Interleukin-15 is able to suppress the increased DNA fragmentation associated with muscle wasting in tumour-bearing rats

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Abstract Administration of interleukin-15 (IL-15) to rats bearing the Yoshida AH-130 ascites hepatoma (a tumour that induces an important cachectic response) resulted in a significant reduction of muscle wasting, both measured as muscle weight and as protein content of different types of skeletal muscle. In addition, the administration of the cytokine completely reversed the increased DNA fragmentation observed in skeletal muscle of tumour-bearing animals. Concerning the mechanism(s) involved in the anti-apoptotic effects of IL-15 on skeletal muscle, the administration of the cytokine resulted in a considerable decrease in both R1 (43%) and R2 (64%) TNF-α receptors (TNFRs), and therefore it may be suggested that IL-15 decreases apoptosis by affecting TNF-α signalling. Formation of NO could be the signalling event associated with the activation of apoptosis in muscle of tumour-bearing rats; indeed, administration of IL-15 decreased the inducible nitric oxide synthase protein levels by 73%, suggesting that NO formation and muscle apoptosis during tumour growth are related. In conclusion, IL-15 seems to be able to reduce/suppress protein loss and apoptosis related to muscle wasting during cancer cachexia in experimental animals.

Keywords: Interleukin-15; Skeletal muscle; Apoptosis; Tumour necrosis factor-α; Nitric oxide synthase

1. Introduction

In addition to its role as T-cell proliferation factor [1], interleukin-15 (IL-15) has considerable metabolic effects involving both adipose tissue and skeletal muscle. Indeed, IL-15 depletes fat tissue by inhibiting both lipoprotein lipase activity and de novo lipogenesis [2]. Interestingly, the effects of the cytokine on adipose tissue seem to be direct, the presence of the different receptor subunits having been reported in adipose tissue [2].

IL-15 also exerts important effects in skeletal muscle, where it behaves as an anabolic cytokine both in vitro [3] and in vivo [4]. Indeed, addition of the cytokine to muscle cell cultures increases the expression of the myosin heavy-chain together with total myofibrillar content [3]. Interestingly, IL-15 exerts its effects in muscle cells by both decreasing the rate of protein degradation and increasing the rate of proliferation [3]. Previous work from our laboratory has shown that administration of IL-15 to cachectic tumour-bearing animals results in an amelioration of the muscle wasting. IL-15 decreases the activity of the ubiquitin/proteasome pathway (the main proteolytic pathway activated during cancer cachexia) in skeletal muscle of tumour-bearing animals [5]. In addition to increase muscle protein degradation during cancer growth, the presence of the tumour also induces an increased rate of DNA fragmentation in skeletal muscle both in rats and mice [6]. This increased rate of fragmentation seems to be activated by tumour necrosis factor-α (TNF-α) [7].

IL-15 has been shown to be anti-apoptotic in different cell types [8–10]. Bearing this in mind, the main aim of the present study was to investigate if administration of IL-15 to cachectic tumour-bearing animals could reduce/neutralize the increased apoptotic rate previously observed in skeletal muscle of tumour-bearing animals.

2. Materials and methods

2.1. Animals, tumour inoculation and treatment

Male Wistar rats (Interfauna, Barcelona, Spain) weighing approximately 110–135 g were used. The animals were maintained on a regular light–dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) and had free access to food and water. The diet consisted of 54% carbohydrate, 17% protein and 5% fat (the residue was non-digestible material). Rats were divided into two groups, controls and tumour hosts. The latter received an intraperitoneal inoculum of 10^8 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours [11]. Both groups were further divided into treated and untreated, the former being administered a daily s.c. dose of IL-15 (100 $\mu g\,kg^{-1}$ b.w. dissolved in physiological saline solution), the latter a corresponding volume of vehicle. Recombinant human IL-15 was kindly provided by Immunex Corporation (Seattle, WA, USA).

On day 7 after the tumour transplantation, animals were weighed and anaesthesized with ketamine:xylacine. The tumour was harvested from the peritoneal cavity, its volume and cellularity evaluated. Tissues were rapidly excised, weighed, and frozen in liquid nitrogen [11,12].

2.2. RNA isolation and Northern blot analysis

Total RNA from gastrocnemius (GSN) muscles was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi [13]. RNA samples (20 μg) were denatured, subjected to 1.2% agarose gel electrophoresis and transferred to Hybond N membrane (Amersham). RNA was fixed to the membranes by Genelinker (BioRad). The RNA in gel and filters was visualized with ethidium bromide and photographed by UV

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transillumination to ensure the integrity of RNA, to check the loading of the equivalent amounts of 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 the hybridization buffer (0.25 M Na_2HPO_4, 7% SDS, 1 mM EDTA, 1% BSA, and 10% dextran sulfate) and denatured labelled probes (10^6–10^7 cpm ml^{-1}) were added. Radiolabelled probes were prepared by random priming method (Roche). The probes used were the entire coding frame for mouse UCP2 [14], a cDNA clone containing the entire coding frame for mouse UCP3 (D.S. Fleury, C.F. Bouillard, GenBank Accession No. AF032902), Bax was generated by PCR (Accession No. U49729), and a 18S rat ribosomal probe was used as hybridization/quantification standard. Blots were quantified on a phosphoimager using the Phoretix 1 D gel analysis (Phoretix Int. Ltd., England).

3. RT-PCR

Analysis of mRNA levels for TNF-α, TNFR1, TNFR2 and Bcl-2 were performed with primers designed to detect gene products. TNF-α used primers 5′-TACTGAACTTCGGGGTGATTGGTC-3′ and 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′, resulting in a 298-bp product. TNFR1 used primers 5′-CCTGATTTCCATCTACCTCTGACTTTGAGC-3′ and 5′-ACACTGGAAATGCGTCTCACTCAGGTAGCG-3′, resulting in a 594-bp product. TNFR2 used primers 5′-GATGAGAAATCCCAGGATGCAGTAGG-3′ and 5′-GCTACAGACGTTCACGATGCAGG-3′, generating a 256-bp fragment. Bcl-2 used primers 5′-CTGTACGGCCCCAGCATGCG-3′ and 5′-GCTTTGTTTCATGGTACATC -3′, resulting in a 231-bp product. 18S was used as a control of loading and the primers are 5′-CGCAGAATTCCCACTCCCGACCC-3′ and 5′-CCCAAGCTCCAACTACGAGC-3′, resulting in a 212-bp product. RT-PCRs were performed with 0.4 μg of total RNA by a one-step RT-PCR method (Ready-to-Go™ RT-PCR Beads, Amersham Biosciences) as follows: **TNF-α**: 1× (42 °C, 30 min); 1× (92 °C, 2 min); 1× (94 °C, 1 min); 32× (94 °C, 1:30 min; 60 °C, 1 min; 72 °C, 2 min); 1× (72 °C, 4 min). **TNFR1**: 1× (42 °C, 30 min); 1× (92 °C, 2 min); 1× (94 °C, 1 min); 30× (94 °C, 30 s; 60 °C, 30 s; 72 °C, 45 s); 1× (72 °C, 7 min). **TNFR2**: 1× (42 °C, 30 min); 1× (92 °C, 2 min); 1× (94 °C, 3 min); 31× (94 °C, 45 s; 60 °C, 30 s; 72 °C, 1:30 min); 1× 72 °C, 10 min. **Bcl-2**: 1× (42 °C, 30 min); 1× (92 °C, 2 min); 25× (95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min); 1× (72 °C, 7 min). **18S**: 1× (42 °C, 30 min); 1× (92 °C, 2 min); 10× (92 °C, 30 s; 55 °C, 1 min; 72 °C, 2 min); 1× (72 °C, 7 min).

The RT-PCR products were electrophoresed in 2% agarose gels in TBE buffer. The gel was stained with ethidium bromide and quantified by scanning densitometry. Values for TNF-α, TNFRs and Bcl-2 mRNA were corrected by 18S RNA expression and expressed as percentage.

3.1. DNA fragmentation assay

GSN muscles were homogenized and incubated at 48 °C overnight in Kauffman buffer (0.5 M Tris, 2 mM EDTA, 10 mM NaCl, and 1% SDS) in the presence of 200 μg ml^{-1} of proteinase K, and DNA was extracted with phenol/chloroform. After ethanol precipitation, the pellets were resuspended and the DNA integrity was checked in a 2% agarose gel electrophoresis and ethidium bromide staining. The percentage of DNA fragmentation was quantified by scanning densitometry. Liver from 8-h anti-Fas antibody-treated mice [15] was used as a positive control of DNA fragmentation.

3.2. Immunoblotting

GSN frozen muscle samples were homogenized in 6 v/w ice-cooled homogenization buffer A (10 mM Tris-maleate, 3 mM EGTA, 275 mM sucrose, 0.1 mM DTT; 2 μg/ml leupeptin; 100 μg/ml PMSF; 2 μg/ml aprotinin, 1 mg/100 ml pepstatin A, pH 7.2). Samples were then centrifuged at 1000*g* for 30 min. The pellet was discarded and the supernatant was designated as crude homogenate. Total muscle protein level in each sample was determined with the Bradford technique (BioRad Inc., Hercules, CA). Crude muscle homogenates (100 μg per sample) were separated by electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with non-fat milk and incubated overnight with the selective anti-iNOS antibody (Transduction Laboratories Inc., Lexington, KY). To validate equal protein loading among various lanes, PVDF membranes were stripped and re-probed with a monoclonal anti-tubuline antibody (Sigma, St. Louis, MO, USA). Lysates obtained from cytokine-activated macrophages and septic rat diaphragm homogenates were used as positive controls for iNOS protein expression. Specific proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. Blots were scanned with an imaging densitometer and optical densities (OD) of a 130 kDa protein were quantified with the software Diversity database 2.1.1 (BioRad Laboratories, Philadelphia, PA) from all the samples.

3.3. Statistical analysis

Statistical analysis of the data was performed by means of one-way analysis of variance (ANOVA).

4. Results and discussion

The implantation of the rapidly growing tumour Yoshida AH-130 resulted in a decrease of liver (40%), white adipose tissue (WAT) (71%) and carcass weight (39%) (Table 1). Administration of IL-15 to tumour-bearing rats promoted a significant increase in liver and carcass weights, while no changes were observed concerning WAT (Table 1). Administration of the cytokine did not result in any changes in tumour cell content. IL-15 treatment promoted a significant decrease in liver weight (9%) and WAT (36%) in non-tumour-bearing animals (Table 1). This result has been reported previously, IL-15 decreasing WAT mass by inhibiting fatty acid synthesis in the adipocyte [16].

Tumour burden inflicted an important decrease in muscle weights: GSN (35%), soleus (28%), tibialis (35%) and heart (32%) (Table 1). Interestingly, administration of the cytokine was able to partially revert the decrease in muscle weights inflicted by the presence of the tumour (Table 1). No effects of the cytokine were observed on the muscle weights of non-tumour-bearing animals (Table 1). These results support previous studies [5] clearly demonstrating that IL-15 does indeed act as an anabolic cytokine on skeletal muscle. The mechanism associated with the effects of the cytokine on muscle involves a decrease in the rate of protein degradation, as previously described [5].

Previous studies from our laboratory indicated that, during experimental cancer cachexia, increased DNA fragmentation could be observed in skeletal muscle, suggesting that protein wasting in the muscle mass is accompanied by an active apoptotic process [7]. Bearing this in mind, we decided to test if

Table 1
Body and tissue weights

	C (4)	CT (4)	T (5)	TT (6)	ANOVA
IBW	111 ± 3	111 ± 3	115 ± 4	114 ± 3	ns
Muscle weights					
GSN	683 ± 13^{a}	677 ± 13^{a}	442 ± 13^{b}	489 ± 7^{c}	$P < 0.001$
Tibialis	215 ± 3^{a}	206 ± 5^{a}	140 ± 4^{b}	163 ± 3^{c}	$P < 0.001$
Soleus	52.0 ± 0.3^{a}	55.6 ± 1.3^{b}	37.5 ± 1.0^{c}	42.1 ± 0.9^{d}	$P < 0.001$
Heart	532 ± 14^{a}	524 ± 5^{a}	360 ± 13^{b}	387 ± 6^{b}	$P < 0.001$
Liver	7944 ± 123^{a}	7196 ± 56^{b}	4098 ± 105^{c}	4769 ± 200^{d}	$P < 0.001$
WAT	838 ± 54^{a}	539 ± 52^{b}	269 ± 30^{c}	246 ± 21^{c}	$P < 0.001$
Carcass	108 ± 1^{a}	104 ± 1^{b}	66 ± 1^{c}	72 ± 1^{d}	$P < 0.001$
Tumour cell content			4528 ± 156	4895 ± 312	ns
Tumour volume			54 ± 2	57 ± 3	ns

Results are means ± S.E.M. for the number of animals indicated in brackets. Initial body weight (IBW) is expressed in grams. Tissue weights are expressed as mg per 100 g of IBW. Tumour cell content is expressed in millions of cells. Tumour volume is expressed in ml. GSN, gastrocnemius; WAT, white adipose tissue; C, control; CT, control-treated; T, tumour; TT, tumour-treated. Statistical significance of the results by one-way ANOVA; different superscripts indicate significant differences between groups by post-hoc Duncan test. ns, non-significant differences.

IL-15 was also able to influence the degree of muscle apoptosis. Previously, it has been shown that IL-15 behaves like an anti-apoptotic cytokine in immune cells [8–10,17]. However, the anti-apoptotic mechanism is still far from being understood [8,17].

The results presented in Fig. 1 clearly show that tumour growth induced an active skeletal muscle DNA fragmentation. Very interestingly, in the animals treated with IL-15 the effect of tumour burden on DNA fragmentation was abolished, therefore suggesting that the anti-apoptotic effects of IL-15 can be extended to muscle tissue. We also analyzed the gene expression of Bcl-2 (an anti-apoptotic protein) and Bax (a proapoptotic protein) in skeletal muscle of tumour-bearing rats. The results presented in Fig. 2 show that both markers were significantly increased (80% for Bax, 61% for Bcl-2) in GSN muscles of tumour-bearing rats as compared with the non tumour-bearing animals. Interestingly, IL-15 treatment decreased both Bax (64%) and Bcl-2 (50%) mRNA levels in tumour-bearing rats (Fig. 2). In fact, the presence of the tumour results in an increase in the Bax/Bcl-2 ratio, suggesting an increase in proapoptotic activity (Fig. 2). Cytokine treatment completely reverses the increased ratio (Fig. 2).

In tumour-bearing rats, one of the signals that seems to activate the apoptotic process in skeletal muscle is the cytokine TNF-α. Indeed, in animals chronically treated with TNF-α there is an increased DNA fragmentation in skeletal muscle [7]. Similarly, TNFR1-deficient mice do not show an increased rate of DNA fragmentation in skeletal muscle when the animals were inoculated with the cachetic Lewis lung carcinoma [7]. Indeed, TNF-α behaves like a clear apoptotic signal in numerous cell types [18,19], the apoptotic cascade involving specifically TNFR1 [18,20]. Bearing this in mind, we decided to analyze whether IL-15 exerts apoptotic effects by altering TNF-α signalling in skeletal muscle. As can be seen in Fig. 3, the presence of the tumour induced an important increase in TNFR1 (49%) and TNFR2 (71%), while the expression of the cytokine was significantly decreased (73%). Interestingly, the administration of IL-15 completely reversed the increased mRNA content of TNFRs in skeletal muscle of tumour-bearing rats, while no changes were observed in TNF-α mRNA content in IL-15-treated rats (Fig. 3). These results suggest that the anti-apoptotic mechanism of IL-15 involves a downregulation of TNFRs in skeletal muscle.

One of the major findings of the present study is that systemic administration of IL-15 to the rats markedly reduced protein levels of the inducible nitric oxide synthase (iNOS) in GSN muscles of the cachectic group of animals. The proinflammatory cytokine TNF-α was shown to induce apoptosis via iNOS expression and nitric oxide (NO) production in neonatal cardiomyocytes [21]. Conversely, the absence of iNOS enzyme in those cells (iNOS deficient mice) led to a dramatic reduction in the TNF-α-induced apoptosis. It has been also pointed out that oxidative stress could be involved in

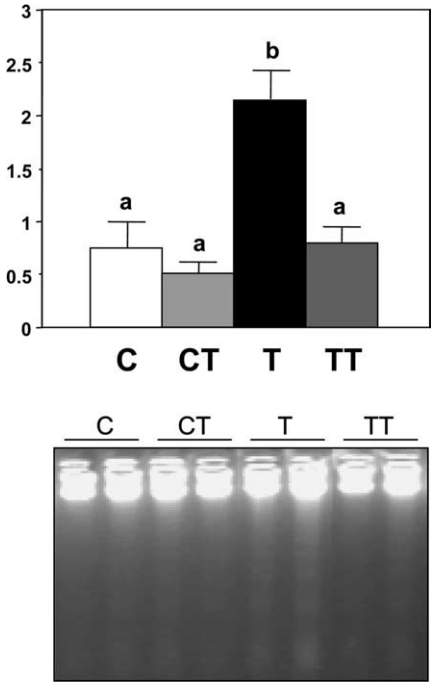

Fig. 1. DNA fragmentation in GSN muscles from tumour-bearing rats. For more details, see Section 2. Results are means ± S.E.M. for five different animals in each experimental group. DNA fragmentation was assessed by monitoring the laddering in an agarose gel. C, control; CT, control treated; T, tumour; TT, tumour treated. Statistical significance of the results by one-way ANOVA, between groups: $P = 0.0002$; different superscripts indicate significant differences between groups by post-hoc Duncan test.

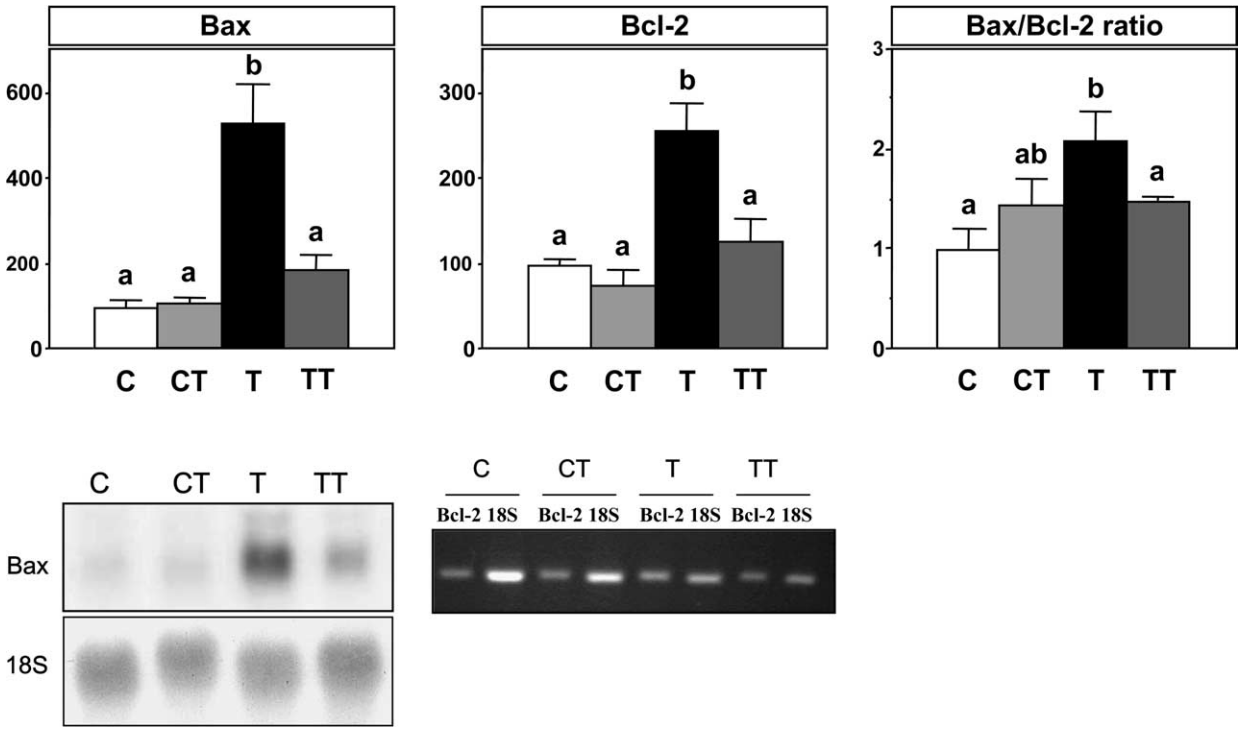

Fig. 2. Expression of Bcl-2 and Bax in skeletal muscle of tumour-bearing rats. For more details, see Section 2. Results are expressed as means ± S.E.M. for $n = 4$ and 6 for non-tumour and tumour-bearers, respectively. C, control; CT, control-treated; T, tumour; TT, tumour-treated. Statistical significance of the results by one-way ANOVA, $P = 0.0003$ (Bax), $P = 0.0008$ (Bcl-2) and $P = 0.0412$ (Bax/Bcl-2); different superscripts indicate significant differences between groups by post-hoc Duncan test.

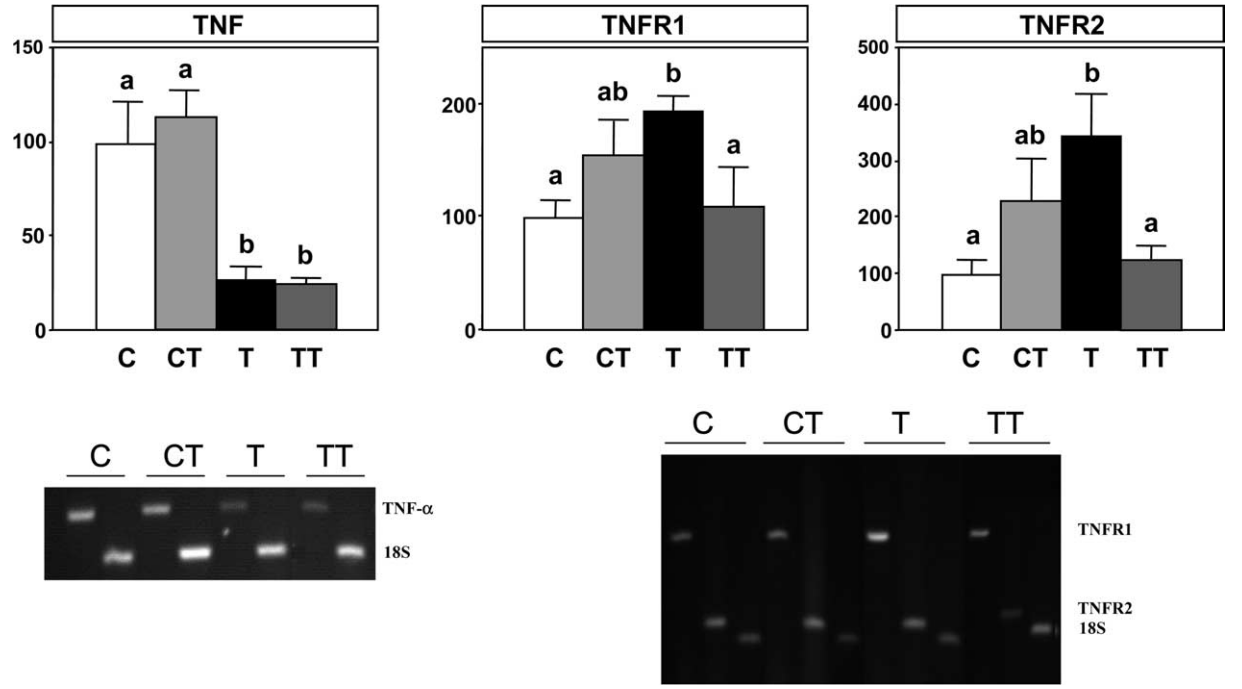

Fig. 3. Expression of TNF-α and its receptors in GSN muscles from rats bearing the Yoshida AH-130 ascites hepatoma. For more details, see Section 2. Results are expressed as means ± S.E.M. for $n = 4$ and 6 for non-tumour and tumour-bearers, respectively. RT-PCR electrophoresis of GSN muscle samples showing the different bands corresponding to TNF-α and its receptors and quantification of both TNF-α and its receptors in skeletal muscle on tumour-bearing rats. C, control; CT, control-treated; T, tumour; TT, tumour-treated. Statistical significance of the results by one-way ANOVA, $P = 0.0001$ (TNF-α), $P = 0.0689$ (TNFR1) and $P = 0.0176$ (TNFR2); different superscripts indicate significant differences between groups by post-hoc Duncan test.

apoptosis [22]. In fact, during oxidative stress NO is formed and has been related with the muscle wasting during cancer cachexia [23]. Under normal conditions, NO is usually synthesized by the neuronal NO synthase (nNOS) within the skeletal muscle fibre. However, the iNOS is by far the most important contributor to NO production under inflammatory conditions provided that this enzyme also bears the highest rate of NO synthesis as compared to the constitutive isoforms. One of the most potent and well known molecules capable of increasing iNOS expression is TNF-α through binding to its receptors (TNFRs) 1 and 2, via the activation of the NF-κB transcription factor. Taking this into consideration, we decided to analyze the content of iNOS, the main enzyme that participates in NO formation. The results presented in Fig. 4 demonstrate that tumour burden increased iNOS (81%) in skeletal muscle. Very interestingly, administration of IL-15 reverted the increased iNOS protein content, suggesting that this enzyme is also involved in the anti-apoptotic signalling cascade associated with the cytokine (Fig. 4). Accordingly,

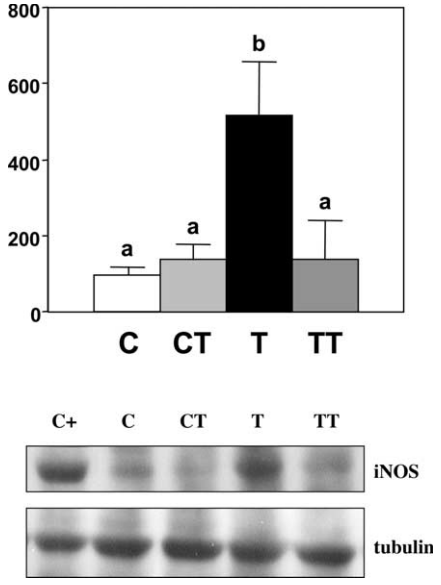

Fig. 4. iNOS protein levels in skeletal muscle of Yoshida AH-130 bearing rats. For more details, see Section 2. Results are expressed as means ± S.E.M. for $n = 4$ animals. C+, positive control; C, control; CT, control-treated; T, tumour; TT, tumour-treated. Statistical significance of the results by one-way ANOVA, between groups: $P = 0.0308$; different superscripts indicate significant differences between groups by post-hoc Duncan test.

based on the increased mRNA expression of both TNFR1 and TNFR2, we conclude from our study that one possible mechanism to account for the augmented DNA fragmentation present in the muscle fibres of tumour-bearing animals is the increased TNF-α-mediated iNOS enzyme content induction. Interestingly, IL-15 administration induced a marked reduction in iNOS protein content only within the muscle fibres of the cachectic animals, showing no change regarding its protein levels in the fibres of those IL-15-treated control rats. One likely explanation of the involvement of iNOS in muscle apoptosis is that excessive NO synthesis in cancer cachectic muscles would lead to the formation of the highly reactive oxygen species (ROS) peroxynitrite, developing nitrosative stress [24].

Uncoupling proteins (UCPs) have been associated with the disruption of the proton gradient across the inner mitochondrial membrane, therefore having a potential energy-dissipating role [25,26]; however, other functions for these proteins have been reported [27]. Among these functions, a role as a mechanism for a compensating oxidative stress has been described [28,29]. Indeed, uncoupling the respiratory chain from oxidative phosphorylation results in less ROS formation. On these lines, several studies confirm that an increase in UCP2 gene expression is related to a decreased generation of H_2O_2 [30]. In fact, UCP2 induction has been suggested as a counter-mechanism in pathophysiological conditions leading to increased oxidative stress [31–33].

Taking all this into consideration, it is very interesting to analyze the effects of IL-15 treatment on the expression of these uncoupling proteins. Indeed, as can be seen in Table 2, tumour growth induced an increase in the expression of these proteins in skeletal muscle, both UCP2 (12%) and UCP3 (67%). Administration of IL-15 promoted an increased expression on both UCP2 (30%) and UCP3 (22%) in tumour-bearing rats, again suggesting that the mechanism associated with the anti-apoptotic action of IL-15 could well involve TNF-α signalling since IL-15 is able to influence TNFRs content in skeletal muscle. Additionally, the cytokine could exert its effects by decreasing oxidative stress, possibly by influencing content (and activity) of uncoupling proteins in skeletal muscle.

In conclusion, from the results presented here it can be suggested that IL-15, in addition to partially reverting muscle wasting associated with tumour-growth, completely reverts the increased DNA fragmentation observed in skeletal muscle of tumour-bearing animals. This makes the cytokine a potentially important drug for the treatment of muscle wasting associated with neoplasies.

Table 2
Expression of uncoupling proteins in skeletal muscle of tumour-bearing rats

Experimental group	IL-15 treatment	UCP 2	UCP 3
Control	No	100 ± 8^{a}	100 ± 18^{a}
	Yes	83 ± 13^{a}	115 ± 34^{a}
Tumour	No	114 ± 13^{a}	298 ± 35^{b}
	Yes	162 ± 10^{b}	381 ± 23^{c}
ANOVA (*P*)		0.0011	<0.001

Expression of the UCP2 and UCP3 mRNAs was detected after hybridization with cDNA probes. Loading correction was carried out by performing blots with the rat 18S ribosomal subunit probe. Autoradiographs were subjected to scanning densitometry. Results are means ± S.E.M. for $n = 6$ and 5 for non-tumour and tumour-bearers, respectively. Statistical significance of the results by one-way ANOVA; different superscripts indicate significant differences between groups by post-hoc Duncan test.

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