at 30°C, the reaction was stopped by the addition of 200  $\mu$ l 1 N HCl to each sample. The phosphatidylinositol 3-phosphate (PI3P) was extracted with 160  $\mu$ l chloroform-methanol (1:1). The phases were separated by centrifugation, and the lower organic phase was removed and separated by TLC. The radioactivity incorporated into PI3P was determined by phosphorimaging of the TLC plates.

**Determination of IRS-1 tyrosine phosphorylation and IRS-1 binding to p85 by immunoprecipitation and immunoblotting.** A total of 300  $\mu$ g of cell lysate was immunoprecipitated with rabbit antiserum to IRS-1, rocking overnight at 4°C. A 50- $\mu$ l slurry of protein A-Sepharose was added to the immunoprecipitates, and incubation was continued for 1 h at 4°C followed by brief centrifugation at 9,000 rpm. The agarose

pellets were then washed three times with immunoprecipitation buffer. A 50- $\mu$ l sample of 2 $\times$  Laemmli buffer was added, and the samples were boiled for 5 min at 100°C. Immunoprecipitates were run on 8% SDS-PAGE blotted to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) following the same procedure as that used for p42<sup>MAPK</sup> and p44<sup>MAPK</sup>. The blots were then probed with anti-phosphotyrosine PY20, rabbit polyclonal anti-IRS-1, and mouse polyclonal anti-p85 antibodies followed by an incubation with their respective secondary antibodies bound to horseradish peroxidase. Immunodetection was performed by enhanced chemiluminescence (ECL; Amersham) following the manufacturer's instructions.

**Determination of p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation.** A total of 100  $\mu$ g of cell lysate was mixed with 2 $\times$  Laemmli buffer, boiled at 100°C for 5 min, loaded on 10% SDS-PAGE under reducing conditions, and blotted to Immobilon-P PVDF membranes (Millipore) for 3 h with 300 mA current at 4°C. Membranes were blocked for 90 min in blocking buffer [10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 plus 5% nonfat dry milk (1% BSA for phosphotyrosine blots)]. The blot was then probed with anti-active MAPK polyclonal antibody to recognize phosphorylated forms of both p42<sup>MAPK</sup> and p44<sup>MAPK</sup>. An anti-rabbit antibody bound to horseradish peroxidase was then used, and immunodetection was performed by ECL.

**Determination of glucose uptake by  $C_2C_{12}$  skeletal muscle cells.** Glucose uptake was assayed using 2-DG. Glucose uptake measurements were performed in duplicate and in three independent experiments. After 5 h of serum starvation, cells were incubated with insulin (100 nM) for 30 min, with TNF- $\alpha$  (10 ng/ml) for 1 h, or with TNF- $\alpha$  for 1 h followed by insulin for 30 min. After TNF- $\alpha$  and insulin treatment, cells were washed two times with wash buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM  $MgSO_4$ , and 1 mM  $CaCl_2$ ). Cells were then incubated in buffer transport solution (wash buffer containing 0.5 mCi 2-[ $^3H$ ]DG/ml and 10 mM 2-DG) for 10 min. Uptake was terminated by aspiration of the solution. Cells were then washed three times, and radioactivity associated with the cells was determined by cell lysis in 0.05 M NaOH, followed by scintillation counting. Aliquots of cell lysates were used for protein content determination. 2-DG uptake was expressed as picomoles per minute per milligram of protein.

**Statistics.** Statistical analysis was performed by using the MIXED procedure for the Statistical Analysis System (SAS Institute). Group differences were determined using a one-way ANOVA. All of the variables are expressed as means  $\pm$  SE. An alpha level of 0.05 was used to determine statistical significance.

## RESULTS

**Effects of TNF- $\alpha$  on IRS-1-mediated PI 3-kinase activity in insulin-stimulated  $C_2C_{12}$  myotubes.** It has been suggested that PI 3-kinase activation is a critical step in the regulation of the insulin signaling pathway and glucose uptake (29). Therefore, we questioned whether TNF- $\alpha$  would decrease insulin stimulation of PI 3-kinase activation. Values were expressed as percentage of insulin-stimulated activity. Insulin treatment alone for 3 min increased ( $P < 0.001$ ) IRS-1-mediated PI 3-kinase activation (Fig. 1, lane 2 vs. lane 1, 100 vs.  $11 \pm 5\%$ ). TNF- $\alpha$  alone did not alter IRS-1-mediated PI 3-kinase activity when compared with nontreated control cells (Fig. 1, lane 3 vs. lane 1,  $13 \pm 6$

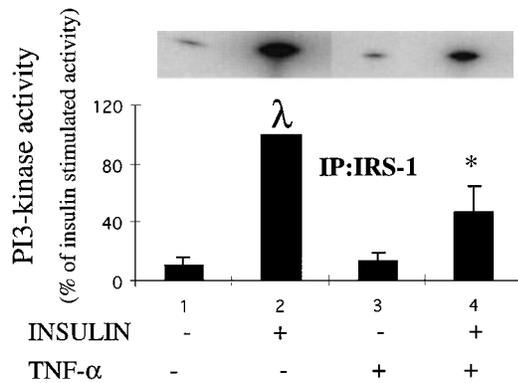


Fig. 1. Effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on insulin receptor substrate (IRS)-1-mediated phosphatidylinositol 3-kinase (PI 3-kinase) activity in insulin-stimulated  $C_2C_{12}$  myotubes. Serum-free starved myotubes were untreated (lane 1) or exposed to 100 nM insulin for 3 min (lane 2), 10 ng/ml TNF- $\alpha$  for 1 h (lane 3), or TNF- $\alpha$  for 1 h followed by 100 nM insulin for 3 min (lane 4). Treatments were performed in duplicate and in 3 independent experiments. IRS-1-mediated PI 3-kinase activity was determined as detailed in METHODS. Radioactivity incorporated in phosphatidylinositol 3-phosphate was quantified by phosphorimaging of the TLC plates. PI 3-kinase activity is expressed as a percentage of insulin-stimulated activity. Results represent the means  $\pm$  SE of 3 independent experiments.  $\lambda$  Increased ( $P < 0.001$ ) with respect to basal. \* Decreased ( $P < 0.05$ ) with respect to insulin treatment alone.

vs.  $11 \pm 5\%$ ). However, pretreatment with TNF- $\alpha$  for 1 h resulted in a significant decrease ( $P < 0.05$ ) in IRS-1-mediated PI 3-kinase activity (Fig. 1, lane 2 vs. lane 4, 100 vs.  $46 \pm 18\%$ ).

**Effects of TNF- $\alpha$  on IRS-2-mediated PI 3-kinase activity in insulin-stimulated  $C_2C_{12}$  myotubes.** We then examined whether IRS-2 could provide an alternative signaling pathway for TNF- $\alpha$  downregulation of IRS-1-mediated PI 3-kinase activation. Similar results as those obtained for IRS-1-mediated PI 3-kinase activation were observed for IRS-2. Insulin increased ( $P < 0.001$ ) IRS-1-mediated PI 3-kinase activation (Fig. 2, lane 2 vs. lane 1, 100 vs.  $10 \pm 4\%$ ). As observed in the data from IRS-1, TNF- $\alpha$  alone had no effect on IRS-2-

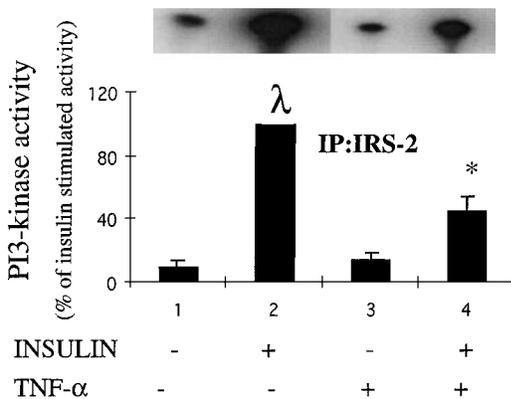


Fig. 2. Effect of TNF- $\alpha$  on IRS-2-mediated PI 3-kinase activity in insulin-stimulated  $C_2C_{12}$  myotubes. IRS-2-mediated PI 3-kinase activity assays were performed as in Fig. 1. Treatments of the serum-free starved myotubes are indicated below the lanes. PI 3-kinase activity is expressed as a percentage of insulin-stimulated activity. Results represent the means  $\pm$  SE of 3 independent experiments.  $\lambda$  Increased ( $P < 0.001$ ) with respect to basal. \* Decreased ( $P < 0.05$ ) with respect to insulin treatment alone.

mediated PI 3-kinase activity (Fig. 2, lane 3 vs. lane 1,  $14 \pm 5$  vs.  $10 \pm 4\%$ ). However, TNF- $\alpha$  did decrease ( $P < 0.05$ ) insulin stimulation of IRS-2-mediated PI 3-kinase activation (Fig. 2, lane 2 vs. lane 4, 100 vs.  $45 \pm 10\%$ ). Although it has been suggested that IRS-2 provides alternative pathways to compensate for impaired IRS-1 in IRS-1-deficient mice (25, 31), our study suggests that IRS-2 does not provide alternative insulin signaling to overcome the downregulation of IRS-1 by TNF- $\alpha$ .

**Effects of TNF- $\alpha$  on IRS-1 tyrosine phosphorylation.** Tyrosine phosphorylation of IRS-1 has been shown to be critical in the activation of PI 3-kinase by insulin (29). Therefore, we tested whether TNF- $\alpha$  inhibition of insulin-activated PI 3-kinase would be accompanied by decreased IRS-1 tyrosine phosphorylation. As seen in Fig. 3A, insulin increased ( $P < 0.001$ ) IRS-1 tyrosine phosphorylation (lane 2 vs. lane 1, 100 vs.  $4 \pm 3\%$ ). TNF- $\alpha$  alone did not alter IRS-1 tyrosine phosphorylation when compared with control cells (Fig. 3A, lane 3 vs. lane 1,  $5 \pm 3$  vs.  $4 \pm 3\%$ ). However, TNF- $\alpha$  decreased ( $P < 0.05$ ) insulin stimulation of IRS-1 tyrosine phosphorylation in cultured  $C_2C_{12}$  muscle cells (Fig. 3A, lane 2 vs. lane 4, 100 vs.  $60 \pm 8\%$ ). We also examined whether exposure of these cells to TNF- $\alpha$  would induce changes in IRS-1 protein content. IRS-1 total protein content (Fig. 3B) was not altered after insulin or TNF- $\alpha$  treatment, suggesting that TNF- $\alpha$  impairs IRS-1 tyrosine phosphorylation without altering IRS-1 protein synthesis under acute conditions.

**Effects of TNF- $\alpha$  on IRS-1-p85 binding in insulin-stimulated  $C_2C_{12}$  myotubes.** The enzyme PI 3-kinase is composed of two subunits, a regulatory p85 subunit

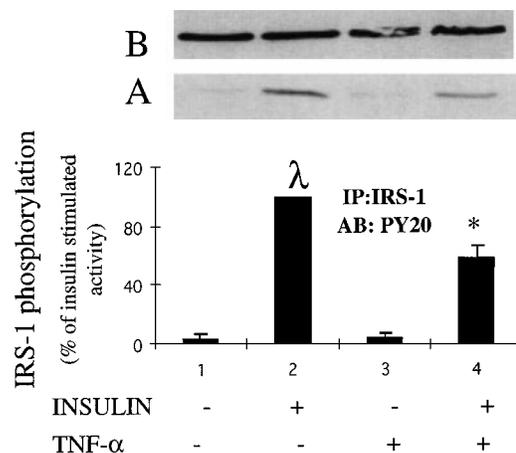


Fig. 3. Effect of TNF- $\alpha$  on IRS-1 tyrosine phosphorylation (A) and IRS-1 total protein (B) in insulin-stimulated myotubes. Serum-free starved myotubes were treated as indicated. A 300- $\mu$ g sample of cell lysate was immunoprecipitated with anti-IRS-1 antibody, followed by SDS-PAGE, and transferred to nitrocellulose. IRS-1 tyrosine phosphorylated protein (A) and IRS-1 protein content (B) were detected by immunoblotting. Membrane was then exposed to X-ray film to detect the chemiluminescent signal. IRS-1 tyrosine phosphorylation is expressed as a percentage of insulin-stimulated activity. Results represent the means  $\pm$  SE of 3 independent experiments. AB, antibody.  $\lambda$  Increased ( $P < 0.001$ ) with respect to basal. \* Decreased ( $P < 0.05$ ) with respect to insulin treatment alone.

and a catalytic p110 subunit (5). IRS-1 binding to the regulatory p85 subunit is critical for the activation of PI 3-kinase (29). Thus, to determine the effect of TNF- $\alpha$  on insulin stimulation of IRS-1-p85 binding, cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibody, and Western blot analysis was performed with an anti-p85 antibody. Insulin treatment for 3 min increased ( $P < 0.001$ ) IRS-1-bound PI 3-kinase (Fig. 4, lane 2 vs. lane 1, 100 vs.  $6 \pm 3\%$ ). In accordance with the data obtained for IRS-1 and PI 3-kinase, TNF- $\alpha$  impaired insulin-induced binding of IRS-1-p85 (Fig. 4, lane 2 vs. lane 4, 100 vs.  $42 \pm 13\%$ ). TNF- $\alpha$  alone did not alter IRS-1-p85 binding when compared with control cells (Fig. 4, lane 3 vs. lane 1,  $15 \pm 5$  vs.  $6 \pm 3\%$ ).

**Effect of TNF- $\alpha$  on p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation.** Both p42<sup>MAPK</sup> and p44<sup>MAPK</sup> are downstream molecules in the insulin signaling pathway and have been reported to be involved in the regulation of glycogen synthesis (3, 5, 8). Insulin treatment for 15 min increased ( $P < 0.001$ ) tyrosine phosphorylation on p42<sup>MAPK</sup> and p44<sup>MAPK</sup> in the cultured C<sub>2</sub>C<sub>12</sub> myotubes used in these experiments (Fig. 5, lane 2 vs. lane 1, 100 vs.  $16 \pm 6\%$  and 100 vs.  $10 \pm 4\%$ , respectively). TNF- $\alpha$  alone did not alter tyrosine phosphorylation on p42<sup>MAPK</sup> and p44<sup>MAPK</sup> when compared with control cells (Fig. 5, lane 3 vs. lane 1,  $8 \pm 5$  vs.  $16 \pm 6\%$  and  $9 \pm 7$  vs.  $10 \pm 4\%$ , respectively). However, TNF- $\alpha$  treatment decreased ( $P < 0.01$ ) insulin-stimulated p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation (Fig. 5, lane 2 vs. lane 4, 100 vs.  $19 \pm 10\%$  and 100 vs.  $21 \pm 8\%$ , respectively).

**Effects of TNF- $\alpha$  on insulin-stimulated glucose uptake.** To further investigate the effects of TNF- $\alpha$  on insulin action in cultured skeletal muscle, we evaluated whether TNF- $\alpha$  downregulation of the insulin signaling pathway is accompanied by impaired insulin stimulation of glucose uptake. Treatment with insulin

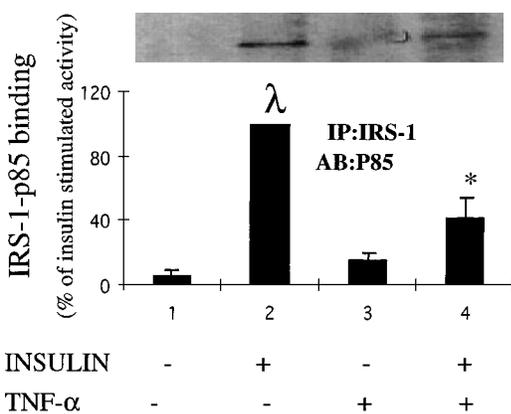


Fig. 4. Effect of TNF- $\alpha$  on p85 $\alpha$  binding to IRS-1 in insulin-stimulated C<sub>2</sub>C<sub>12</sub> myotubes. Serum-free starved myotubes were treated as indicated. A 300- $\mu$ g sample of cell lysate was immunoprecipitated with anti-IRS-1 rabbit polyclonal antibody followed by SDS-PAGE and transferred to nitrocellulose. IRS-1 binding to p85 was detected by immunoblotting using anti-p85 antibodies. Membrane was then exposed to X-ray film to detect the chemiluminescent signal. IRS-1-bound PI 3-kinase is expressed as a percentage of insulin-stimulated activity. Results represent the means  $\pm$  SE of 3 independent experiments.  $\lambda$  Increased ( $P < 0.001$ ) with respect to basal. \* Decreased ( $P < 0.05$ ) with respect to insulin treatment alone.

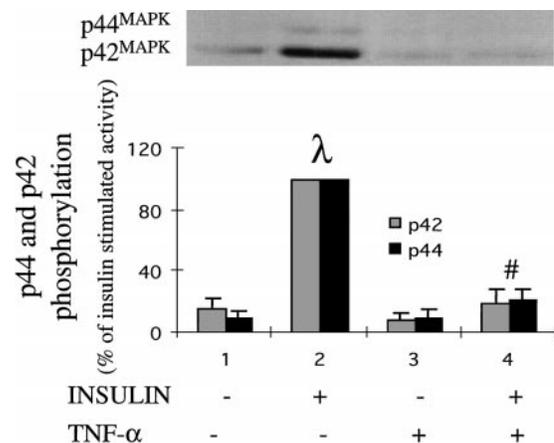


Fig. 5. Effect of TNF- $\alpha$  on p42<sup>MAPK</sup> and p44<sup>MAPK</sup> in insulin-stimulated C<sub>2</sub>C<sub>12</sub> myotubes. Serum-free starved myotubes were either untreated (lane 1) or exposed to 100 nM insulin for 15 min (lane 2), 10 ng/ml TNF- $\alpha$  for 1 h (lane 3), or 10 ng/ml TNF- $\alpha$  for 1 h followed by 100 nM insulin for 15 min (lane 4). Tyrosine-phosphorylated p42<sup>MAPK</sup> and p44<sup>MAPK</sup> were detected with an anti-active p42<sup>MAPK</sup> and p44<sup>MAPK</sup> polyclonal antibody. Immunodetection was performed by enhanced chemiluminescence, and signal was detected by X-ray autoradiography. p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation is expressed as a percentage of insulin-stimulated activity. Results represent the means  $\pm$  SE of 3 independent experiments.  $\lambda$  Increased ( $P < 0.001$ ) with respect to basal. # Decreased ( $P < 0.01$ ) with respect to insulin treatment alone.

(100 nM) for 30 min increased ( $P < 0.05$ ) 2-DG uptake with respect to the nontreated cell ( $45 \pm 5$  vs.  $60 \pm 4$  pmol $\cdot$ min<sup>-1</sup> $\cdot$ mg<sup>-1</sup>). Furthermore, TNF- $\alpha$  (10 ng/ml) decreased ( $P < 0.03$ ) basal 2-DG uptake ( $44 \pm 6$  vs.  $25 \pm 4$  pmol $\cdot$ min<sup>-1</sup> $\cdot$ mg<sup>-1</sup>) and insulin-stimulated 2-DG uptake ( $P < 0.05$ ;  $60 \pm 4$  vs.  $44 \pm 6$  pmol $\cdot$ min<sup>-1</sup> $\cdot$ mg<sup>-1</sup>; Fig. 6).

## DISCUSSION

Insulin resistance is a complex metabolic abnormality that affects the ability of peripheral tissues to respond to insulin. TNF- $\alpha$ , a cytokine produced by mononuclear cells during stress (4, 34), has been closely associated with inhibition of the insulin signaling pathway and insulin resistance (10, 15). TNF- $\alpha$  has previously been reported to diminish insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation in hepato-

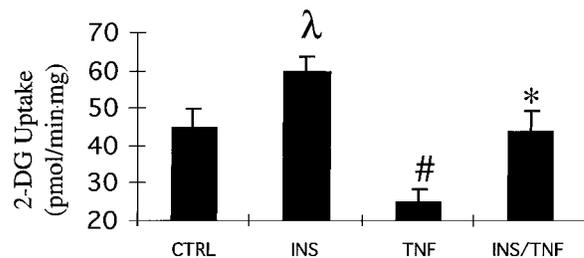


Fig. 6. Effect of TNF- $\alpha$  on insulin-stimulated 2-deoxyglucose (2-DG) uptake in C<sub>2</sub>C<sub>12</sub> myotubes. Serum-starved cells were treated with insulin (INS, 100 nM) for 30 min, with TNF- $\alpha$  (10 ng/ml) for 1 h, or with TNF- $\alpha$  (10 ng/ml) for 1 h followed by insulin (100 nM) for 30 min. Results are expressed as the means  $\pm$  SE of 3 independent experiments performed in duplicate. CTRL, control.  $\lambda$  Increased ( $P < 0.05$ ) with respect to basal. # Decreased ( $P < 0.03$ ) with respect to basal. \* Decreased ( $P < 0.05$ ) with respect to insulin-treated cells.

cytes and adipocytes, resulting in impaired insulin action (16, 18, 26). It has been suggested that PI 3-kinase activation by IRS-1 and IRS-2 is essential for insulin-mediated glucose uptake by the cell (12, 24). Therefore, we examined the effects of TNF- $\alpha$  on PI 3-kinase activation to better understand the cross-talk between TNF- $\alpha$  and insulin action in muscle cells. Skeletal muscle is the major site for insulin-mediated glucose disposal (9). We show that insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activity is decreased by TNF- $\alpha$  in cultured C<sub>2</sub>C<sub>12</sub> myotubes. Furthermore, TNF- $\alpha$  decreased insulin stimulation of cellular glucose uptake. Thus impaired insulin stimulation of PI 3-kinase activation by elevated levels of TNF- $\alpha$  after the stress of tissue damage could result in decreased glucose uptake and transient insulin resistance.

It has been suggested that IRS-1 is a pivotal molecule in the regulation of the insulin signaling pathway (29). Insulin activation of IRS-1 through tyrosine phosphorylation results in IRS-1 binding with the p85 regulatory subunit of PI 3-kinase. Pretreatment with TNF- $\alpha$  for 1 h impaired insulin stimulation of IRS-1 and IRS-1-p85 binding, with no change in IRS-1 total protein. These results are strongly supported by previous studies performed in adipocytes and hepatocytes (10, 14, 18) and in models in vivo (14), in which insulin-induced tyrosine phosphorylation of IRS-1 is impaired by TNF- $\alpha$ . However, our results extend these findings to skeletal muscle, which is responsible for >85% of insulin-mediated glucose disposal in the body (9).

It has been suggested that PI 3-kinase activation is required for insulin-mediated glucose uptake (6). Furthermore, impaired PI 3-kinase activation has been associated with decreased GLUT-4 translocation (11) and insulin resistance (13). Thus PI 3-kinase activation is key in the regulation of insulin-mediated glucose uptake. PI 3-kinase is activated by tyrosine-phosphorylated IRS in response to insulin binding to its receptor in insulin-dependent tissues (17, 29). Two IRS complexes, IRS-1 and IRS-2, have been identified and have been shown to be structurally and functionally similar (30). Therefore, we questioned whether TNF- $\alpha$  could impair insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activation in skeletal muscle cells. We found that insulin-dependent PI 3-kinase activation was impaired by TNF- $\alpha$  in both IRS-1- and IRS-2-mediated PI 3-kinase activation. Although it has been suggested that IRS-2 provides an alternative pathway for insulin signal transduction in IRS-1-deficient mice (25, 32), it has also been reported that insulin-induced PI 3-kinase activation is significantly decreased in muscle, but not in liver, from IRS-1-deficient mice compared with those in wild-type mice (35). In our study, pretreatment with TNF- $\alpha$  repressed insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activation by 54 and 55%, respectively, in myotubes stimulated with insulin, indicating that IRS-2 does not compensate for the downregulation by TNF- $\alpha$  of IRS-1-activated PI 3-kinase. These data suggest that IRS-1-

and IRS-2-mediated PI 3-kinase activation are equally diminished by TNF- $\alpha$  in muscle cells. Previous studies have suggested that insulin stimulation of IRS-2-mediated PI 3-kinase activation occurs faster and more transiently than insulin stimulation of IRS-1-mediated PI 3-kinase activation (23). Our study suggests that IRS-1 and IRS-2 activation is synchronously stimulated by insulin and is impaired by TNF- $\alpha$  in our model of C<sub>2</sub>C<sub>12</sub> myotubes. Thus, in differentiated muscle, insulin stimulation of both IRS-1- and IRS-2-mediated PI 3-kinase activation is minimized by TNF- $\alpha$ . Impaired insulin activation of PI 3-kinase could lead to a decrease in glucose uptake and therefore insulin resistance.

To further investigate the potential role played by TNF- $\alpha$  on insulin resistance, we evaluated the effects of TNF- $\alpha$  on insulin-mediated glucose uptake. TNF- $\alpha$  downregulation of PI 3-kinase activation was accompanied by impairment of insulin-stimulated glucose uptake. These results suggest that elevated TNF- $\alpha$  may be a key component in the development of insulin resistance in skeletal muscle by inhibiting IRS-1 tyrosine phosphorylation and PI 3-kinase activation, leading to decreased insulin stimulation of glucose uptake. TNF- $\alpha$  also caused significant reductions in basal glucose uptake. Previous investigations have shown a decrease in GLUT-1 protein content in 3T3-L1 adipocytes exposed to TNF- $\alpha$  (28). GLUT-1 is the glucose transporter responsible for basal glucose uptake (5). Thus a decrease in GLUT-1 protein content after TNF- $\alpha$  treatment could be responsible for the reductions observed in basal glucose uptake in our model of C<sub>2</sub>C<sub>12</sub> skeletal muscle cells.

IRS-1 tyrosine phosphorylation has also been implicated in signal transduction from the insulin receptor downstream to the MAPK, p42<sup>MAPK</sup> and p44<sup>MAPK</sup>. These two isoforms of MAPK have been associated with the regulation of glycogen synthesis (5, 8, 27). In the present study, TNF- $\alpha$  impairs insulin-induced p42<sup>MAPK</sup> and p44<sup>MAPK</sup> tyrosine phosphorylation in cultured muscle cells. Furthermore, the extent to which TNF- $\alpha$  downregulates p42<sup>MAPK</sup> and p44<sup>MAPK</sup> (81%) is greater than TNF- $\alpha$  impairment of IRS-1-mediated PI 3-kinase activation (54%). These data suggest that the intracellular components involved in insulin-mediated glycogen synthesis may be more sensitive to the effects of TNF- $\alpha$  than the upstream molecules of the insulin signaling pathway involved in glucose uptake. Therefore, inhibition of p42<sup>MAPK</sup> and p44<sup>MAPK</sup> could be one of the major components of the insulin signaling pathway downregulated by TNF- $\alpha$ .

In summary, these data indicate that TNF- $\alpha$  impairs insulin stimulation of IRS-1- and IRS-2-mediated PI 3-kinase activation, p42<sup>MAPK</sup>/p44<sup>MAPK</sup> tyrosine phosphorylation, and 2-DG uptake in muscle cells. IRS-2 was unable to compensate for the downregulation of IRS-1-mediated PI 3-kinase activation by TNF- $\alpha$ , suggesting that IRS-2 is not an alternative pathway to IRS-1 in the presence of increased TNF- $\alpha$  in muscle cells. Impaired insulin activation of p42<sup>MAPK</sup>/p44<sup>MAPK</sup> by elevated levels

of TNF- $\alpha$  could result in decreased glycogen synthesis. Thus less glucose available for storage and impaired glycogen synthesis activity could result in a failure to synthesize glycogen stores in skeletal muscle. Further studies need to be performed to determine whether TNF- $\alpha$  is a key factor in the development of the systemic insulin resistance resulting from muscle tissue damage in vivo.

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## REFERENCES

1. **Asp, S., J. R. Dugaard, S. Kristiansen, B. Kiens, and E. A. Richter.** Eccentric exercise decreases maximal insulin action in humans: muscle and systemic effects. *J. Physiol. (Lond.)* 494: 891–898, 1996.
2. **Asp, S., and E. A. Richter.** Decreased insulin action on muscle glucose transport after eccentric contractions in rats. *J. Appl. Physiol.* 81: 1924–1928, 1996.
3. **Begum, N., and L. Ragolia.** Altered regulation of insulin signaling components in adipocytes of insulin-resistant type II diabetic Goto-Kakizaki rats. *Metabolism* 47: 54–62, 1998.
4. **Cannon, J. G., S. N. Meydani, R. A. Fielding, M. A. Fiatarone, M. Meydani, M. Farhangmehr, S. F. Orencole, J. B. Blumberg, and W. J. Evans.** Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis. *Am. J. Physiol.* 260 (Regulatory Integrative Comp. Physiol. 29): R1235–R1240, 1991.
5. **Cheatham, B., and C. R. Kahn.** Insulin action and the insulin signaling network. *Endocr. Rev.* 16: 117–142, 1995.
6. **Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis, and C. R. Kahn.** Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol. Cell. Biol.* 14: 4902–4911, 1994.
7. **Costill, D. L., D. D. Pascoe, W. J. Fink, R. A. Robergs, S. I. Barr, and D. Pearson.** Impaired muscle glycogen resynthesis after eccentric exercise. *J. Appl. Physiol.* 69: 46–50, 1990.
8. **Cross, D. A., D. R. Alessi, J. R. Vandenheede, H. E. McDowell, H. S. Hundal, and P. Cohen.** The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* 303: 21–26, 1994.
9. **DeFronzo, R. A., R. Gunnarsson, O. Bjorkman, M. Olsson, and J. Wahren.** Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J. Clin. Invest.* 76: 149–155, 1985.
10. **Feinstein, R., H. Kanety, M. Z. Papa, B. Lunenfeld, and A. Karasik.** Tumor necrosis factor- $\alpha$  suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J. Biol. Chem.* 268: 26055–26058, 1993.
11. **Goodyear, L. J., F. Giorgino, L. A. Sherman, J. Carey, R. J. Smith, and G. L. Dohm.** Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J. Clin. Invest.* 95: 2195–2204, 1995.
12. **Heller-Harrison, R. A., A. M. Morin, A. Guilherme, and M. P. Czech.** Insulin-mediated targeting of phosphatidylinositol 3-kinase to Glut4-containing vesicles. *J. Biol. Chem.* 271: 10200–10204, 1996.
13. **Heydrick, S. J., D. Jullien, N. Gautier, J.-F. Tanti, S. Giorgetti, E. van Obberghen, and Y. le Marchand-Brustel.** Defect in skeletal muscle phosphatidylinositol 3-kinase in obese insulin-resistant mice. *J. Clin. Invest.* 91: 1358–1566, 1993.
14. **Hotamisligil, G. S., A. Budavari, D. Murray, and B. M. Spiegelman.** Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor- $\alpha$ . *J. Clin. Invest.* 94: 1543–1549, 1994.
15. **Hotamisligil, G. S., D. L. Murray, L. N. Choy, and B. M. Spiegelman.** Tumor necrosis factor  $\alpha$  inhibits signaling from the insulin receptor. *Proc. Natl. Acad. Sci. USA* 91: 4854–4858, 1994.
16. **Hotamisligil, G. S., P. Peraldi, A. Budavari, R. Ellis, M. F. White, and B. M. Spiegelman.** IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- $\alpha$ - and obesity-induced insulin resistance. *Science* 271: 665–668, 1996.
17. **Kahn, C. R., and F. Folli.** Molecular determinants of insulin action. *Horm. Res.* 39: 93–101, 1993.
18. **Kanety, H., R. Feinstein, M. Z. Papa, R. Hemi, and A. Karasik.** Tumor necrosis factor  $\alpha$ -induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J. Biol. Chem.* 270: 23780–23784, 1995.
19. **Kirwan, J. P., R. E. Bourey, W. M. Kohrt, M. A. Staten, and J. O. Holloszy.** Effects of treadmill exercise to exhaustion on the insulin response to hyperglycemia in untrained men. *J. Appl. Physiol.* 70: 246–250, 1991.
20. **Kirwan, J. P., R. C. Hickner, K. E. Yarasheski, W. M. Kohrt, B. V. Wiethop, and J. O. Holloszy.** Eccentric exercise induces transient insulin resistance in healthy individuals. *J. Appl. Physiol.* 72: 2197–2202, 1992.
21. **Krishnan, R. K., J. M. Hernandez, D. L. Williamson, D. J. O’Gorman, W. J. Evans, and J. P. Kirwan.** Age-related differences in the pancreatic  $\beta$ -cell response to hyperglycemia after eccentric exercise. *Am. J. Physiol.* 275 (Endocrinol. Metab. 38): E463–E470, 1998.
22. **Lang, C. H., C. Dobrescu, and G. J. Bagby.** Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology* 130: 43–52, 1992.
23. **Ogihara, T., B. C. Shin, M. Anai, H. Katagiri, K. Inukai, M. Funaki, Y. Fukushima, H. Ishihara, K. Takata, M. Kikuchi, Y. Yazaki, Y. Oka, and T. Asano.** Insulin receptor substrate (IRS)-2 is dephosphorylated more rapidly than IRS-1 via its association with phosphatidylinositol 3-kinase in skeletal muscle cells. *J. Biol. Chem.* 272: 12868–12873, 1997.
24. **Okada, T., Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui.** Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J. Biol. Chem.* 269: 3568–3573, 1994.
25. **Patti, M. E., X. J. Sun, J. C. Bruening, E. Araki, M. A. Lipes, M. F. White, and C. R. Kahn.** 4PS/insulin receptor substrate (IRS)-2 is the alternative substrate of the insulin receptor in IRS-1-deficient mice. *J. Biol. Chem.* 270: 24670–24673, 1995.
26. **Paz, K., R. Hemi, D. LeRoith, A. Karasik, E. Elhanany, H. Kanety, and Y. Zick.** A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J. Biol. Chem.* 272: 29911–29918, 1997.
27. **Ragolia, L., and N. Begum.** Protein phosphatase-1 and insulin action. *Mol. Cell. Biochem.* 182: 49–58, 1998.
28. **Stephens, J. M., and P. H. Pekala.** Transcriptional repression of the Glut-4 and C/EBP genes in adipocytes by tumor necrosis factor  $\alpha$ . *J. Biol. Chem.* 266: 21839–21845, 1991.
29. **Sun, X. J., D. L. Crimmins, M. G. Myers, Jr., M. Miralpeix, and M. F. White.** Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. *Mol. Cell. Biol.* 13: 7418–7428, 1993.

30. **Sun, X. J., L. M. Wang, Y. Zhang, L. Yenush, M. G. Myers, Jr., E. Glasheen, W. S. Lane, J. H. Pierce, and M. F. White.** Role of IRS-2 in insulin and cytokine signaling. *Nature* 377: 173–177, 1995.
31. **Tobe, K., H. Tamemoto, T. Yamauchi, S. Aizawa, Y. Yazaki, and T. Kadowaki.** Identification of a 190-kDa protein as a novel substrate for the insulin receptor kinase functionally similar to insulin receptor substrate-1. *J. Biol. Chem.* 270: 5698–5702, 1994.
32. **Tobe, K., K. Ueki, H. Tamemoto, Y. Kaburagi, R. Honda, and T. Kadowaki.** The role of phosphorylation cascade in insulin action. *Nippon Rinsho* 52: 2673–2685, 1994.
33. **White, M. F., R. Maron, and C. R. Kahn.** Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature* 318: 183–186, 1985.
34. **Yamamura, M., X. H. Wang, J. D. Ohmen, K. Uyemura, T. H. Rea, B. R. Bloom, and R. L. Modlin.** Cytokine patterns of immunologically mediated tissue damage. *J. Immunol.* 149: 1470–1475, 1992.
35. **Yamauchi, T., K. Tobe, H. Tamemoto, K. Ueki, Y. Kaburagi, R. Yamamoto-Honda, Y. Takahashi, F. Yoshizawa, S. Aizawa, Y. Akanuma, N. Sonenberg, Y. Yazaki, and T. Kadowaki.** Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. *Mol. Cell. Biol.* 16: 3074–3084, 1996.

