

Tumor Necrosis Factor- α Induces Skeletal Muscle Insulin Resistance in Healthy Human Subjects via Inhibition of Akt Substrate 160 Phosphorylation

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Most lifestyle-related chronic diseases are characterized by low-grade systemic inflammation and insulin resistance. Excessive tumor necrosis factor- α (TNF- α) concentrations have been implicated in the development of insulin resistance, but direct evidence in humans is lacking. Here, we demonstrate that TNF- α infusion in healthy humans induces insulin resistance in skeletal muscle, without effect on endogenous glucose production, as estimated by a combined euglycemic insulin clamp and stable isotope tracer method. TNF- α directly impairs glucose uptake and metabolism by altering insulin signal transduction. TNF- α infusion increases phosphorylation of p70 S6 kinase, extracellular signal-regulated kinase-1/2, and c-Jun NH₂-terminal kinase, concomitant with increased serine and reduced tyrosine phosphorylation of insulin receptor substrate-1. These signaling effects are associated with impaired phosphorylation of Akt substrate 160, the most proximal step identified in the canonical insulin signaling cascade regulating GLUT4 translocation and glucose uptake. Thus, excessive concentrations of TNF- α negatively regulate insulin signaling and whole-body glucose uptake in humans. Our results provide a molecular link between low-grade systemic inflammation and the metabolic syndrome. *Diabetes* 54:2939–2945, 2005

Low-grade systemic inflammation is a feature of obesity and insulin resistance (1). There is now an increasing appreciation that type 2 diabetes and systemic inflammation are related and that elevated cytokine levels may contribute to insulin resistance (2–4). Type 2 diabetes is associated with elevated

levels of the proinflammatory cytokine tumor necrosis factor- α (TNF- α) in skeletal muscle (5), adipose tissue (6), and plasma (7–9). However, the clinical consequence of elevated TNF- α concentrations on hepatic insulin sensitivity and peripheral glucose uptake is unknown. In isolated rat skeletal muscle, TNF- α had no effect on insulin-mediated glucose uptake, even at very high doses (10). Although this may imply the inhibitory effects of TNF- α on insulin action are targeted exclusively at the vascular tissue, cell culture studies provide direct evidence that TNF- α inhibits insulin signaling and glucose metabolism (11–13).

Excessive TNF- α levels cause insulin resistance through modifications of insulin signal transduction. In cultured cells, TNF- α induces insulin resistance through increased serine phosphorylation of insulin receptor substrate-1 (IRS-1), which subsequently converts IRS-1 to an inhibitor of insulin receptor tyrosine kinase activity (14). IRS-1 plays a critical role in mediating insulin signal transduction along pathways governing metabolic responses, such as GLUT4 translocation and glucose uptake in skeletal muscle (15). Direct evidence for a role of TNF- α as a negative regulator of insulin action is revealed from functional studies in knockout mice (16). Ablation of TNF- α improves insulin sensitivity in dietary-induced obese and *ob/ob* mice by preventing obesity-related reductions in insulin signaling in skeletal muscle and adipose tissue (16).

Evidence implicating TNF- α in the development of insulin resistance in humans is correlative (17). TNF- α is produced locally in adipose tissue (6) and skeletal muscle (5) and may function in an autocrine manner to inhibit insulin signaling and glucose transport. Attempts to improve insulin sensitivity in obese type 2 diabetic patients by neutralizing TNF- α , using anti-TNF- α antibodies (18) or recombinant TNF receptors, have been ineffective (19). The lack of efficacy may be attributable to the treatment strategy because only a single injection of a neutralizing antibody toward TNF/TNF receptor was evaluated (18,19). Alternatively, the neutralizing antibodies may be ineffective in skeletal muscle. Although animal studies provide convincing evidence for a role of TNF- α in promoting insulin resistance, results from human studies are inconclusive.

We determined the effects of TNF- α infusion on whole-body insulin-mediated glucose uptake and signal transduction in healthy humans. Furthermore, we assessed the effects of insulin and TNF- α on phosphorylation of Akt substrate 160 (AS160). Phosphorylation of AS160, a protein containing a Rab GAP (GTPase activating protein)

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Received for publication 5 April 2005 and accepted in revised form 23 June 2005.

AS160, Akt substrate 160; ERK, extracellular signal-regulated kinase; IL, interleukin; IRS, insulin receptor substrate; JNK, c-Jun NH₂-terminal kinase; S6K, p70 S6 kinase; TNF- α , tumor necrosis factor- α .

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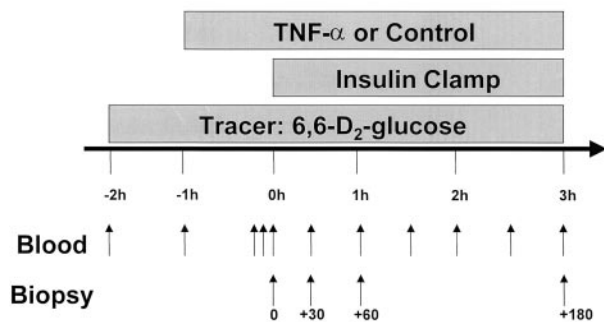


FIG. 1. Study protocol. Whole-body glucose metabolism was measured by a combination of the euglycemic insulin clamp and stable isotope tracer methodology. A schematic representation of the study protocol is shown to outline the infusion conditions and the time points for blood and skeletal muscle biopsy sampling.

domain (20), is the most proximal step in the insulin signaling cascade identified to date, and it promotes GLUT4 exocytosis to the plasma membrane to facilitate glucose uptake and metabolism (21,22).

RESEARCH DESIGN AND METHODS

Nine healthy men participated in this study. Before the two experimental days, the subjects underwent a thorough clinical examination and a standard blood chemistry analysis. Volunteers were aged 26.3 ± 1.7 years (mean \pm SE) and had a height of 181.7 ± 1.7 cm, weight of 78.8 ± 2.2 kg, and BMI of 23.9 ± 0.7 kg/cm². Subjects received oral and written information regarding the potential hazards and discomfort of the experiment. The information was approved by the local ethical committee (no. KF 01-006/04) in accordance with the Helsinki Declaration.

In vivo protocol. The study protocol is depicted in Fig. 1. Subjects underwent two separate experiments with 4-h saline or human recombinant TNF- α (Beromun; Boeringer-Ingelheim) infusion. On each occasion, both arms were catheterized in the antecubital vein. One side was used for infusion and the other for blood sampling. A priming dose of the glucose tracer [6,6-²H₂]glucose (22 μ mol/kg; Cambridge Isotope Laboratories) was given, and the infusion was started at a rate of $0.22 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 2 h to achieve equilibrium in the glucose pool for subsequent determination of glucose turnover. During the insulin infusion, the tracer infusion rate was reduced to $0.11 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. The placebo or TNF- α (rate: 1,000 ng/h per m²) infusion was started at 60 min, and TNF- α was administered in saline with 20% human albumin. Placebo consisted of saline infusion with 20% human albumin. After 2 h of tracer infusion and 1 h of TNF- α /placebo, a hyperinsulinemic-euglycemic clamp was started. The infusion rate of insulin was 20 mU/min per m², and the plasma glucose was kept at 5 mmol/l by infusion of 20% glucose enriched to 2.5% with the glucose tracer. Plasma glucose was measured frequently (EML 105 radiometer) every 5 min the 1st h and every 10 min thereafter to maintain the glucose concentration at fasting levels. Blood samples and biopsies from the vastus lateralis of musculus quadriceps were obtained at the designated time points (Fig. 1). Skeletal muscle biopsies were obtained at 0, 30, 60, and 180 min after insulin infusion. Biopsies were frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was separated into plasma and stored at -80°C .

Stable isotopes. Isotope enrichment was measured, using liquid chromatography-mass spectrometry (Finnegan aQa). First, the glucose was derivatized by benzylation. Then, 20 μ l of plasma was vigorously mixed (5 min) together with 1 mol/l K₂HPO₄, 20 μ l of 8 mol/l NaOH, and 10 μ l benzoyl chloride. Thereafter, 10 μ l of 1.4 mol/l H₃PO₄ and 600 μ l ethylacetate was added, and the samples were vortexed. The ethylacetate phase (75 μ l) containing the benzyolated glucose was transferred to a new tube and dried under a stream of nitrogen. The derivate was solubilized in the liquid chromatography buffer (80:20 acetonitril:water), filtrated, and transferred to a liquid chromatography vial. The derivate was separated by liquid chromatography using a column flow at 0.05 ml/min isocratic 80:20 acetonitril:water; the water contained 125 mmol/l NH₄-acetate for optimal ionization. The ionization source was electrospray ionization at 37 kV and a temperature of 250°C for soft fragmentation. The fragment of the derivate contained both deuterium atoms and had a mass at 231 m/z. The area under the curves for the non-enriched M-peak (231 m/z) and the enriched M+2-peak (233 m/z) were measured. The tracer-to-tracee ratio was calculated as the ratio of the M and M+2 area under the curve minus the natural background enrichment.

Clinical chemistry. Hormone analysis was performed using commercially available kits. Insulin and C-peptide were measured using enzyme-linked immunosorbent assay kits from DAKO; the glucagon kit was from Linco Research, and enzyme immunosorbent assay cortisol kits were from Diagnostic Systems Laboratories. TNF- α and interleukin (IL)-6 were measured using kits from R&D Systems. Free fatty acids were measured using a nonesterified fatty acid kit from Wako Chemicals, and glycerol was assessed using a method previously described (23). Both assays were automated and performed on a Cobas Fara robot (Roche).

Calculations. The glucose release and uptake from the circulation was calculated using a single-pool non-steady-state model, modified after the Steele equation. The exogenous glucose infused during the clamp was enriched to 2.5%, thereby improving the sensitivity of the method. The volume of glucose distribution was set at 145 ml/kg. Endogenous glucose production (rate of appearance [R_a]) and glucose uptake (rate of disappearance [R_d]) was calculated using the following:

$$R_a(t) = \frac{F}{\frac{E_1 + E_2}{2}} - \frac{pV \left(\frac{C_1 - C_2}{2} \right) \left(\frac{E_2 - E_1}{t_2 - t_1} \right)}{\frac{E_1 + E_2}{2}} + \frac{E_{\text{exo}}}{E_1 + E_2} G_{\text{inf}} - G_{\text{inf}}$$

and

$$R_d(t) = \frac{F}{\frac{E_1 + E_2}{2}} - \frac{pV \left(\frac{C_1 - C_2}{2} \right) \left(\frac{E_2 - E_1}{t_2 - t_1} \right)}{\frac{E_1 + E_2}{2}} + \frac{E_{\text{exo}}}{E_1 + E_2} G_{\text{inf}} - pV \left(\frac{C_2 - C_1}{t_2 - t_1} \right)$$

where F is the tracer infusion rate ($\mu\text{mol/kg} \times \text{min}$), pV is the distribution volume of the tracee (ml/kg), C is the concentration ($\mu\text{mol/ml}$), E is the enrichment (tracer-to-tracee ratio), E_{exo} is the enrichment of exogenous glucose, G_{inf} is the glucose infusion rate ($\mu\text{mol/min} \times \text{kg}$), and t is time (min).

Determination of protein phosphorylation. Muscles biopsies were homogenized at 4°C in 20 mmol/l Tris-HCl (pH 7.4), 140 mmol/l NaCl, 10 mmol/l EDTA, 4 mmol/l NaVO₄, 100 mmol/l NaF, 10 mmol/l pyrophosphate, and 1% Nonidet P-40, supplemented with protease inhibitors. Lysates were centrifuged (12,000g for 15 min) in lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with a freshly prepared cocktail of protease inhibitors (ICN Pharmaceuticals). Total protein was determined using the Bradford method (Bio-Rad). Aliquots of muscle lysate (50 μ g protein) were separated by SDS-PAGE, and proteins were transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight with anti-phospho-p70S6 kinase, anti-phospho-extracellular signal-regulated kinase (ERK)-1/2 (recognizing ERK-1/2 phosphorylation at Thr202 and Tyr204), anti-phospho-c-Jun NH₂-terminal kinase (JNK; recognizing phosphorylation of the 46- and 54-kDa isoforms of JNK at Thr183 and Tyr185), or anti-phospho-(Ser/Thr) Akt substrate antibody (Cell Signaling Technology). For IRS-1 phosphorylation, aliquots of muscle lysate (500 μ g protein) were immunoprecipitated with anti-IRS-1 and subjected to SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes and probed with polyclonal

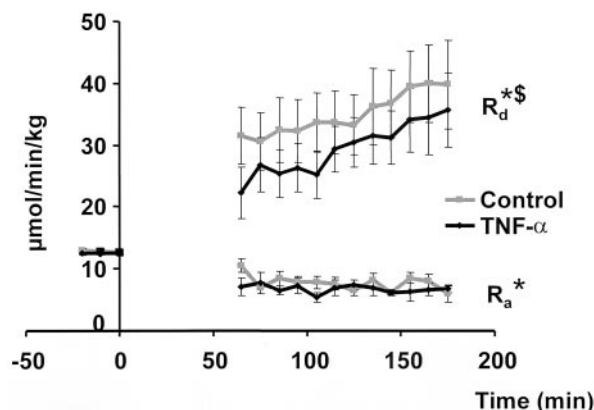


FIG. 2. Effect of TNF- α infusion on whole-body glucose uptake and hepatic glucose production. The graph shows the rate of disappearance (R_d) and appearance (R_a) for glucose ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) during the trials. Insulin increased R_d ($P < 0.001$) and decreased R_a ($P < 0.001$). Insulin-mediated glucose uptake was reduced during TNF- α administration. *Difference from the basal condition; \$difference between saline and TNF- α trial.

anti-phosphotyrosine, anti-phospho-Ser312, or anti-phospho-Ser639 (Cell Signaling Technology). Blots were stripped and reprobed with anti-IRS-1 to normalize for equal protein amount. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL Plus; Amersham) and quantified by densitometry, using Molecular Analyst software (Bio-Rad). Results are reported in arbitrary units, relative to each respective basal biopsy obtained during saline infusion.

Statistics. When data were normally distributed, arithmetical means \pm SE were presented. Otherwise, a logarithmic transformation was performed, and geometric means \pm SE were presented. The statistical comparisons in the stable isotope experiments are based on the area under the curve measurements obtained during the basal (-20 to 0 min) and insulin-stimulated (60 – 180 min) conditions. Student's paired t test was used to assess differences between saline and TNF- α treatment when only one variable was compared. To evaluate the difference between groups, a two-way repeated-measurement ANOVA was performed and a two-sample t test was used as a post hoc test. To assess the effect of time, a one-way repeated-measurement ANOVA was performed. A paired t test was applied as post hoc analysis to compare change from the "pre" sample. Both tests were Bonferroni corrected. Systat version 8.0 for Windows (SPSS) was used for the statistical analysis. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of TNF- α infusion on whole-body glucose uptake and hepatic glucose production. To investigate the effects of TNF- α on glucose homeostasis, nine healthy human volunteers received an infusion of either TNF- α or placebo for 4 h (Fig. 1). Before TNF- α infusion, $[6,6\text{-}^2\text{H}_2]\text{glucose}$ was administered to allow for the determination of glucose turnover. A euglycemic insulin clamp was initiated after 1 h of TNF- α infusion. TNF- α infusion inhibited whole-body glucose uptake (Fig. 2). R_a decreased in response to the insulin treatment, without any effect of TNF- α administration. R_d increased in response to insulin infusion in both trials ($P < 0.001$). During insulin infusion, the R_d largely reflects insulin-mediated glucose uptake in skeletal muscle. Importantly, hepatic glucose production was unaltered by TNF- α infusion (Fig. 2), demonstrating a direct effect of TNF- α on peripheral tissues.

TABLE 1
Effect of TNF- α infusion on hormones, metabolites, and clinical parameters

	Infusion						
	-120 min	-60 min	0 min	30 min	60 min	120 min	180 min
Hormones							
Insulin (pmol/l)							
Control	42.6 ± 5.5	37.6 ± 4.8	42.1 ± 7.6	$201.5 \pm 13.3^*$	$192.9 \pm 10.3^*$	$200.3 \pm 12.7^*$	$198.8 \pm 10.7^*$
TNF- α	36.4 ± 7.5	31.3 ± 4.2	45.5 ± 8.4	$186.8 \pm 16.5^*$	$180.5 \pm 13.6^*$	$171.0 \pm 7.2^*$	$171.4 \pm 12.4^*$
C-peptide (pmol/l)							
Control	667.6 ± 112.3	$628.9 \pm 112.0^*$	$606.1 \pm 112.9^*$	$568.0 \pm 118.5^*$	$509.7 \pm 116.2^*$	$441.8 \pm 107.4^*$	$426.8 \pm 107.7^*$
TNF- α	629.2 ± 128.4	616.2 ± 142.7	579.9 ± 136.4	586.9 ± 161.5	$503.2 \pm 142.4^*$	$408.1 \pm 126.1^*$	$411.6 \pm 124.1^*$
Glucagon (pmol/l)							
Control	98.8 ± 14.6	77.7 ± 6.6	67.7 ± 9.9	74.5 ± 4.4	61.5 ± 4.3	$51.4 \pm 4.1^*$	$46.4 \pm 4.0^*$
TNF- α	82.9 ± 5.7	75.8 ± 22.9	94.5 ± 12.9	67.0 ± 14.5	66.4 ± 13.6	58.3 ± 13.4	56.9 ± 13.4
Cortisol ($\mu\text{g/dl}$)							
Control	12.1 ± 1.6	$7.7 \pm 1.3^*$	$6.8 \pm 1.1^{\dagger}$	8.3 ± 1.6	8.9 ± 1.3	$6.5 \pm 1.0^*$	$7.4 \pm 1.1^{\dagger}$
TNF- α	12.7 ± 1.6	$7.4 \pm 1.1^*$	11.8 ± 0.8	14.3 ± 1.3	14.2 ± 1.8	11.2 ± 1.7	14.8 ± 2.0
Metabolites							
Glucose (mmol/l)							
Control	5.4 ± 0.2	5.3 ± 0.1	5.3 ± 0.1	4.9 ± 0.1	5.2 ± 0.1	5.0 ± 0.1	5.0 ± 0.1
TNF- α	5.1 ± 0.1	5.0 ± 0.1	5.0 ± 0.1	5.1 ± 0.2	5.0 ± 0.1	4.8 ± 0.1	5.0 ± 0.1
Lactate (mmol/l)							
Control	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.1
TNF- α	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.1
Free fatty acids ($\mu\text{mol/l}$)							
Control	528.4 ± 38.7	451.2 ± 32.3	621.3 ± 54.8	496.1 ± 71.7	$253.1 \pm 25.8^*$	$169.6 \pm 13.4^*$	$149.5 \pm 9.3^*$
TNF- α	614.7 ± 50.9	501.8 ± 34.8	665.9 ± 66.1	440.2 ± 33.5	$280.3 \pm 23.6^*$	$225.6 \pm 25.5^*$	$213.9 \pm 25.8^*$
Glycerol ($\mu\text{mol/l}$)							
Control	64.2 ± 4.8	51.1 ± 2.7	65.6 ± 4.0	43.9 ± 4.7	$35.3 \pm 3.3^*$	$22.8 \pm 1.4^*$	$21.6 \pm 1.5^*$
TNF- α	74.3 ± 5.6	50.9 ± 3.3	64.6 ± 6.7	$37.4 \pm 2.4^*$	$32.1 \pm 2.6^*$	$29.5 \pm 3.0^*$	$26.4 \pm 2.9^*$
Cytokines							
Control	2.3 ± 0.9	2.2 ± 0.8	2.2 ± 0.9	2.2 ± 0.9	2.1 ± 0.8	2.6 ± 1.3	2.3 ± 0.9
TNF- α	2.2 ± 0.7	2.3 ± 0.8	$14.3 \pm 0.8^{\dagger}$	$16.9 \pm 0.8^{\dagger}$	$16.0 \pm 1.0^{\dagger}$	$15.6 \pm 0.8^{\dagger}$	$15.9 \pm 0.9^{\dagger}$
IL-6 (pg/ml)							
Control	1.7 ± 0.4	1.6 ± 0.4	1.7 ± 0.3	1.8 ± 0.3	2.1 ± 0.3	2.2 ± 0.4	2.5 ± 0.3
TNF- α	1.8 ± 0.9	1.6 ± 0.6	5.3 ± 2.0	$13.2 \pm 3.8^*$	$14.0 \pm 3.1^{\dagger}$	$23.9 \pm 5.3^{\dagger}$	21.7 ± 6.5
Clinical parameters							
Temperature ($^{\circ}\text{C}$)							
Control	36.3 ± 0.1	$36.4 \pm 0.1^*$	$36.6 \pm 0.1^*$	$36.7 \pm 0.1^*$	$36.7 \pm 0.1^*$	$36.8 \pm 0.1^*$	$36.9 \pm 0.1^*$
TNF- α	36.2 ± 0.1	$36.5 \pm 0.1^*$	$37.0 \pm 0.1^*$	$37.2 \pm 0.1^*$	$37.3 \pm 0.1^{\dagger}$	$37.4 \pm 0.2^*$	$37.6 \pm 0.2^*$
Heart rate (bpm)							
Control	62.9 ± 5.5	60.0 ± 4.2	63.1 ± 4.0	61.3 ± 3.4	63.4 ± 4.1	62.6 ± 3.5	62.4 ± 4.0
TNF- α	61.0 ± 3.4	60.8 ± 3.8	67.1 ± 4.6	66.9 ± 3.6	$69.8 \pm 3.9^*$	$73.2 \pm 4.2^*$	$74.7 \pm 4.2^*$
Blood pressure (MAP)							
Control	86.1 ± 2.9	81.9 ± 2.8	86.7 ± 3.1	87.3 ± 3.3	89.3 ± 2.1	87.5 ± 3.4	89.5 ± 4.1
TNF- α	91.1 ± 3.0	89.4 ± 3.4	91.9 ± 2.6	87.3 ± 3.0	85.6 ± 2.8	85.4 ± 3.0	84.3 ± 3.3

Data are means \pm SE. *Difference from the basal condition; \dagger difference between saline and TNF- α trial. MAP, mean arterial pressure (mmHg).

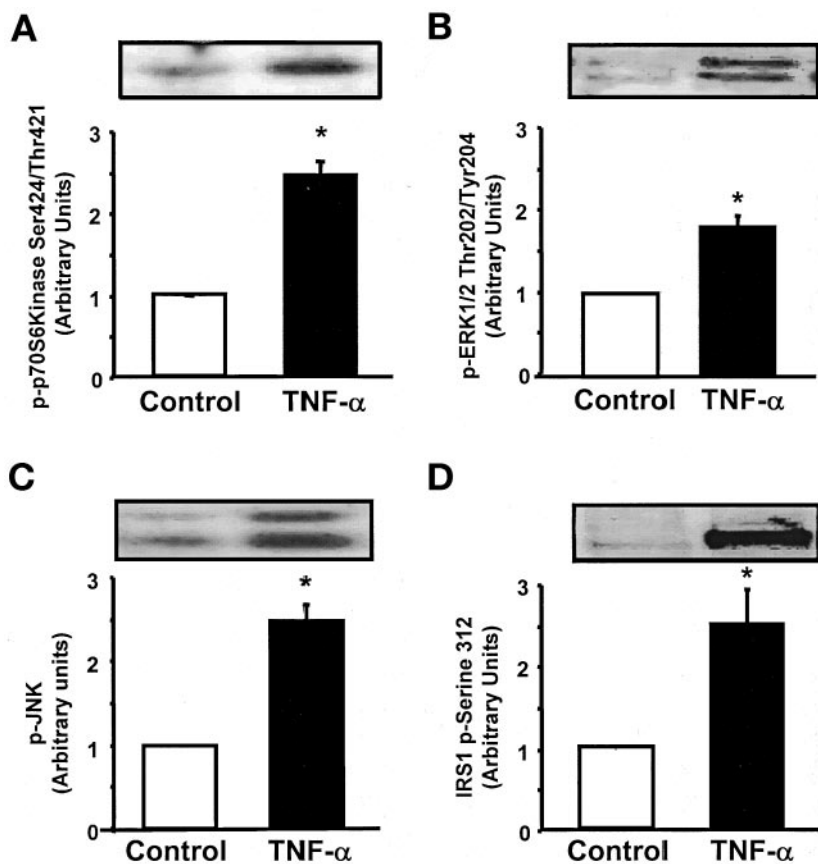


FIG. 3. Effect of 1 h of TNF- α infusion on protein phosphorylation. Phosphorylation of S6K (A), ERK-1/2 (B), JNK (C), and IRS-1 on serine site 312 (D) was assessed in skeletal muscle after the 1-h infusion of TNF- α or saline. For A–C, results are expressed relative to basal phosphorylation (time 0) in saline-treated skeletal muscle and a representative immunoblot (upper panel). The means \pm SE in arbitrary units (graph) are shown. *Significant difference between TNF- α and saline infusion.

Clinical chemistry and subject characteristics during the in vivo study. Plasma TNF- α concentration increased eightfold during the infusion (Table 1). Plasma IL-6 concentration was gradually elevated in response to TNF infusion, with a 12-fold increase noted at the end of the infusion (Table 1). TNF- α infusion induced a minor increase in body temperature and heart rate (Table 1), but volunteers did not experience any adverse effects or major changes in metabolic hormones. The plasma insulin concentration during the euglycemic clamp corresponded to levels generally obtained after a light meal. Insulin infusion suppressed free fatty acids and glycerol concentrations (Table 1).

Effect of TNF- α infusion on signal transduction in skeletal muscle. To determine the mechanism underlying the TNF- α -induced inhibition of insulin-stimulated peripheral glucose uptake, biopsies of vastus lateralis quadriceps muscle were obtained (Fig. 1). Here, we show that TNF- α infusion for 1 h, before the administration of exogenous insulin, increased phosphorylation of p70 S6 kinase (S6K), ERK-1/2, and JNK in skeletal muscle (Fig. 3A, B, and C, respectively), concomitant with increased serine phosphorylation of IRS-1 at site 312 (Fig. 3D). These targets have been implicated in the negative regulation of insulin signal transduction (24). Given this profile, we hypothesized that insulin action on signal transduction would be blunted by TNF- α .

TNF- α infusion altered insulin action on IRS-1 by interfering with tyrosine and serine phosphorylation. Insulin-stimulated tyrosine phosphorylation of IRS-1 was prevented by TNF- α infusion (Fig. 4A), whereas IRS-1 serine phosphorylation was increased at two different sites (Fig. 4B and C). TNF- α increased serine phosphorylation on site 312 and 636/639 with different kinetics. Under

insulin-stimulated conditions, TNF- α infusion further increased serine phosphorylation of IRS-1 with the most dramatic response at 180 min (Fig. 4B). Phosphorylation of IRS-1 on site 636/639 was unaltered by TNF- α infusion. Under insulin-stimulated conditions, TNF- α infusion was associated with a dramatic increase in serine phosphorylation of IRS-1 on site 636/639 at 30 min (Fig. 4C). The latter findings were observed in parallel with impaired phosphorylation of IRS-1 on tyrosine residues (Fig. 4A). Moreover, using a polyclonal affinity-purified antibody that recognizes Akt phosphorylation motif peptide sequences [RXRXX(T*/S*)] (20,21), we show TNF- α infusion impairs insulin action of AS160 (Fig. 4D). Reduced AS160 phosphorylation may provide a mechanism for TNF- α -induced insulin resistance on glucose uptake because GLUT4 exocytosis is dependent on AS160 phosphorylation (20,21).

DISCUSSION

In healthy humans, infusion of physiologically relevant systemic concentrations of TNF- α inhibits peripheral insulin-mediated glucose uptake, without influencing endogenous glucose production from the liver. This observation is consistent with the notion that inflammatory pathways are coupled to metabolic control in humans. Our results indicate that TNF- α infusion primarily compromises skeletal muscle insulin action. We resolved this impairment to the level of insulin signaling. The TNF- α concentrations achieved during the in vivo infusion were slightly higher than typically found in patients with low-grade systemic inflammation, such as type 2 diabetes and cardiovascular diseases, but considerably lower than in septic diseases (25). Our finding that a relatively minor elevation in the

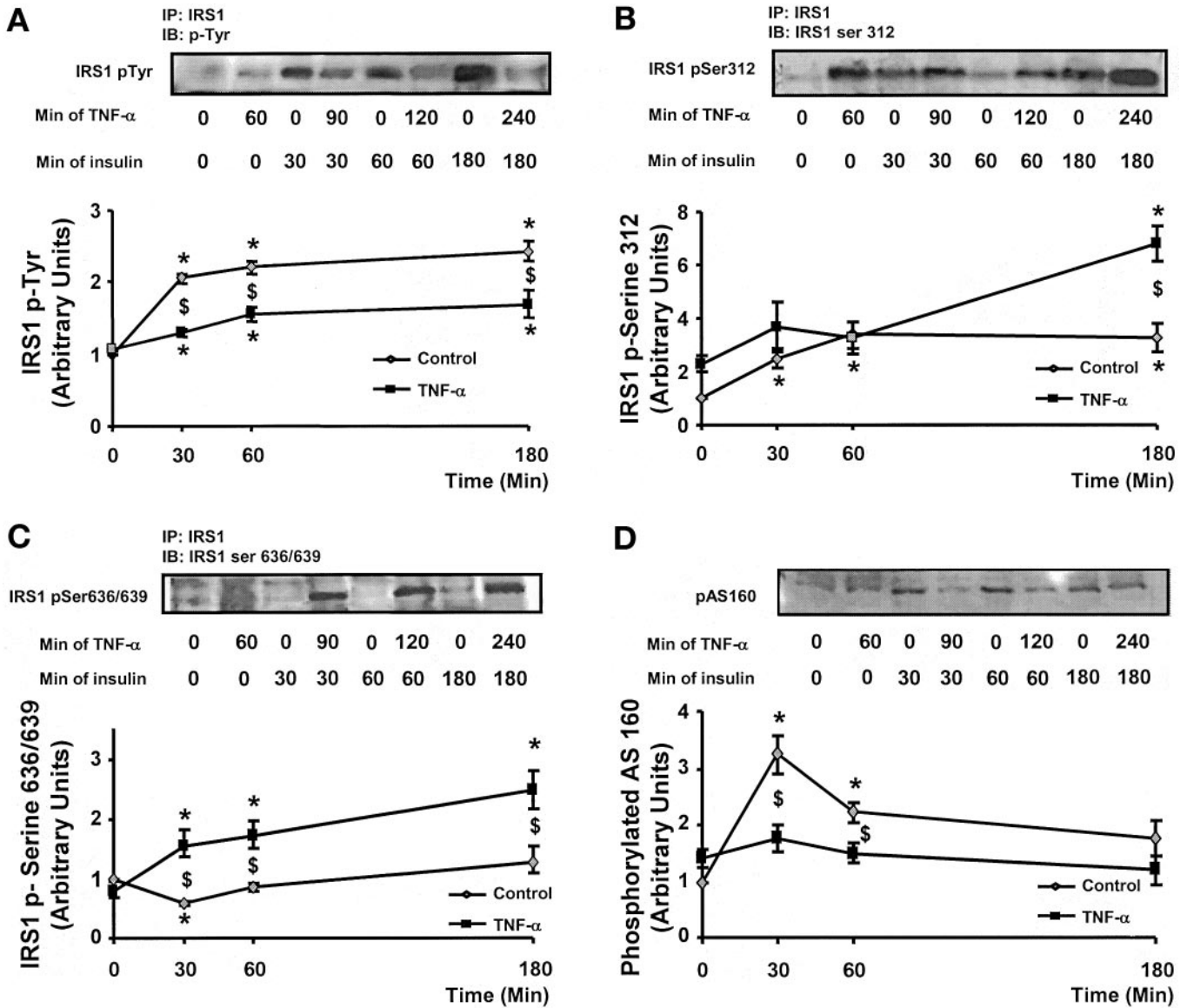


FIG. 4. Effect of TNF- α infusion on insulin signaling. IRS-1 tyrosine phosphorylation (A), IRS-1 serine phosphorylation (sites 312 and 636/639) (B and C, respectively), and AS160 phosphorylation (D) were determined in skeletal muscle. Representative immunoblot (upper panel) and means \pm SE in arbitrary units (graph) are shown. Results are expressed relative to basal phosphorylation (time 0) in saline-treated skeletal muscle. *Difference from the basal condition; \$difference between saline and TNF- α trial.

TNF- α concentration has deleterious effects on peripheral insulin action is of clinical relevance and highlights fundamental differences between rodents and humans in sensitivity to cytokines (26).

TNF- α has been shown to have a direct (insulin-independent) effect on S6K (27) and ERK-1/2 (28) in cultured cells. Here, we provide evidence that TNF- α infusion increases phosphorylation of S6K, ERK-1/2, and JNK, concomitant with increased serine phosphorylation of IRS-1. These direct effects of TNF- α raise the question of whether a prolonged exposure of this cytokine would induce insulin resistance. However, blood glucose concentration was unaltered in fasting subjects after a 3-h TNF- α infusion (29). Furthermore, here, we provide evidence that basal glucose uptake is unaltered after 1 h of TNF- α infusion. Thus, under these acute in vivo conditions, TNF- α has a direct insulin-independent effect on signal transduction in human skeletal muscle that does not appear to modify basal hepatic or peripheral glucose metabolism.

TNF- α impairs insulin-stimulated rates of glucose storage in cultured human muscle cells (30) and whole-body insulin-mediated glucose uptake in rats (31). Here, we provide evidence that TNF- α infusion negatively regulates insulin signaling and whole-body glucose uptake in humans. In cultured cells (14,32) and rodents (33), TNF- α impairs insulin signaling by increasing serine phosphorylation of IRS-1. Phosphorylation of IRS-1 at Ser 307 (site 312 in human IRS-1) is critical for the inhibitory effect of TNF- α , which appears to be mediated by an association of activated JNK with IRS-1 and the insulin receptor, thereby providing a mechanism to partly explain the development of insulin resistance in response to excessive cytokine production (34,35). Under insulin-stimulated conditions, infusion of TNF- α prevented tyrosine phosphorylation of IRS-1 and increased serine phosphorylation of IRS-1 at sites 312 and 636/639 in human skeletal muscle. The impairment in IRS-1 signaling was associated with reduced phosphorylation of AS160, a Rab GTPase-activating protein (20) that plays a role in insulin-stimulated GLUT4 translocat-

tion/exocytosis to the plasma membrane in 3T3-L1 adipocytes (20,21). Insulin-stimulated AS160 phosphorylation is also impaired in skeletal muscle from type 2 diabetic patients (36). Collectively, these studies provide evidence that AS160 plays a role in the regulation of glucose metabolism and represent a target for insulin resistance in response to excessive TNF- α exposure. Thus, TNF- α infusion rapidly promotes skeletal muscle insulin resistance by negatively regulating insulin signal transduction to glucose uptake.

In addition to effects on glucose metabolism, TNF- α has been implicated in endothelial dysfunction. An acute exposure of cultured endothelial cells to TNF- α inhibits insulin signaling and nitric oxide production, concomitant with impaired insulin-stimulated vasodilation (37). The effect of low-grade systemic TNF- α concentration on blood flow in humans is unknown. TNF- α and insulin oppose each other, such that insulin action is most vulnerable to TNF- α -mediated inhibition when insulin concentrations are low (as in the present study) and least vulnerable when insulin concentrations are high (38). In rats, an acute *in vivo* TNF- α infusion completely blocked the hemodynamic effects of insulin on blood flow and capillary recruitment, and it inhibited skeletal muscle glucose uptake (31). Moreover, in a human perfused forearm model, a 200-fold "local" increase in plasma TNF- α concentration was associated with impaired insulin-mediated endothelium-dependent vasodilation and skeletal muscle glucose uptake (39). Clearly, effects of TNF- α on endothelial function may contribute to defects in glucose metabolism. However, our results provide evidence that TNF- α also has a direct effect in skeletal muscle. Phosphorylation of S6K, ERK-1/2, JNK, and IRS-1 at site 312 was directly increased in response to the TNF- α infusion, and this was associated with negative regulation along the canonical insulin signaling cascade and a subsequent impairment in glucose uptake.

TNF- α and IL-6 are early mediators of inflammation. These two cytokines are tightly linked, such that TNF- α induces IL-6 production, which consequently inhibits TNF- α gene expression (25). We observed a gradual rise in IL-6 concentrations during the TNF- α infusion, which could conceivably contribute to the development of skeletal muscle insulin resistance. Elevations in IL-6 may lead to hepatic insulin resistance (40); however, effects on insulin signaling and glucose metabolism in skeletal muscle and adipose tissue are equivocal. IL-6 has been reported to enhance (41) and suppress (42) insulin-stimulated glucose transport in myotubes and adipocytes. In the present study, the deleterious effect of TNF- α on insulin action was localized to skeletal muscle, rather than liver. Thus, we propose that TNF- α , rather than IL-6, is the driver behind the observed impairment in insulin signaling and glucose uptake in skeletal muscle. This interpretation is strengthened by clinical observations in healthy humans stating that endogenous glucose production and peripheral glucose uptake are unaltered by acute infusion of recombinant human IL-6 (43).

Our results provide a molecular link between low-grade systemic inflammation and the metabolic syndrome. Systemic inflammation and insulin resistance accompany a cluster of clinically related diseases that constitute the metabolic syndrome, as well as autoimmune and infectious diseases (1,44,45). The regulation of insulin-mediated glucose uptake in humans is extremely sensitive to cytokines. Excessive TNF- α concentrations negatively regulate

insulin signaling and whole-body glucose uptake in humans. The identification of the molecular targets that mediate the deleterious effects of TNF- α on insulin signaling and glucose metabolism opens the possibility for new therapeutic strategies for treatment of a global clinical health problem.

ACKNOWLEDGMENTS

This study funded from the Commission of the European Communities (contract no. LSHM-CT-2004-005272 Exgenesis), the Swedish Medical Research Council, the Swedish Foundation for Strategic Research, the Novo Nordic Foundation, and the Swedish Diabetes Association. The Centre of Inflammation and Metabolism was supported by a grant from the Danish National Research Foundation (DG 02-512-555). The Copenhagen Muscle Research Centre was supported by grants from the University of Copenhagen Faculty of Health Sciences and the Copenhagen Hospital Corporation. This study also received support from the National Institutes of Health Clinical Nutrition Research Unit (DK 56341) and the Danish Diabetes Association.

We thank Professor Christian Torp-Pedersen, University of Copenhagen, for donating rhTNF- α .

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