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Direct Effects of Tumor Necrosis Factor Alpha (TNF-α) on L6 Myotubes

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Tumor necrosis factor (TNF)-α is a pleiotropic cytokine responsible for a diverse range of signaling events within cells. We studied direct effects of TNF-α on skeletal muscle protein content. Incubation of the L6 myotubes with 1 to 10 U/ml of TNF-α resulted in a significant increase of total and myofibrillar protein contents as compared with the control. However, incubation with lower (0.001-0.1 U/ ml) or higher (100-300 U/ml) doses of TNF-α resulted in a decrease of protein content. These findings suggest that TNF-α may elicit both anabolic and catabolic effects on L6 myotubes in a dose dependent manner. The anabolic effect of TNF-α was mediated at least in part by mitogen activated protein kinase (MAPK), especially by an extracellular-regulated kinases (ERK). This divergent effect of TNF-α may be crucial to elucidate the complexity of TNF-α action on the skeletal muscle.

The proinflammatory cytokine tumor necrosis factor (TNF) -α is a pleiotropic cytokine with a broad range of diverse actions that include proliferative effects as cell growth and differentiation [16], inflammatory effects, mediation of immune responses [23], and destructive cellular outcomes such as apoptotic and necrotic cell death mechanisms [3]. The expression level of TNF-α elevated in conditions associated with muscle pathology such as inflammatory myopathy [20], Duchenne muscular dystrophy [21], and cancer cachexia [24].

Actions of TNF-α are mediated by its binding to the specific receptors, TNF-α receptors 1 and 2 (TNFR-1 and TNFR-2), expressed on the surface of most cell types [4]. TNF-α stimulates a complex array of post receptor signaling events, mostly through three major pathways. One pathway stimulates apoptosis *via* interaction with the TNF-α receptor complex and the Fas-associated protein with death domain. The second pathway activates the Jun-N-terminal kinase, a mitogen activated protein kinase (MAPK) pathway, and the third pathway activates the nuclear factor κ B (NF-κB), a primary mediator of transcriptional control and a major candidate for catabolic signaling [6]. In addition, activation of phosphatidylinositol 3 (PI3)-kinase-Akt/PKB pathway via TNFR-2 was recently reported in fibroblasts [15], cardiac [13], and retinal cells [10].

A divergent effect of TNF-α on skeletal muscle protein has been reported, mostly using the C2C12 mouse skeletal muscle cell line. In this study, we examined the direct effects of different TNF-α concentrations on total muscle protein content as well as myofibrillar protein content of the L6 rat skeletal muscle cells. We also studied the effects of various kinase inhibitors to clarify the intracellular signaling pathways of TNF-α stimulation.

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MATERIALS AND METHODS

Cell Culture

L6 myoblasts were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal bovine serum (FBS) till they reach 80% confluency. To induce differentiation, cells were further cultured in DMEM containing 2 %FBS for 7 days. Cell viability was assessed by the Trypan blue viability test. Myogenic differentiation to myotube was confirmed both morphologically and biochemically. Morphological parameters of differentiation (alignment, elongation, and fusion) were assessed by light microscopy after staining with May-Grünwald Giemsa. Myogenic differentiation was determined biochemically by measurement of muscle creatine kinase (CK) activity, using a spectrophotometric-based kit. Specific CK activity was calculated after correction for total protein.

TNF-α Treatment and Measuring Protein Content

To study the effects of TNF-α on total protein content, cells were treated with either 2 % DMEM alone, or with recombinant rat TNF-α in various concentrations (0.001, 0.01, 0.1, 1, 10, 100, and 300 U/ml) for 24 hours. The effect of 1 or 10 U/ml TNF-α was further studied at 0.5, 6, 12, 24 or 72 hours. To conduct inhibitor studies, cells were pretreated for 30 minutes with various inhibitors of signal transduction pathway prior to stimulation with 1 or 10 U/ml of TNF-α for 6 hours. Inhibitors used were PI3kinase inhibitors: LY294002 and wortmannin, MAPK inhibitors: PD98059 (ERK 1/2 inhibitor) and SB203580 (p38 MAPK inhibitor), and a NF-κB inhibitor: MG-132. Control dishes were supplied with medium containing the least amount of DMSO used to dissolve the blockers.

After incubation, monolayers were lysed in 1 % Triton X-100. Lysates were centrifuged for 5 min at 12,000 g and the supernatant was stored at –80 °C for determination of protein content or CK activity. Total protein content was determined using the BCA protein assay kit (Pierce Chemical Co.). Myofibrillar proteins were extracted from the cell samples according to standard procedures [5]. Briefly, the cell pellet was re-suspended in 150 µl of a high-salt buffer (300 mM NaCl, 100 mM NaH_2PO_4, 50 mM Na_2HPO_4, 10 mM $Na_4P_2O_7$, 10 mM EDTA, 1 mM $MgCl_2$, pH 6.5, with 0.1 % mercaptoethanol). After extraction on ice for 40 min, samples were centrifuged at 13,000 g for 30 min. Supernatants were diluted 10 times in filament formation buffer (1 mM EDTA, 0.1 % mercaptoethanol), and incubated on ice overnight to precipitate the myofibrillar proteins. The sample was centrifuged at 13,000 g for 30 min, and the pellet was re-suspended in sample buffer (500 mM NaCl, 12.5 mM NaH_2PO_4, pH 7.0, with 0.1 mg/ml pepstatin and leupeptin) and then incubated on ice overnight to dissolve. Myofibrillar protein content was determined using the BCA protein assay kit.

Western Blotting

For Western blotting evaluation, 2x Laemmli sample buffer (0.1 M DTT, and 0.01% [w/v] bromophenol blue) was added to samples. Approximately 10 mg of protein was loaded per lane and separated on 7 or 10% polyacrylamide gel followed by transfer to a nitrocellulose membrane. Nitrocellulose blots were washed in TBS-Tween20 (0.05 %), and probed with the primary antibody, monoclonal anti-skeletal myosin (fast) antibody MY-32 (SIGMA), the antibody agaiinst myosin heavy chain protein fast twitch (MyHCf) at 1:100 dilution, followed by incubation with horseradish peroxidase-labeled anti-mouse IgG antibody (used at 1:5,000 dilution). Blots were subsequently detected with enhanced chemiluminescence system (Amersham Biosciences).

Materials

L6 rat skeletal muscle myoblasts were obtained from the Japanese Cancer Research

Resource Bank. All materials and chemicals were obtained from commercial sources.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Statistical differences between data were determined by ANOVA followed by Dunnett's multiple comparison test conducted with statistical program GraphPad Prism, version 4.00 (GraphPad Inc.).

RESULTS

Seven days after induction of myogeninc differentiation, cultured cells showed apparent morphological changes to myotube formation. CK activity was modestly increased within 2 days after induction of differentiation, and then further increased gradually.

Effect of TNF on Total and Myofibrillar Protein Content

As shown in Figure 1A, total protein content in L6 myotubes significantly ($p<0.05$) increased after 24 hours incubation with 1 U/ml TNF-α as compared with the control of vehicle alone. Either a low concentration (0.001U/ml) or a high concentration (300 U/ml) of TNF-α significantly ($p<0.05$) decreased total protein content in L6 myotubes.

The effects of TNF-α on the abundance of myofibrillar proteins in differentiated L6 myotubes were also shown in Figure 1B. A 24 hours incubation with 10 U/ml TNF-α resulted in significant increase ($p<0.01$) in myofibrillar protein concentration. Conversely, TNF-α in low concentrations (0.001, 0.01, 0.1 U/ml) decreased myofibrillar protein content. Again, 300 U/ml TNF-α reduced myofibrillar protein concentration. Western blot analysis to myosin heavy chain protein (MyHCf) revealed that MyHCf expression was increased in moderately high concentration (10 U/ml) of TNF-α (Figure 1C).

Time Course of TNF Effect on Total Protein Concentration

Incubation of L6 myotubes with 1 or 10 U/ml TNF-α caused a progressive increase in total protein concentration starting as early as 30 min reaching its peak after 6 hours and was still detected after 72 hours of incubation as shown in Figure 2. The 6 hours of incubation was chosen as a time point to further assess this positive effect.

Effect of Different Pathway Blockers

Modifications of the stimulatory effects of TNF-α on protein concentrations of L6 myotubes by various inhibitors for signaling pathways are shown in Figure 3. When the L6 myotubes were pretreated with 30 mM PD98059, TNF-α failed to increase total protein concentration of L6 myotubes. Either pretreatment with 5 μM of SB203580 or 40 μM of MG-132 had no statistically significant effect on the TNF-α action. Neither with 50 μM of LY294002 nor 100 μM of wortmannin affected TNF-α-induced increase in total protein concentration of L6 myotubes.

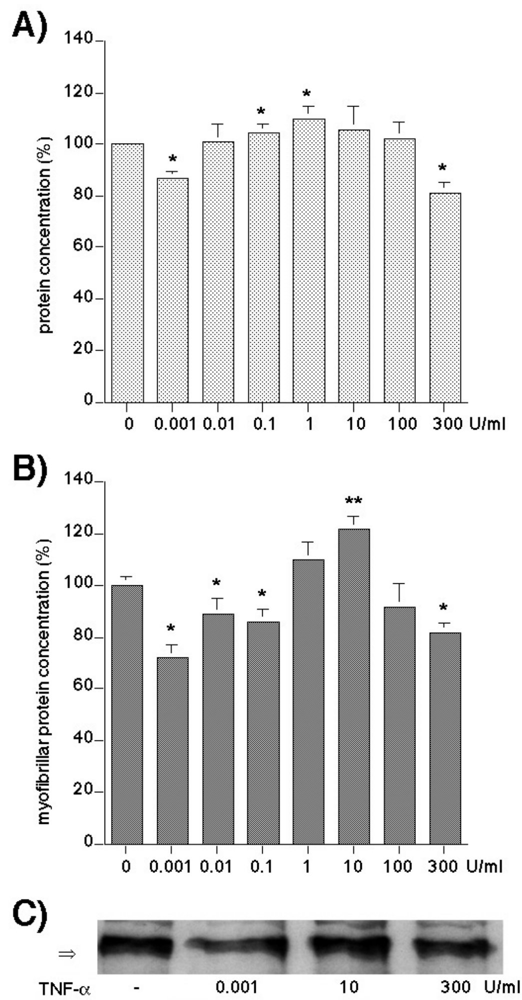

Figure 1. Effects of different TNF-α concentrations on total (A) and myofibrillar (B) protein contents, and expression of myosin heavy chain (C) in L6 myotubes. Myotubes were exposed to various TNF-α concentrations for 24 hours. Results are the means of four experiments. Protein values are expressed as percentage of the control (untreated cells), and the error bars express the standard deviations. TNF-α has tri-phasic effects on protein content and myofibrillar protein content in L6 myotubes. ✶ $p<0.05$, ✶✶ $p<0.01$: Statistically significant from control values. Western blotting study revealed that 10 U/ml of TNF-α increased expression of myosin heavy chain (indicated by the arrow) in L6 myotubes.

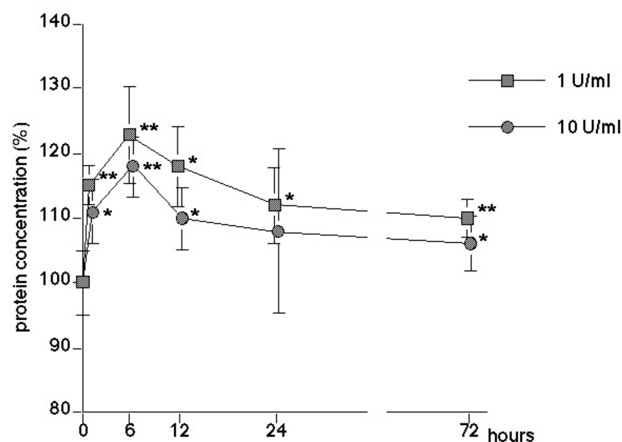

Figure 2. Time course of TNF-α stimulation on total protein content in L6 myotubes.

Myotubes were incubated for (30 minutes, 6, 12, 24 or 72 hours), with TNF-α (1, 10 U/ml). Results are the means (±SD) of four experiments. Protein values are expressed as percentage of the control (untreated cells). ✶ $p<0.05$, ✶✶ $p<0.01$: Statistically significant from control values.

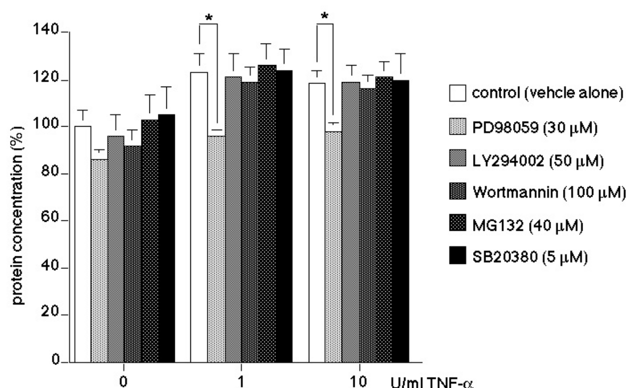

Figure 3. Effects of different inhibitors on TNF-α stimulated protein accumulation in L6 myotubes.

Myotubes were pretreated for 30 minutes with PD98059, LY294002, wortmannin, MG-132, or SB203580 before addition of TNF-α. Results are the means ± standard deviation of four experiments. Protein values are expressed as percentage of the control (vehicle alone). Pretreatment with PD98059 inhibited the stimulatory effect of TNF-α on protein concentration in L6 myotubes. Statistical differences: ✶ $p<0.05$.

DISCUSSION

Our study presented a dose dependent tri-phasic effect of TNF-α on protein content in L6 myotubes. TNF-α at low (< 0.1 U/ ml) and high (>100 U/ml) concentrations directly decreased total and myofibrillar protein, as well as myosin heavy chain level. However, moderately high TNF-α concentrations (1 or 10 U/ml) increased total and myofibrillar protein contents, and increased MyHCf level in L6 myotubes. Because of its accelerated breakdown, myosin heavy chain has been used as a muscle-specific structural protein to assess TNF-α induced effects [1]. This suggests that TNF-α at a concentration range from 1 to 10 U/ml has an anabolic effect on protein content.

We investigated the pathways by which the anabolic effect of 1 or 10 U/ml of TNF-α was exerted on L6 myotubes. PI3K activation is central to a number of important cellular processes, including protection from apoptosis, increased translation, and protein synthesis in skeletal muscle [12]. In our study, PI3-kinase inhibitors, LY294002 and wortmannin, failed to abolish the protein increase in L6 myotubes induced by TNF-α. These results suggested that elements other than the PI3-kinase cascade might mediate TNF-α-induced increase in protein content in L6 myotubes.

Another pathway involves the mitogen activated protein kinase (MAPK) family, there are at least three distinct MAPK signal transduction pathways, one leads to activation of ERK1 and ERK2, which are usually activated by growth factors like insulin-like growth factor I (IGF-I) [22]. In the present study, PD98059, a specific ERK1/2 inhibitor, significantly attenuated the TNF-α effect. It was suggested that ERK1/2 involves in the signaling process of protein increase in L6 myotubes by TNF-α. Proinflammatory cytokines typically trigger the MAPK signal transduction pathway through activation of p38. However, addition of SB203580, a p38 MAPK inhibitor, did not result in any significant effect. NFκB is known to be a major candidate for catabolic signaling, while it is also capable of stimulating cell survival and is involved in IGF-II or insulin stimulated signaling pathways [14]. Addition of MG132, that prevents NFκB activation by preventing the degradation of its inhibitor (Iκ B), caused no significant effect. These results suggested that the p38 and the NFκB are not involved in the positive effect of TNF-α on protein accumulation in L6 myotubes.

Indeed, there is a large body of evidence associating elevated TNF-α levels with muscle pathology. Protein breakdown was enhanced after chronic administration of recombinant TNF-α [9]. Animals expressing a TNF-α transgene also showed significant muscle loss [7]. Together with its role in diseases like cachexia [24] and inflammatory myopathy [20], TNF-α is considered to play a pathophysiological role in skeletal muscle. Contrary to these negative effects, TNF-α was expressed in regenerating muscle fibers in both inflammatory and non-inflammatory myopathy [17, 8]. TNF-α was also implicated in the regeneration process of skeletal myocyte after certain forms of stress and injury [25].

Results of in vitro studies were also divergent. Using murine cell cultures, it was reported that TNF-α induced protein loss attributed to increase in protein degradation [19], or decrease in protein synthesis [11]. Another study demonstrated a bimodal effect of TNF-α, that is, decrease of protein content with low doses of TNF-α and increase with its high doses [2]. In a more recent study, TNF-α increased total protein content of C2C12 myotubes [18]. In the present study, we also demonstrated that medium concentration of TNF-α had anabolic effect on L6 myotubes.

Effects of TNF-α on skeletal muscle are varied, depending on cell lines and species. Differences in culture conditions might also cause different response even in the same cell line. Li et al reported that 10 ng/ml (50 U/ml) of TNF-α decreased total protein content in C2C12 myotubes [19]. On the contrary, Langen et al. [18] reported stimulatory effect of

TNF-α on protein content in the same cell line. Langen et al. [18] used Matrigel coated dishes, while Li et al. [19] used the 2% horse serum. These observations together with our results suggest that TNF-α might have a beneficial role in skeletal muscle in some circumstances. However, this beneficial effect seems to be limited by many factors including amount of TNF-α, time of exposure, cell type, culture conditions, state of the cell (normal or deranged), and its stage of differentiation.

In conclusion, our findings implied that at certain time points, certain concentrations of TNF-α have a positive effect on total and myofibrillar protein accumulation in L6 myotubes, and this action is mediated at least in part by ERK1/2, one of the distinct MAPK signal transduction pathways. More understanding of this pathway and its upstream and downstream elements is necessary to clarify the basic mechanisms that regulate physiological and pathophysiological effects of TNF-α in skeletal muscle.

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