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Acute treatment with tumour necrosis factor-α induces changes in protein metabolism in rat skeletal muscle

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Abstract

Acute treatment of rats with recombinant tumour necrosis factor (TNF-α) caused an enhanced proteolytic rate – measured as tyrosine released in the presence of cycloheximide – in *soleus* muscle (34%). The cytokine treatment also decreased the rate of protein synthesis in this muscle (22%) while it had no effect upon the same parameter in *extensor digitorum longus (EDL)* (26%) muscle. In addition, treatment of rats with TNF-α increased amino acid uptake by transport system A in the incubated muscles both in *soleus* (45%) and *EDL* (99%) in the presence of insulin in the incubating medium. This effect was not associated with a direct action of TNF on muscle since the addition of different concentrations of the cytokine to the preparations did not alter the uptake of α-(methyl)-aminoisobutyric acid by the incubated muscles. It can be concluded that acute TNF-α treatment causes changes in protein metabolism in red-type muscles – such *soleus* – while little effects are seen in white-type muscles – such as EDL. The results presented may, to some extent, be related to the cachectic response associated with cancer and inflammation. (Mol Cell Biochem **125:** 11–18, 1993)

Key words: tumor necrosis factor, cytokines, protein metabolism, skeletal muscle

Introduction

The development of cachexia is associated with cancer and several other chronic and inflammatory diseases [1, 2]. In response to neoplasic and infectious stimuli, a variety of cells, including stimulated macrophages and lymphocytes, secrete cytokines which can alter the host's metabolism. It has been suggested that two of these cytokines, interleukin-1 (IL-1) and tumour necrosis factor (TNF-α) mediate the metabolic changes associated with the cachectic process [3]. Weight loss, enhanced urinary nitrogen excretion, acute-phase protein synthesis and muscle wasting are among the most important changes associated with this pathological state.

While the effects of TNF-α on lipid metabolism have been well documented, several studies have failed to

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consistently reveal any changes in protein metabolism in skeletal muscle tissue incubated *in vitro* with recombinant TNF-α [4]. On the other hand, studies involving administration of TNF-α *in vivo* have shown an increase in nitrogen efflux from skeletal muscle in non-weight losing humans with disseminated cancer [5, 6], loss of body protein in growing rats [7] and a reduced rate of protein synthesis in rat muscle [8]. Flores *et al.* [9] infused ^{14}C-leucine to rats and calculated protein breakdown in muscle from the relation of the tissue to circulating radioactive amino acid. In their study infusion of recombinant TNF-α significantly enhanced protein degradation, this effect being synergistically augmented by recombinant IL-1 treatment. Conversely, the administration of an acute TNF-α dose to rats did not induce an enhanced proteolysis or PGE_2 production in *soleus* or *EDL* muscles subsequently incubated [10]. Moldawer *et al.* [11] administered recombinant IL-1 to mice and observed, after incubating their *EDL* muscles, that there was an increase in PGE_2 production although the rates of both protein synthesis and degradation were unaffected, thus concluding that the rise in PGE_2 was not associated with a rise in protein turnover. Very recently, Goodman [12], measuring both tyrosine and 3-methylhistidine release by incubated *EDL* muscles from recombinant TNF-treated rats, concluded that TNF-α does indeed activate skeletal muscle protein degradation in the rat. The reasons for these conflicting results are not clear. It is for this reason that the aim of the present investigation was to study the effects of acute TNF-α treatment on rat skeletal muscle protein metabolism. Bearing this in mind, the effects of an acute TNF-α treatment on the rates of protein synthesis and degradation together with the capacity for amino acid uptake by the A system, have been investigated.

Experimental

Animals

Female Wistar rats from our own colony were used. Their body mass (60–70 g) was chosen so that their muscles could be incubated whole, without being under anoxia. *Soleus* and *extensor digitorum longus (EDL),* were chosen because they were representative of red and white muscles, respectively. The animals were housed in collective polypropylene cages (5 animals per cage) maintained at 22–23° C with a 12 hr-light/12 hr-dark cycle. They were fed Purina Laboratory chow *ad libitum.*

Biochemicals

All enzymes and coenzymes were either obtained from Boehringer Mannheim S.A. (Barcelona, Spain) or from Sigma Chemical, St. Louis, U.S.A. Recombinant-derived TNF-α was generously given by BASF/Knoll A.G., Ludwigshafen, Germany.

Cytokine administration

The animals were injected with 6 nmol of recombinant-derived TNF-α in 0.5 ml of Krebs-Henseleit saline intravenously through the dorsal tail vein under light diethyl ether anaesthesia; control animals received 0.5 ml of vehicle. All injections were administered between 09:00 and 10:00 h on the day of the experiment, with the animals being used 1 h later; food was available during this period.

Muscle preparations and incubations

The dissection and isolation of the *extensor digitorum longus (EDL)* and *soleus* were carried out under pentobarbital anaesthesia as previously described [13]. Their weights were 25.6 ± 1.9 and 28.5 ± 2.2 mg respectively. The isolated muscles were fixed to a stainless-steel clip in order to maintain the muscle under slight tension (making it comparable to resting length) during the incubation. Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 3 hr incubation period. The muscles were incubated in a shaking-thermostatised water bath at 37° C for 3 hr in 3 ml of Krebs-Henseleit physiological saline pH 7.4, containing 5 mM glucose, bovine serum albumin (2 mg/ml) and 20 mM HEPES. After the addition of the muscles to the vials, these were stoppered and the incubation started at a shaking rate of 70 cycles/min. Vials were gassed with O_2/CO_2 (19 : 1) during the whole incubation period. The incubation medium was kept for no longer than 90 min and was renewed thereafter with fresh medium with the same composition as described above but with insulin (200 nM) where stated.

Rates of protein synthesis

After the same incubations referred to above, muscles were transferred to 1.5 ml of the same composition fresh medium with the addition of 0.277 μC_i of [^{14}C]phenylalanine (150 nmol/ml L-phenylalanine). In the last 30 min of incubation, the muscles were quickly rinsed in cold saline (0.9% NaCl), blotted briefly on filter paper, frozen in liquid N_2 and weighed. They were later homogenized in cold saline and deproteinized by means of 40% trichloroacetic acid and later placed in ether/ethanol (1 : 1). The pellet was dried under N_2 and digested in 0.25 ml of tissue solubilizer (*Protosol,* Biotechnology Systems, Dupont, U.S.A.) at 50° C in Teflon-sealed vials for 2–3 hr. Since a high concentration of phenylalanine in the medium was used, the extracellular specific radioactivity of [^{14}C]phenylalanine was used for the calculation of the amount of amino acid incorporated into the protein fraction and, consequently, the estimation of the rates of protein synthesis.

Rates of protein degradation

Net protein degradation by the isolated muscles was calculated as the rate of tyrosine released into the medium. Tyrosine was measured fluorimetrically as previously described [15]. Total protein degradation was determined in the presence of cycloheximide to block the reincorporation of tyrosine into tissue protein.

Amino acid uptake by transport system A

For the estimation of α-(methyl)aminoisobutyric acid (MeAIB) uptake – a non-metabolizable amino acid analog only transported by system A –, after the 150 min incubation period muscles were blotted and transferred to a vial with 1.5 ml of Krebs-Henseleit saline solution pH 7.4, containing 5 mM glucose, bovine serum albumin (1 mg/ml), 20 mM HEPES, 200 nM insulin – where stated – and 0.1 mM α-[1-^{14}C]-(methyl)-aminoisobutyric acid (800 μC_i/mmol), 1 mM [^{3}H]mannitol (333 μC_i/mmol). The incubation took place for 30 min in the same conditions as previously stated. After this period the muscles were quickly rinsed in cold saline (0.9% NaCl), blotted briefly on filter paper and frozen in liquid N_2 using the same procedure described above. Muscle digests and samples of the incubation media were placed in scintillation vials containing 10 ml of scintillation cocktail and processed for simultaneous ^{3}H and ^{14}C counting. The amount of each radioisotope present in the samples was determined, and this information was used to calculate the extracellular space. The intracellular concentration of ^{14}C-labelled amino acid analogue was calculated by substracting its amount in the extracellular space from the total label found in tissue, as previously reported [13].

Lactate released by the muscles was determined spectrophotometrically [14]. Student's *t* test was used for statistical analysis of the data.

Results and discussion

Protein metabolism

Treatment of the animals with an acute TNF-α resulted in an increased basal tyrosine release in *EDL* (34%) and an increased tyrosine release in *soleus* in the presence of cycloheximide in the incubation medium (25%) (Table 1). Tyrosine is an amino acid found in almost all proteins, and its release from incubated muscles should give a reasonable estimate of the average breakdown rate of mus-

Table 1. Proteolytic rate in isolated muscles from TNF-treated animals

Pretreatment	Additions	Type of muscle incubated	
		EDL	Soleus
Control	none	105 ± 5 (5)	170 ± 10 (6)
	insulin	74 ± 3 ††† (6)	120 ± 10 †† (6)
	cycloheximide	110 ± 6 (6)	151 ± 5 (6)
	insulin + cycloheximide	89 ± 3 †† (7)	142 ± 11 (7)
TNF-α	none	131 ± 10 * (6)	160 ± 12 (8)
	insulin	88 ± 9 †† (6)	138 ± 14 (9)
	cycloheximide	118 ± 6 (6)	203 ± 16 ** (6)
	insulin + cycloheximide	88 ± 3 ††† (6)	158 ± 10 (6)

For more details see the Experimental section. Insulin (200 nM) and cycloheximide (500 nM) were present during the incubations where stated. The results are expressed as nmoles tyrosine/g × h and are mean ± S.E.M. with the number of animals used in the different groups indicated in parentheses. Statistical significance of the results: * $p < 0.05$, ** $p < 0.01$ (control v.s. TNF-α); †† $p < 0.01$, ††† $p < 0.001$ (v.s. no insulin).

cle proteins. Cycloheximide was used to block the reincorporation of tyrosine into tissue protein, and thus, the proteolytic rate measured in the presence of this compound provides an estimate of total protein degradation. But, because cycloheximide may block prostaglandin synthesis, which is important for prostaglandin-mediated skeletal protein synthesis, net protein degradation was also determined in the absence of cycloheximide (net proteolytic rate). The addition of insulin to the incubated muscles reduced both total and net protein degradation in EDL only (Table 1). In the presence of the hormone, TNF-α treatment did not have any effect on the proteolytic rates. Previous studies that have evaluated the effects of TNF on muscle proteolysis, using incubated muscles, have led to contradictory results. Kettelhut & Goldberg [10] found no differences in rat muscle proteolysis, neither in *soleus* nor *EDL,* 4 hr after intraperitoneal administration of 20 μg/kg recombinant human TNF-α. However, Goodman [12] has recently reported an increase in the release of both tyrosine and 3-methylhistidine – an indicator of the rate of protein breakdown of myofibrillar proteins – 8 hr after an acute intravenous administration (200 μg/kg) of human recombinant TNF-α. The conclusion that can be drawn from our results indicates that TNF-α does indeed increase muscle proteolysis and that this effect can be observed very shortly after its administration. Experiments concerning administration of other cytokines such as IL-1 (α and β forms) have proved that these were not able to increase the rate of proteolysis in incubated muscles. In addition, *in vivo* estimations of the rate of rat muscle protein breakdown using infusion of [^{14}C]leucine [9] have demonstrated an increased protein breakdown following chronic TNF-α treatment. Using the method of protein loading with [^{14}C]bicarbonate, we have reached similar conclusions after a 10 day chronic TNF-α treatment to rats (Llovera, López-Soriano & Argilés, unpublished results).

Concerning protein synthesis, synthetic rates were derived from the linear incorporation of [^{14}C]phenylalanine into acid-precipitated, and expressed as nmoles phenylalanine incorporated per gram of fresh muscle weight per 30 min. Estimates of protein synthesis have been based on media specific radioactivities. The intracellular amino acid concentration and the media specific radioactivity remained constant over the incubation period and did not differ from the acid-soluble specific radioactivity obtained at the end of the incubations (results not shown). TNF-α treatment caused a marked reduction of [^{14}C]phenylalanine incorporation into the protein fraction of the incubated muscles in *soleus* muscles both in the presence (13%) or absence (22%) of insulin in the incubation medium (Table 2). Following TNF treatment, a decreased protein synthesis was also observed in *EDL* (21%) in the presence of insulin in the incubation medium. Kettelhut & Goldberg [10] found no changes in the rat of protein synthesis in either incubated rat *EDL* or *soleus* muscles following human recombinant TNF-α treatment. Moldawer *et al.* [11] did not observe any changes in the rate of protein synthesis in *EDL* muscle preparations, following IL-1 treatment to mice. However, Charters & Grimble [8] estimated the *in vivo* rates of protein synthesis in rat *tibialis* muscles and found that an acute human recombinant TNF-α treatment caused a decrease in total protein synthesis both at TNF-α doses of 30 and 300 μg/kg, 8 hr after the administration of the cytokine. The same authors found no alterations in the rate of protein synthesis over a longer interval (24 hr). Similarly, Flores *et al.* [9] found no changes in the rates of fractional protein synthesis in skeletal muscle of rats infused with human recombinant TNF-α. The results presented in Table 2 seem to suggest a rapid effect of acute TNF-α treatment on rat incubated muscles. Differences in muscle type, animal species and dose and mode of cytokine administration may account for the discrepancies observed by different authors.

Table 2. Rates of protein synthesis in isolated muscles from TNF-treated animals

Pretreatment	Additions	Type of muscle incubated	
		EDL	Soleus
Control	none	25 ± 3 (5)	36 ± 2 (4)
	insulin	43 ± 1 ††† (5)	53 ± 2 ††† (4)
TNF-α	none	24 ± 1 (4)	28 ± 2 * (4)
	insulin	32 ± 2 †† *** (4)	46 ± 2 ††† * (4)

For more details see the Experimental section. Insulin (200 nM) was present during the incubations where stated. The results are expressed as nmoles of [^{14}C]phenylalanine incorporated into protein/g × 30 min and are mean ± S.E.M. with the number of animals used in the different groups indicated in parentheses. Statistical significance of the results: * $p < 0.05$ (control v.s. TNF-α); †† $p < 0.01$, ††† $p < 0.001$ (v.s. no insulin).

Amino acid transport

In order to see if the changes observed in both protein synthesis and degradation were associated with changes in the rate of amino acid transport, we estimated the uptake of α-[1-^{14}C]-(methyl)-aminoisobutyric acid (MeAIB) by the incubated muscles. This amino acid analogue is taken up in skeletal muscle exclusively by system A, the main amino acid transport system in this tissue. The uptake of the amino acid analogue was linear during the incubation period (results not shown). The data presented in Table 3 indicate that the addition of insulin clearly stimulated MeAIB uptake in both muscles, as previously described [13], and that TNF-α treatment caused an increase in MeAIB uptake, in the presence of insulin, in both *soleus* (45%) and *EDL* (99%) muscles. To our knowledge, this is the first report concerning the effects of TNF-α treatment on the rates of system A activity in skeletal muscle. We have previously studied the effects of the cytokine on the *in vivo* amino acid uptake by both skeletal and cardiac muscle [16], concluding that TNF-α was capable of reducing amino acid uptake. The results presented here do not, therefore, correlate with the described *in vivo* effects. It has to be pointed out, however, that the increase in amino acid transport is only manifested in the presence of insulin in the incubating medium. One can, therefore, speculate as to whether the cytokine is actually able to increase the sensitivity of skeletal muscle for the hormone. Future work will consequently be focused on this point.

The production of lactate by the incubated muscles – both *soleus* and *EDL* – was significantly increased by insulin in the control animals; however, this effect was not seen in muscles of the animals that received TNF-α.

In vitro *effects of TNF-α*

In order to see if the effects of the cytokine treatment of amino acid uptake were actually the result of a direct action on the incubated muscles, we incubated both *soleus* and *EDL* muscles in the presence of different concentrations of the cytokine (Fig. 1) and estimated MeAIB uptake, tyrosine release and lactate production in the presence of 200 nM insulin. The presence of TNF-α receptors has been demonstrated in most tissues [17, 18]. In skeletal muscle, the cytokine is able to induce changes in the electrogenic potential of the plasma membrane [19]. Since the activity of A system is indeed dependent on the transmembrane potential, it was of particular interest to assess any possible direct effects of the cytokine. The results, however, show that TNF-α did not promote any changes in MeAIB uptake at any of the concentrations tested (Fig. 1); consequently a direct action of the cytokine can be excluded. In addition, the cytokine did not alter the rates of protein degradation, as expected. Indeed, a variety of investigations have not been able to describe any direct effects of TNF-α – or

Table 3. Rates of α-(methyl)-aminoisobutyric acid uptake by isolated muscles from TNF-treated animals

Pretreatment	Additions	Type of muscle incubated	
		EDL	Soleus
Control	none	53 ± 6 (6)	40 ± 3 (6)
	insulin	101 ± 12 †† (6)	73 ± 4 ††† (5)
TNF-α	none	61 ± 6 (6)	58 ± 11 (9)
	insulin	147 ± 10 ††† ** (7)	145 ± 23 †† * (8)

For more details see the Experimental section. Insulin (200 nM) and cycloheximide (nM) were present during the incubations where stated. The results are expressed as nmoles/g × h and are mean ± S.E.M. with the number of animals used in the different groups indicated in parentheses. Statistical significance of the results: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (control v.s. TNF-α); †† $p < 0.01$, ††† $p < 0.001$ (v.s. insulin).

Table 4. Lactate released by isolated muscles from TNF-treated animals

Pretreatment	Additions	Type of muscle incubated	
		EDL	Soleus
Control	none	7.6 ± 1.1 (6)	9.0 ± 1.3 (4)
	insulin	12.6 ± 0.8 †† (5)	14.5 ± 1.3 † (5)
TNF-α	none	9.8 ± 1.8 (6)	7.7 ± 1.6 (7)
	insulin	11.3 ± 0.8 (4)	11.6 ± 1.0 (7)

For more details see the Experimental section. Insulin (200 nM) was present during the incubations where stated. The results are expressed as μmoles/g × hour and are mean ± S.E.M. with the number of animals used in the different groups indicated in parentheses. Statistical significance of the results: * $p < 0.05$ (control v.s. TNF-α); † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ (v.s. no insulin).

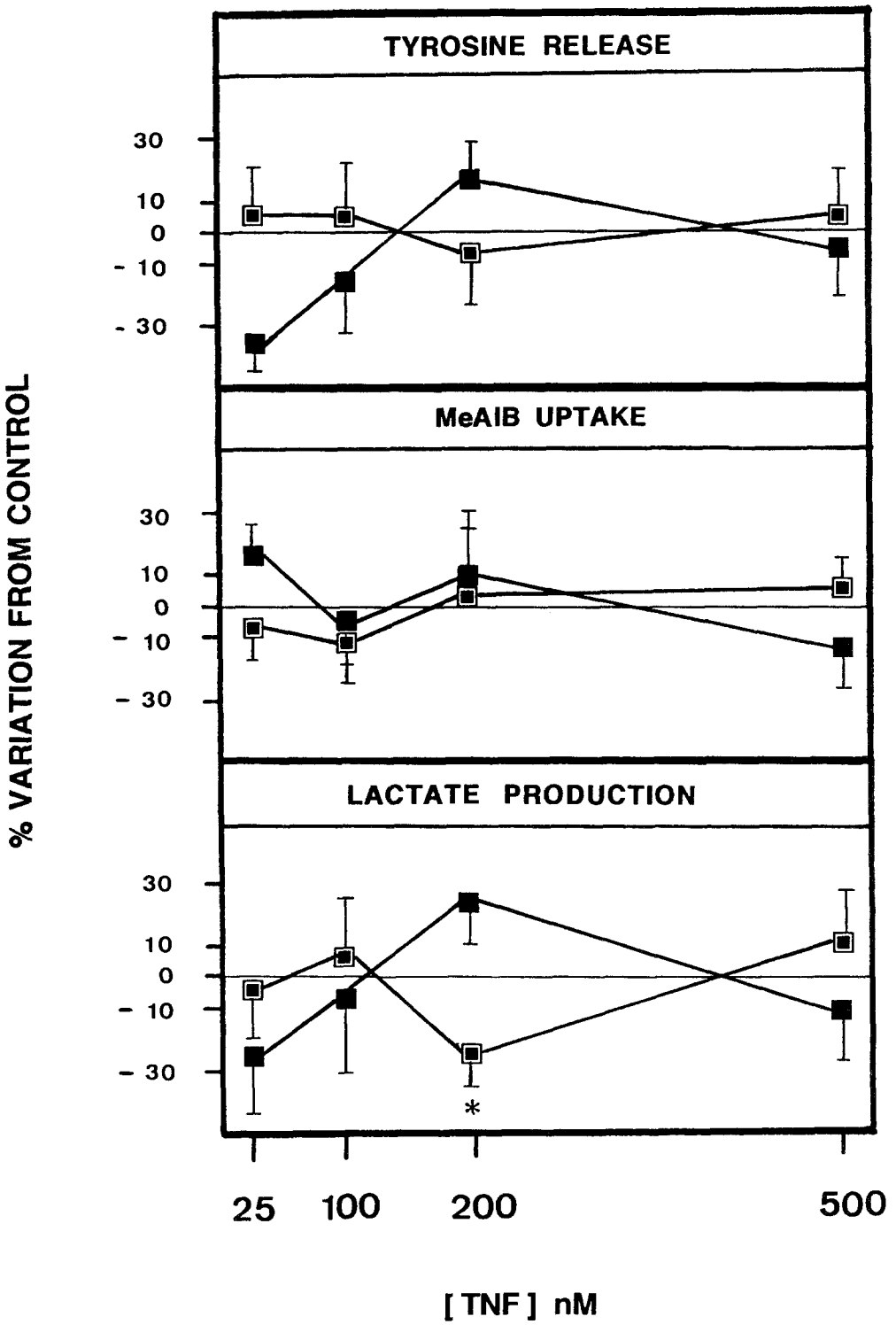

Fig. 1. Muscles isolated from non-treated rats (■-■ *EDL* and □-□ *soleus*) were incubated in the presence of different TNF-α concentrations (* $p < 0.05$).

other cytokines – on protein breakdown in muscle preparations [4]. Indeed, work from several laboratories indicated that circulating factor(s) released by activated macrophages during infection signaled enhanced muscle protein breakdown [20]. Futhermore, it was shown that a partially purified human IL-1 preparation, when

added to rat muscles *in vitro,* stimulated proteolysis [21]. Therefore, a still unidentified factor distinct from the known cytokines is responsible for activation of muscle protein breakdown during infection. On the same lines, Rofe *et al.* [22] did not see any increased alanine release in hemidiaphragms in the presence of human recombinant TNF-α. Our results shown, however, a slightly decreased lactate production by *soleus* muscles at a 200 nM TNF-α concentration.

Concluding remarks

The results presented here support the view that TNF-α may indeed be related with muscle wasting as observed in response to sepsis, trauma and malignancy. Whereas an effect of TNF-α on lipid metabolism had been well documented, contradictory information has previously been published regarding TNF-α effects on skeletal muscle. From our data, it can be speculated that there is a short-term and rapid action of the cytokine on muscle protein metabolism both decreasing synthesis and increasing protein breakdown. In addition, a novel action of the cytokine is described in stimulating amino acid uptake by transport system A in the presence of insulin which may suggest that TNF-α could change insulin sensitivity in skeletal muscle. This observation may open a field for promising future research.

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