Cytokines and endotoxin induce cytokine receptors in skeletal muscle

YAN ZHANG,1 GENEVIEVE PILON,2 ANDRÉ MARETTE,2 AND VICKIE E. BARACOS1
1Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta T6G 2P5; and 2Department of Physiology and Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Quebec, Canada G1V 4G2

Received 14 September 1999; accepted in final form 1 February 2000

Zhang, Yan, Geneviève Pilon, André Marette, and Vickie E. Baracos. Cytokines and endotoxin induce cytokine receptors in skeletal muscle. Am J Physiol Endocrinol Metab 279: E196–E205, 2000.—Proinflammatory cytokines are important factors in the regulation of diverse aspects of skeletal muscle function; however, the muscle cytokine receptors mediating these functions are uncharacterized. Binding kinetics (dissociation constant = 39 ± 4.7 × 10−9 M, maximal binding = 3.5 ± 0.23 × 10−12 mol/mg membrane protein) of muscle tumor necrosis factor-α (TNF-α) receptors were obtained. Skeletal muscle was found to express mRNAs encoding interleukin-1 type I and II receptors, interleukin-6 receptor (IL-6R), and interferon-γ receptor by RT-PCR, but these receptors were below limits of detection of ligand-binding assay (≥1 fmol binding sites/mg protein). Twenty-four hours after intraperitoneal administration of endotoxin to rats, TNF receptor type II (TNFRII) and IL-6R mRNA were increased in skeletal muscle (P < 0.05). In cultured L6 cells, the expression of mRNA encoding TNFRII and IL-6R receptors was induced by TNF-α, and all six cytokine receptor mRNA were induced by a mixture of TNF-α, IFN-γ, and endotoxin (P < 0.05). This suggests that the low level of cytokine receptor expression is complemented by a capacity for receptor induction, providing a clear mechanism for amplification of cytokine responses at the muscle level.

Tumor necrosis factor-α

Injection of bacteria or their endotoxin [lipopolysaccharide (LPS)] in animals results in metabolic alterations in skeletal muscle, including enhanced protein breakdown, decreased protein synthesis (18), decreased fatty acid uptake and oxidation (30), insulin resistance (24), and increased expression of inducible nitric oxide synthase (iNOS; see Ref. 24). It has been proposed that these metabolic alterations are mediated mainly by four proinflammatory cytokines [interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ); see Refs. 2, 18, 30], at least in vivo.

Cytokines belong to a family of small proteins mainly produced by activated macrophages and monocytes after infection (13, 16). Injection of the proinflammatory cytokines in animals can reproduce many manifestations seen in infection (18, 30), and specific anticytokine treatment can prevent or attenuate part of the pathological process (25). Furthermore, these cytokines have various direct actions on skeletal muscle cell lines, including 1) stimulation of the synthesis of nitric oxide through induction of iNOS by TNF-α, IFN-γ, and IL-1 (2, 41), 2) induction of insulin resistance by TNF-α and IFN-γ (2), and 3) suppression of protein synthesis by TNF-α (14).

The work cited above suggests the presence of cytokine production and cytokine response in skeletal muscle. As proteins, cytokines exert their biological effects via binding to specific membrane receptors. There are two distinct cell surface binding sites for TNF-α, a type I receptor (p55–60 kDa) and a type II receptor (p75–80 kDa), which differ in their intracellular domains and thus mediate distinct cellular responses (20). IL-1 has two unique receptors; the type I receptor (IL-1RI) transduces a signal, whereas the type II receptor (IL-1RII) binds IL-1 but does not transduce a signal. The IL-1RII acts as a sink for IL-1 and has been termed a “decoy” receptor (10). There is only one receptor form each for IL-6 and IFN-γ (16, 19). This information comes from other cell types, and skeletal muscle cytokine receptors have been barely studied to date. There is one report of specific binding of TNF-α to myotubes (5), and another more recent study established the presence of mRNAs of TNF type I receptor (TNFRI) and TNF type II receptor (TNFRII) in skeletal muscle (21).

The first objective of our study was to establish the presence of receptors or receptor mRNA for the four proinflammatory cytokines thought to modulate muscle metabolism by radioligand binding assay and RT-PCR. Because binding assays require large amounts of muscle membrane, this experiment was first conducted on porcine sarcolemma. Commercially available125I-labeled cytokines are either human or murine types; however, there is sufficient homology between gene sequences or amino acid sequences of human/mouse and porcine cytokines/cytokine receptors for binding to take place (9, 26, 33, 41). The binding results from
porcine sarcolemma were confirmed by using murine sarcolemma. Our initial results demonstrated the presence of specific binding sites for TNF-α on sarcolemma by radioligand binding assay. By contrast, specific binding of IL-1, IL-6, and IFN-γ was below the limits of detection of this assay (~1 fmol receptor/mg membrane protein); however, receptor mRNAs could be detected by RT-PCR.

Endotoxin and cytokines are known to modulate tissue and cell cytokine receptors in vivo and in vitro, and both downregulation and upregulation have been noted, depending on the specific cell type (22, 34, 38). We hypothesized that, as shown for other cell types, a low basal level of cytokine receptors on muscle cells might be complemented by a capacity for receptor upregulation. It is also known that synergy among cytokines is essential for specific biological responses. For example, stimulation of iNOS requires an interaction between IFN-γ and TNF-α or IL-1β (2, 41), and further combination of cytokines with endotoxin enhanced the induction (2). To determine whether cytokine receptor gene expression on skeletal muscle is modulated, we employed the following two approaches: 1) cytokine stimulation of cultured muscle cells using a cell line (L6) that was derived from neonatal rat thigh muscle and retains many morphological, biochemical, and metabolic characteristics of skeletal muscle (42), and 2) endotoxin injection in laboratory rats to stimulate cytokine production in vivo.

MATERIALS AND METHODS

Reagents. Recombinant cytokines (murine IL-1β, human IL-6, TNF-α, and IFN-γ) were obtained from Peprotech (Peprotech, Rocky Hill, NJ). Murine TNF-α (ED50: 0.02–0.05 ng/ml) and rat IFN-γ (4 × 10−6 U/μg) were purchased from R&D Systems. 125I-labeled cytokines (murine IL-1β, human IL-6, TNF-α, and IFN-γ) were purchased from Du Pont-New England Nuclear (Boston, MA). Endotoxin (LPS: Escherichia coli O55:B5), aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, wheat germ agglutinin, and BSA (fraction V) were purchased from Sigma (St. Louis, MO). Iscove’s modified Dulbecco’s medium, MEM (Eagle’s), FBS, penicillin, streptomycin, Taq polymerase, TRIZol reagent, restriction endonucleases, and the random-primer-labeling kit were purchased from Gibco (Grand Island, NY). Expend-RT was from Boehringer Mannheim (Laval, Quebec). [32P]dATP was a product of Amersham (Oakville, Ontario).

Experimental animals. Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Sprague-Dawley rats, mice, and domestic pigs were obtained from breeding colonies maintained at the University of Alberta. Male animals were used for skeletal muscle membrane isolation and purification. Rats were housed in light-dark cycle and were fed laboratory chow (Continental Grain, Chicago, IL) containing 24% crude protein. Total RNA membrane isolation and purification. Rats were housed in a product of Amersham (Oakville, Ontario).

Cell culture. The murine fibroblast tumor cell line L929 and epithelial tumor cell line NOB-1 were provided by Dr. L. Guilbert (University of Alberta, Edmonton, Canada) and were cultured in DMEM supplemented with 10% FBS. The murine OKT 4 cell line (CRL-8002) and human HeLa 229 cell line (CCL-2.1) were purchased from American Type Culture Collection (Rockville, MD). OKT 4 cells and HeLa cells were cultured in Iscove’s modified Dulbecco’s medium plus 20% FBS and Eagle’s MEM with 10% FBS, respectively. L6 skeletal muscle cells were used as a purified myogenic cell culture. Cells were grown and maintained in monolayer culture in α-MEM containing 2% (vol/vol) FBS in an atmosphere of 5% CO2 at 37°C. L6 myoblasts were plated in 10-cm dishes at 20,000 cells/ml and were used after complete differentiation to myotubes (7 days postplating). L6 myotubes were then incubated with or without 1) murine TNF-α (10 ng/ml) or 2) murine TNF-α (10 ng/ml) + rat IFN-γ (50 ng/ml) + endotoxin (10 μg/ml) for 24 h before the RNA isolation. All of the above culture media contained 1% (vol/vol) antibiotic/antimycotic solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B).

Membrane Preparation

Group I. Plasma membranes from skeletal muscle were isolated using a procedure reported by Ohlendieck et al. (31) as in our previous work with muscle insulin and insulin-like growth factor I receptors (28). The procedure is based on subcellular fractionation by differential centrifugation, density gradient centrifugation, and wheat germ agglutination. The isolated plasma membrane is essentially devoid of sarcoplasmic reticulum and T tubular markers (31). Membrane after the density-gradient-centrifugation step was suspended in PBS buffer and stored at −70°C for further analysis.

Group II. Plasma membranes were from cell lines described by Bird et al. (6). Cells (~106) were centrifuged at 500 g for 5 min, and the pellet was resuspended in 5 ml hypotonic buffer, disrupted in Dounce glass-glass homogenate made up to 35 ml with 0.25 M sucrose containing 5 mM Tris · HCl and 1 mM MgCl2, and centrifuged at 150,000 g for 30 min. The pellet was resuspended in PBS and stored at −70°C. Membrane protein was determined after solubilization with 1 N NaOH by the method of Bradford, using BSA (fraction V; Sigma) as standard.

Radioligand Binding Assay

As described previously (6, 28), 50 μl of porcine or murine membrane suspension (2 μg protein/μl, after density-gradient centrifugation) were incubated with individual 125I-labeled cytokines (murine IL-1β, human IL-6, TNF-α, and IFN-γ) in microcentrifuge tubes at increasing concentrations. Alternatively, the membrane was incubated with a constant amount of 125I-labeled cytokine and increasing concentrations of unlabeled cytokine (as indicated in Figs. 1–6). The final volume was 100 μl, and all samples were done in triplicate. Nonspecific binding is defined as radioactivity bound to membrane fractions in the presence of 500-fold excess of unlabeled cytokine. The mixture was incubated in a shaking water bath for 4 h at 25°C. The binding reaction was stopped by centrifugation at 12,000 g for 5 min after agglutination of the vesicles with 100 μl of 1 mg/ml wheat germ agglutinin. The supernatant was discarded, and the pellet was washed twice with chilled 0.25 M sucrose. The tips
of the tubes containing the pellets were cut off and counted in a Packard Cobra Auto-Gamma counter. Nonspecific binding was ~40% of total binding, and this was uniform across all of the binding assays conducted.

RT-PCR

Total RNA was extracted by the guanidinium isothiocyanate/phenol/chloroform method with TRIzol Reagent based on the method developed by Chomczynski and Sacchi (8). RNAs for PCR were harvested from differentiated rat muscle tissue, as well as from the cultured rat muscle cell line (L6). Primers were based on published sequences in the literature or according their mRNA sequences submitted to the National Gene Bank with the assistance of computer programs [Genejockey II from Biosoft (Ferguson, MO) and Amplify1.2]. The primer sequences are listed in Table 1 and are specific for rat genes.

For qualitative PCR assay, total RNA (1 µg) was reverse transcribed into cDNA in the presence of 50 units Expand-RT (5–10 µl RNase inhibitor, 0.5 µM sequence-specific antisense primer, 10 mM dithiothreitol, and 1× Expand RT buffer (first strand) in a total volume of 20 µl by the procedure recommended by the manufacturer. The RT reaction system contained 5 µl × 10 PCR buffer, an aliquot of RT (5–10 µl) product, 1.5 µl of 50 mM MgCl2, 1 µl of 10 mM dNTP mixture, 1.25 units Taq DNA polymerase, and 2 µl each of 10 µM sense and antisense primers in a total volume of 50 µl. Amplification was carried out as follows: for IL-1RI, IL-6 type II receptor, IL-6 receptor (IL-6R), IFN-γ receptor (IFNR), TNFRI, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 1 × 3 min at 94°C, 1 min at 60°C, and 3 min at 72°C; 45 × 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; and 1 × 7 min at 72°C. For IL-1R1 and TNFRII, the “Hot-start” protocol provided by GIBCO-BRL was employed (1 × 3 min at 94°C) before addition of Taq polymerase, followed by addition of enzyme at 80°C, then 35 × 45 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C and 1 × 10 min at 72°C. A portion (20 µl) of RT-PCR product was electrophoresed in 1% agarose gel in Tris-borate-EDTA (TBE) buffer. The gel was stained with ethidium bromide and photographed with Gel-Doc 1000 (Bio-Rad).

Restriction Enzyme Digestion of PCR Products

The PCR products were purified with the Gene clean Kit (Bio 101) from agarose gels. Restriction enzymes (5–10 units) and buffers adequate to optimize the reactive condition for each restriction enzyme were added to the purified PCR product. The volume of each reaction was adjusted to 20 µl with DNase-free water. These reaction mixtures were incubated at 37°C for 2 h and electrophoresed in 1% agarose gel in TBE buffer. The gel was stained and photographed as described above.

Semiquantitative RT-PCR Assay

To examine the possibility of semiquantitative analysis using RT-PCR, the samples with different amounts of total RNA (0.1–0.5 µg) were assayed for cytokine receptor mRNA by RT-PCR. The RT-PCR condition was as described above, except that the cycle number was reduced (24 for IL-1RI, 26 for IL-1RII, 24 for IL-6R, 22 for TNFRI and IFNR, and 28 for TNFRII) so that PCR reaction was conducted in the exponential phase. All of the PCR reactions were repeated in triplicate. The amplified products were detected by Southern blotting. After 1% agarose gel electrophoresis, the PCR product was transferred to nitrocellulose membranes and hybridized overnight with a random-primer 32P-labeled specific cDNA probe, which was generated by PCR reaction and purified with the Gene clean kit from agarose gel. The hybridized filters were then washed, exposed to Fuji Phosphoimage plates, and analyzed using a Fuji Bioimaging analyzer BAS1800. A similar semiquantitative method has been used previously to test mRNA abundance of IL-6 and IL-6R in rat hypothalamus (15).

Data Analysis

Dissociation constant ($K_d$) and maximal binding (B$_{max}$) values were calculated from binding data using the GraphPad program (GraphPad Software). The results of each treatment are presented as means ± SE. Differences were analyzed statistically by the unpaired Student’s t-test for comparisons between groups.

RESULTS

Evidence for the Presence of TNF-α Receptors in Sarcolemma

125I-labeled TNF-α bound specifically to porcine sarcolemma and membrane from a control TNF-responsive cell line (L929) in a direct binding assay at different cytokine concentrations (Fig. 1A). Competition

Table 1. Summary of primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFRI</td>
<td>Sense: 5’-CCTGATTTCATCTTCATCTTGACTTTTGAGC-3’</td>
<td>M63122</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-ACACTGGAAATTCGGTCCTCAGTCAAGGCG-3’</td>
<td></td>
</tr>
<tr>
<td>TNFRII</td>
<td>Sense: 5’-GATGAGAAATCCGCAGGATGCAGTACG-3’</td>
<td>U55849</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-GCTACAGACCTCGACATGACAGG-3’</td>
<td></td>
</tr>
<tr>
<td>IL-1RI</td>
<td>Sense: 5’-AGATGGAAGGACCTATGATG-3’</td>
<td>M95578</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-TGAGAGCATCTGAGCAGAGCAAG-3’</td>
<td>Z22812</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>Sense: 5’-GGCAAGGGAATACACATCAC-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-TGCGACACTGTCGACAGGACAGAACA-3’</td>
<td>M58587</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Sense: 5’-TGCCAACTCGTGTGATACCCGTCC-3’</td>
<td>U68272</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-TGGATGGCTTTGAGTTCAGG-3’</td>
<td></td>
</tr>
<tr>
<td>IFNR</td>
<td>Sense: 5’-GCAAGGGAAGGAGGACAGCAGAC-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CTCTGACTGCTTCTCAGTAC-3’</td>
<td>X02231</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5’-TATGCGTGAGGATCAGTCAAGGCG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CTCTGACTGCTTCTCAGTAC-3’</td>
<td></td>
</tr>
</tbody>
</table>

TNF, tumor necrosis factor; IL, interleukin; IFN, interferon-γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, receptor; RI, type I receptor; RII, type II receptor. Accession no. is the number used to access the corresponding gene sequence stored in GenBank of the National Center for Biotechnology Information.
binding studies were also employed (Fig. 1B). Human TNF-α competed with the binding of 125I-labeled TNF-α in a dose-dependent manner. Scatchard plots were generated from the competition binding data (Fig. 1, C and D). The $K_d$ and $B_{\text{max}}$ values, respectively, were $39 \pm 6.6 \times 10^{-9}$ M and $3.5 \pm 0.23 \times 10^{-12}$ mol binding sites/mg membrane protein for porcine sarcolemma and $20 \pm 2.7 \times 10^{-9}$ M and $3.7 \pm 0.19 \times 10^{-12}$ mol binding sites/mg protein for L929 cell membrane. Analysis of the binding data of human TNF-α on porcine sarcolemma fit a one-site model, and analysis as a two-site model did not reduce the error term. These binding sites likely reflect reaction of the ligand to TNFRI, since the human 125I-TNF-α employed in this...
Evidence for the Presence of IL-1, IL-6, and IFN-γ Receptors

There was no specific binding of 125I-labeled murine IL-1, human IL-6, and IFN-γ to either porcine (data not shown) or murine sarcolemma. IL-6 binding on murine sarcolemma and membranes from a control cell line (OKT 4) are shown in Fig. 2. All three 125I-labeled cytokines did specifically bind to control cell membranes (NOB-1: IL-1β; OKT 4: IL-6; HeLa: IFN-γ); therefore, the failure of detection of these receptors is not due to technical problems. Direct binding assay was effective in detecting ≥1 fmol binding sites/mg protein, so these cytokine receptors, if present on sarcolemma, may be beyond their limit of sensitivity.

The ligand binding assays suggest that skeletal muscle either does not express receptors for IL-1, IL-6, and IFN-γ or expresses very low levels of these receptors. To explore the latter possibility, we employed RT-PCR to amplify cytokine receptor mRNAs and also to determine whether receptor mRNA expression in skeletal muscle was subject to upregulation by cytokines, as has been shown in other cell types expressing low levels of receptor. Four transcripts of expected size were identified by RT-PCR in RNA from mature rat skeletal muscle [IL-1RI, IL-1RII, IL-6R, and IFNR (Fig. 3A)]. The PCR products were further confirmed by digestion using appropriate restriction enzymes, and digested products of the predicted lengths appeared (Fig. 3A). Cultured muscle cells are free of other cell types, and this source of RNA was used to determine that cytokine receptors are expressed by muscle cells per se. L6 cells expressed mRNA encoding both IL-1 receptor isoforms as well as IL-6R and IFNR (Fig. 3B).

Validity of Semiquantitative RT-PCR

To evaluate the ability of this method to measure the relative abundance of receptor mRNA, a group of concentration curves was generated for each of the cytokine receptor genes that we measured under the RT-PCR conditions defined in MATERIALS AND METHODS. GAPDH gene was used as an internal control, and its level was evaluated in the same RNA samples as described (2). Data for IL-1RI and GAPDH are shown in Fig. 4. The intensity of the bands was related linearly with the initial RNA concentration in the range of 0.1–0.4/0.5 μg. A concentration within the linear range (0.25 μg) was chosen for further analysis.

Regulation of Muscle Cytokine Receptors in Endotoxemia and the Role of TNF-α

Eight rats were randomly divided into two groups to receive saline injection or endotoxin (400 μg/kg body wt). This dose induces well-characterized symptoms, including anorexia, lethargy, weight loss, and muscle wasting (18). Significant loss of body weight was observed in the endotoxic rats compared with the healthy group at 24 h after injection (−15.6 ± 2.5% initial body weight vs. −4.7 ± 0.5%, n = 4, P < 0.001). Muscle samples were collected 24 h after injection, and the levels of six cytokine receptor mRNAs were measured by semiquantitative RT-PCR. The densitometric quantification of the specific cytokine receptor signals obtained, corrected for the constitutively expressed GAPDH gene, is represented in Fig. 5A. There was a significant increase of IL-6R and TNFRII mRNA in the endotoxin-treated group (P < 0.05), whereas the mRNA level of other receptor genes was unchanged. After injection of endotoxin or bacteria in vivo, TNF-α is usually the first cytokine to appear in plasma, followed by IL-1 and IL-6 (12, 37). TNF-α is viewed as an initiator for production of other cytokines. Because TNF receptors were the only abundant cytokine receptors found in skeletal muscle, we suspected that the changes observed in the endotoxin-injected animals might have been mediated by TNF. To evaluate the role of TNF-α in such regulation, L6 myotubes were treated with TNF-α alone for 24 h (Fig. 5B). TNF-stimulated myotubes showed an identical pattern of cytokine receptor mRNA expression as muscles from endotoxin-injected rats, with increased mRNA levels of IL-6R and TNFRII but not other receptors.
Regulation of Cytokine Receptors by Cytokines and Endotoxin Treatment in L6 Cells

It has been established that synergy among cytokines exists for specific biological responses. For example, stimulation of iNOS activity in skeletal muscle requires an interaction between IFN-γ and TNF-α or IL-1β (2, 41), and combination of cytokines with endotoxin further enhanced the induction (2). To investigate the synergy between cytokines and endotoxin, L6 cells were treated with a combination of TNF-α, IFN-γ, and endotoxin for 24 h. Six proinflammatory cytokine receptor mRNAs were examined by semiquantitative RT-PCR. The expression of all six receptor mRNAs was clearly increased by this treatment \((P < 0.05, Fig. 6)\), whereas TNF alone had induced expression of only IL-6R and TNFRII mRNAs \((P < 0.05, Fig. 5B)\).

DISCUSSION

We have identified the presence of specific binding sites and/or mRNAs encoding receptors for four proinflammatory cytokines in skeletal muscle tissue and L6 cells. Specific modulation of cytokine mRNA expression was observed after stimulation of muscle by cytokines. TNF-α had been previously reported to bind to myotubes (5). Our ligand-binding studies show that \(^{125}\)I-TNF-α bound specifically to highly purified muscle plasma membrane, suggesting that differentiated skeletal muscle and transformed muscle cell types in culture express cytokine receptors. mRNAs for two types of TNF-α receptors had been reported in skeletal muscle (21). We confirmed this result by RT-PCR in both muscle cell lines and differentiated muscle tissue. The \(K_d\) value for muscle TNF receptors falls in the range of the \(K_d\) values for L929 cell TNF receptors and showed characteristics of one-site binding, as has been shown for other cell types (39).

Specific TNF-α-binding sites on sarcolemma \((3.5 \pm 0.23 \times 10^{-12}\) mol/mg membrane protein) are ~10-fold less abundant than those that bind insulin \((B_{max} = 1–5 \times 10^{-11}\) mol binding sites/mg sarcolemma protein; see Ref. 28). RT-PCR revealed the presence of mRNAs encoding four other proinflammatory cytokine receptors (IL-1RI, IL-1RII, IL-6R, and IFNR) in skeletal muscle; however, the levels of these receptors were...
and the other cytokines (TNF-α, IL-1, IL-6) may not be proportional to the density of their receptors on the cell membrane. The physiological contribution of individual cytokines in regulating muscle function is not clear. Most or all of the injurious sequelae of septic shock syndrome are attributable to the effects of TNF-α and the cytokine cascade triggered by TNF-α (13, 37). TNF-α appears early in the plasma after infection (13, 37), and early blockade of TNF-α inhibited the production of IL-1 and IL-6 (13, 37). At the same time, IFN-γ, but not TNF-α, was essential to formulate a functional cytokine cocktail inducing iNOS from skeletal muscle cells (41). IL-6, not TNF-α, was found to shorten the half-life of long-lived proteins in C2C12 myotubes (11). These results point to a high degree of complexity in cytokine responses by skeletal muscle.

Our studies showed that mRNA levels for TNFRII (p75) and IL-6R were increased in skeletal muscle by itself was not transparent for an increase in the corresponding protein levels or any correlation between receptor mRNA level and net biological effects. TNFRI, IL-1RI, IL-1RII, and IFN-γ did not respond to TNF-α at the concentration used within 24 h of stimulation. Additionally, it remains unknown how long the induction of TNFRII and IL-6R is sustained and when the peak response occurs. More experiments on the time course may elucidate these points. The concentration of TNF-α (10 ng/ml) used in the experi-

below the limits of detection of the radioligand-binding assay. Cytokines are thought to be capable of activating a signal transduction pathway, even at very low receptor levels (10). IL-1 signal transduction has been observed in cells expressing <10 type I receptors per cell. The physiological contribution of individual cytokines may not be proportional to the density of their membrane receptors, so the significance of the discrepancy between the densities of specific binding sites for TNF-α (3.5 ± 0.23 × 10⁻¹² mol/mg membrane protein) and the other cytokines (<1 × 10⁻¹² mol/mg membrane protein) remains unclear.

The relative abundance of TNF-α receptors on sarcolemma suggests that TNF-α might play a primary direct role in regulating muscle function. Most or all of the injurious sequelae of septic shock syndrome are attributable to the effects of TNF-α and the cytokine cascade triggered by TNF-α (13, 37). TNF-α appears early in the plasma after infection (13, 37), and early blockade of TNF-α inhibited the production of IL-1 and IL-6 (13, 37). At the same time, IFN-γ, but not TNF-α, was essential to formulate a functional cytokine cocktail inducing iNOS from skeletal muscle cells (41). IL-6, not TNF-α, was found to shorten the half-life of long-lived proteins in C2C12 myotubes (11). These results point to a high degree of complexity in cytokine responses by skeletal muscle.

Our studies showed that mRNA levels for TNFRII (p75) and IL-6R were increased in skeletal muscle samples from endotoxic animals. TNF-α is able to regulate its own receptors as well as other cytokine receptors, and the regulation pattern is cell type specific (22, 34, 38). Our work showed that TNF-α by itself was capable of upregulating the expression of TNFRII and IL-6R mRNA in L6 cells after 24 h incubation, implying that this cytokine may be the mediator of the adaptation seen in vivo after endotoxin injection. No response to TNF-α was seen at earlier time points (within 12 h; data not shown). Compared with other cell types, skeletal muscle is a tissue that responds to cytokines slowly (41). iNOS mRNA expression in myotubes was seen after 6 h incubation with cytokines, and iNOS protein was detectable only after 12 h stimulation by cytokines (32). By contrast, such induction occurs within 2 h in macrophages (41). TNF-α down-regulated TNFRI mRNA within 30 min and increased the expression of IFN-γ and TNF-α mRNA within 4 h in rat tracheal epithelial cells (1). Therefore, it may take a longer time to observe the muscle’s response to cytokines compared with fast-reacting cell types.

The various biological activities of TNF-α are thought to be mediated by two isotypes of receptors. It is not clear why only the mRNA level of TNFRII, not TNFRI, is modified in response to TNF-α stimulation and endotoxin challenge. Most of the known TNF-α responses are mediated via TNFRI (p55), and this notion has been confirmed in vivo. Mice deficient in TNFRI were resistant to endotoxic shock (20) and tumor-induced protein breakdown in skeletal muscle (29). However, TNFRII is associated with thymocyte proliferation, and cytotoxicity may be a function of TNFRII alone or together with TNFRII (20). Furthermore, TNF-α upregulated the mRNA abundance of TNFRII but not TNFRI in human malignant epithelial cells (22). Based on the present results, it is hard to draw any conclusion on the participation of individual cytokines in modulating muscle metabolism. The up-regulation of IL-6R is not surprising because TNF-α induces the production of IL-6 (13, 37). It can be speculated that some of the effects assumed to be caused by TNF-α are mediated by IL-6.

Soluble receptors of the TNF receptor and IL-6R families are known to exist, and the circulating levels of soluble receptors are increased in various pathophysiological conditions (19, 37). Soluble receptors may act as agonists or antagonists of cytokine action (36, 37). Taking all of these factors into consideration, the significance of the mRNA expression for cytokine receptors described in the present study should be interpreted with caution, because no evidence has been provided for an increase in the corresponding protein levels or any correlation between receptor mRNA level and net biological effects.

TNFRII, IL-1RI, IL-1RII, and IFN-γ did not respond to TNF-α at the concentration used within 24 h of stimulation. Additionally, it remains unknown how long the induction of TNFRII and IL-6R is sustained and when the peak response occurs. More experiments on the time course may elucidate these points. The concentration of TNF-α (10 ng/ml) used in the experi-

Fig. 4. Quantification of cytokine receptor mRNA levels by RT-PCR. Samples of different RNA content (0.1–0.5 μg) were analyzed for IL-1RI and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are expressed as arbitrary units obtained by quantitative densitometry. Bottom: Southern blot of the RT-PCR signal.
ment is common for in vitro studies. The level used here is higher than physiological concentrations but relatively low compared with other published studies (14). Peak serum TNF-α levels were ~2 ng/ml in patients injected with endotoxin (7), whereas TNF-α exerted its highest inhibitory effect on protein synthesis in human myoblasts at the concentration of 100 ng/ml (14). Further dose-response experiments may reveal

Fig. 5. Modulation of cytokine receptor mRNA expression by endotoxin injection or TNF-α in skeletal muscle tissues (A) and L6 myotubes (B). Endotoxin or saline was injected in rats, and epitrochlearis muscle samples were collected 24 h later. L6 cells were incubated with TNF-α (10 ng/ml) for 24 h and collected for RNA isolation. mRNA levels of six cytokine receptors (IL-1RI, IL-1RII, IL-6R, IFNR, TNFRI, and TNFRII) were measured. All RT-PCR reactions were done in triplicate. A: densitometric analysis of specific RT-PCR signals of cytokine receptors from endotoxin treatment (n = 4/group). B: densitometric analysis of specific RT-PCR signals of cytokine receptors from TNF-α treatment. Data have been corrected for GAPDH mRNA signal and are expressed as percentage compared with control (n = 2). *P < 0.05.

Fig. 6. TNF-α + IFN-γ + endotoxin induce cytokine receptor mRNAs in L6 myotubes. L6 myotubes were incubated with the cytokines and endotoxin (TNF-α: 10 ng/ml; IFN-γ: 50 ng/ml; endotoxin: 10 μg/ml) for 24 h, and mRNA levels of six cytokine receptors (IL-1RI, IL-1RII, IL-6R, IFNR, TNFRI, and TNFRII) were measured. Bottom: Southern blot of RT-PCR signal of cytokine receptors and GAPDH. Data are corrected for the GAPDH mRNA signal and are expressed as percentage of the controls (n = 2). *P < 0.05.
the threshold concentration of TNF-α necessary for in vitro stimulation of cytokine receptor mRNA expression in L6 cells and the concentration for maximal stimulation. Under conditions of maximal stimulation, it may be possible hereafter to study at the protein level with ligand-binding experiments. To fully evaluate the significance of cytokine receptor modulation in skeletal muscle, the upregulation of gene expression of cytokine receptors has to be associated with the modification of specific biological activities.

We noted that the mRNA level of all six cytokine receptors was stimulated by the combination of cytokines with endotoxin. The synergy between cytokines and other factors such as endotoxin may be necessary to see the full scope of a biological response, as in the stimulation of nitric oxide release through iNOS induction (2, 41). The synergy seems to be operative at the level of cytokine receptor mRNA expression and may provide a mechanism for sensitization of cytokine responses at the tissue level. The exact role of each cytokine in muscle is intriguing and as yet difficult to elucidate. Further study with gene knock-out animals may be helpful in revealing the relations between the regulation of muscle biological activity and specific cytokine(s).

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES


