

Inhibition of muscle insulin-like growth factor I expression by tumor necrosis factor- α

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Fernández-Celemín, Laura, Nevi Pasko, Valérie Blomart, and Jean-Paul Thissen. Inhibition of muscle insulin-like growth factor I expression by tumor necrosis factor- α . *Am J Physiol Endocrinol Metab* 283: E1279–E1290, 2002. First published July 2, 2002; 10.1152/ajpendo.00054.2002.—The role of TNF- α in muscle catabolism is well established, but little is known about the mechanisms of its catabolic action. One possibility could be that TNF- α impairs the production of local growth factors like IGF-I. The aim of this study was to investigate whether TNF- α can directly inhibit IGF-I gene and protein expression in muscle. First, we investigated whether the acute inflammation induced by endotoxin injection changes IGF-I and TNF- α mRNA in rat tibialis anterior muscle. Endotoxin rapidly increased TNF- α mRNA (7-fold at 1 h, $P < 0.001$) and later decreased IGF-I mRNA (–73% at 12 h, $P < 0.001$). Furthermore, in a model of C₂C₁₂ myotubes, TNF- α strongly inhibited IGF-I mRNA and protein (–73 and –47% after 72 h, $P < 0.001$ and $P < 0.01$, respectively). Other proinflammatory cytokines failed to inhibit IGF-I mRNA. The effect of TNF- α on IGF-I mRNA was not mediated by nitric oxide, and the activation of NF- κ B was insufficient to inhibit IGF-I expression. Taken together, our data suggest that TNF- α induced in muscle after LPS injection can locally inhibit IGF-I expression. The inhibition of muscle IGF-I production could contribute to the catabolic effect of TNF- α skeletal muscle; lipopolysaccharide; C₂C₁₂ cells

A DECREASE IN LEAN BODY MASS is commonly observed in pathological conditions such as sepsis, cancer, AIDS, or chronic inflammatory diseases. This change in body composition may lead to functional alterations that increase mortality and hamper a patient's rehabilitation. The loss of lean mass is mainly due to a loss of skeletal muscle (4). The mechanisms of muscle catabolism involve complex interactions among several mediators and remain nowadays only partially unraveled. Hence, a better understanding of these interactions is important for developing successful therapies able to prevent the decline in lean body mass and reduce morbidity and mortality.

Tumor necrosis factor (TNF)- α is a proinflammatory cytokine that is essential for a successful response against invading pathogens (19, 41, 43). On the other

hand, TNF- α is considered to play a major role in muscle catabolism. First, circulating levels of TNF- α are markedly increased in catabolic states (3). Second, enhanced protein degradation and muscle loss have been observed in TNF- α -transgenic animals (29) after chronic administration of the cytokine (23, 54) or in animals bearing TNF- α -producing tumors (12). Furthermore, administration of anti-TNF- α antibodies or of TNF- α -soluble receptor attenuates the catabolic reaction (28, 42).

Catabolic actions of TNF- α in skeletal muscle can be exerted in different ways. One possibility is that TNF- α acts directly on skeletal muscle to induce muscle catabolism. Indeed, TNF- α has been reported to inhibit protein synthesis and myogenesis in myoblasts (22, 25, 34). Recent observations indicate that TNF- α can also stimulate the proteolysis of myosin heavy chains in C₂C₁₂ myotubes by activating the ubiquitin-proteasome pathway (36). Besides, TNF- α increases apoptotic cell death in skeletal muscle (39). Alternatively, TNF- α can act indirectly to stimulate skeletal muscle catabolism by modifying hormones that regulate protein turnover (15, 53), by increasing the production of other cytokines (53), or by inducing anorexia (54).

Insulin-like growth factor I (IGF)-I is an anabolic growth factor responsible for normal growth and development (18). Autocrine IGF-I production has been demonstrated to play a crucial role in muscle growth (47, 56). In skeletal muscle, IGF-I acts as an anabolic growth factor stimulating protein synthesis (5) as well as proliferation and differentiation of satellite cells (18). IGF-I has also been reported to suppress proteolysis (21, 27) and to inhibit the ubiquitin-proteasome system (11). Finally, IGF-I exerts antiapoptotic effects in muscle, favoring the survival of differentiated cells (35).

Sepsis and endotoxin (LPS) injection, a classical model of acute catabolism, are associated with an increase in circulating TNF- α levels (3) and a decrease in muscle IGF-I (16). Taking into consideration the opposite actions of these two molecules in the control of muscle mass, we hypothesized that the catabolic effects of TNF- α might result from a direct inhibition of

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muscle IGF-I gene expression. The aims of this study were first to assess whether the acute inflammation caused by LPS is associated with a reduction in muscle IGF-I mRNA and an increase in muscle TNF- α mRNA and second to investigate the role of TNF- α in the decrease of muscle IGF-I caused by LPS.

MATERIALS AND METHODS

Materials. For in vivo experiments, lipopolysaccharide of *Escherichia coli* (LPS, serotype 0127:B8) was obtained from Sigma Chemical (St. Louis, MO) and was diluted to 1 mg/ml in sterile, endotoxin-free saline. For in vitro experiments, recombinant murine TNF- α , interleukin-1 β , and interferon- γ (rmTNF- α , rmIL-1 β , rmIFN- γ) and recombinant rat IL-6 were purchased through R&D Systems (Abingdon, UK). NUNCLOON plastic dishes were purchased from NUNC (Roskilde, Denmark), and Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, horse serum (lot: 3010240D), nonessential amino acids, and L-glutamine were purchased from GIBCO-BRL Life Technologies (Paisley, UK).

In vivo experiment. Male Wistar rats (Katholieke Universiteit of Leuven, Leuven, Belgium) $\sim$ 8 wk of age (218 ± 9.2 g; mean $\pm$ SD) were maintained for 1 wk under standardized conditions of light (12:12-h light-dark cycle) and temperature ($22 \pm 2^\circ\text{C}$). Access to animal chow was available only between 6:00 PM and 9:00 AM, whereas access to water was unrestricted. The morning of the 8th day, rats were injected intraperitoneally with LPS (750 $\mu\text{g}/100$ g body wt) and killed at different times after the injection. Tibialis anterior muscles were dissected, flash-frozen in liquid nitrogen, and stored at -80°C . The choice of tibialis anterior, a fast muscle, was based on the observation that fast muscles, composed mainly of white fiber and weakly resistant to fatigue, are more sensitive than slow muscles to catabolic stimuli (26, 17).

Muscle incubations. Male Wistar rats (221 ± 12 g, 7 wk old, $n = 4$; Janvier Breeding, Le Genest-Saint-Isle, France) were anesthetized with a mixture of Ketalar/Rompum (75:15 mg/kg body wt ip), and the extensor digitorum longus (EDL) muscles were gently dissected with intact tendons and mounted on supports at approximate resting length. Individual muscles were preincubated for 30 min and then incubated for 3 h in 20 ml of oxygenated Krebs-Ringer bicarbonate solution at 25°C in the presence or absence of LPS (10 $\mu\text{g}/\text{ml}$). Previous studies had demonstrated that, at 25°C , the histochemical and mechanical properties of muscles are better conserved than at temperatures above 25°C (45). After the 3-h incubation, muscles were deep-frozen in liquid nitrogen, and RNA extraction and Northern blot were performed as described in *RNA extraction and Northern blot analysis*.

C₂C₁₂ cell culture. Myoblasts from the muscle-derived C₂C₁₂ cell line were obtained from American Type Culture Collection (Manassas, VA). The seeding density used throughout the experiments was 0.4×10^6 cellules/plate of 10 mm diameter. Undifferentiated cells were grown in DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin (100 U $\cdot$ 100 $\mu\text{g}^{-1} \cdot \text{ml}^{-1}$), 1% nonessential amino acids, and 2% L-glutamine at 37°C in the presence of 5% CO₂. This medium will be referred to as growth medium (GM). When cells reached 90–100% confluence (after 3 days), 10% heat-inactivated fetal calf serum was replaced by 2% heat-inactivated horse serum to induce myogenic differentiation. This medium will be referred to as differentiation medium (DM). Muscle cells were examined for evidence of myotube formation and growth by use of an inverted Olympus IMT microscope (Olympus Optical, Hamburg, Germany). The me-

dium was changed every 48 h, and differentiation was allowed to continue for 96 h (4 days) before the experimentation period. To preserve the characteristics of the C₂C₁₂ cell line, the splitting of cells was done up to a maximum of seven times. The experiments lasted from 24 to 72 h, and the cytokines or other molecules used were added to the DM every 24 h at the doses indicated in the figure legends.

RNA extraction and Northern blot analysis. For in vivo experiments, RNA was extracted from tibialis anterior muscle with TRIzol reagent (Life Technologies, Paisley, UK) following the instructions of the manufacturer. Briefly, 100 mg of tibialis anterior muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-labor-technik, Staufen, Germany) in 1 ml of TRIzol reagent and centrifuged at 12,000 g for 10 min at 4°C . The supernatant was then added with chloroform, thoroughly vortexed, incubated for 10 min at room temperature, and centrifuged at 12,000 g for 15 min at 4°C . The aqueous phase containing the RNA was retained, and RNA was precipitated with isopropanol. The RNA pellet was washed with 75% ethanol, dried, resuspended in diethyl pyrocarbonate-treated water, and stored at -80°C . The concentration of total RNA was determined by spectrophotometry. For in vitro experiments, the cells were washed once with $1 \times$ PBS, pH 7.4, and then each plate was collected in 1.5 ml of TRIzol and processed as described for the in vivo experiments.

Twenty micrograms of total RNA from each sample were denatured in formaldehyde-MOPS and subjected to electrophoresis on 1% agarose gels. Homogeneity of the loading was assessed by UV transillumination of the gels after staining with ethidium bromide. The RNA was transferred to nylon membranes (Hybond N; Amersham, Buckinghamshire, UK) by capillary overnight blotting, and levels of IGF-I, IGF-II, or TNF- α mRNA were determined by hybridization with specific probes. For IGF-I, a 194-bp *Avall-HinfI* rat IGF-I exon 4 cDNA (15) was inserted into the plasmid vector Bluescript pBSM13+ (Stratagene, La Jolla, CA) and linearized with *HindIII*. This probe includes only the coding region for the mature IGF-I peptide and detects all IGF-I mRNAs. For IGF-II, a 275-bp *RSA1* rat IGF-II exon 3 (coding region of IGF-II) cDNA was ligated into the pBS+ (Stratagene) and linearized with *EcoRI*. Specific ^{32}P -labeled RNA antisense probes were generated from linearized plasmids with uridine 5'-[α - ^{32}P]triphosphate (800 Ci/mmol; Amersham) by use of T7 (IGF-I) or T3 (IGF-II) RNA polymerase. Levels of TNF- α mRNA were determined by hybridization with a random-primed [^{32}P]dCTP-labeled TNF- α probe (p6r TNF- α) encompassing the rat TNF- α coding sequence (amino acids 44–231) (40). To verify uniform loading, control hybridization was performed with a 23-mer 18S oligonucleotide end-labeled with adenosine [γ - ^{32}P]triphosphate by T4 polynucleotide kinase (Amersham).

For IGF-I and IGF-II, blots were prehybridized for 3–4 h at 65°C in buffer containing NaCl (0.8 M), Na phosphate (50 mM), EDTA (0.5 mM), Denhardt's (2 g/l each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), salmon sperm DNA (200 $\mu\text{g}/\text{ml}$), and formamide (50%). For TNF- α , blots were prehybridized for 4 h at 50°C in buffer containing NaCl (0.75 M), Na citrate (75 mM), SDS (0.25%), Denhardt's (2 g/l each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), salmon sperm DNA (200 $\mu\text{g}/\text{ml}$), and formamide (50%). They were then hybridized with the radiolabeled antisense RNA probe or cDNA probe (1×10^6 cpm/ml of buffer) for 16–18 h in a shaking water bath at 65°C (for IGF-I or IGF-II) and at 50°C (for TNF- α). After hybridization, blots were washed several times. For IGF-I or IGF-II, blots were washed rapidly once with 300 ml of washing buffer (in mM: 20 Na

phosphate, 50 NaCl, and 1 EDTA and 0.1% SDS) at room temperature and then more extensively twice for 30 min in a shaking waterbath at 70°C. For TNF- α , blots were washed twice for 20 min with 300 ml of washing *buffer I* (0.3 M NaCl, 30 mM Na citrate, 0.1% SDS) at room temperature and then twice with 300 ml of washing *buffer II* (0.03 M NaCl, 3 mM Na citrate, 0.25% SDS) for 20 min in a shaking water bath at 55°C. Membranes were finally exposed to XAR-5 Kodak film for several hours to several days at -80°C. The mRNA levels were quantified by densitometric scanning of the hybridization signal (LKB Ultrosan XL laser densitometry; LKB, Bromma, Sweden) with the use of software (Gel Scan, Pharmacia & Upjohn, Brussels, Belgium). IGF-I mRNA transcripts, as visualized by Northern blot analysis, presented a complex picture consisting of a large (7.5 kb) transcript, a group of transcripts ranging from 0.8 to 1.2 kb, and two additional minor transcripts of 1.7 and 4.7 kb. Because all of these transcripts may potentially be translated into IGF-I precursors, we performed a densitometric analysis of the four bands visible on the blot. For IGF-II, we considered 1.5- to 2.2-kb transcripts and a 4.6-kb transcript for the densitometric analysis. Densitometric values were normalized by assigning to the mRNA levels observed after 96 h postdifferentiation (D4, day on which experiments were initiated) an arbitrary value of 100%.

Cytoplasmic and nuclear extracts. After cells were washed twice with ice-cold PBS and harvested in 500 μ l of cold *buffer A* (10 mM HEPES-KOH, pH 7.9, 5% glycerol, 100 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μ g/ml leupeptin, and 2 μ g/ml aprotinin), cytoplasmic extracts were prepared by subjecting the cells to three cycles of freezing-thawing. Supernatants were then collected after samples were centrifuged for 10 s in a microcentrifuge. Nuclear extracts were prepared by resuspending the pellets in cold *buffer B* (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μ g/ml leupeptin, and 2 μ g/ml aprotinin). The resuspended pellets were mixed and incubated at 4°C for 20 min and centrifuged for 10 min in a microcentrifuge at 4°C. Supernatants were then collected and stored at -80°C. Protein content of the cytoplasmic and nuclear extracts was determined using Bradford's protein assay (Bio-Rad, Munich, Germany).

Electrophoretic mobility shift assay. For the electrophoretic mobility shift assay (EMSA), preannealed, chromatography-purified, double-stranded oligonucleotides containing the consensus DNA-binding sequence for NF- κ B (5'-TCGAGGGCT-GGGGATTCCCCATCTC-3') were radiolabeled with [γ -³²P]-ATP, and EMSA was performed as previously described (48). Briefly, 10 μ g of nuclear proteins (prepared as described) were incubated with radiolabeled DNA probe for 20 min at room temperature and then electrophoresed on a 5% polyacrylamide gel. To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotide was incubated with the nuclear extracts for 5 min at room temperature before the addition of the radiolabeled probe and then incubated for 20 min.

For supershift, 4 μ l (0.8 μ g/ml) of anti-p50- or anti-p65-specific polyclonal antibody for the NF- κ B subunits were incubated with the extract samples for 5 min before the addition of the labeled probe. NF- κ B p65 and p50 antibodies are, respectively, rabbit and goat affinity-purified polyclonal antibodies raised against a peptide mapping the carboxy terminus of NF- κ B p65 or p50 of human origin and identical to the corresponding mouse sequence (Santa Cruz Biotechnology, Santa Cruz, CA). Gels were dried and exposed for 20 h to a Hyperfilm MP (Amersham).

Creatine kinase activity. Myogenic differentiation was assessed biochemically on cytoplasmic extracts (prepared as described) via determination of muscle creatine kinase (CK) activity with a SYNCHRON LX Systems reagent kit (Beckman Coulter) following manufacturer's instructions. Specific CK activity was calculated after correction for total protein.

Nitrite determination. Nitrite (NO₂⁻) is a stable end product used extensively as an indicator of NO production by cultured cells. In our experimental conditions, nitrite accumulation was assayed by the Griess reaction, according to the method described previously (20). Briefly, 400 μ l of cell culture medium were mixed with three times the amount of Griess reagent (1% sulfanilamide, 0.1% naphthylendiamine, 2.5% H₃PO₄). Samples were incubated at room temperature for 10 min, and absorbance was subsequently read at 543 nm by a spectrophotometer. Nitrite concentrations were calculated compared with a sodium nitrite (NaNO₂) standard curve.

End point PCR of TNF- α receptors. For PCR assay, 1 μ g of total RNA from C₂C₁₂ myotubes was reverse transcribed into cDNA in the presence of 1 $\times$ RT-PCR buffer (Superscript kit no. 18053-07, Life Technologies), 4.44 mM of dNTPs (no. 1581295, Roche Diagnostics), 88.8 pM of random hexamers (Clinical Molecular Biology Unit, St Luc Hospital, Brussels, Belgium), 11 mM DTT, and 50 U of superscript reverse transcriptase (Superscript Kit, Life Technologies) per reaction. The final volume was 18 μ l, and cDNA synthesis was performed in the GeneAmp PCR system 2400 (Perkin-Elmer, Foster City, CA) as follows: 22°C for 10 min, 42°C for 1 h, and 99°C for 5 min.

End point PCR amplification was then carried out on 3 μ l of the cDNA product, with 1 $\times$ PCR buffer (Perkin-Elmer kit N808-0161), 1 mM MgCl₂, 0.8 mM dNTP (no. 1581295; Roche Diagnostics), 0.5 mM of specific primers, and 3.5 U/reaction of *Taq* DNA polymerase (PWO-expand; Roche Diagnostics); the final volume was 50 μ l. End point PCR was performed in the GeneAmp PCR system 2400 (Perkin-Elmer) as follows: 94°C for 1 min followed by 45 cycles [94°C for 1 min; 60°C (p60) and 58°C (p80) for 2 min; 72°C for 3 min]. The primer pairs used to amplify TNF- α Rp60 and Rp80 cDNA (GenBank nos. M59377 and M59378, respectively) are shown in Table 1. Negative control reverse transcription and amplification reactions were also run without RNA input or cDNA input. A portion (20 μ l) of RT-PCR product was electrophoresed in 1% agarose gel in 1 $\times$ Tris-borate-EDTA buffer (89 mM Tris base, 89 mM boric acid, 200 mM EDTA), to-

Table 1. Summary of primer sequences

End Point PCR	Sequences	Amplicon
TNFR1	Forward 5'-gcc cga agt cta ctc cat cat ttg tag gg-3' Reverse 5'-cat cca cca cag cat aca gaa tcg caa gg-3'	371 bp
TNFR2	Forward 5'-ata cta tga cag gaa ggc tca gat gtg c-3' Reverse 5'-ccc ttg gta ctt tgt tca ata atg ggg g-3'	636 bp

TNFR1 and -2, TNF- α receptors 1 and 2, respectively.

gether with positive controls (mouse spleen and WEG-1 cell cDNAs) and DNA molecular weight markers from 8 to 587 bp (Roche, Mannheim, Germany). The gel was stained with ethidium bromide and photographed under UV transillumination.

IGF-I RIA. Culture medium concentrations of IGF-I were measured by RIA after acid extraction of medium samples with 0.5 N HCl in silicic acid columns (C₁₈ Sep-Pak cartridges from Waters, Milford, MA), as described previously (13). The rabbit IGF-I antiserum (UB-487) was used at a dilution of 1:40,000 and was a kind gift from Dr. L. E. Underwood (Chapel Hill, NC).

Statistical analysis. Statistical significance between samples was determined by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparisons test. For in vivo experiments, each point is the mean $\pm$ SE of seven or eight animals. For in vitro experiments, each point is the mean $\pm$ SE of values obtained in three or six separate experiments, each performed in one new culture of C₂C₁₂ cells. Statistical significance was set at $P < 0.05$.

RESULTS

Regulation of muscle expression of IGF-I and TNF- α by LPS. In a first step, we investigated the changes produced by LPS injection in muscle TNF- α mRNA and IGF-I mRNA.

Basal TNF- α gene expression was very low in tibialis anterior muscle of intact animals. LPS administration induced a rapid peak of TNF- α mRNA between 1 and 3 h, with a 7-fold increase at 1 and 2 h and a 5.5-fold increase at 3 h ($P < 0.001$ vs. time-matched saline group). Afterward, TNF- α transcripts decreased to reach basal values by 12 h (Fig. 1A). Animals continued

to display symptoms of infection (gastric dilatation, decreased mobility, piloerection) until 12 h. Furthermore, LPS injection resulted in a progressive inhibition of tibialis anterior IGF-I gene expression that became significant after 6 and 12 h (61 and 73% reduction, respectively, $P < 0.001$ vs. time-matched saline group; Fig. 1B). This reduction was not due to the anorectic effect of LPS, as LPS-injected and saline-treated animals fasted throughout the day of the experiment.

Although LPS significantly decreased IGF-I gene expression in skeletal muscle, it did not seem to affect IGF-II expression (data not shown).

Muscle TNF- α and IGF-I mRNA levels in saline-injected animals were not different from those in non-injected controls (time 0 h), indicating that the injection procedure had no effect by itself on TNF- α and IGF-I gene expression (Fig. 1).

In vitro experiments with EDL muscles incubated in the presence or absence of LPS demonstrated that LPS is capable of rapidly inducing TNF- α gene expression in skeletal muscle (2-fold increase vs. control, $P < 0.001$; Fig. 2).

Regulation of myotube expression of IGF-I by TNF- α . Initial experiments served to characterize the time frame of C₂C₁₂ cell differentiation after replacement of GM with DM. This was done through analysis of cell morphology and muscle-specific differentiation markers. By day 4 of differentiation, myotubes were long and multinucleated and displayed contractile properties. The activity of CK, total cytoplasmic protein content, and myosin heavy-chain (MHC) content increased

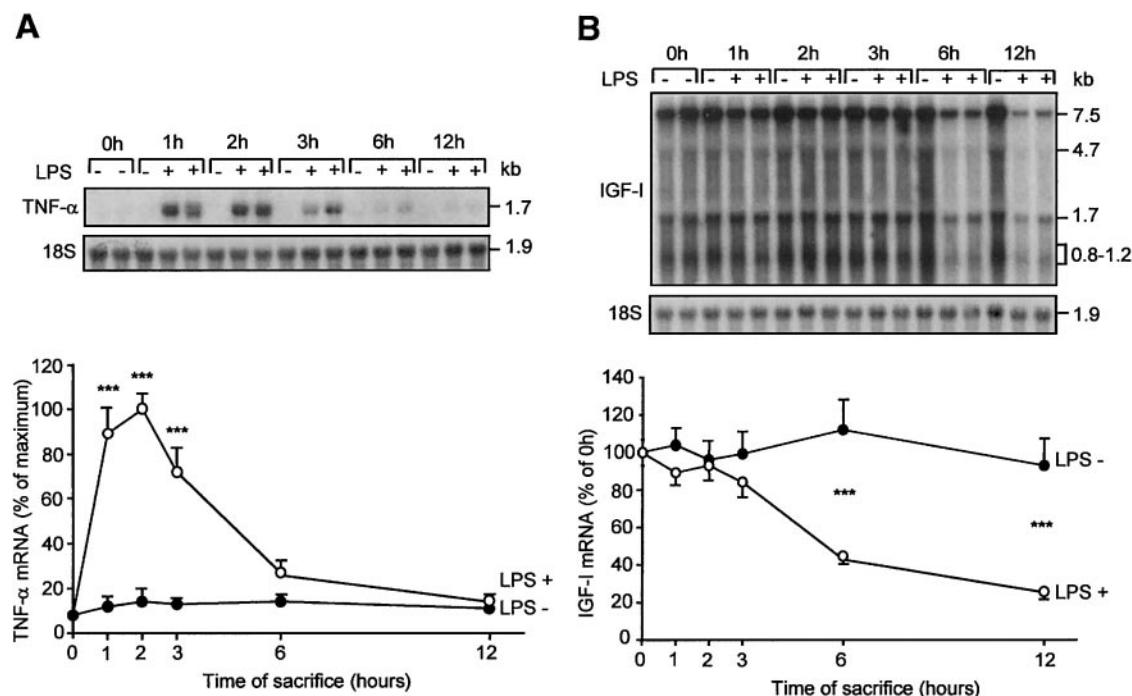


Fig. 1. Effect of LPS injection on TNF- α (A) and IGF-I (B) mRNA in rat tibialis anterior muscle. Male Wistar rats were injected ip with either 750 μ g LPS/100 g body wt ($n = 7-8$) or saline ($n = 4$) and killed 1, 2, 3, 6, or 12 h after the injection. After tibialis anterior muscle RNA was extracted, 10 μ g of total RNA were electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE. *** $P < 0.001$ vs. time-matched saline.

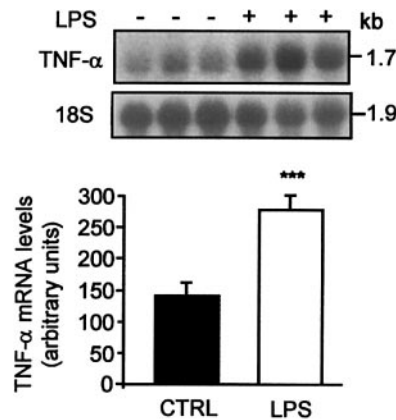


Fig. 2. Induction of TNF- α mRNA in extensor digitorum longus (EDL) muscles incubated with LPS. Individual EDL muscles from normal rats were incubated for 3 h in the presence or absence of LPS (10 μ g/ml). After the incubation, muscles were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 4 separate experiments. *** P < 0.001 vs. control.

with differentiation (34). Because all of these parameters were stable between *day 4* and *day 8* of differentiation, experimental procedures were performed from *day 4*.

Inhibition of IGF-I gene and protein expression by TNF- α . Because *in vivo* results suggested a causal relationship between the increase in TNF- α expression and the reduction of IGF-I mRNA levels, we began by investigating the effect of this proinflammatory cytokine on the IGF-I gene expression in the C₂C₁₂ myotubes. In this model, TNF- α has been reported to exert a catabolic action leading to decrease in MHC content (37).

Four-day-differentiated myotubes were incubated for 2, 4, 6, 8, or 10 h in the presence or absence of TNF- α (5 ng/ml). TNF- α resulted in a progressive de-

crease of IGF-I mRNA levels to 48% of the time-matched control values at 8 h and to 62% at 10 h (Fig. 3A). A longer exposure of myotubes to TNF- α emphasized its inhibitory effect, further diminishing IGF-I mRNA levels by 66% at 24 h (P < 0.05), 69% at 48 h (P < 0.001), and 73% at 72 h (P < 0.001) vs. time-matched control expression (Fig. 3B). As expected from the decrease in IGF-I mRNA levels, exposure of myotubes to TNF- α for 72 h reduced IGF-I medium concentrations by 47% (control: 1.55 ± 0.25 ng/ml vs. TNF- α : 0.82 ± 0.08 ng/ml; P < 0.01, n = 6).

TNF- α decreased IGF-I mRNA levels in a dose-dependent manner. Incubation of 4-day-differentiated myotubes with 1 ng/ml of the cytokine decreased IGF-I mRNA by 27% compared with control values (P < 0.05). A dose of 5 ng/ml, which was used throughout the experiments unless otherwise specified in the figure legends, resulted in a 70% reduction of IGF-I transcripts (P < 0.001 vs. control). Higher doses of TNF- α (≤ 50 ng/ml) did not inhibit further IGF-I gene expression (Fig. 4 and data not shown).

Because mature C₂C₁₂ myotubes continue to express IGF-II, we studied whether TNF- α was able to cause IGF-II gene expression to decrease. Exposure of myotubes to 5 ng/ml TNF- α for 24, 48, or 72 h did not affect IGF-II transcripts, suggesting that TNF- α effect on IGF-I mRNA did not result from a generalized inhibitory effect on gene expression (Fig. 5).

Reversibility and relation to differentiation state of the inhibition of IGF-I gene expression by TNF- α . The inhibition of IGF-I mRNA levels induced by 24-h exposure to TNF- α (5 ng/ml) was totally reversed by removal of the cytokine from the conditioned medium. Indeed, IGF-I mRNA values were restored to normal 24 h after TNF- α was removed (Fig. 6). This result indirectly indicates that the TNF- α inhibitory effect on IGF-I mRNA is independent of a cytotoxic effect of the cytokine.

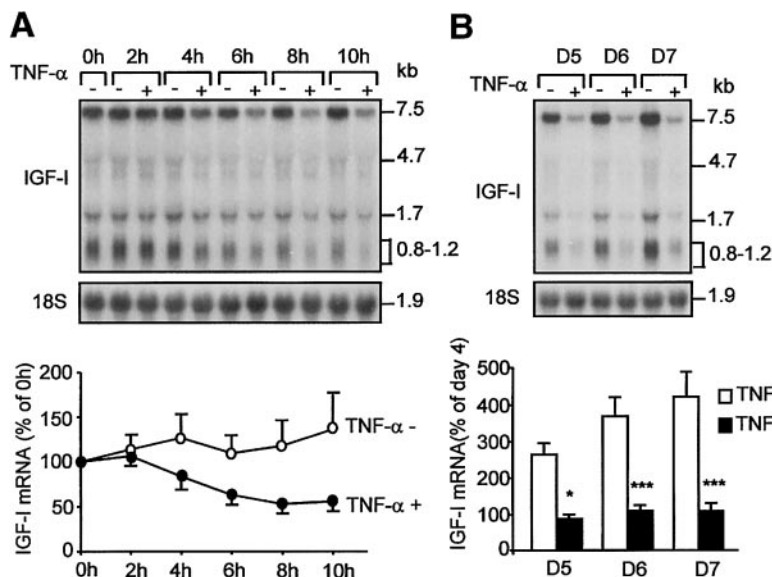


Fig. 3. Time-dependent effect of TNF- α on IGF-I mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for a short-term period (A) or a long-term period (B) with TNF- α (5 ng/ml). D5, D6, and D7, 24, 48, and 72 h of incubation, respectively. At each time point, cells were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 (A) or 6 (B) separate experiments. * P < 0.05 and *** P < 0.001 vs. time-matched control.

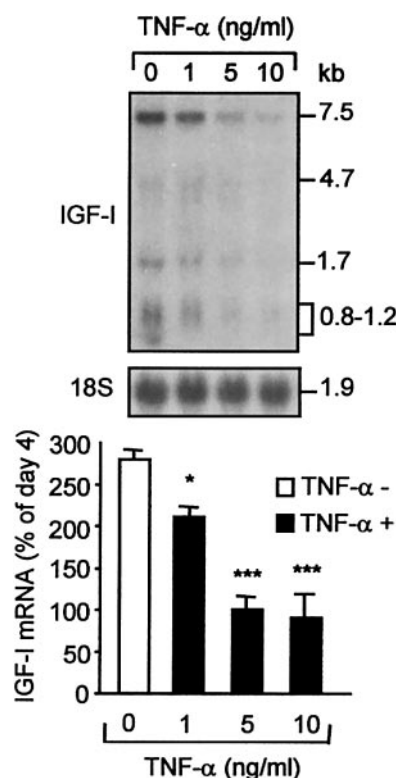


Fig. 4. Dose-dependent effect of TNF- α on IGF-I mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 h with 1, 5, or 10 ng/ml of TNF- α . At each point, cells were collected and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 separate experiments. * P < 0.05 and *** P < 0.001 vs. control (0 ng/ml TNF- α).

After 72 h of TNF- α treatment (5 ng/ml), myotube CK activity was slightly but significantly decreased (P < 0.01 vs. control; Fig. 7), suggesting a modest inhibitory effect of TNF- α on cell differentiation.

Effect of other proinflammatory cytokines and LPS on IGF-I gene expression. To determine whether other proinflammatory cytokines might be involved in the decrease of muscle IGF-I mRNA observed in vivo in response to LPS, we tested the effect of IL-1 β , IL-6, and IFN- γ (5 ng/ml) on 4-day-differentiated myotubes. Although, as expected, TNF- α decreased IGF-I mRNA levels at all times tested (P < 0.05 at 24 h, P < 0.01 at 48 h, and P < 0.001 at 72 h vs. time-matched control group), none of the other cytokines (IL-1 β , IL-6, or IFN- γ) modified consistently the IGF-I mRNA levels in muscle cells (Fig. 8A). All together, these observations indicate that, of all the cytokines tested, TNF- α is the only one to significantly inhibit IGF-I mRNA.

Because LPS injection resulted in decreased IGF-I mRNA levels in vivo, we studied whether LPS could directly influence IGF-I mRNA in differentiated myotubes. After 24 h of exposure to LPS (2 μ g/ml), IGF-I gene expression was slightly decreased, albeit in a nonsignificant way (Fig. 8B).

Effect of the combination of TNF- α and IFN- γ on IGF-I gene expression. Because IFN- γ can potentiate some TNF- α actions (50), we investigated the effect of the combination of these two cytokines on IGF-I gene expression in C₂C₁₂ myotubes. Four-day-differentiated myotubes were treated for 24 h with TNF- α (3 ng/ml) and IFN- γ (5 ng/ml) alone or in combination. As expected, IFN- γ did not significantly modify IGF-I mRNA (29% decrease, P > 0.05), whereas TNF- α alone caused a 74% decrease of IGF-I mRNA levels (P < 0.01 vs. control). When myotubes were challenged with the combination of TNF- α and IFN- γ , the IGF-I mRNA levels were further decreased to 90% of the control values (P < 0.001 vs. control and P < 0.05 vs. TNF- α alone), suggesting more an additive action of both cytokines than a potentiation (Fig. 9).

Expression of TNF- α receptors on C₂C₁₂ myotubes. To assess the nature of TNF- α receptor isoforms expressed in C₂C₁₂ myotubes, total cDNA was prepared and amplified for transcripts encoding the different TNF- α receptor isoforms p60 (TNFR1) and p80 (TNFR2). Both types of TNF- α receptors (TNFR1 and TNFR2) were found to be expressed at the mRNA level in this cell line (Fig. 10).

Stimulation of NO production by TNF- α and IFN- γ . Inducible nitric oxide synthase (iNOS) plays an important role in the transduction of TNF- α signal in catabolic states (7). Hence, we examined the possibility that TNF- α inhibition of the IGF-I gene would be mediated through NO production. Myotubes were stimulated for 24 h with TNF- α (3 ng/ml), IFN- γ (5 ng/ml), or their combination. Neither TNF- α nor IFN- γ exposure increased nitrite production in C₂C₁₂ myo-

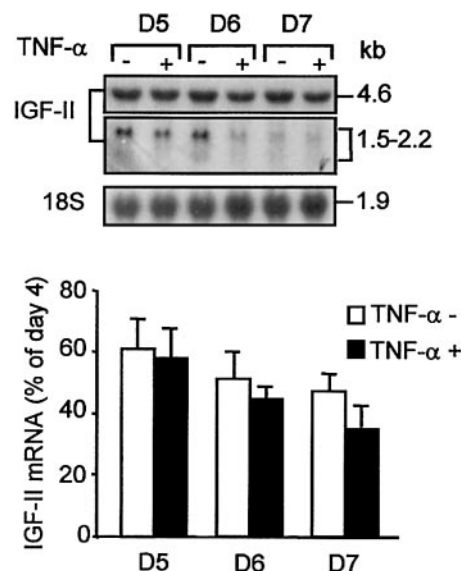


Fig. 5. Effect of TNF- α on IGF-II mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 (D5), 48 (D6), or 72 h (D7) with TNF- α (5 ng/ml). At each time point, cells were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 separate experiments.

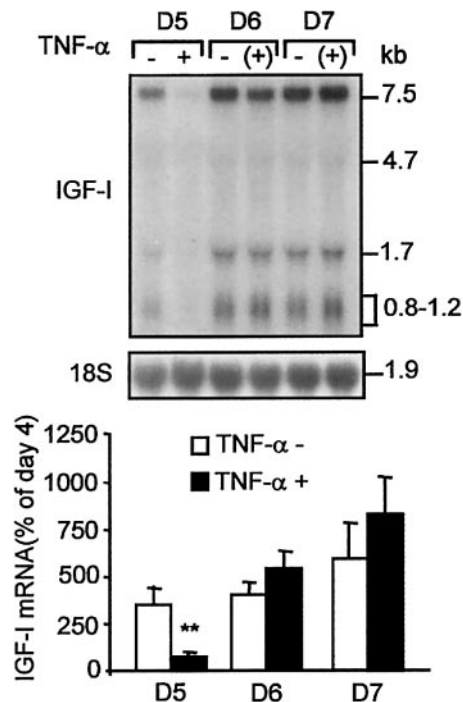


Fig. 6. Reversibility of TNF- α effect on IGF-I mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 h (D5) with TNF- α (5 ng/ml). After 24 h, medium was changed to control medium, and cells were incubated for another 24 (D6) or 48 h (D7) without the cytokine. At each time point, cells were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 separate experiments. ** P < 0.01 vs. time-matched control.

tubes. However, marked nitrite production occurred only when myotubes were stimulated with the combination of TNF- α and IFN- γ (47-fold the control values; P < 0.001; Table 2). This result clearly shows a synergy of action between TNF- α and IFN- γ on NO production in our model.

Activation of NF- κ B by TNF- α and IL-1 β . Because NF- κ B activation is essential for the inflammatory response (51), we assessed whether this transcription factor plays a role in the inhibitory effect of TNF- α on IGF-I gene expression. Our data show that exposure of 4-day-differentiated myotubes for 30 min to TNF- α (3 ng/ml) activates NF- κ B (Fig. 11). The incubation of nuclear extracts with specific antibodies demonstrated that the signal was indeed NF- κ B and that it was composed mainly of the subunits p50 and p65. Further incubation of the nuclear extracts with an excess of cold, homologous probe or with a heterologous Sp1 probe allowed us to confirm the specificity of the NF- κ B oligonucleotide complex formed (Fig. 11).

To assess the requirement of NF- κ B activation for the inhibition of IGF-I mRNA by TNF- α , we tested whether IL-1 β , another proinflammatory cytokine that was unable to inhibit IGF-I expression, activated NF- κ B transcription factor. Incubation of myotubes for 30 min with IL-1 β (3 ng/ml) was, in fact, capable of NF- κ B activation (Fig. 12). Because both TNF- α and

IL-1 β activated NF- κ B but only TNF- α inhibited IGF-I gene expression, we can conclude that NF- κ B activation by TNF- α is insufficient to inhibit IGF-I gene expression.

DISCUSSION

Our results show that LPS injection induces a rapid increase of TNF- α expression and a later inhibition of IGF-I expression in skeletal muscle, suggesting the possibility of a causal relationship between these two changes. This hypothesis was confirmed in a model of differentiated C₂C₁₂ myotubes, where we observed that TNF- α inhibits IGF-I gene and protein expression.

In our *in vivo* model, TNF- α mRNA levels are induced rapidly and transiently in tibialis anterior after LPS administration. This indicates that the stimulation of TNF- α gene expression by LPS is not restricted to diaphragm tissue, about which it was originally described (46), but is probably a generalized response of skeletal muscles. These observations also support the hypothesis that the catabolic action of TNF- α in skeletal muscle is exerted in an autocrine/paracrine way. Our data do not allow us to identify the nature of the cells expressing TNF- α in skeletal muscle. Although the possibility exists that TNF- α is produced, at least partially, by the myocytes (15, 44), in our model of C₂C₁₂ myotubes, LPS failed to induce significant TNF- α expression (data not shown). In contrast, TNF- α expression was stimulated in whole muscles incubated in the presence of LPS. From these observations, we speculate that cells other than myocytes, probably immune cells, are the main contributor of TNF- α production in muscles exposed to LPS.

The mechanisms for increased TNF- α expression in skeletal muscle after LPS injection are still unknown. This effect could result from a direct effect of LPS on skeletal muscle, or it could be mediated by circulating TNF- α produced principally by the liver in response to LPS. The first possibility is strongly supported by our observation that LPS can act directly on muscle to increase TNF- α expression. On the other hand, the second possibility is suggested by the fact that TNF- α

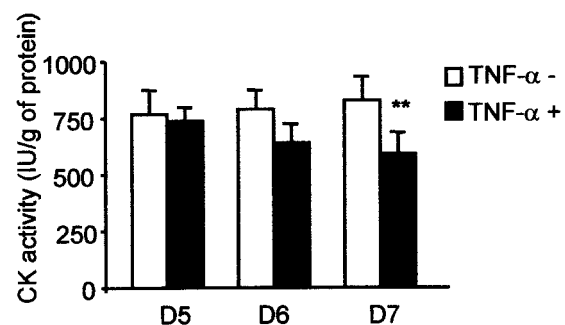
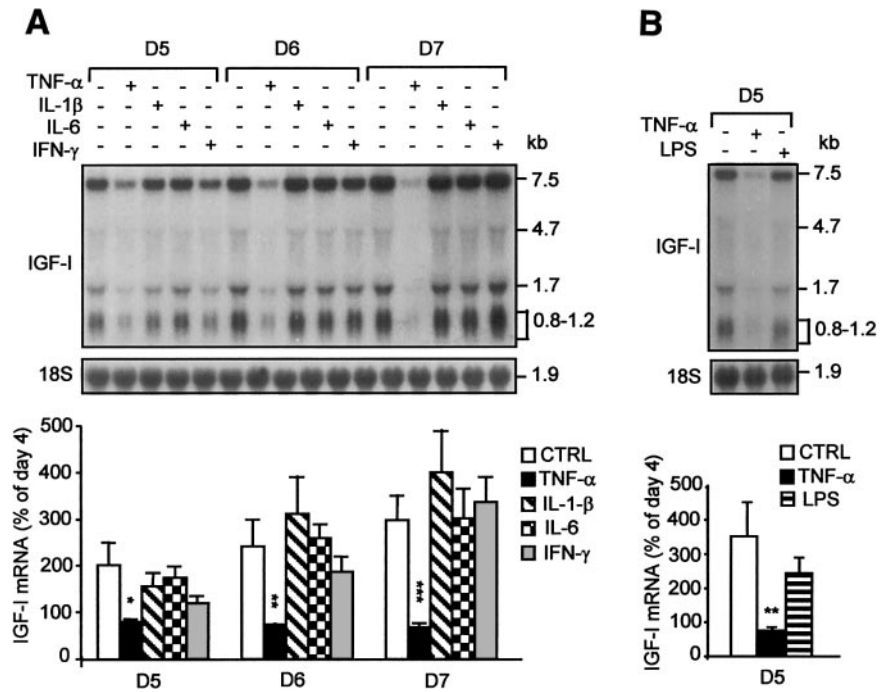


Fig. 7. Effect of TNF- α on creatine kinase (CK) activity of C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 (D5), 48 (D6), or 72 h (D7) with TNF- α (5 ng/ml). CK activity was measured in cytoplasmic extracts, as indicated in MATERIALS AND METHODS, and normalized for total protein. Data are expressed as means $\pm$ SE of 3 separate experiments. ** P < 0.01 vs. time-matched control.

Fig. 8. Effect of different proinflammatory cytokines and LPS on IGF-I mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 (D5), 48 (D6), or 72 h (D7) with 5 ng/ml of TNF- α , IL-1 β , IL-6, or IFN- γ (A) or for 24 h (D5) with 2 μ g/ml of LPS (B). At each time point, cells were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 separate experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. time-matched control.



induces its own expression in vitro, both in immune cells and in myocytes (1, 15, 53, 57). However, the concomitant induction of TNF- α in liver and muscle

after LPS injection favors a major direct role of LPS in the induction of muscle TNF- α .

LPS injection caused a decrease in muscle IGF-I mRNA levels. This is in agreement with previous work showing that LPS injection decreases immunoreactive IGF-I and IGF-I mRNA in gastrocnemius muscle (31). Because gastrocnemius is a mixed-fiber muscle and tibialis anterior a fast-fiber muscle, the decrease in IGF-I gene expression after LPS does not seem to be restricted to a specific type of muscle.

The time course of TNF- α induction and IGF-I inhibition caused by LPS in skeletal muscle suggests a causal relationship between these two changes. This hypothesis is strengthened by the parallelism with the liver situation, where LPS increases TNF- α and de-

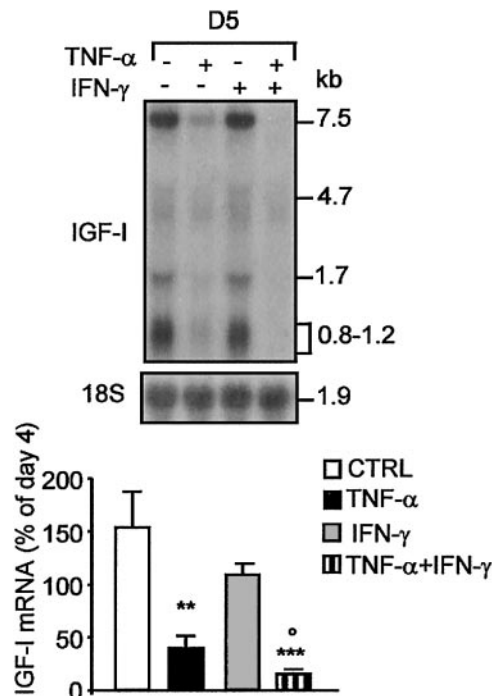


Fig. 9. Effect of TNF- α and IFN- γ on IGF-I mRNA in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 h (D5) with TNF- α (3 ng/ml), IFN- γ (5 ng/ml), or their combination. Cells were collected, and total RNA was extracted. Total RNA (20 μ g) was electrophoresed in each lane, and Northern blot analysis was performed as indicated in MATERIALS AND METHODS. Top: autoradiography; bottom: densitometric analysis. Data are expressed as means $\pm$ SE of 3 separate experiments. ** P < 0.01, *** P < 0.001 vs. control, and ° P < 0.05 vs. TNF- α alone.

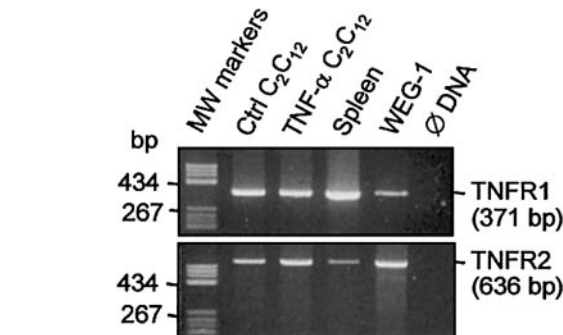


Fig. 10. Presence of TNF- α receptor isoforms in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 h in the presence or absence of TNF- α (5 ng/ml). Then, myotube total RNA was extracted, and RT-PCR was performed on total RNA for the expression of TNF- α receptors TNFR1 (p60) and TNFR2 (p80), as described in MATERIALS AND METHODS. Positive controls included mouse spleen and WEG-1 cells. Amplicon size (MW) markers are indicated in the left margin; p60 and p80 amplicon sizes are indicated in the right margin.

Table 2. Cytokine induction of nitrite production by C₂C₁₂ myotubes

	NO Concentration, μ M
CTRL	0.6 $\pm$ 0.0
TNF- α	0.6 $\pm$ 0.2
IFN- γ	0.5 $\pm$ 0.0
TNF- α + IFN- γ	27.1 $\pm$ 2.5*

Results are expressed as total nitrite produced per plate (means $\pm$ SE of 3 separate experiments). After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 24 h (D5) with TNF- α (3 ng/ml), IFN- γ (5 ng/ml), or their combination. * P < 0.001 vs. all groups.

creases IGF-I production (14) and by the fact that TNF- α inhibits IGF-I mRNA in hepatocytes (52). Because the time courses of the IGF-I mRNA decrease coincide in muscle and in liver, it is likely that the mechanism of IGF-I gene inhibition by LPS is the same in both tissues. Therefore, the decrease in muscle IGF-I expression might be secondary to the muscle TNF- α induction by LPS.

To demonstrate this hypothesis, we assessed the effects of TNF- α on IGF-I gene and protein expression in muscle cells in vitro. We show here that TNF- α inhibits IGF-I gene and protein expression in differentiated C₂C₁₂ myotubes. The use of differentiated myotubes rather than myoblasts strengthens the relevance of our observations, as it is more likely for the same effects to be found in mature muscle (32).

Although muscle IGF-I mRNA concentrations are decreased in vivo by LPS and in vitro by TNF- α , IGF-II mRNA is not affected by these stimuli. Because IGFs share many biological properties, one could question the physiological consequences on muscle mass of a local IGF-I decline. However, several lines of evidence

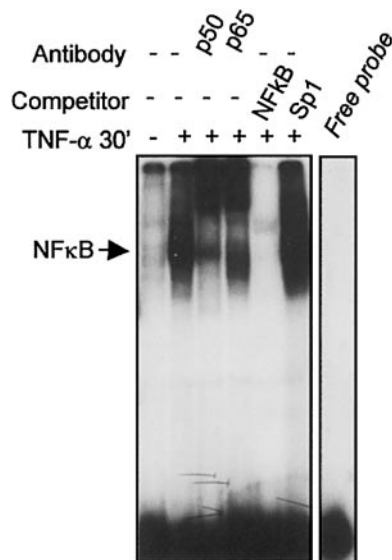


Fig. 11. Activation of NF- κ B by TNF- α in C₂C₁₂ myotubes. After 4 days of differentiation (D4), C₂C₁₂ myotubes were incubated for 30 min with TNF- α (3 ng/ml). Gel shift assay and supershift, using p50 and p65 specific antibodies, were performed on nuclear extracts, as described in MATERIALS AND METHODS. These results are from 1 of 3 separate experiments.

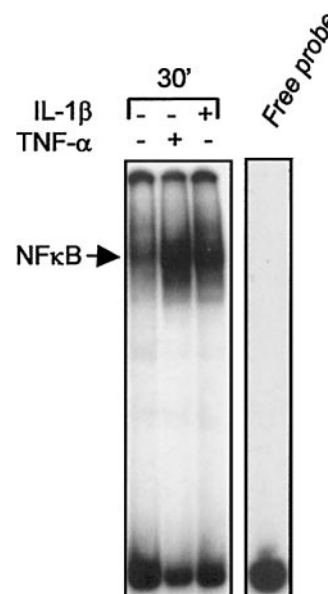


Fig. 12. Activation of NF- κ B by TNF- α and IL-1 β in C₂C₁₂ myotubes. After 4 days of induced differentiation (D4), C₂C₁₂ myotubes were incubated for 30 min with TNF- α or IL-1 β (3 ng/ml). Gel shift assay was performed on nuclear extracts as described in MATERIALS AND METHODS. These results are from 1 of 3 separate experiments.

indicate that the respective actions of IGF-I and IGF-II on the muscle are not “interchangeable.” *igf1*-Null mice present a generalized skeletal muscle hypoplasia that occurs despite normal IGF-II expression (8). Furthermore, although *igf1*-transgenic mice are characterized by an increase in muscle growth, *igf2*-transgenic mice have normal muscle mass and body growth (18, 49). Taken together, these data support a different role for IGF-I and IGF-II regarding the control of muscle mass and imply that the decline in muscle IGF-I production can impair muscle mass despite normal IGF-II expression.

Although TNF- α can lead to cell death in different cell systems, this was not the case in our model. First, the morphological aspect of myotubes exposed for 72 h to TNF- α was similar to that of controls. Second, the decrease in IGF-I expression caused by TNF- α was rapidly reversed by removal of the cytokine from the culture medium, thus confirming that IGF-I inhibition is not secondary to cell death. Moreover, several research groups have reported that C₂C₁₂ myotubes are resistant to the apoptotic action of TNF- α , even after 72 h of incubation (37). Although induction of TNF-soluble receptors can block TNF- α action, this seems not to be the case in our model, as the TNF- α inhibitory effect on IGF-I was sustained for $\geq$ 72 h.

The inhibition of IGF-I mRNA by TNF- α could result from dedifferentiation of myotubes caused by the cytokine (34). Indeed, TNF- α clearly impairs the differentiation of myoblasts into myotubes by inhibiting several myogenic markers (25). In the present study, TNF- α treatment significantly decreased CK activity, a marker of differentiation, but this occurred only after 72 h of treatment. Others have reported similar late effects of TNF- α on the myogenic differentiation of

myotubes (34). However, we cannot exclude the possibility that changes in CK activity detected at 72 h reflect early changes in steady-state levels of CK mRNA. These observations suggest that the rapid inhibitory action of TNF- α on IGF-I gene expression is probably independent of myotube dedifferentiation, although this phenomenon could be involved at later stages.

The TNF- α concentration capable of causing a decrease in IGF-I expression can be considered as "physiological," i.e., in the range of those found in inflammatory states or after LPS injection (3, 38) and close to the affinity of the TNF- α receptor for its ligand (30). Besides, when possible paracrine interaction between the cytokine-producing cells and myocytes is considered, local concentrations could be even higher than those found in circulation after LPS injection or in inflammatory states. In addition, the kinetics of the IGF-I mRNA decrease after TNF- α treatment of myotubes are close to those observed *in vivo* after LPS injection. All of these observations plead for a direct autocrine/paracrine effect of TNF- α , produced by LPS, on IGF-I in skeletal muscle.

Whereas TNF- α is not the sole cytokine capable of inducing a catabolic state (38) and whereas some actions of TNF- α have been shown to be mediated by other cytokines (2), we studied whether IL-1 β , IL-6, or IFN- γ were capable of inhibiting IGF-I expression. All of these cytokines fail to decrease IGF-I mRNA levels significantly in C₂C₁₂ myotubes. However, our data allow us to demonstrate the responsiveness of C₂C₁₂ myotubes to IL-1 β and IFN- γ , as the former activated NF- κ B and the latter potentiated the TNF- α effect on nitrite production. A similar conclusion can be drawn for IL-6, as C₂C₁₂ myotube sensitivity to IL-6 has recently been reported (2). Our data suggest that the TNF- α effect on IGF-I expression is not mediated by IL-1, IL-6, or IFN- γ . Nevertheless, the mechanism by which TNF- α exerts its inhibitory effect on IGF-I expression might still be an indirect one, needing other mediators or a combination of different cytokines (15, 57).

LPS administration did not result in a significant inhibition of IGF-I expression in differentiated myotubes. This apparent insensitivity might result from the absence of Toll-like receptors capable of LPS recognition in C₂C₁₂ myotubes. However, this is unlikely, as other biological responses to LPS have been described in the same model (24). Therefore, the lack of effect of LPS is most likely due to its failure to induce significant TNF- α expression in C₂C₁₂ myotubes.

Because most of the TNF- α catabolic actions are mediated through TNFR1, we searched for the presence of this receptor in C₂C₁₂ myotubes. This cell line actually expresses both TNF- α receptor isoforms, TNFR1 and TNFR2. We cannot, therefore, affirm the nature of the TNF- α receptor isoform whose activation causes IGF-I gene expression to decrease.

Although NO induction seems to play a role in some catabolic effects of TNF- α , the role of the NO pathway in the IGF-I gene inhibition by TNF- α can be excluded,

as TNF- α alone fails to increase NO production in C₂C₁₂ myotubes. This is not surprising, because previous work showed that iNOS activation in C₂C₁₂ myocytes required stimulation by a combination of cytokines, among which IFN- γ appears to be essential (55). This seems to be also the case in our model, where only the combination of TNF- α and IFN- γ resulted in a dramatic NO production, as assessed by nitrite concentration in the medium. The essential role of IFN- γ in inducing NO production might result from its ability to upregulate TNF- α receptor expression (9) or from a synergy operated at the transcriptional level (10). A synergistic action between TNF- α and IFN- γ was not observed on IGF-I mRNA. Both cytokines together decreased IGF-I gene expression more than each one did alone, supporting an additive effect rather than a potentiation.

Activation of NF- κ B is a crucial step for many TNF- α actions (51). Our data show that TNF- α indeed activates NF- κ B in myotubes, confirming the results reported by Li et al. (37). Nevertheless, our observations extend their findings by showing that NF- κ B is composed mainly of p50 and p65 subunits, which constitute the main heterodimer activated in the inflammatory response (51). The rapid activation of NF- κ B by TNF- α might suggest that this transcription factor plays a role in the inhibition of IGF-I expression caused by TNF- α . However, IL-1 β also activates NF- κ B in C₂C₁₂ myotubes, similarly to TNF- α but without inhibiting IGF-I expression. Activation of NF- κ B is therefore not sufficient to inhibit IGF-I gene expression.

Taken together, our data indicate that TNF- α induced by LPS in skeletal muscle may exert its catabolic effects in an autocrine/paracrine manner by reducing IGF-I gene and protein expression.

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