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Tumour necrosis factor-α increases the ubiquitinization of rat skeletal muscle proteins

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An acute intravenous administration of 100 μg/kg body weight of recombinant tumour necrosis factor-α (TNF) resulted in a time-dependent increase in the levels of both free and conjugated ubiquitin in rat skeletal muscle. The effects of the cytokine were more pronounced in the red muscle soleus than in the white muscle EDL. In the former muscle type, TNF-treatment also resulted in a time-dependent increase in the percentage of free ubiquitin. The results suggest that the ubiquitin system for non-lysosomal protein degradation could have a very important role in the mechanism triggered by TNF which is responsible for enhanced muscle proteolysis in sepsis and other pathological states.

TNF; Protein metabolism; Ubiquitin; Cachexia

1. INTRODUCTION

Cachexia is a poorly understood syndrome characterized by anorexia, weight loss, profound metabolic abnormalities and progressive host wasting which may result in death [1–3]. Tissue wasting involves mainly adipose tissue and skeletal muscle. Numerous reports attribute the cachectic state of the host to cytokines releases as a result of invasive stimuli (see Evans et al. [4] for review). However, while the role of cytokines, particularly tumour necrosis factor-α (TNF), on adipose tissue dissolution seems to be clear [5–7], the effects of the cytokine on muscle metabolism lead to more confusing results (see Argilés et al. [8] for review).

Muscle wasting is generally accepted to be caused by an increase in protein breakdown. Chronic treatment of rats with recombinant TNF resulted in depletion of body protein compared with pair-fed control animals [9]. Indeed, chronic treatment with either recombinant TNF or interleukin-1-β (IL-1) resulted in a body protein redistribution and a significant decrease in muscle protein content associated with coordinate decreases in muscle mRNA levels for myofibrillar proteins [10]. Studies involving administration of recombinant TNF in vivo have shown an increase in nitrogen efflux from skeletal muscle of non-weight losing humans with disseminated cancer [11]. Flores et al. [12], by infusing [^{14}C]leucine to rats, showed that chronic recombinant TNF administration significantly enhanced muscle protein breakdown. Recently, Goodman [13], measuring both tyrosine and 3-methylhistidine release by incubated rat muscles of animals acutely treated with the cytokine, concluded that TNF was involved in activating muscle proteolysis.

The precise mechanism by which intracellular proteins are degraded is largely unknown, although it is accepted that proteolysis can occur both within and outside of lysosomes. In particular, lysosomic proteinases, mainly cathepsins, do not seem to be involved in the degradation of myofibrillar proteins in rat skeletal muscle [14]. Ubiquitin, a 8,600-Da peptide, is involved in the targeting of proteins undergoing cytosolic ATP-dependent proteolysis. In the cell, ubiquitin can be found free or conjugated in an isopeptide linkage to other cellular proteins. Proteins with multiple ubiquitins are the ones targeted for degradation by an ATP-dependent protease [15–18]. The ubiquitin system is postulated to account for the turnover of 'short-lived' normal proteins [19] and abnormal proteins formed during stress such as heat-shock [20]. The role of ubiquitin-dependent proteolysis can be tested by exploiting the ubiquitin moiety as an immunological probe [21,22] of substrate specificity. Bearing this in mind, the objective of the present study was to evaluate the possible role of the ubiquitin-mediated sytem in TNF-induced proteolysis in rat skeletal muscle. It has been studied by Western blot analysis of both free and conjugated ubiquitin in muscle tissue of TNF-treated animals.

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2. MATERIALS AND METHODS

2.1. *Animals*

Female Sprague–Dawley rats (160–180 g) from our colony (Faculty

of Medicine, University of Barcelona) were used. The animals were housed in individual polypropylene cages, maintained at 22–23°C (with a 12 h-light/12 h-dark cycle). They were fed Purina laboratory chow ad libitum.

2.2. *Biochemicals*

All enzymes and coenzymes were either obtained from Boehringer Mannheim, (Barcelona, Spain) or from Sigma (St. Louis, USA). Recombinant-derived TNF was generously given by BASF/Knoll (Ludwigshafen, Germany). Polyclonal rabbit anti-rat-ubiquitin was provided by Dr. Arthur L Haas, Department of Biochemistry, Medical College of Wisconsin, Milwaukee (Wisconsin, USA).

2.3. *TNF treatment*

Two different treatments were used. In the chronic study, TNF was given intraperitoneally for 8 days at a dose of 100 μg/kg (twice a day, at 08.00 and 20.00 h). Control animals received 0.5 ml of vehicle (Krebs–Henseleit saline). In the acute study, a single dose of TNF (100 μg/kg) was administered intravenously through one of the tail veins under light diethylether anaesthesia. Control animals received 0.5 ml of vehicle (same as above). All injections were administered between 8.00 and 9.00 h, with the animals being killed either 1, 3, or 8 h later. Food was available during this period.

2.4. *Isolation and preparation of muscle tissue for quantification of free and conjugated ubiquitin*

The red soleus and white extensor digitorum longus (EDL) were removed under penthobarbital anaesthesia Excised muscles were quickly weighed and rapidly frozen in liquid N_2. Ubiquitin pools in muscle extracts were determined by immunochemical analysis. Individual muscles were finely homogenized using a Polytron homogenizer in 2 ml of ice-cold 50 mM Tris-HCl buffer containing 0.25 mM sucrose, 5 mM EDTA, 1% (w/v) sodium dodecyl sulphate, 0.1 trypsin inhibitor U/ml aprotinin, 1 μM leupeptin, 1 μM pepstatin, 5 mM *N*-ethylmaleimide and 1 mM PMSF pH 7 4. The resulting homogenates were centrifuged at 4°C for 20 min at 15,000 × *g* to remove cell debris. Aliquots of the supernatants were stored at −80°C. Protein content was measured using the Bradford assay [23] using bovine serum albumin as a standard.

2.5. *Western blots*

Aliquots of the supernatants were diluted with 0.5 volumes of 30 mM phosphate buffer pH 7.0 containing 7.5% (w/v) sodium dodecyl sulphate and 0.15% (w/v) dithiotreitol, 0.05% (w/v) Bromophenol blue and 30% glycerol. Samples were boiled for 5 min, 50 μg of protein samples being assayed for ubiquitin conjugates. Sodium dodecyl sulphate (SDS)-15% polyacrylamide gel electrophoresis was performed on muscle samples. Proteins were transferred to Immobilin in 25 mM Tris buffer containing 144 mM glycine, 25% methanol, 0.02% SDS for 4 h. The filter was blocked for 1 h at room temperature in buffer A (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 25 mg/ml BSA and 0.02% sodium azide) and incubated for 90 min with an anti-ubiquitin polyclonal antibody which recognized free and conjugated ubiquitin at a concentration of 10 μg/ml in buffer A. Detection of antibody-antigen complexes was carried out using [^{125}I]protein A according to standard procedures. Quantitation of the different autoradiographs was done by densitometry using a scanning microdensitometer. Data for both free and conjugated ubiquitin were normalized to extract protein concentration determined by the Bradford assay [23]. Statistical comparisons between groups were carried out by means of the Student's *t*-test.

3. RESULTS AND DISCUSSION

Increased muscle catabolism is an important component of the metabolic response to acute and chronic inflammatory processes accompanying infections [24], trauma [25], tissue injury [26] and cancer cachexia [27]. Although the protein synthesis mechanisms are well known, those involved in protein degradation remain somewhat obscure. Protein degradation takes place by two major pathways. The lysosomal pathway is of poor selectivity and seems to be responsible for basal degradation of long-life proteins in normal dietary conditions and also for enhanced degradation in pathological states [28]. The cytosolic pathway is ATP-dependent and shows high specificity for short-life proteins [29]. In this second degradative pathway, the protein ubiquitin seems to be involved in targeting those proteins undergoing ATP-dependent degradation by the formation of ubiquitin conjugates with ulterior regeneration of free, functional ubiquitin. Since cells contain a substantial pool of proteolytically stable conjugates, ubiquitin binding cannot indicate absolute commitment to degradation; however, the proteolytic rate seems to be dependent on the formation of the conjugates [30]. It was the objective of this study to prove if the ubiquitin system for protein degradation was involved as a possible mechanism for the proposed action of TNF on muscle proteolysis. In order to test this hypothesis, the animals received repetitive cytokine administrations.

3.1. *Chronic TNF administration*

Chronic administration of 100 μg of recombinant-derived TNF resulted in a significant decrease in percentual body weight increase at day 1; however, successive cytokine administrations produced no changes in food intake or body weight for the rest of the period studied (results not shown). This is in agreement with other studies where repetitive TNF administrations have failed to promote changes in body mass deposition. Stovroff et al. [31] saw that repetitive administration of recombinant TNF to rats resulted only in a transient weight loss followed by rapid recovery 24 h after the initiation of the treatment. It is possible that the tolerance developed in all these studies may be due to the formation of antibodies against TNF, specially if species-specific TNF is not used. However, it has been demonstrated that when human recombinant TNF was injected into rats, antibody formation did not begin until the tenth day of injection. Another factor to be taken into consideration is that episodic administration of TNF may not be a physiologically sound test of the normal function of the cytokine. In this way, only when TNF was administered using osmotic minipumps [32], a significant reduction of body weight has been reported. Alternatively, Oliff et al. [33] after cloning the human TNF gene into a mammalian expression vector under the control of a constitutive promoter, and transfecting it into a tumorogenic cell line, were able to demonstrate progressive weight loss after transplantation of the tumour cells into nude mice.

TNF treatment did not result either in changes in liver or gastrointestinal tract weights (results not

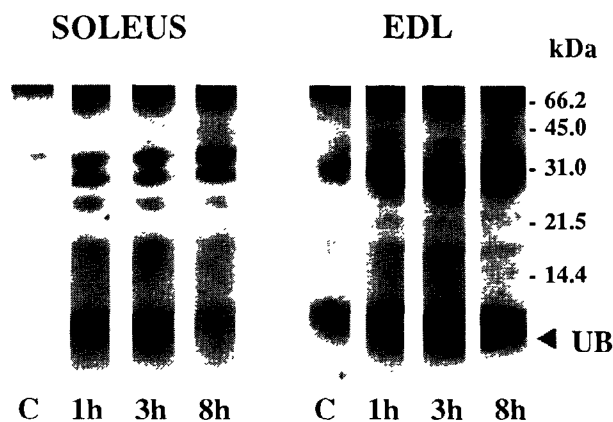

Fig. 1. Western blots of muscle (soleus and EDL) extracts from rats following a single dose of recombinant TNF (100 μg/kg) administration. 50 μg of protein was loaded in all wells. Ubiquitin conjugates of different molecular weights are shown. UB = free ubiquitin.

shown). Although there was a tendensy for soleus muscles to be smaller in the TNF-treated (46 ± 2 mg/100 g b.w.) than in control animals (52 ± 2 mg/100 g b.w.), it did not reach statistical significance. EDL muscle weight was also unchanged following TNF treatment (49 ± 2 mg/100 g b.w. (control) and 49 ± 2 mg/100 g b.w. (TNF-treated)). Total and specific protein content of both soleus and EDL muscles was not affected by TNF treatment (results not shown). As would be expected, no changes in either free or conjugated ubiquitin were seen either in soleus or EDL muscles (Table I). As commented above, repeated administration of cytokines, TNF in particular, may not be a physiologically sound test of the actions of these compounds. This is related to the fact that TNF has a very short life once in the circulation [34] and also to the fact that animals become tolerant after episodic administrations. Bearing this in mind, we decided to carry out acute administrations to see if the cytokine could activate ubiquitin synthesis and conjugation before any tolerance had developed in the animal.

Table I

Free and conjugated ubiquitin after chronic TNF administration

Type of muscle	Fraction	Treatment	
		Control	TNF
Soleus	FUB	0.130 ± 0.037 (4)	0.117 ± 0.021 (4)
	CUB	1.380 ± 0.266 (5)	1.750 ± 0.034 (5)
EDL	FUB	0.223 ± 0.071 (4)	0.172 ± 0.014 (5)
	CUB	5.622 ± 0.582 (4)	4.582 ± 0.645 (5)

For further details, see section 2. All data are expressed in arbitrary units/50 μg of protein and represent the mean ± S.E.M. with the number of different animals indicated in parentheses. FUB = free ubiquitin, CUB = conjugated ubiquitin.

3.2. *Acute TNF administration*

The results presented in Table II show an increased presence of both free and conjugated ubiquitin in soleus muscles of TNF-treated rats. The increase is significant both at 3 and 8 h after the administration of the cytokine thus indicating relatively long-term effects. Concerning EDL muscles, a significant rise in free ubiquitin can also be observed 3 h after the administration of the cytokine, the conjugated ubiquitin fraction being elevated still 8 h after administration (Table II, Fig. 1). The greater increases in both free (4.2-fold) and conjugated (2.1-fold) ubiquitin are observed in soleus muscles, while EDL ones show a more moderate increase for both free (1.5-fold) and conjugated (1.3-fold) ubiquitin, 8 h after the administration of the cytokine (Table II, Fig. 1). Different studies suggest that red muscles have a higher rate of total protein turnover than white muscles [35]. This observation is consistent with our results indicating that soleus muscles are affected to a larger extent in the increase in total ubiquitin than EDL ones (Table II).

3.3. *Concluding remarks*

Although several studies have clearly demonstrated the involvement of TNF either in inducing muscle proteolysis [12,13,36] or reducing amino acid uptake [37], the identification of the actual mechanisms taking place in skeletal muscle has received little attention. While Hall-Angeras et al. [24] have demonstrated that both myofibrillar proteinase and cathepsin B activities are increased in skeletal muscle of septic rats, this effect being mediated by TNF [36], concomitant with stimulated total and myofibrillar protein breakdown, others have demonstrated that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal

Table II

Free and conjugated ubiquitin after an acute TNF administration

Hours after TNF	Type of muscle			
	Soleus		EDL	
	FUB	CUB	FUB	CUB
1	1.81 ± 0.34	1.48 ± 0.28	1.30 ± 0.13	0.97 ± 0.08
3	4.87 ± 0.67**	2.14 ± 0.13**	1.63 ± 0.09**	1.14 ± 0.05
8	4.23 ± 0.53**	2.12 ± 0.12**	1.46 ± 0.18	1.28 ± 0.06*

For further details, see section 2. All data are expressed as arbitrary units/50 μg of protein divided by the values of time 0 h. The values represent the mean ± S.E.M. for four different animals. FUB = free ubiquitin, CUB = conjugated ubiquitin. Statistical comparisons between control and TNF groups were carried out using the Student's t-test. $*P < 0.05$, $**P < 0.01$.

muscle. The present study thus clearly contributes to the elucidation of the mechanism by which TNF stimulates muscles protein breakdown, the ubiquitin system being involved. Whether the cytokine is capable of activating the ubiquitinization of muscle proteins directly (the presence of TNF receptors in most tissues has been reported [38]), or indirectly (via glucocorticoids or other hormonal changes) remains to be investigated and will, no doubt, provide work for future promising investigations.

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