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Direct effects of tumor necrosis factor alpha (TNF-alpha) on murine skeletal muscle cell lines. Bimodal effects on protein metabolism

Abstract

Incubation of murine C2C12 myotubes with tumour necrosis factor-alpha (TNF-alpha) leads to significant changes in protein content and turnover, suggesting that the cytokine exerts direct effects in skeletal muscle. The effects of the cytokine on protein content show a clear bimodal behaviour. At low concentrations (1 U/ml or less), TNF-alpha decreases both total and myofibrillar

protein content, while at relatively high concentrations (100 U/ml or more), the effects are opposite and TNF-alpha increases the total and myofibrillar protein content in C2C12 myotubes. The mechanisms responsible for this latter, unexpected anabolic effect of the cytokine on muscle cells are related to a 40% increase in the rate of protein synthesis and to a significant decrease (14%) in the rate of protein degradation. At high concentrations, TNF-alpha decreased the expression of the mRNA of components of both the ATP- (ubiquitin, E2, C8) and Ca2+-dependent (m-calpain) proteolytic systems. The effects of TNF-alpha (10 U/ml or higher) on protein content of cultured murine myotubes (differentiated myogenic cells) were similar to those induced by insulin (1 or 5 mug/ml), but the effects of TNF-alpha and those of insulin were not additive. Experiments using inhibitors of the signalling pathways mediated by PI3K and MAP kinases (MAPKs) ERK1/2 and p38 suggest that insulin and TNF-alpha may share some intracellular signalling pathways involving MAPKs in the enhanced protein accretion observed in the muscle cell cultures.

Full text

INTRODUCTION

Tumour necrosis factor-alpha (TNF-alpha) is a pleiotropic cytokine which exerts a variety of effects, such as growth promotion or inhibition, cytotoxicity, angiogenesis, inflammation and immunomodulation. Although TNF-alpha is mainly produced by immune cells in response to infection, tumour growth and other invasive stimuli, many cell types have been reported to produce and release TNF-alpha [1]. TNF-alpha is synthesized as a 26 kDa, membrane-bound precursor that is cleaved proteolytically to a mature 17 kDa form with the prosequence polypeptide remaining associated with the membrane [2]. This peptide is bioactive as a 51 kDa trimer, which can be recognized by two different receptors, TNFR1 (or p55) and TNFR2 (or p75).

Bearing in mind that skeletal muscle constitutes approximately 45% of body weight, and that it represents the main source of protein loss during wasting diseases, studies concerning the effects of TNF-alpha on this tissue have proliferated over the last decade. However, many of the findings are divergent and therefore generate doubts as to whether or not this cytokine does have a role in muscle wasting [3, 4]. One of the very first observations concerning an action of TNF-alpha in skeletal muscle was reported by Tracey *et al.*, when it was

observed that the cytokine could alter the membrane potential of the muscle cell [5]. Miller *et al.* studied the effects of the cytokine on human muscle cell cultures and demonstrated that TNF-alpha exerts different effects on myoblasts and myotubes [6]. Thus, TNF-alpha added to myoblasts could prevent both the expression of muscle-specific genes (both skeletal and cardiac alpha-actin and myosin heavy-chain) and fusion into terminally-differentiated myotubes. The authors speculate that, since TNF-alpha has been implicated in the mammalian response to stress and in the pathogenesis of septic shock and cachexia, the target of TNF-alpha action in skeletal muscle may be the satellite cell population, which has the capacity to proliferate under conditions of muscle injury. Under these conditions, TNF-alpha may function to inhibit satellite cell differentiation while sparing differentiated muscle cells. Similarly, Szalay *et al.* have shown that TNF-alpha, although having a positive effect on the early steps of myogenesis by enhancing proliferation and aggregation of murine C2 myoblasts, strongly inhibits the expression of myogenic transcription factors (myoD and myogenin); these factors are known to be responsible for inducing the expression of muscle-specific genes [7].

A large body of evidence suggests that TNF-alpha participates in skeletal muscle protein wasting and loss of nitrogen associated with cachectic situations. Chronic treatment of rats with recombinant TNF-alpha resulted in a depletion of body protein compared with pair-fed control animals [8]. Indeed, chronic treatment with either recombinant TNF-alpha or interleukin-1 (IL-1) resulted in a body protein redistribution and a significant decrease in muscle protein content, associated with coordinated decreases in muscle mRNA levels for myofibrillar proteins [8]. Studies involving *in vivo* administration of recombinant TNF-alpha have shown an increase in nitrogen efflux from skeletal muscle of non-weight losing humans with disseminated cancer [9]. Flores *et al.*, by infusing ^{14}C-leucine into rats, showed that chronic recombinant TNF-alpha administration significantly enhanced muscle protein breakdown [10]. Goodman, measuring both tyrosine and 3-methylhistidine release by incubated rat muscles of animals acutely treated with the cytokine, concluded that TNF-alpha was involved in activating muscle proteolysis [11]. The mechanisms underlying such actions are now becoming clear. Our research group has demonstrated that TNF-alpha treatment enhances protein degradation measured *in vivo* in rat skeletal muscle [12, 13]. In addition, we have described that, at least during tumour growth, muscle wasting is associated with the activation of non-lysosomal, ubiquitin-dependent proteolytic activity [14, 15],

and that this activation seems to be mediated *via* TNF-alpha [16-18]. We have also reported that *in vivo* administration of TNF-alpha to rats results in an increased skeletal muscle proteolysis associated with an increase in both gene expression and higher levels of free and conjugated ubiquitin [17, 18]. Concerning a possible direct action of TNF-alpha on muscle proteolysis, the presence of both TNFR1 and TNFR2 receptors in muscle has been described [19], and our group has demonstrated that the action of the cytokine on the induction of ubiquitin-dependent proteolysis can be direct [20]. Other cytokines, such as IL-1 or interferon-gamma (IFN-gamma), are also able to activate ubiquitin gene expression [21]. Therefore, TNF-alpha, alone or in combination with other cytokines, seems to mediate most of the changes concerning skeletal muscle nitrogen metabolism associated with cachectic states.

The effects of TNF-alpha upon protein turnover have also been investigated in muscle cell culture systems. Frost *et al.* have reported that transient exposure of human myoblasts to TNF-alpha inhibits IGF-I-stimulated protein synthesis in a dose-dependent manner [22]. In this study, TNF-alpha acted in a similar manner on human myotubes. In contrast, exposure of myoblasts to TNF-alpha had no effect on IGF-I binding or on its ability to stimulate ^{3}H-thymidine uptake. The authors concluded that the anabolic actions of IGF-I on muscle protein synthesis may be impaired during catabolic conditions in which TNF-alpha is overexpressed. In connection with this study, Fan *et al.* observed that exogenous administration of TNF-alpha to experimental animals reduced muscle IGF-I content [23].

While *in vivo* chronic TNF-alpha treatment leads to muscle protein wasting, the mechanisms involved deserve further investigation since neither experiments carried out with isolated incubated muscle or with muscle cell cultures have shown very clear results. Bearing this in mind, it was the aim of the present work to investigate the effects of the cytokine on muscle protein content and metabolism in cultured, differentiated muscle cells.

EXPERIMENTAL

Materials

Recombinant murine TNF-alpha and recombinat murine IL-6 were obtained from PeproTech (London, UK). SB302580 and PD98059 were from Calbiochem (Merck KGaA, Darmstadt, Germany). Radiochemicals were purchased from Amersham Int. (Amersham, Bucks., UK). All other biochemicals were reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA) or from Roche Diagnostic SL (Barcelona, Spain).

Cell culture

C2C12 mouse skeletal muscle cells were obtained from the American Type Culture Collection. Cells were passaged in high glucose DMEM suplemented with 10% FBS, 100 units penicillin/ml, 100 mug streptomycin/ml, 25 ng fungizone/ml, 110 mug sodium pyruvate/ml, and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37° C. For experimental analyses, cells were seeded at 3.7 x 10^4 cells/ml or cm^3 in 10% FBS/DMEM until they reached 90-100% confluence 24 hours later. At this time, the medium was replaced by DMEM containing 10% HS for induction of differentiation. Abundant myotube formation, monitored microscopically, occurred after 4 days in 10% HS/DMEM. Such fused myotubes cultures were utilized for experimental analyses, with hour 0 considered the time of TNF-alpha, IGF-I or insulin addition to the cultures; cultures were exposed to the different treatments for 1-72 hours and all cells were collected at the end of the incubations, being in all cases day 7 after transferring cells to 10% HS/DMEM.

L6E9 rat skeletal muscle myotubes were also obtained from the American Type Culture Collection. Cells were grown similarly to C2C12, but they were seeded at 1.1 x 10^4 cells/ml and differentiation was induced by switching to DMEM containing 2% FBS when confluence was 80%.

Culture medium, growth factors and cytokines were replaced daily.

Total and myofibrillar protein content

For total protein determination, cells were plated in 24-well plates. After various incubation periods, monolayers were washed twice with ice-cold PBS (200 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.47 mM, KH_2PO_4, pH 7.4). Then, 10% trichloroacetic acid (TCA) was added to lyse cells and precipitate cell proteins. After at least 1 hour at 4 °C, monolayers were rinsed once with ice-cold 10% TCA

and proteins were dissolved in 0.5 M NaOH containing 0.1% Triton X-100 at 37 °C (at least 1 hour) and the monolayers collected quantitatively. Protein content was determined using the BCA assay (Pierce Chemical Co.).

Myofibrillar protein content was determined as described by Clark *et al.* [24] with some modifications. Cells were grown in 6-well plates and at the end of the treatments, monolayers were washed with ice-cold PBS, scraped and collected in 200 mul low salt buffer (40 mM NaCl, 1 mM dithiotreitol, 0.1 mM EGTA, 5 mM Na_2SO_4, pH 6.8) containing 1% Triton X-100 and freshly added proteases inhibitors (10 mg/ml leupeptin, 2 mug/ml aprotinin, 0.1 mM PMSF, 5 mM NEM, 2 muM pepstatin). After centrifuging for 10 min (1,500 g, 4 °C), the pellet containing the myofibrillar proteins fraction was washed twice with 200 mul low salt buffer w/o Triton X-100. Finally, 200 mul of sample buffer were added (2% SDS, 10% glycerol, in 62.5 mM Tris buffer, pH 6.8) and proteins were solubilized by boiling for 3 min. Protein content was determined using the BCA assay.

Equal volumes of the lysates were loaded onto 10% SDS-PAGE gels and after protein separation, gels were stained with Coomassie Blue. Specific proteins were identified from their mobilities relative to pre-stained molecular weight standards and bands were quantitated by densitometry.

Rates of protein synthesis and degradation

For the measurement of protein degradation, the cells were grown in 6-well plates, and proteins were radiolabeled by incubating 4 day-old myotubes with 1 muCi/ml L-^{3}H-phenylalanine [2-6] (^{3}H-Phe; 143 Ci/mmol, Amersham) for 24 hours in DMEM supplemented with 10% HS. The cells were rinsed twice in 2 ml of ice-cold Hank's balanced salt solution (1.7 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 200 mM NaCl, 0.42 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.6 mM D-glucose) containing 2 mM non-radioactive phenylalanine (HBSS-Phe) and then incubated for 2 hours in a chase medium, composed of DMEM containing 10% FBS and 2 mM non-radioactive phenylalanine, to allow the degradation of short-lived proteins. The myotubes were rinsed twice in ice-cold HBSS-Phe and then transferred to the degradation medium (3 ml), consisting of DMEM + 10% HS + 2 mM Phe, supplemented or not with TNF-alpha. After 6, 24 and 48 hours, 0.5 ml aliquots were sampled to measure the radioactivity of [^{3}H] in the non-TCA-precipitable fraction in the medium released from the cells using a liquid scintillation counter (LSC). After 48 hours, the remaining radioactivity in

the attached cells was counted by dissolving the monolayers in 0.5 M NaOH containing 0.1% Triton X-100 and then subjecting this to LSC. The percentage of protein degraded at any point was computed by dividing the radioactivity in the amino acid fraction of the released medium at that time, divided by the initial radioactivity. Initial radioactivity was calculated as the sum of the radioactivity in the TCA soluble fraction of the medium plus the radioactivity in the non-TCA-soluble fraction in the medium plus the remaining radioactivity in the cells.

To determine the rates of protein synthesis, myotubes in 6-well plates were incubated in non-radioactive experimental medium (DMEM supplemented with 10% HS with or without TNF-alpha) for various periods of time. The culture medium was replaced with experimental medium containing 1 muCi of [^{3}H]Phe/ml for the final hour of incubation. Cells were rinsed twice in ice-cold HBSS-Phe. Monolayers were precipitated with 1 ml 10% TCA for at least 1 hour at 4 °C and, after washing once with ice-cold 10% TCA, the precipitated protein was dissolved by incubation for at least 1 hour at 37 °C in 1 ml of 0.5 M NaOH containing 0.1% Triton X-100. Radioactivity was determined using an LSC, and protein content was determined using the BCA assay.

RNA isolation and Northern blot analysis

Myotubes were grown in 10 mm dishes and treated with or without TNF-alpha for different periods. RNA was extracted from seven day-old myotubes using the acid guanidinium isothiocianate/phenol/choloroform method [25]. RNA samples were denatured, subjected to electrophoresis in 1.2% agarose gels containing 6.3% formaldehide and transferred to Hybond N membranes (Amersham). RNA was fixed to the membranes by UV crosslinking. The RNA in gels and on filters was visualized by ethidium bromide staining and photographed by UV transillumination to ensure the integrity of the RNA and to confirm proper transfer. RNA was transferred in 20x standard saline citrate (SSC; 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). Hybridization was done at 65 °C overnight in hybridization buffer (0.25 M Na_2HPO_4, 7% SDS, 1 mM EDTA, 1% BSA, 10% dextran sulfate), and denatured labeled probes (10^6-10^7 cpm/ml) were added. Radiolabeled probes were prepared by the random primer method (Roche). The ubiquitin probe used was a cDNA clone (provided by Dr. M.J. Schlesinger), containing 12 pairs of the second coding sequence, plus a complete third and fourth ubiquitin coding sequence, and 120 base pairs of the 3'-untranslated region of the chicken polyubiquitin gene UBI [26]. The membranes were also

hybridized with cDNA probes encoding rat m-calpain (provided by John S. Elce, Department of Biochemistry, Queen's University, Kinston, Ontario, Canada), rat cathepsin B (provided by Dr. S.J. Chan, Baylor College of Medicine, Division of Neuroscience, Huston, USA) and the proteasome subnunit C8 (provided by Dr. Tanaka, Intitute for Enzyme Research, University of Tokuhima, Japan). Loading correction was carried out by performing Northern blots with the rat 18S ribosomal subunit probe [27]. Filters were exposed to Hyperfilm-MP (Amersham) at - 80 °C for 1-4 days, and the films quantified by scanning densitometry (Phoretix 2.51, Phoretix International Ltd.).

Statistical analysis

Statistical analysis of the data was performed by means of Student's *t* test (two-tailed, unpaired).

RESULTS

Figure 1 depicts the effects of various TNF-alpha concentrations on total protein content in C2C12 myotube cell cultures. The effects of the cytokine are dependent on its concentration; at low TNF-alpha concentrations (0.001 to 0.1 U/ml), the cytokine significantly ($p < 0.005$) decreases protein content, while at concentrations of 10 U/ml or above, TNF-alpha exerts an opposite effect, increasing myotube protein content by 20% at the higher dose (Figure 1). The decrease in myotube protein content associated with low TNF-alpha concentrations is consistent with previous studies implicating TNF-alpha in muscle protein loss [12]. However, the increase in myotube protein at high TNF-alpha concentrations was paradoxical and was investigated further. The effects of the cytokine on protein deposition were already observed at 24 hours, and were still observed even after 72 hours of administration. (Figure 2). The effects of high TNF-alpha concentrations on muscle cell protein content were also observed in another type of myogenic cell, the L6E9 line (Figure 3), suggesting therefore that the effects of the cytokine at high concentrations were not limited to a single myogenic cell line. Figure 4 shows that the increase in total protein content induced by TNF-alpha was accompanied by an increase in myofibrillar protein content. Indeed, treatment of C2C12 myotubes for 48 hours with 1,000 U/ml of TNF-alpha resulted in over 30% increases in actin, myosin and desmin (Figure 4). Again, a similar increase in the myofibrillar content was observed in L6E9 cells (data not shown). There was a corresponding marked

decrease in the content of the myofibrillar fraction is induced by incubating the myotubes with low doses of the cytokine (Figure 4).

We next determined protein turnover, both protein synthesis and degradation (Figure 5). Using the method of incorporation of ^{3}H-phenylalanine into protein, we were able to see that only 1 hour following the addition of high-dose cytokine to the cell cultures (1,000 U/ml), there was a significant increase in the rate of protein synthesis, which was 40% higher as compared to the control cells, 72 hours following addition of TNF-alpha (Figure 5a). Interestingly, the highest effect of the cytokine on the rate of protein synthesis was observed at 6h (65% increase) (Figure 5a). The cytokine had also effects on the rate of protein degradation as measured by pre-loading of proteins with ^{3}H-phenylalanine and determination of free amino acid release. Indeed, TNF-alpha treatment (1,000 U/ml) resulted in a significant decrease in the rate of degradation 24 hours following addition (Figure 5b). There was no effect on protein degradation 6h after addition of the cytokine (Figure 5b), thus suggesting that the effects of TNF-alpha on protein synthesis were faster than those observed on protein degradation (Figure 5). The decrease in protein degradation was accompanied by a decrease in both the 2.4 and the 1.2 kb ubiquitin mRNA transcripts (over 50% decrease) observed 48 hours after the addition of the cytokine (Table 1), therefore suggesting that the ATP-dependent proteolytic pathway could be mediating the effects of TNF-alpha on protein degradation. The levels of expression of the C8 proteasome subunit and the E2 conjugating enzyme were also decreased (Table 1). TNF-alpha addition to myotubes also resulted in decreased levels of m-calpain mRNA (which is associated with the Ca^{2+}-dependent protein degradation) and on cathepsin B gene expression (associated with the lysosomal proteolytic system), but only 2 hours after the addition of the cytokine (Table 1).

The magnitude of the high dose-TNF-alpha effects on protein accretion in C2C12 myotubes was compared with the well-characterized anabolic actions of insulin [28] used at concentrations at which it activates both insulin receptors and type-1 IGF receptors. We incubated cells with either 1 or 5 mg of the hormone/ml and observed a clear increase in protein content 48 hours after addition to the cell cultures (Figure 6). At the lowest concentration, the effects of the hormone were very similar to those observed with TNF-alpha. Interestingly, the effects of TNF-alpha and insulin were not additive at any of the insulin concentrations (Figure 6). The increase in total protein content

promoted by both insulin and TNF-alpha was accompanied by an increase in the rate of protein synthesis (Figure 7); again, the the effects of TNF-alpha and insulin were not additive (Figure 7).

Since the effects of TNF-alpha and insulin were not additive, this suggested that the two factors may share some signalling pathways. To examine this possibility, we used wortmannin (an inhibitor of PI3K activated pathway), PD98059 (an inhibitor of the ERK1/2 MAPKs pathway) and SB203580 (an inhibitor of the p38 stress-activated MAPK pathway). These compounds inhibit signalling intermediates which are involved in the biological actions activated by insulin and/or TNF-alpha (Figure 8). C2C12 myotubes were incubated for 48 hours with either TNF-alpha (1,000 U/ml) or insulin (5 mug/ml) in the presence or absence of the inhibitors (1 muM wortmannin, 5 muM PD98059, 5 muM SB203580). The results (Table 2) show that while inhibition of PI3K with wortmannin did not have any effects on either the TNF-alpha- or insulin-mediated increase in protein content in C2C12 cells, addition of PD98059 caused a significant decrease in insulin-stimulated protein accretion in the cell cultures and abolished the effects TNF-alpha. Addition of SB203580 also prevented the effects of TNF-alpha, while it resulted in a significant increase (17%) in the effects of insulin (Table 2).

DISCUSSION

Muscle wasting and cachexia are responsible for the death of many cancer patients since they are associated with a decrease in treatment efficiency and an increase in both psychological and cardiac complications, among others. Cachexia occurs in more than two thirds of patients who die with advanced cancer and is estimated to be responsible for the death of 22% of cancer patients [29]. Indeed, the degree of cachexia is inversely correlated with the survival time of the patient and it always implies a poor prognosis [30-32]. At the biochemical level, various explanations can be found to account for cancer-induced cachexia, but basically the presence of both tumoral and humoral compounds (mainly cytokines, TNF-alpha in particular) is associated with depletion of fat stores and muscular tissues [3]. The balance between proinflammatory cytokines, their soluble receptors and the anti-inflammatory cytokines plays a key role in the development of the cachectic syndrome [33].

Although there is little doubt that TNF-alpha (and other cytokines, such as IL-6 [34]) is involved in muscle wasting in many pathological conditions in addititon to cancer cachexia [33], it is unclear whether the actions of the cytokine on muscle cells are direct through binding of the cytokine to its two distinct receptors. The presence of both TNFR1 and TNFR2 receptors in muscle has been described [19]. Using incubated rat soleus and EDL muscles, Llovera *et al.* have demonstrated that the action of the cytokine on the induction of ubiquitin-dependent proteolysis can be direct [20]. Other studies using murine muscle cell cultures have confirmed these results [22, 35]. As can be seen from the results presented in Table 1, both the lysosomal and the Ca^{2+}-dependent proteolytic systems are affected by TNF-alpha, suggesting a wider action of the cytokine on proteolysis. The situation is less clear in humans, and some studies have not supported the concept of a direct action of TNF-alpha on skeletal muscle metabolism. Thus, De Blaauw *et al.* studied six patients undergoing isolated limb perfusion with TNF-alpha for irresectable soft-tissue sarcoma or in-transit melanomas, and observed no changes in muscle glucose or protein metabolism, suggesting that the cytokine has no direct metabolic effect on human muscle [36].

Our results show that at low concentrations, 0.1 U/ml (0.01 ng/ml) or lower, the effects of TNF-alpha on total and myofibrillar protein content in two lines of cultured muscle cells are clearly catabolic. This is in agreement with a clear activation of the proteolytic pathways, as described by the studies of Li *et al.* [35] who reported that TNF-alpha is able to decrease protein content in C2C12 myotubes by increasing protein degradation through activation of the ubiquitin-dependent pathway *via* an activation of the NF-kappaB-dependent signalling pathway. The experiments presented here show that the action of TNF-alpha on these cells is dependent on the concentration of the cytokine. Indeed, 10^{-3}-0.1 U/ml (10^{-4}-0.01 ng/ml) of TNF-alpha decreases myofibrillar protein content; however, higher concentrations (10-1,000 U/ml, 1-100 ng/ml) of TNF-alpha increase total protein content through an increase in total protein synthesis and a decrease in degradation (Figure 5). Even higher increases were observed in myofibrillar protein content after treatment with high doses of TNF-alpha. This is the first report of a bimodal action of TNF-alpha on skeletal muscle and a further understanding of the nature of this bimodal response to TNF-alpha may be crucial to developing a strategy to inhibit the cachectic effects of TNF-alpha on muscle tissue.

Circulating TNF-alpha levels in different pathological catabolic conditions such as sepsis, trauma, cancer and injury, are subject to great variability. For instance, during human cancer cachexia, the levels of circulating TNF-alpha measured can be high [37] or undetectable [38]. Circulating levels of TNF-alpha are subject to a considerable degree of diurnal variation [39]. In addition, the determination of the cytokine using ELISA leads to disappointing results due to the lack of reproducibility [40]. In experimental cancer cachexia, however, high circulating TNF-alpha levels are detectable, in the order of 500 pg/ml [41].

However, skeletal muscle concentrations may be considerably higher since several studies have shown that skeletal muscle is able to express TNF-alpha mRNA [42], together with mRNA of its two receptors [43]. Some reports show that in several pathophysiological situations, in which high TNF-alpha concentrations are implicated, plasma levels of TNF-alpha do not correlate with high local concentrations. TNF-alpha concentrations as high as 15 ng/ml were found in the bronchoalveolar fluid of patients with adult respiratory distress, while concentrations in the plasma rarely exceeded 100 pg/ml [44]. In patients with peritonitis, TNF-alpha in the peritoneal fluid exceeded 10 ng/ml, while plasma levels were undetectable by immunoassays or bioassays [45]. In pathological conditions where there is a clear loss of skeletal muscle, there is an increased expression of the TNF-alpha gene [46]. In addition, TNF-alpha is able to regulate the expression of the receptors in cultured muscle cells [47].

The increase in protein content brought about by TNF-alpha, was comparable to that found for IGF-I (data not shown). This growth factor has previously been shown to be important for stimulating muscle proliferation, differentiation and hypertrophy [28]. In addition, it is interesting to note that the anabolic effects of high dose TNF-alpha on skeletal muscle are similar in magnitude to those mediated by addition of insulin (Figures 6 and 7). The increase in protein content and rate of protein synthesis induced by both the cytokine and the hormone are comparable. The effects are not additive, suggesting therefore that insulin and TNF-alpha may use common intracellular signalling pathways. It was for this reason that we performed several experiments using inhibitors of the signalling pathways that may have been involved. Indeed, previous studies have shown that TNF-alpha is able to interact with insulin signalling. Thus, Hotamisligil *et al*. have shown that TNF-alpha has the ability to decrease tyrosine kinase activity of the insulin receptor [48]. Treatment of cultured murine adipocytes with TNF-alpha was shown to induce serine phosphorylation of insulin receptor substrate-

1 (IRS-1) and convert IRS-1 into an inhibitor of the insulin receptor tyrosine kinase *in vitro*, thus indicating that TNF-alpha induces insulin resistance through an unexpected action of IRS-1 to attenuate insulin receptor signalling. Some of the insulin effects are mediated *via* activation of IRS-1 phosphorylation with subsequent PI3K activation. Thus, we used wortmannin to inhibit this intracellular signalling pathway. Other insulin effects are mediated *via* the MAP kinase cascade. There are at least three distinct mitogen-activated protein kinase (MAPK) signal transduction pathways in mammalian cells, each leading to activation of either the extracellular-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK-SAPK) or p38. We used the inhibitor PD98059 to block activation of ERK 1/2. This activation can take place *via* ras-GTP and Raf (insulin), or *via* the ceramide pathway and ulterior activation of Raf (TNF-alpha) (Figure 8). Finally, we used the inhibitor SB203508 to block p38 activation, a pathway typically triggered by proinflammatory cytokines, which is activated by SEK *via* MEKK1 (Figure 8). The results obtained with the different inhibitors (Table 2 and Figure 8) allow us to suggest that the PI3K pathway is not involved in the insulin or TNF-alpha effects on protein accumulation at 48 hours in C2C12 myotubes, since the addition of wortmannin did not result in any significant effects (Table 2). PD98056 abolished the effects of TNF-alpha on protein content, while it only promoted a 60% decrease in the effects of insulin. The addition of SB203580 abrogated the effects on protein content induced by TNF-alpha, while surprisingly causing a 17% increase in insulin-stimulated protein accumulation. These results suggest that all three MAPKs pathways may be involved in the effects of insulin and TNF-alpha on protein content in C2C12 cells.

Finally, and although we have shown that the effects of the cytokine on cultured muscle cells are direct, another cytokine or mediator may also be involved. Thus, we have observed that addition of TNF-alpha to the muscle cells results in an significant increase in IL-6 expression (unpublished results), so that this cytokine could also be mediating some of the effects of TNF-alpha on protein accumulation. Indeed, human muscle satellite cell proliferation *in vitro* seems to be regulated by autocrine secretion of IL-6 [49]. This could also be the case in our experimental system and therefore investigations in our laboratory are currently underway to ascertain this point.

CONCLUSION

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Figures

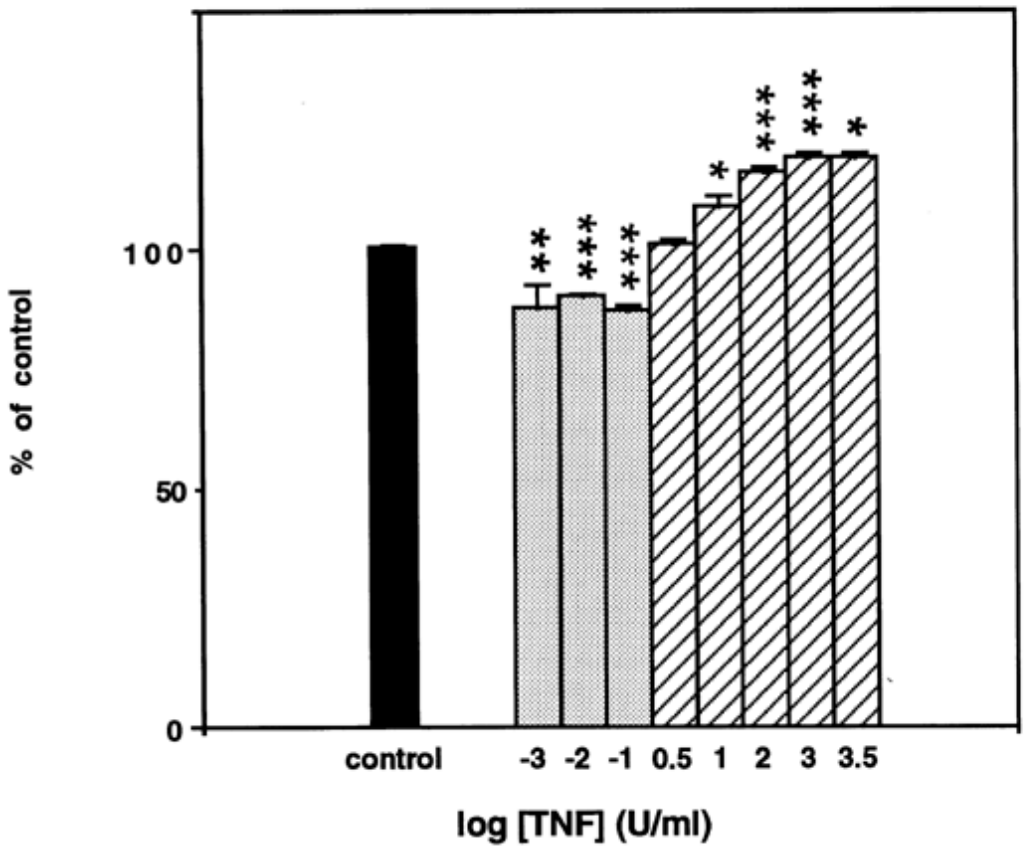


Figure 1
Effects of different TNF-alpha concentrations on total protein content in C2C12 myotubes.

Myotubes were exposed to various concentrations of TNF-alpha for 72 hours. Total protein values as expressed as a percentage of control (untreated) cells, and are means ± S.E.M. of mug of protein/plate. Statistical significance of the results (Student's t-test): * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

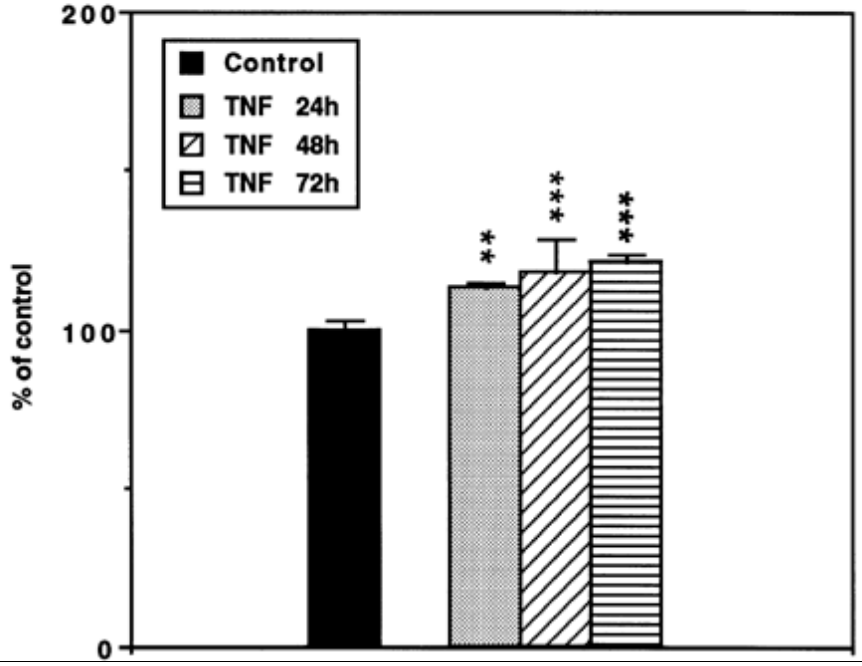


Figure 2
Time course of high-dose TNF-alpha effects on protein content in C2C12 myotubes.

Total protein values are expressed as a percentage of control (untreated) cells and are means ± S.E.M. TNF-alpha was administered at a concentration of 1,000 U/ml and cells were exposed to 1,000 U/ml of TNF-alpha for different periods. Statistical significance of the results (Student's t-test): ** $p < 0.005$.

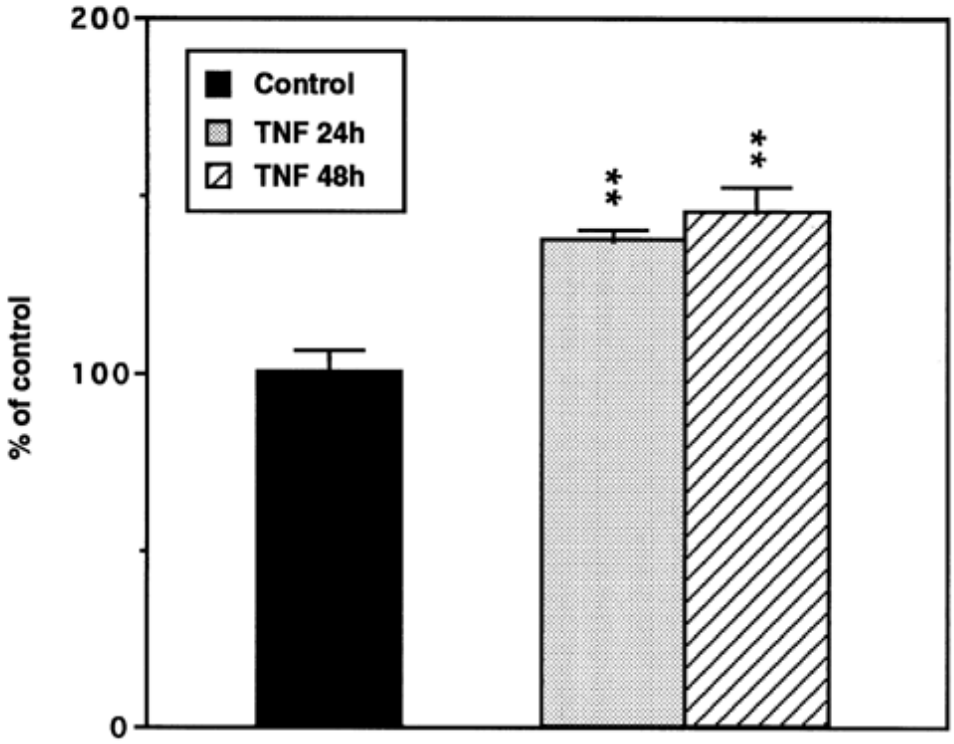


Figure 3
Effects of high-dose TNF-alpha on total protein content in L6E9 myotubes.

Total protein values are expressed as a percentage of control (untreated) cells and are means ± S.E.M. TNF-alpha was administered at a concentration of 1,000 U/ml and cells were exposed to 1000 U/ml of TNF-alpha for different periods. Statistical significance of the results (Student's t-test): ** $p < 0.005$, *** $p < 0.001$.

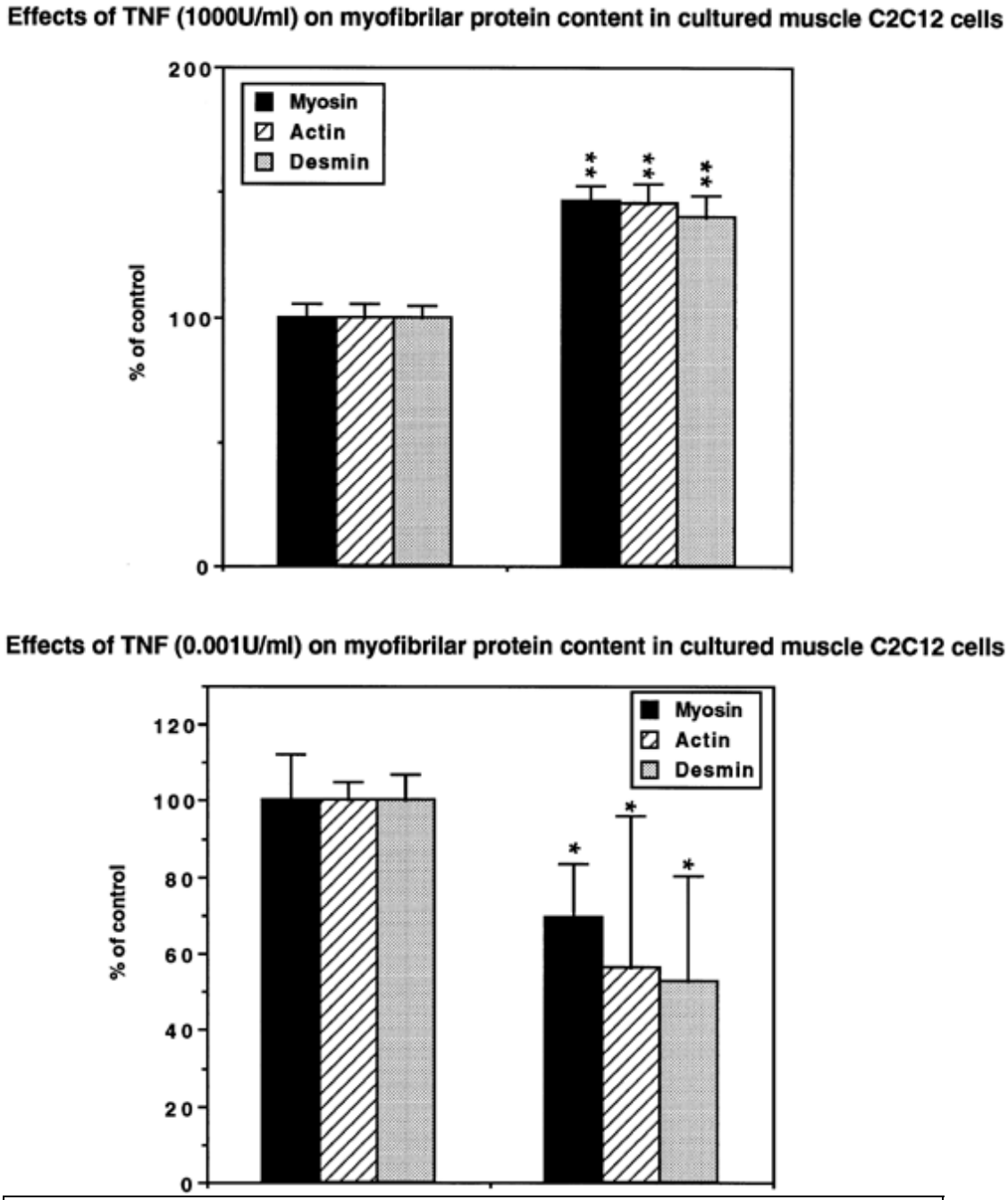
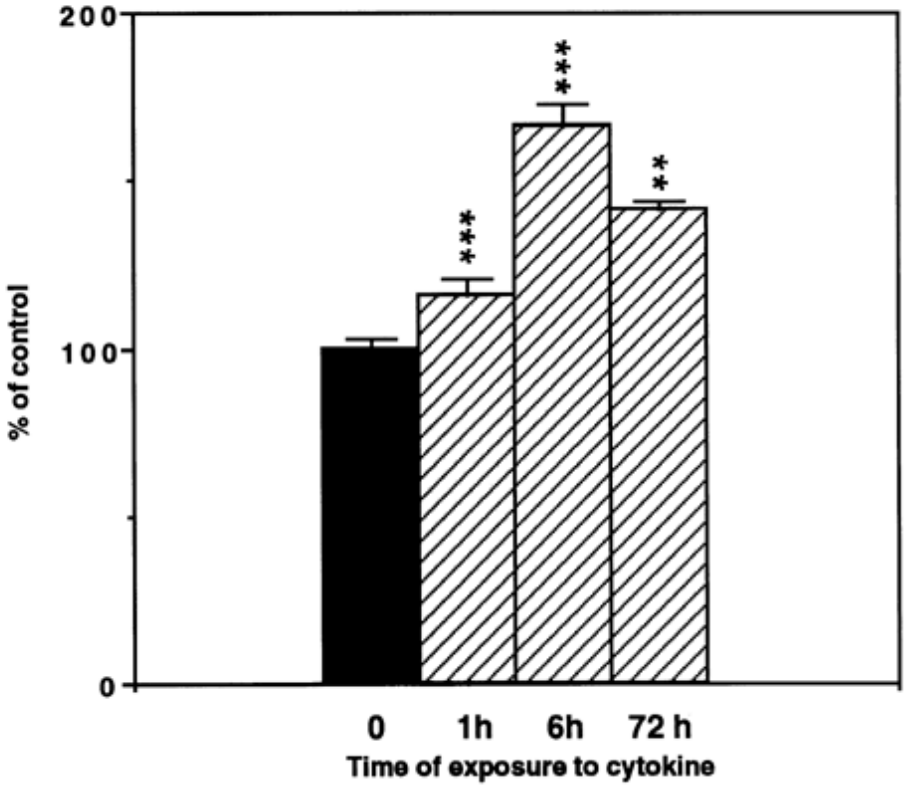


Figure 4
Effects of low and high TNF-alpha concentrations on myofibrillar protein content.

Myotubes were treated for 72 hours with TNF-alpha at 1,000 U/ml. At the end of the experiments, myofibrillar protein was extracted and fractioned by SDS-PAGE. Individual proteins were quantitated by densitometry on the Coomassie-blue stained gels. Values are expressed as a percentage of control (untreated) cells and are

means ± S.E.M. Statistical significance of the results (Student's t-test): * $p < 0.05$, *** $p < 0.001$.

Effects of TNF (1000 U/ml) on the rate of protein synthesis in C2C12 cells



Effects of TNF (1000 U/ml) on the rate of protein degradation in C2C12 cells

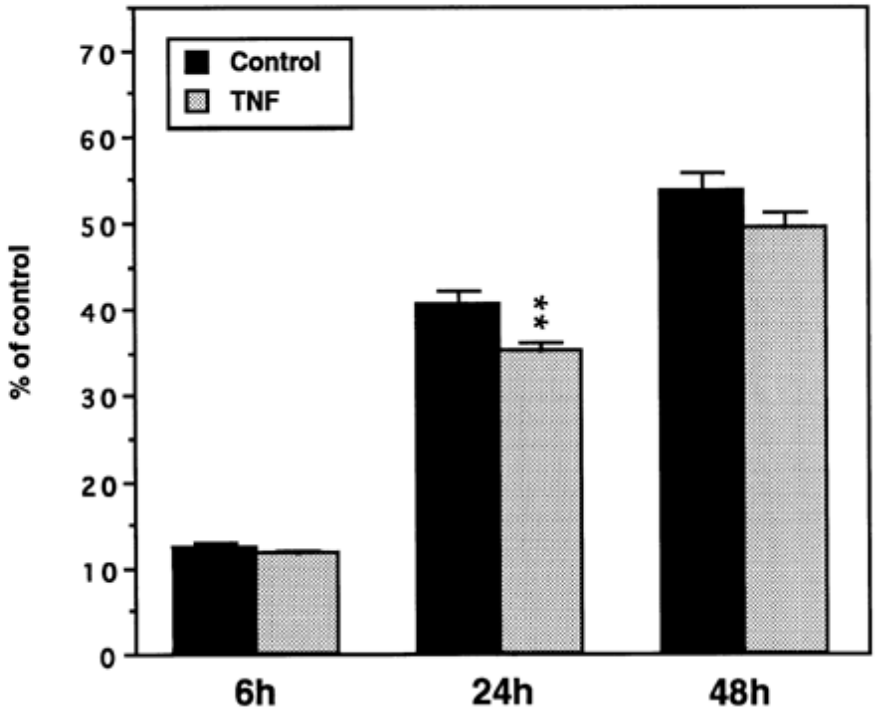


Figure 5
Effects of TNF-alpha on the rates of protein synthesis and degradation.

For protein synthesis rate determination, myotubes were treated with TNF-alpha at 1,000 U/ml for various periods and ^{3}H-Phe was added during the final hour of incubation. Values for protein synthesis were assessed as radioactivity incorporated into protein/mug of total protein and are expressed as a percentage of control (untreated) cells ± S.E.M. For protein degradation rate determination, myotubes were treated for 48 hours with ^{3}H-Phe before exposure to TNF-alpha. Values for protein degradation represent the percentage of radioactivity in the amino acids in the medium versus the total radioactivity incorporated into protein at time 0). TNF-alpha was present at a concentration of 1,000 U/ml. All data are means ± S.E.M. Statistical significance of the results (Student's t-test): ** $p < 0.005$.

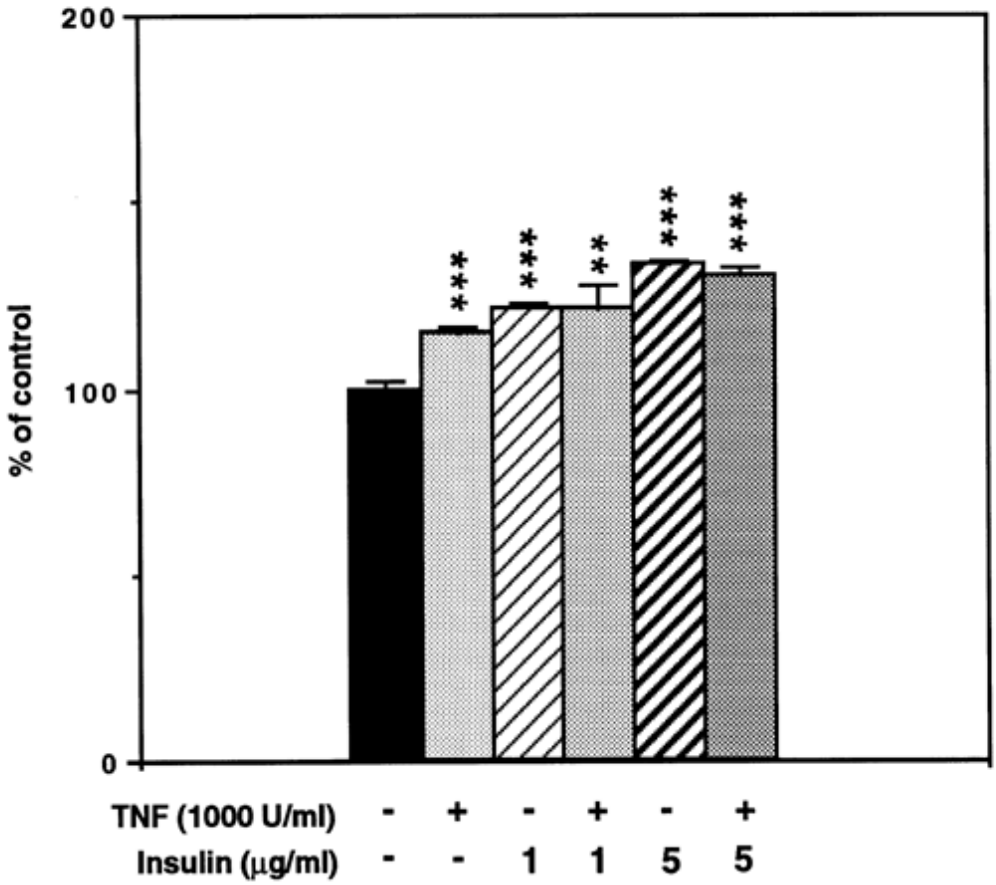


Figure 6
Effects of insulin and TNF-alpha on protein accumulation in C2C12 cells.

Values are expressed as a percentage of control (untreated) cells and are means ± S.E.M. of mug of protein/plate. Insulin 1 or insulin 5 represent insulin at concentrations of 1 or 5 mug/ml respectively.TNF-alpha was present at a concentration of 1,000 U/ml. Myotubes were exposed to TNF-alpha and/or insulin for 48 hours. Statistical significance of the results (Student's t-test): **p < 0.01, ***p < 0.001.

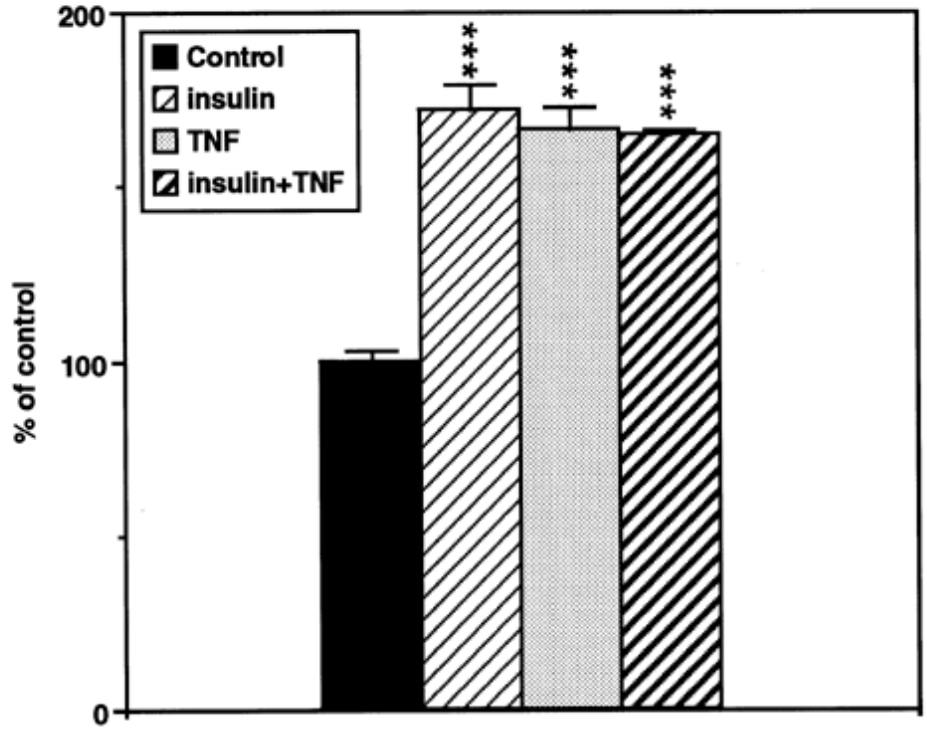


Figure 7
Effects of insulin and TNF-alpha on the rate of protein synthesis in C2C12 cells.

For protein synthesis rate determination, myotubes were treated with TNF-alpha at 1,000 U/ml and/or insulin at 5 mug/ml for various periods and ^{3}H-Phe was added at the final hour of incubation. Values for protein synthesis were assessed as radioactivity incorporated into protein/mug of total protein and are expressed as a percentage of control (untreated) cells ± S.E.M. Statistical significance of the results (Student's t-test): *** $p < 0.001$.

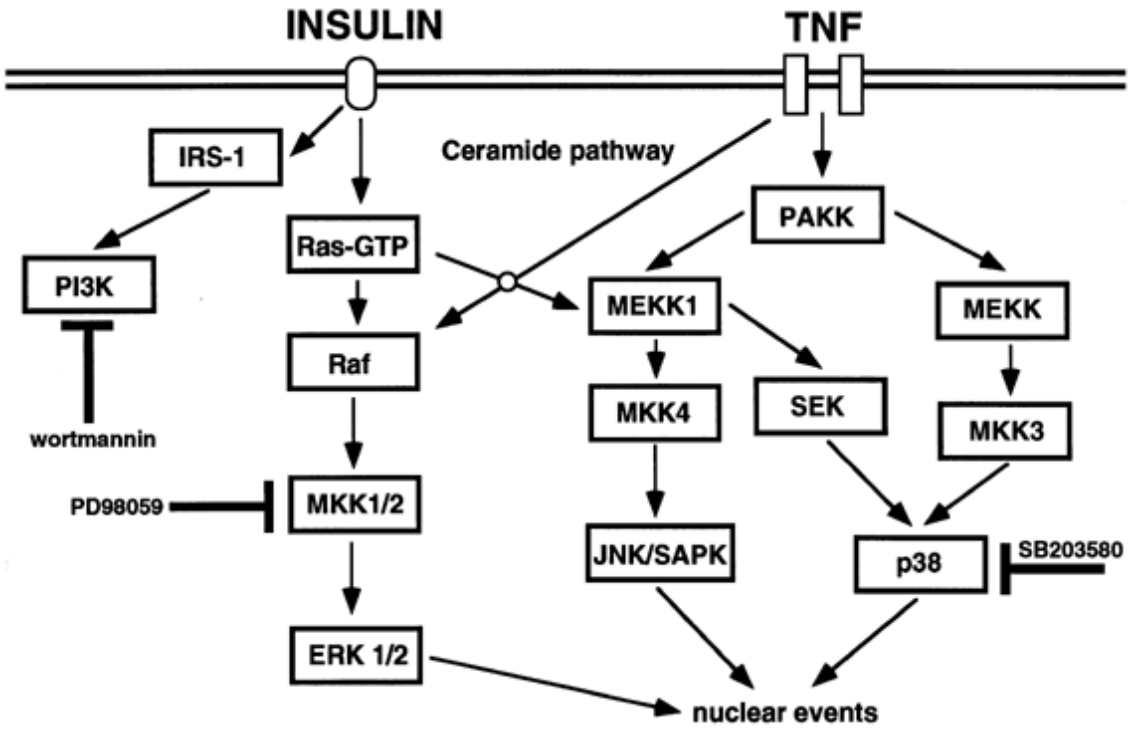


Figure 8
Signalling pathways: inhibiting the MAPKs.

Insulin and TNF-alpha have several pathways in common affecting the MAP kinase cascade. The different inhibitors shown here were tested to elucidate signalling pathways that may account for the effects of the hormone and the cytokine on protein accumulation in cultured murine muscle cells. Wortmannin is an inhibitor of phosphatidylinositol-3-kinase (PI3 kinase), which is directly linked to the action of the insulin phosphorylation cascade through IRS-1. PD98059 is a selective and potent inhibitor of the p44/42 MAPK cascade by binding to inactive MEK and preventing phosphorylation and activation by Raf; this compound does not inhibit SAPK, p38 or PI3K. SB203580 is a selective and potent inhibitor of the MKK3, which phosphorylates p38.

Table 1
Effects of 1,000 U/ml TNF-alpha on mRNA of various proteolytic systems components

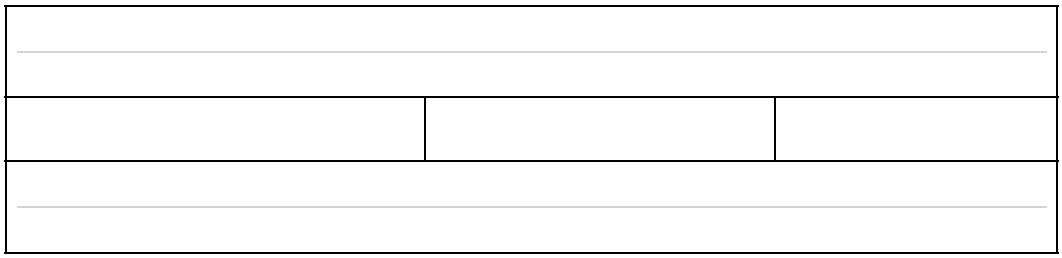
mRNA	Treat-ment	2 hours	48 hours
Ubiquitin 2.4 kb	None	225 ± 15.2	228 ± 15.3
	TNF-alpha	162 ± 7.04*	92.9 ± 9.66**
Ubiquitin 1.2 kb	None	179 ± 7.88	279 ± 74.4
	TNF-alpha	191 ± 23.2	143 ± 26.4 *
C8 proteasome subunit	None	4,193 ± 312	1,183 ± 279
	TNF-alpha	2,596 ± 303*	1,051 ± 222
E2 conjugating enzyme	None	1,190 ± 83	842 ± 163
	TNF-alpha	536 ± 73.7*	601 ± 105
m-calpain	None	1,189 ± 131	757 ± 46.6
	TNF-alpha	758 ± 64.9	507 ± 69.5*
Cathepsin B	None	3,802 ± 451	3,288 ± 497
	TNF-alpha	2,521 ± 221*	2,517 ± 471

C2C12 cells mRNAs were isolated either 2 or 48 hours following treatment. Results are expressed as arbitrary units and are means ± S.E.M. for five different experiments. Statistical significance of the results (Student's t-test): * $p < 0.05$, ** $p < 0.005$.

Table 2
Effects of PI3K and MAPKs inhibitors on the TNF-alpha- and insulin-mediated increase in protein content in C2C12 cells

	Addition	
	TNF-alpha	Insulin
Inhibitor added		
None	121 ± 0.49	121 ± 3.07
Wortmannin	114 ± 3.66	127 ± 1.26
None	115 ± 7.69	118 ± 4.25
PD98059	86.9 ± 2.93**	107 ± 3.06**
None	123 ± 5.13	149 ± 4.47
SB203580	98.8 ± 3.49**	174 ± 3.73**

C2C12 cells were incubated with either TNF-alpha or insulin for 48 hours, in the presence or absence of the inhibitors. The data show total protein content values and are expressed as percentage of the corresponding control values (control for TNF-alpha and insulin groups are cells with no treatment, and control for TNF-alpha + inhibitors and insulin + inhibitors are cells with the corresponding inhibitor but no treatment). Values are means ± S.E.M. for at least five different experiments. Statistical significance of the results (Student's t-test): ** $p < 0.005$ for TNF-alpha or INS with inhibitor *versus* without inhibitor.



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Safety, pharmacokinetics and pharmacodynamics of recombinant human tumour necrosis factor-binding protein-1 (Onercept) injected by intravenous, intramuscular and subcutaneous routes into healthy volunteers

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P. Pal Singh, S. Singh
Plasmodium berghei, protection, rmGM-CSF, met-enkephalin.

Semiautomated and simultaneous analysis of the interleukin-10 gene microsatellites IL-10G and IL-10R by fluorescence-based polymerase chain reaction reveals significant differences in allele distributions between Caucasians (Germany) and Africans (Gabon)

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