Short-Term Exposure to Tumor Necrosis Factor-α Does Not Affect Insulin-Stimulated Glucose Uptake in Skeletal Muscle

Lorraine A. Nolte, Polly A. Hansen, May M. Chen, Jane M. Schluter, Eric A. Gulve, and John O. Holloszy

It has been hypothesized that increased production of tumor necrosis factor-α (TNF-α) plays a role in causing the insulin resistance associated with obesity. Obesity with insulin resistance is associated with increased production of TNF-α by fat cells. Exposure of 3T3-L1 adipocytes to TNF-α for 3–4 days makes them insulin resistant. TNF-α has also been reported to rapidly (15–60 min) cause insulin resistance, with a decrease in insulin-stimulated tyrosine phosphorylation, in a number of cultured cell lines. Because skeletal muscle is the major tissue responsible for insulin-stimulated glucose disposal, we performed the present study to determine if acute exposure to TNF-α causes insulin resistance in muscle. We found that exposure of soleus muscles to 6 nmol/l TNF-α for 45 min in vitro had no inhibitory effect on insulin-stimulated tyrosine phosphorylation of the insulin receptor or insulin receptor substrate 1 (IRS-1) or on phosphatidylinositol 3-kinase association with IRS-1. Incubation of epitrochlearis and soleus muscles with 6 nmol/l TNF-α for 45 min or 4 h had no effect on insulin-stimulated 2-deoxyglucose (2-DG) uptake. Treatment of epitrochlearis muscles with 2 nmol/l TNF-α for 8 h also had no effect on insulin-stimulated 2-DG uptake. We conclude that in contrast to Fao hepatoma cells and 3T3-L1 fibroblasts, skeletal muscle does not become insulin resistant in response to short-term exposure to TNF-α. *Diabetes* 47: 721–726, 1998

Obesity is a major cause of insulin resistance. It has been hypothesized that when hypertrophying fat cells attain some critical mass, they start to secrete an insulin resistance factor to protect themselves against continued fat storage and further hypertrophy (1). In support of this hypothesis, it has been reported that hypertrophied fat cells from a variety of animal models of obesity (2,3) and obese humans (1,4) secrete the inflammatory cytokine tumor necrosis factor-α (TNF-α) at an approximately twofold higher rate than do fat cells of lean rodents and humans. Because skeletal muscle is responsible for >90% of whole-body insulin-stimulated glucose disposal (5), it is muscle, rather than adipose tissue, that is primarily responsible for the insulin resistance associated with obesity. The muscle insulin resistance associated with obesity has been explained in the context of the TNF-α hypothesis as being due to exposure to TNF-α released by the fat cells (2,3,6). In addition, it has been reported that skeletal muscle expresses TNF-α and that cultured muscle cells from diabetic patients produce more than twice as much TNF-α as those from nondiabetic subjects (7), suggesting that endogenous TNF-α production could play a role in muscle insulin resistance in obesity and diabetes.

Studies of the mechanism by which TNF-α inhibits insulin action have been conducted on cells exposed to the cytokine in culture for a number of days (8–12). It has been reported that 3–5 days of exposure of 3T3-L1 or 3T3-F442A adipocytes to TNF-α results in marked reductions in insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation in response to a maximal insulin stimulus (8,9). It has also been shown that 3–4 days of exposure of 3T3-L1 adipocytes to TNF-α results in large decreases in GLUT4, IR, and IRS-1 mRNA and protein (11,12). It has been proposed that these reductions in insulin-stimulated tyrosine phosphorylation and/or the decreases in IR, IRS-1, and GLUT4 proteins are responsible for TNF-α–mediated insulin resistance.

A number of investigators have reported that, like long-term exposure, brief periods of treatment with TNF-α, in the range of 15 to 60 min, induce decreases in insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in Fao hepatoma cells (13,14) and NIH3T3 fibroblasts (15), as well as a decrease in insulin-stimulated glucose transport in L6 myocytes (16). Because skeletal muscle is the major site of insulin-stimulated glucose disposal, it appeared of interest to determine whether TNF-α also has a rapid effect on the response to insulin in this tissue. In the present study, we examined the effect of short-term exposure to TNF-α on insulin-stimulated tyrosine phosphorylation of the IR and IRS-1, on phosphatidylinositol (PI) 3-kinase associated with IRS-1, and on glucose transport in isolated rat skeletal muscles.

RESEARCH DESIGN AND METHODS

Materials. Recombinant human TNF-α and okadaic acid were purchased from LC Laboratories (Woburn, MA). Pork insulin was purchased from Eli Lilly (Indianapolis, IN). 2-Deoxy-D-[1,2-^{3}H] glucose was obtained from American Radiolabeled Chemicals (St. Louis, MO), and donkey anti-rabbit ^{125}IgG and [U-^{14}C]mannitol were obtained from DuPont-NEN (Boston, MA). The anti-phosphotyrosine, anti-phosphatidylinositol 3-kinase, and anti-rat COOH-terminal IRS-1 antibodies

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri.

Address correspondence and reprint requests to Dr. John O. Holloszy, Washington University School of Medicine, 4566 Scott Ave., 2nd Floor, West Bldg., Campus Box 8113, St. Louis, MO 63110. E-mail: jhollosz@imgate.wustl.edu.

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BSA, bovine serum albumin; 2-DG, 2-deoxyglucose; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; IR, insulin receptor; IRS-1, insulin receptor substrate 1; KHB, Krebs-Henseleit bicarbonate buffer; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; TNF-α, tumor necrosis factor-α.

were purchased from Upstate Biotechnology (Lake Placid, NY). The phosphospecific anti-pp42/pp44 mitogen-activated protein kinase (MAPK) antibody was purchased from Promega (Madison, WI). Horseradish peroxidase (HRP) conjugated-donkey anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Arlington Heights, IL). The bicinchoninic acid protein assay kit was purchased from Pierce Chemical (Rockford, IL). All other chemicals were obtained from Sigma (St. Louis, MO).

Treatment of animals and muscle preparation. The experimental protocol was approved by the Washington University Animal Studies Committee. Colony-bred Wistar rats were maintained on a 12 h/12 h light/dark schedule and given rat chow and water ad libitum. All food was removed at 5:00 P.M. on the day before the experiment. The following morning, between 9:00 and 10:00 A.M., animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body wt), and the epitrochlearis and soleus muscles were removed. The epitrochlearis is a small thin muscle, <0.2 mm thick, consisting predominantly (85%) of fast-twitch fibers, that is suitable for measurement of glucose transport activity in vitro (17,18). Soleus muscles were split before incubation to allow adequate diffusion of oxygen and substrates, as described previously (18).

Muscle treatments for insulin-stimulated tyrosine phosphorylation. After dissection, split soleus muscles were incubated for 30 min at 30°C in 1 ml of Krebs-Henseleit bicarbonate buffer (KHB) containing 0.1% radioimmunoassay-grade bovine serum albumin (BSA), 8 mmol/l glucose, and 32 mmol/l mannitol. Muscles were then incubated in medium of the same composition, in the presence or absence of 6 nmol/l TNF-α, for 45 min. Finally, muscles were transferred to identical medium and incubated in the presence or absence of 600 nmol/l insulin for 4 min. Muscles were immediately blotted and then clamp-frozen using tongs precooled in liquid N_2.

It has been suggested that TNF-α inhibits tyrosine phosphorylation of IRS-1 by increasing serine phosphorylation of this protein (14) by a mechanism similar to that of the phosphatase inhibitor okadaic acid (19). Therefore, as a positive control, experiments were performed to assess the effect of okadaic acid on insulin-stimulated IRS-1 tyrosine phosphorylation. In these experiments, split soleus muscles were treated as described above, except that muscles were incubated in the presence or absence of 500 nmol/l okadaic acid for 15 min before the 4-min exposure to 600 nmol/l insulin.

Preparation of muscle extracts. Muscle samples were pulverized using a mortar and pestle cooled by liquid N_2 and maintained on dry ice. The powdered muscle was immediately homogenized in ice-cold buffer containing 50 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l $MgCl_2$, 1.0 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 2.0 mmol/l Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mmol/l phenylmethylsulfonyl fluoride. Homogenates were incubated with end-over-end rotation at 4°C for 1 h and then centrifuged at 200,000*g* for 50 min at 4°C.

Immunoprecipitation and immunoblotting. Equal amounts of supernatant protein were precleared for 1 h using 40 μl protein A-Sepharose (50% slurry), followed by overnight incubation (4°C) with anti–IRS-1 antibody. The immune complexes were precipitated by incubation with protein A-Sepharose (40 μl) for 1 h at 4°C. The pellet was washed four times by brief centrifugation and gentle resuspension in ice-cold wash buffer (0.5% Triton X-100, 50 mmol/l HEPES [pH 7.4], 150 mmol/l NaCl, and 200 μmol/l Na_3VO_4). After the final wash, the antibody conjugates were released from the Sepharose by addition of 2 × Laemmli buffer (20) containing 100 mmol/l dithiothreitol and heating in a boiling water bath for 5 min.

Immunoprecipitates were subjected to SDS-PAGE (6.25% resolving gel), followed by transfer to polyvinylidene difluoride membranes at 90 V for 90 min by the method of Towbin et al. (21), but with 0.02% SDS added to the transfer buffer. The membrane was cut in half horizontally, using the 120 kDa molecular weight marker as a guide. The top portion of the membrane was blocked overnight at 4°C in TNT buffer (10 mmol/l Tris [pH 7.5], 150 mmol/l NaCl, 0.1% Tween 20) with 1% BSA and used for detection of tyrosine-phosphorylated IRS-1 using a polyclonal anti-phosphotyrosine antibody (1.5 μg/ml) followed by donkey anti-rabbit ^{125}IgG and exposure to X-ray film for 12–48 h. Band intensity was analyzed by densitometry. The bottom half of the PVDF membrane was blocked overnight in 5% nonfat dried milk in phosphate-buffered saline containing 0.1% Tween-20, then used to assess IRS-1–immunoprecipitable PI 3-kinase. Membranes were incubated with a monoclonal antibody directed against the NH_2-terminal SH2 region of the 85-kDa regulatory subunit of the kinase (p85; 1:3,000 dilution), followed by HRP-conjugated donkey anti-mouse-IgG. The antibody-bound p85 protein was visualized using ECL. Intensity of the p85 band was analyzed by densitometry.

For analysis of insulin receptor β-subunit tyrosine phosphorylation, aliquots of the muscle extract were treated with 2 × Laemmli buffer containing 100 mmol/l dithiothreitol and boiled for 5 min. Samples containing 100 μg protein were subjected to SDS-PAGE and Western blotting exactly as described above for the IRS-1 immunoprecipitates.

Measurement of MAPK activation. TNF-α has been shown to activate the pp42 and pp44 MAPKs in human diploid skin fibroblasts (22). An experiment was performed to evaluate the bioactivity of the human recombinant TNF-α used in this study in rat skeletal muscle. Split soleus muscles were incubated in 1 ml of KHB containing 0.1% BSA, 8 mmol/l glucose, and 32 mmol/l mannitol for 30 min at 30°C. Next, muscles were transferred to another vial containing medium of the same composition in the presence or absence of 6 nmol/l TNF-α and incubated for 20 min at 30°C. Muscles were clamp frozen. Muscle extracts were prepared as described above.

For determination of phosphorylated pp42/44 MAPK, 80 μg of protein from each sample was subjected to SDS-PAGE (10% resolving gel), followed by transfer to PVDF membranes as described above for the IRS-1 immunoprecipitates. Membranes were incubated with a polyclonal antibody (Promega V6671) made against a dual phosphorylated peptide that corresponds to the active form of the pp42/44 MAPK enzymes, followed by donkey anti-rabbit ^{125}IgG and exposure to X-ray film for 12–24 h.

Muscle incubations for glucose transport. Epitrochlearis or split soleus muscles were incubated at 30°C for 15 min, 4 h, or 8 h in 2 ml of KHB containing 0.1% BSA, 8 mmol/l glucose, 32 mmol/l mannitol, and 0.5, 2.0, or 6.0 nmol/l TNF-α. When muscles were incubated for 8 h, the medium was supplemented with essential amino acids and glutamine (23), penicillin (50 U/ml), and streptomycin (5 μg/ml), and the medium was changed every 2 h. The vials were incubated with shaking and a gas phase of 95% O_2/5% CO_2.

After the initial incubation, muscles were transferred to 2 ml KHB buffer with 0.1% BSA, 2 mmol/l sodium pyruvate, 36 mmol/l mannitol, and the same concentration of TNF-α as in the previous step, with or without 450 pmol/l or 12 nmol/l of insulin, and incubated for 30 min before measurement of glucose transport activity.

To determine the effect of okadaic acid on basal and insulin-stimulated glucose transport, split soleus muscles were allowed to recover in KHB medium containing 8 mmol/l glucose and 32 mmol/l mannitol for 30 min. Muscles were then transferred to a flask containing KHB with 0.1% BSA, 2 mmol/l sodium pyruvate, and 36 mmol/l mannitol, with or without 12 nmol/l of insulin and 500 nmol/l okadaic acid, and incubated for 60 min. Okadaic acid was prepared as a 2 mmol/l stock solution in DMSO. The concentration of DMSO in the incubation medium never exceeded 0.01%. Identical concentrations of DMSO were also added to the control incubation medium.

Measurement of 2-deoxyglucose uptake. 2-Deoxyglucose (2-DG) uptake was measured using a modification of a previously described procedure (24). Unless otherwise specified in the table legend, 2-DG uptake was assayed in 1.0 ml KHB containing 0.1% BSA, 4 mmol/l 2-deoxy-D-[1,2-^{3}H]glucose (1.5 μCi/ml), 36 mmol/l [U-^{14}C]mannitol (0.3 μCi/ml), and the same concentrations of TNF-α, okadaic acid, and insulin present in the previous incubations. Extracellular space and intracellular 2-DG ($\mu mol \cdot ml^{-1} \cdot 20\ min^{-1}$) were determined as previously described (24).

Statistical analysis. Data are presented as means ± SE. Comparisons of multiple groups were analyzed using a one-way analysis of variance (ANOVA). Unpaired *t* tests were used to assess the effects of TNF-α or okadaic acid on insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 or on PI 3-kinase associated with IRS-1. $P < 0.05$ was the accepted level for statistical significance.

RESULTS

Insulin-stimulated tyrosine phosphorylation and IRS-1–associated PI 3-kinase. Incubation with 600 nmol/l of insulin for 4 min induced an approximately twofold increase in tyrosine phosphorylation of the IR and an approximately sevenfold increase in tyrosine phosphorylation of IRS-1 in soleus muscles. IRS-1–associated PI 3-kinase increased ~13-fold in the insulin-stimulated muscles. Incubation of soleus muscles with 6 nmol/l TNF-α for 45 min before the insulin treatment had no effect on insulin-stimulated tyrosine phosphorylation of the IR or IRS-1 or on the increase in IRS-1–bound PI 3-kinase in response to insulin (Fig. 1).

Stimulation of 2-DG uptake by insulin. As shown in Table 1, pretreatment with 6 nmol/l TNF-α for 45 min had no effect on stimulation of 2-DG uptake by a maximally effective insulin stimulus (12 nmol/l) in either the epitrochlearis or split soleus. Exposure of epitrochlearis or split soleus muscles to TNF-α at concentrations ranging from 0.5 to 6.0 nmol/l for 4 h also had no inhibitory effect on the stimulation of glucose uptake by either a maximally effective (12 nmol/l; Table 2) or a submaximally effective (450 pmol/l; Table 3) insulin concentration. Thus, TNF-α had no effect on either the respon-

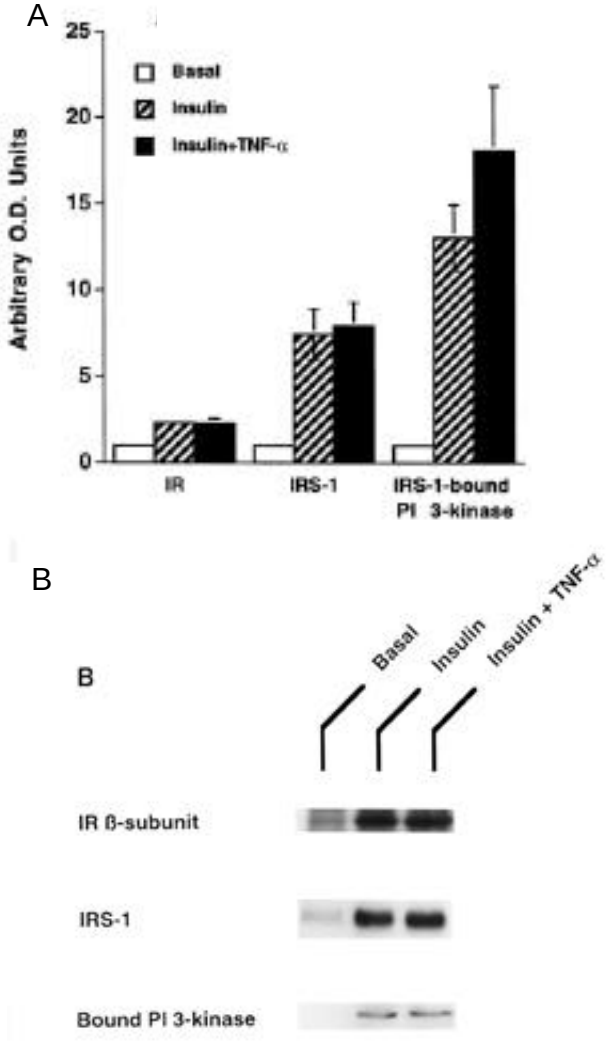

FIG. 1. Effect of 45 min exposure to 6 nmol/l TNF-α on insulin signaling in the split soleus muscle. *A*: Basal and insulin-stimulated tyrosine phosphorylation of the IR or IRS-1 and of IRS-1–bound PI 3-kinase. Results are expressed as arbitrary optical density (O.D.) units relative to control (basal = 1.00). *B*: Representative Western blots showing tyrosine phosphorylation of the β-subunit of the IR and IRS-1 and of IRS-1–bound PI 3-kinase (p85) in muscles not treated with insulin or TNF-α (basal), treated with 600 nmol/l insulin, or treated with 6 nmol/l TNF-α + 600 nmol/l insulin.

siveness or the sensitivity of the glucose transport process to insulin.

Incubation of epitrochlearis muscles for 8 h in the presence of 2 nmol/l TNF-α had no effect on the stimulation of 2-DG uptake by 450 pmol/l of insulin (Table 4).

Effect of okadaic acid. Insulin-stimulated tyrosine phosphorylation of the IR was unaffected by exposure of soleus muscles to the serine phosphatase inhibitor okadaic acid (Fig. 2). However, insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased by nearly 50%, and the stimulation of PI 3-kinase association with IRS-1 by insulin was reduced by more than 30%, in muscles exposed to okadaic acid (Fig. 2). Okadaic acid also had an inhibitory effect on insulin-stimulated 2-DG uptake, even though okadaic acid by itself had a small stimulatory effect (Fig. 3). These data are in agreement with previously published results on the effect of okadaic acid in skeletal muscle (25).

TABLE 1
Effect of 45-min exposure to 6 nmol/l TNF-α on maximally insulin-stimulated 2-DG uptake (12 nmol/l) in isolated epitrochlearis and split soleus muscles

Insulin (12 nmol/l)	TNF-α (6 nmol/l)	2-DG uptake (μmol · ml^{-1} · 20 min^{-1})	
		Epitrochlearis	Soleus
–	–	0.58 ± 0.02	0.93 ± 0.12
+	–	2.39 ± 0.34	4.44 ± 0.27
+	+	2.75 ± 0.14	4.38 ± 0.35

Data are means ± SE for five to seven muscles per treatment.

Activation of pp42/44 MAPK. The negative results in our experiments on the effects of TNF-α on insulin signaling and insulin-stimulated glucose transport raised the question of whether or not the human recombinant TNF-α we used was biologically active in rat skeletal muscle. Therefore, we determined the effect of incubating soleus muscles with TNF-α on the phosphorylation (activation) of MAPKs pp42 and pp44, which are known to be activated by treatment of cells with TNF-α (22). Exposure of soleus muscles to TNF-α (6 nmol/l) for 20 min resulted in a ~70% increase in phosphorylation of pp42 and pp44 (Fig. 4), providing evidence that our TNF-α preparation is active in rat skeletal muscle.

DISCUSSION

Our results show that treatment of skeletal muscle with high concentrations of TNF-α for 45 min has no effect on the stimulation of tyrosine phosphorylation of the IR and IRS-1 by insulin. The ability of insulin to stimulate association of PI 3-kinase with IRS-1 is also unaffected in muscle. These results differ from those of previous studies on cultured Fao hepatoma cells (13,14) and 3T3 fibroblasts (15), in which 15–60 min of treatment with TNF-α was reported to result in large decreases in insulin-stimulated tyrosine phosphorylation of the IR and IRS-1. It has been proposed that TNF-α interferes with tyrosine phosphorylation of IRS-1 by an action similar to that of the serine phosphatase inhibitor okadaic acid (14). An inhibitory effect of okadaic acid on tyrosine phosphorylation of IRS-1 was clearly evident in the present study. This finding demonstrates that our methodology would have detected an effect of TNF-α on tyrosine phosphorylation if one had occurred. Thus, the differences between our results on muscle and those reported on Fao cells (13,14) and 3T3 fibroblasts (15) are probably explained by the major differences between skeletal muscle and these cultured cell lines. Because these cell lines express the GLUT1, rather than the insulin-regulatable GLUT4, isoform of the glucose transporter, they are not suitable models for studying insulin resistance of the glucose transport process. It is interesting in this context that in contrast to the response of fat cells, TNF-α treatment induces an approximately eightfold increase in glucose transport activity in 3T3 fibroblasts by both inducing GLUT1 translocation and increasing GLUT1 expression (26).

We found that although okadaic acid induced a 50% decrease in insulin-stimulated glucose transport, exposure of skeletal muscles to TNF-α for periods ranging from 45 min to 8 h had no effect on insulin-stimulated glucose transport. These results differ from those of Begum and Ragolia

TABLE 2
Maximally insulin-stimulated 2-DG uptake rate (12 nmol/l) in isolated epitrochlearis and soleus muscles after 4-h exposure to different concentrations of TNF-α

Insulin (12 nmol/l)	TNF-α (nmol/l)	2-DG uptake (μmol · ml^{-1} · 20 min^{-1})	
		Epitrochlearis	Soleus
–	–	0.79 ± 0.08	1.00 ± 0.08
+	–	3.28 ± 0.27	4.71 ± 0.29
+	0.5	3.10 ± 0.16	4.59 ± 0.34
+	2.0	3.18 ± 0.32	4.68 ± 0.74
+	6.0	3.46 ± 0.46	4.68 ± 0.49

Data are means ± SE for five or six muscles per treatment.

TABLE 3
Submaximally insulin-stimulated (450 pmol/l) 2-DG uptake rate in isolated epitrochlearis and soleus muscles following 4-h exposure to different concentrations of TNF-α

Insulin (450 pmol/l)	TNF-α (nmol/l)	2-DG uptake (μmol · ml^{-1} · 20 min^{-1})	
		Epitrochlearis	Soleus
–	–	0.62 ± 0.11	0.91 ± 0.05
+	–	1.37 ± 0.18	3.14 ± 0.27
+	0.5	1.25 ± 0.17	3.08 ± 0.32
+	2.0	1.41 ± 0.22	2.78 ± 0.32
+	6.0	1.32 ± 0.10	2.83 ± 0.13

Data are means ± SE for five or six muscles per treatment.

(16), who reported that treatment of L6 myocytes with TNF-α for 60 min inhibited both basal and insulin-stimulated 2-DG uptake by ~50%, with a further decrease in glucose transport to below basal in the presence of insulin after 12 h. If correct, this finding would only provide further evidence that cultured myocytes are not suitable models for studying the regulation of glucose uptake in adult skeletal muscle (27). Contrary to the report by Begum and Ragolia (16), a number of studies have shown that TNF-α stimulates basal glucose transport activity in L6 myocytes (28–30). In these studies, 12 h of exposure to TNF-α induced an approximately threefold increase in glucose transport activity (28,29), whereas 4–8 days of TNF-α treatment resulted in an approximately twofold increase (30). This increase in glucose transport activity appears to be due to induction of GLUT1 protein expression by TNF-α (28,29). Insulin has a minimal stimulatory effect on glucose transport in L6 myocytes; it is not clear whether this small stimulation in glucose transport by insulin is inhibited by TNF-α, with one group observing development of insulin resistance (29) and another finding no effect (30).

It is well documented that 3–4 days of exposure of 3T3-L1 adipocytes to TNF-α causes insulin resistance (8,11,12) and that a large decrease in GLUT4 content plays a major role in the decrease in insulin-stimulated glucose transport (2,11,12,31). Interestingly, obesity is not associated with a decrease in muscle GLUT4 (32,33). It was recently shown that there is also a large decrease in IRS-1 protein in 3T3-L1 adipocytes treated with TNF-α for 3–4 days (11). Whether decreased tyrosine phosphorylation of the IR and the remaining IRS-1 also plays a role in the TNF-α–induced insulin resistance is currently a subject of controversy, with Hotamisligil et al. (8) reporting a reduction and Stephens et al. (11) finding no effect. It is not known if prolonged exposure of muscle to TNF-α also decreases GLUT4 and IRS-1 protein expression and/or IR and IRS-1 tyrosine phosphorylation. A reason for this lack of information is that it has not been possible to maintain skeletal muscle with normal metabolic function in vitro for prolonged periods. Furthermore, primary cultures of skeletal muscle cells revert to a fetal phenotype, with a replacement of GLUT4 with GLUT1 glucose transporters and loss of insulin action on glucose transport (34). Therefore, the only currently available approach is to expose muscle to TNF-α in vivo.

In a study of the mechanism by which TNF-α plays a role in the tissue wasting that occurs in infections, toxemias, and cancer, Lang et al. (35) found that infusion of TNF-α into rats for ~20 h induced a more than twofold increase in basal glucose production and utilization. A euglycemic-hyperinsulinemic clamp showed that the TNF-α–infused rats were insulin resistant, as evidenced by a decrease in insulin-stimulated glucose disposal (35). Evaluation of muscle glucose uptake by injection of 2-DG showed that basal glucose accumulation by muscle was increased, whereas insulin-stimulated glucose uptake was decreased in gastrocnemius and quadriceps muscles (35). It is not possible to determine from these findings whether insulin-stimulated glucose uptake was decreased by a direct effect of TNF-α on muscle, by secondary mechanisms mediated by effects of TNF-α on the vasculature and/or extracellular space that impaired 2-DG delivery, or by the TNF-α–induced increases in catecholamines and glucocorticoids.

It is also not clear what, if any, relevance the findings obtained with high concentrations of TNF-α, such as were used in the in vitro studies (8,12,14,15,30) and in the in vivo studies designed to mimic effects of infections or toxemias (35), have to the insulin resistance associated with obesity. It has been hypothesized that the TNF-α produced by hypertrophied fat cells spills over and causes muscle insulin resistance (2,6). However, because plasma TNF-α levels are not increased in obesity with or without NIDDM (4), it has been proposed that TNF-α released by fat cells may cause muscle insulin resistance by a paracrine effect (2,3,6). This mechanism does not seem possible, as fat and muscle cells do not share a common extracellular space and factors released by the fat cells must first enter the circulation before coming into contact with muscle cells. Therefore, if TNF-α is involved in muscle insulin resistance associated with obesity, it appears that the insulin resistance would have to be mediated by TNF-α produced by the muscle

TABLE 4
Effect of 8-h exposure to 2 nmol/l TNF-α on insulin-stimulated (450 pmol/l) 2-DG transport in epitrochlearis muscles

TNF-α	2-DG uptake (μmol · ml^{-1} · 20 min^{-1})	
	Without insulin	With insulin
–	0.38 ± 0.03	0.82 ± 0.05
+	0.48 ± 0.07	0.89 ± 0.11

Data are means ± SE for five muscles per treatment. Glucose uptake was measured using 1.0 mmol/l 2-DG.

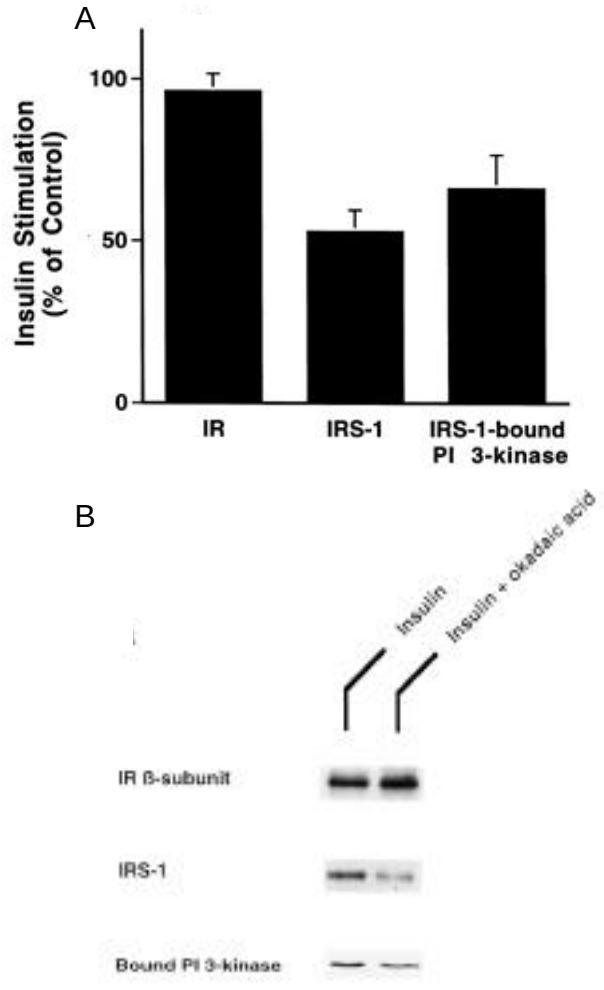

FIG. 2. Effect of treatment with 500 nmol/l okadaic acid on insulin signaling in the split soleus muscle. *A*: Insulin-stimulated tyrosine phosphorylation of the IR or IRS-1 and of IRS-1–bound PI 3-kinase. Results are expressed as percent of control value obtained in the absence of okadaic acid (n = 5 or 6). *B*: Representative Western blots showing insulin-stimulated tyrosine phosphorylation of the β-subunit of the IR and IRS-1 and of IRS-1–bound PI 3-kinase (p85) in the absence or presence of 500 nmol/l okadaic acid.

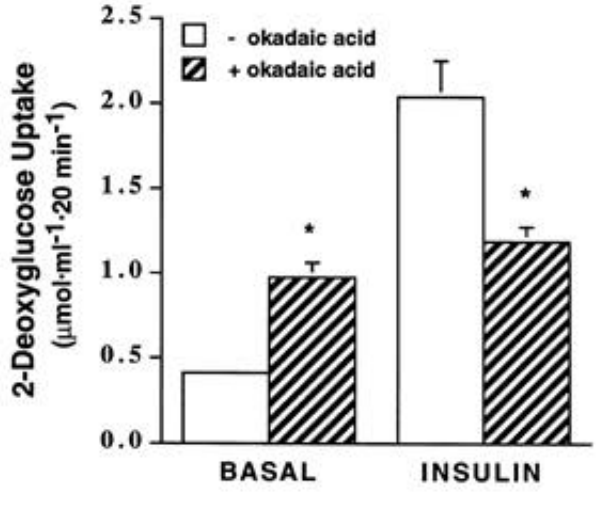

FIG. 3. The effect of 500 nmol/l okadaic acid on insulin-stimulated (12 nmol/l) 2-DG uptake in the split soleus muscle. Results are expressed as mean ± SE for 11 or 12 muscles/treatment. *P < 0.05 compared with no okadaic acid.

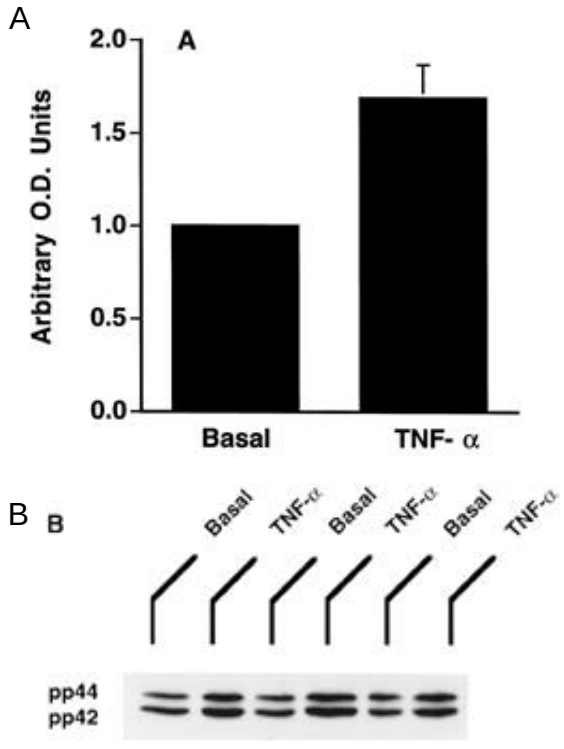

FIG. 4. Effect of 20 min exposure to 6 nmol/l TNF-α on phosphorylation of pp42 and pp44 MAPK in split soleus muscles. *A*: Phosphorylation of pp42 and pp44 MAPK at basal or after TNF-α treatment. Results are expressed as arbitrary optical density (O.D.) units relative to control (basal = 1.0). *B*: Representative Western blot showing phosphorylation of pp42 and pp44 MAPK in the absence or presence of TNF-α.

cells, as hypothesized by Saghizadeh et al. (7). These investigators reported that the expression of TNF-α in muscles of obese patients with NIDDM was fourfold higher than in normal control subjects (7).

In support of a role of TNF-α in muscle insulin resistance, Hotamisligil and co-workers (2,6) reported that neutralization of TNF-α by infusion of a TNF-α receptor–IgG fusion protein reversed insulin resistance in Zucker *fa/fa* rats. However, in subsequent studies on obese humans with NIDDM, intravenous injection of a TNF-α neutralizing antibody (36) or TNF-α receptor–IgG fusion protein (37) had no effect on insulin resistance. Furthermore, Ventre et al. (38) found that in TNF-α knockout mice, the absence of TNF-α did not substantially protect against insulin resistance in the gold-thioglucose model of obesity. Clearly, further research is needed to determine whether or not TNF-α plays a role in the muscle insulin resistance associated with obesity. However, the present results show that if TNF-α does play a role, it does not have a rapid effect that occurs in response to short-term exposure of muscle to this cytokine.

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