Tumour necrosis factor blockade did not prevent the increase of muscular muscle RING finger-1 and muscle atrophy F-box in arthritic rats

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Abstract
Chronic inflammation is associated with a decrease in body weight and cachexia, which is characterized by anorexia and skeletal muscle wasting. The expression of atrogens muscle RING finger-1 (MuRF-1) and muscle atrophy F-box (MAFbx) are increased in muscle atrophy and it is known that tumour necrosis factor (TNF) regulates skeletal muscle loss through TNF receptor p55 (TNFRI). The aim of this study was to examine the effect of polyethylene glycol linked to soluble TNFRI (PEG-sTNFRI) on gene expression of the atrogens MuRF-1 and MAFbx in skeletal muscle of arthritic rats. Rats were injected with Freund’s adjuvant and, 15 days later, arthritic and control rats were injected daily with PEG-sTNFRI (1 mg/kg, s.c.) or saline for 8 days. Arthritis decreased body weight gain, the weight of skeletal muscle and adipose mass. PEG-sTNFRI administration increased body weight gain and adipose mass of arthritic rats; however, it did not modify the skeletal muscle weight. The gene expression of TNF-α, MuRF1 and MAFbx, IGF-I and IGFBP-5 were increased in the skeletal muscle of arthritic rats, and the administration of PEG-sTNFRI did not modify these parameters. These data suggest that the anti-TNF agent PEG-sTNFRI did not prevent the increase in E3 ubiquitin-ligating enzymes, MuRF1 and MAFbx, gene expression in the skeletal muscle of arthritic rats.


Introduction
Adjuvant-induced arthritis is an animal model for chronic inflammation and rheumatoid arthritis characterized by a decrease in body weight and cachexia (Roubenoff et al. 1997, Ibáñez de Cáceres et al. 2000). Cachexia, which also prevails in other situations such as burns, sepsis and cancer, is characterized by anorexia and tissue wasting, reflected essentially in a loss of adipose tissue and skeletal muscle.

The skeletal muscle loss can be due to both the reduction in protein synthesis and the increase in myofibrillar protein breakdown. The increased protein breakdown seems to play a major role in muscle wasting as observed in rats with sepsis (Hasselgren et al. 1989) and in cancer patients (Tisdale 2000). Skeletal muscle contains three major proteolytic systems; one of them is the ubiquitin–proteasome-dependent pathway that mediates the degradation of the most abundant contractile proteins (Mitch & Goldberg 1996). Ubiquitin targets specific protein substrates for degradation and this action involves the ubiquitin−activating enzyme (E1), ubiquitin−conjugating enzymes (E2), and, in general, ubiquitin−protein ligases (E3) (Hershko et al. 2000). There are two new ubiquitin ligases that seem to be specifically involved in myofibrillar protein degradation, the mouse atrophy gene−1 (atrogin−1; Gomes et al. 2001), also described as the rat muscle atrophy F-box (MAFbx; Bodine et al. 2001), and the rat muscle RING finger−1 (MuRF−1; Bodine et al. 2001). Their expression is increased in catabolic conditions that result in muscle atrophy (Bodine et al. 2001, Gomes et al. 2001, Dehoux et al. 2003).

Recently, Lecker et al. (2004) has confirmed that these two atrogens are clearly essential in the process of muscle wasting. In addition, recent studies from our laboratory have provided evidence that arthritis-induced muscle cachexia mainly reflects increased ubiquitin−proteasome−dependent proteolysis and is associated with up−regulated gene expression of E3 ubiquitin−ligating enzymes MuRF−1 and MAFbx in skeletal muscle (Granado et al. 2005).

Muscle proteolysis, in particular, activation of the ubiquitin−proteasome system, has been reported to be regulated by tumour necrosis factor (TNF−α) (Llobera et al. 1997) and insulin-like growth factor (IGF−I) (Chrysis & Underwood 1999). Anti-TNF antibodies prevented increased protein breakdown and increased expression of ubiquitin in skeletal muscle from rats with hepatoma (Costelli et al. 1993, Llobera et al. 1996). Moreover, inhibition of TNF production in vivo by xanthine derivatives, such as pentoxifylline or tobramycin prevented muscle wasting and suppressed the enhanced ubiquitin−proteasome−dependent proteolysis in...
septic and cancer rats (Vary et al. 1999, Combaret et al. 2002). In arthritic rats, the increase in muscular MuRF-1 and MAFbx gene expression was accompanied by the increase in the gene expression of TNF-α. (Granado et al. 2005). Catabolic actions of TNF can be exerted in different ways. One possibility is that TNF-α acts directly on skeletal muscle to induce muscle catabolism. Alternatively, TNF-α can act indirectly, stimulating skeletal muscle catabolism by modifying hormones that regulate protein turnover, such as IGF-I (Fernández-CElemín et al. 2002) or by inducing anorexia (Tracey et al. 1990).

The role of IGF-I in muscle cachexia remains unclear, since this growth factor stimulates muscle protein synthesis, but it does not prevent the sepsis-induced increase in muscle protein breakdown (Fang et al. 2000). However, IGF-I has also been reported to inhibit the ubiquitin–proteasome system in skeletal muscle in a catabolic state induced by dexamethasone (Chrysia & Underwood 1999). The actions of IGF-I are modulated locally by the IGF-binding proteins (IGFBPs). IGFBP-5 binds to the extracellular matrix and modulates the muscle’s response to IGF-I (Schneider et al. 2002). A down-regulation of IGFBP-5 has been reported by microarray studies in several models of muscular wasting (Lecker et al. 2004). However, in arthritic rats, we found that catabolic response in skeletal muscle was associated with increased gene expression of IGF-I and IGFBP-5 (Granado et al. 2005).

Since most of the TNF-α effects on skeletal muscle protein turnover are mediated through TNF receptor p55 (TNFRI) (Llovera et al. 1998), in the present study we tested the hypothesis that administration of high affinity anti-TNF agent, polyethylene glycol linked to soluble TNFRI (PEG-sTNFRI) inhibits the muscular atrophy and the increase in E3 ubiquitin-ligating enzymes MuRF-1 and MAFbx gene expression in skeletal muscle of arthritic rats. Since catabolic response in skeletal muscle was associated with increased gene expression of TNF-α, IGF-I and IGFBP-5 in our previous study (Granado et al. 2005), mRNA levels for TNF-α, IGF-I and IGFBP-5 in gastrocnemius muscle were also determined.

Materials and Methods

Animals and experimental design

Male Wistar rats were housed three or four per cage and were given a regular supply of food and water and allowed to feed ad libitum, under constant conditions of temperature (20–22 °C) and light (lights on from 0730 to 1930 h). The procedures followed the guidelines recommended by the EU for the care and use of laboratory animals. Control and arthritic rats were purchased from Charles River (Barcelona, Spain). Arthritis was induced by an intradermal injection of a suspension of 1 mg heat-killed Mycobacterium butyricum in incomplete Freund’s adjuvant. The injection was given in the plantar surface of the right hind paw under ketamine (75 mg/kg) + diazepam (5 mg/kg) anaesthesia. Control animals were injected with vehicle (paraffin oil). Fifteen days after adjuvant injection, 20 control and 24 arthritic rats were divided into two groups, one injected daily with 1 mg/kg s.c. PEG-sTNFRI (Amgen Inc, Thousand Oaks, CA, USA) and the other with 250 μl saline, until day 22 after adjuvant injection. At this dose, this TNF-binding complex is able to block the systemic TNF-α response (evaluated by its cytotoxicity activity) as well as plasma TNF-α bioactivity in baboons challenged with a lethal Escherichia coli bacteraemia (Solorzano et al. 1998). Assessment of arthritis was performed by measuring the arthritis index of each animal, which was scored by grading each paw from 0 to 4. Grading was determined as the following: 0, neither erythema nor swelling; 1, slight erythema or swelling of one or more digits; 2, swelling of entire paw; 3, erythema and swelling of the ankle; 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16 (Tanaka et al. 1996). Rats having an arthritis index score below 8 on day 15 (1 out of 25 rats) were excluded from the experiment. All rats were weighed daily. Food intake per cage was calculated daily by measuring the difference between the initial and the remaining amount of pellets in the feeder, and expressed as grams per 100 g of body weight throughout the 8 days of treatment.

On day 22 after adjuvant or vehicle injection, all rats were killed by decapitation, 2:5 h after the last PEG-sTNFRI or saline injection. Gastrocnemius muscle from the left paw was frozen in liquid nitrogen and stored at −80 °C. Gastrocnemius muscle from the right paw and epididymal fat were removed, dissected and weighed.

RNA extraction and real-time PCR

Total RNA from skeletal muscle was extracted by the guanidine thiocyanate method using a commercial reagent (Ultraspec RNA, Biotecx Laboratories, Houston, TX, USA). The extracted RNA was dissolved in diethyl pyrocarbonate water with 0:1% SDS and quantified at 260 nm; RNA integrity was confirmed by agarose gel electrophoresis. For RT-PCR analysis, 2 μg skeletal muscle mRNA were reverse transcribed in a total volume of 30 μl at 37 °C for 60 h with 125 units of Moloney murine leukaemia virus RT (Maxim Bioch, San Francisco, CA, USA). Each RT-PCR reaction consisted of 2:5 μl cDNA, SYBR Premix Ex Taq (Takara Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25 μl. Reactions were carried out on a SmartCycler (Cepheid, Sunnyvale, CA, USA). Primers for PCR were obtained from previously published sequences of TNF-α, MuRF-1 and IGF-1 (Dehoux et al. 2003), r18S (Bar et al. 2003) or using the rat GenBank and the EXIQON ProbeLibrary IGFBP-5 and MAFbx. Primers were designed to span a single sequence derived from two exons (i.e. separated by an intron in genomic DNA and primary RNA transcripts to minimize amplification). Parameters included an initial activation of hotStar Taq DNA polymerase at 95 °C for 10 s, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C (MAFbx), 53 °C (MuRF-1 and IGFBP-5), 55 °C (IGF-I), 57 °C (TNF-α), and 60 °C (r18S).
for 30 s, and extension at 72 °C for 30 s. Specific amplification was confirmed by the presence of single peak in the melting curve plots. In addition, the PCR products were analysed in agarose gel electrophoresis. Results were calculated as percentage of control rats injected with saline, using the ΔΔCt method (Livak & Schmittgen 2001) with r18S as the control gene.

Statistical analysis

Statistics were computed using the statistics program STATGRAPHICS plus for Windows (Manugistic, Inc., Rockville, MD, USA). Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA followed by least significant difference (LSD) multiple range test or two-way ANOVA with arthritis and PEG-sTNFRI as factors. When the two-way ANOVA indicated a significant interaction between factors, individual means were compared with LSD multiple range test. Epididymi fat weight and TNF-α mRNA data were subjected to log transformation, since variances showed a non-normal distribution after Kolmogorov–Smirnov test. Statistical significance was set at P<0.05.

Results

As shown in Fig. 1, arthritic rats injected with saline gained less body weight than controls rats, between days 15 and 22 after adjuvant injection (P<0.01). This decrease in body weight gain was concomitant with a significant (P<0.05 and 0.01) decrease in food intake (Fig. 1, upper and lower panel). PEG-sTNFRI administration to arthritic rats induced an increase in body weight gain as compared with arthritic rats injected with saline (P<0.05 on days 1–3 and P<0.01 on days 4–7). The stimulatory effect of PEG-sTNFRI treatment on body weight is associated with an increase in food intake, since from days 2 to 7 of the experiment, food intake was statistically increased in arthritic rats treated with PEG-sTNFRI (P<0.05 and 0.01) as compared with arthritic rats injected with saline. The 8-day administration of PEG-sTNFRI did not modify body weight gain or food intake in control rats (Fig. 1).

Arthritis induced a marked decrease in the gastrocnemius weight and in the epididymi fat weight (P<0.01; Table 1). PEG-sTNFRI administration did not modify the gastrocnemius weight. However, this anti-TNF agent increased the epididymi fat (P<0.05; Table 1).

There was a significant increase (P<0.05) in TNF-α mRNA in the skeletal muscle of arthritic rats injected with saline. PEG-sTNFRI administration did not modify the TNF-α mRNA in arthritic rats, but it increased TNF-α mRNA in control rats even though this increase was not statistically significant (Fig. 2).

As shown in Fig. 3, arthritis induced a significant increase in the two ubiquitin ligases MuRF-1 (P<0.05) and MAFbx (P<0.01) gene expression in the skeletal muscle. After PEG-sTNFRI administration, gene expression of MuRF-1 and MAFbx in the skeletal muscle of control and arthritic rats was similar to values found after saline administration.

The effects of arthritis and PEG-sTNFRI on IGF-I mRNA and IGFBP-5 mRNA in the skeletal muscle are shown in Fig. 4. Arthritis induced an increase in both IGF-I mRNA (P<0.05) and IGFBP-5 mRNA (P<0.01) in the skeletal muscle, whereas PEG-sTNFRI administration did not modify the mRNA of IGF-I and IGFBP-5 in both control and arthritic rats.

Discussion

Our results show that treatment with PEG-sTNFRI resulted in no change in MuRF-1 and MAFbx gene expression in the skeletal muscle of control and arthritic rats.
The effects on MuRF-1 and MAFbx gene expression in gastrocnemius muscle are independent of food intake. The weight lost by the arthritic rats is not secondary to the reduction observed in food intake (Ibañez de Cáceres et al. 2000, Granado et al. 2006). The decrease in body weight in arthritic rats can be seen in the decrease in skeletal muscle weight and in the adipose mass. This muscular atrophy has been reported in adjuvant-arthritic rats (Hamada et al. 2000), septic rats (Cooney et al. 1999) and rats treated with TNF (Hoshino et al. 1991). In TNF-infused rats, the loss of gastrocnemius mass has been ascribed to food intake reduction (Hoshino et al. 1991). This result contrasts with that obtained in the present study, since administration of PEG-sTNFRI to arthritic rats stimulates food intake but does not alter gastrocnemius weight. These results support previous observations (Roubenoff et al. 1997) which demonstrate that although adjuvant arthritis decreases food intake, cachexia is not the result of the decrease in caloric intake. PEG-sTNFRI administration did not change gastrocnemius weight in control or in arthritic rats. Similarly, administration of PEG-sTNFRI administration increased the epididymi fat weight (F_{1,21}=5.04, P<0.05) and the epididymi fat weight (F_{1,21}=5.04, P<0.05).

Two-way ANOVA revealed that there was no interaction between the effects of arthritis and PEG-sTNFRI administration on gastrocnemius weight (F_{1,43}=0.32, P=0.57) and on epididymi fat log weight (F_{1,43}=0.37, P=0.54). Arthritis decreased the gastrocnemius weight (F_{1,41}=886, P<0.01) and the epididymi fat weight (F_{1,41}=100, P<0.01). PEG-sTNFRI administration increased the epididymi fat weight (F_{1,41}=5.04, P<0.05).

### Table 1

Gastrocnemius and epididymi fat weight in control and arthritic (AA) rats treated with saline or PEG-sTNFRI from days 15 to 22 after adjuvant or vehicle injection

<table>
<thead>
<tr>
<th></th>
<th>Control saline</th>
<th>Control PEG-sTNFRI</th>
<th>AA saline</th>
<th>AA PEG-sTNFRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius (mg)</td>
<td>1564±34</td>
<td>1641±42</td>
<td>500±26</td>
<td>535±39</td>
</tr>
<tr>
<td>Epididymi fat log (mg)</td>
<td>3.55±0.04</td>
<td>3.62±0.07</td>
<td>3.10±0.03</td>
<td>3.22±0.03</td>
</tr>
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Figure 2 TTNF-α mRNA in skeletal muscle of control and arthritic (AA) rats treated with PEG-sTNFRI (1 mg/kg s.c.) or saline. There was an interaction between the effects of arthritis and PEG-sTNFRI (F_{1,30}=4.27, P<0.05), as PEG-sTNFRI increased muscular TTNF-α mRNA in control but not arthritic rats. Each bar represents the mean±s.e.m. for 8–10 rats per group. *P<0.05 vs control saline (two-way ANOVA plus LSD multiple range test).

Figure 3 Effect of PEG-sTNFRI (1 mg/kg s.c.) or saline administration on skeletal muscle MuRF-1 (upper panel) and MAFbx (lower panel) gene expression in control and arthritic (AA) rats. There was an interaction between the effects of arthritis and PEG-sTNFRI on muscular MuRF-1 gene expression (F_{1,33}=4.95, P<0.05), as PEG-sTNFRI increased muscular MuRF-1 in arthritic but not in control rats. There was no interaction between the effects of arthritis and PEG-sTNFRI on muscular MAFbx gene expression (F_{1,34}=0.04, P=0.84). Arthritis increased MAFbx (F_{1,34}=11.56, P<0.01) gene expression in the skeletal muscle. Each bar represents the mean±s.e.m. for 8–10 rats per group. *P<0.05 vs control saline, †P<0.001 vs control PEG-sTNFRI (two-way ANOVA plus LSD multiple range test).

this TNF-α inhibitory protein did not modify gastrocnemius weight in control rats, although this inhibitory protein increased it in septic rats (Cooney et al. 1999). The increase in body weight gain in arthritic rats after PEG-sTNFRI administration is reflected in the increase in fat mass. There is evidence that TNF-α can act through the TNF-α receptor (TNFR1) to inhibit adipogenesis (Warne 2003).

Arthritis decreased gastrocnemius weight and increased the gene expression of ubiquitin ligases MuRF-1 and MAFbx in the skeletal muscle as previously shown (Granado et al. 2005). Furthermore, in tumour-bearing mice, the decrease in gastrocnemius muscle mass was associated with increased muscle levels of E3 ubiquitin-ligases (Hitt et al. 2005). These data suggest that the decrease in skeletal muscle weight is secondary to an enhanced protein degradation pathway, the ubiquitin–proteasome proteolytic pathway. PEG-sTNFRI administration to arthritic rats increased food intake, but it did not prevent the activation of ubiquitin ligase gene expression. These results suggest that MuRF-1 and MAFbx gene expression in the skeletal muscle are independent of food intake. PEG-sTNFRI administration did not change muscular MuRF-1 and MAFbx gene expression or gastrocnemius weight in arthritic rats. However, suppression of TNF by pentoxifylline prevented the inhibition of protein synthesis and stimulation of protein degradation as well as the decrease in epitrochlearis muscle weight during sepsis (Vary et al. 1999). These authors suggest that the effect of pentoxifylline in the regulation of protein turnover during sepsis does not appear to be dependent on food intake (Vary et al. 1999).

IGF-I controls muscle mass by enhancing myofibre hypertrophy and stimulating myogenesis (Musaro et al. 2001). However, it has been reported (Foulstone et al. 2001) that the effects of IGF-I on muscle may depend on the cytokine environment in such a way that in the presence of TNF-α, IGF-I induces apoptosis. The increase in IGF-I gene expression in skeletal muscle of arthritic rats does not correlate with the decrease observed in the muscle of lipopolysaccharide (LPS)-treated rats (Lang et al. 2000, Fernández-Celemin et al. 2002) and fasting rats (Dehoux et al. 2004). The increase in MuRF-1 and MAFbx gene expression found in the skeletal muscle of arthritic rats (Granado et al. 2005) has also been found in the muscle of LPS-injected rats (Dehoux et al. 2003). Muscle mRNA content of MAFbx was also increased in other catabolic situations, such as fasting and diabetes (Dehoux et al. 2004).

Since IGF-I has been reported to inhibit MAFbx expression in muscles of fasting and diabetic rats (Dehoux et al. 2004), the increase in IGF-I in the muscle of arthritic rats should be followed by a decrease in muscular MuRF-1 and MAFbx gene expression. Since this is not the case, there is a possibility that arthritic muscles have become resistant to IGF-I, as has been observed in experimental models of sepsis (Fang et al. 2001) and chronic renal failure (Ding et al. 1996). This effect can be exerted by muscular TNF-α, since this cytokine acts on muscle cells inducing a state of IGF-I receptor resistance (Broussard et al. 2003). Moreover, anti-TNF therapy improves glucocorticoid-induced IGF-I resistance in rheumatoid arthritis (Sarzi-Puttini et al. 2006).

On the other hand, muscular IGFBP-5 could also contribute to the loss of the beneficial IGF-I effect, since this protein can inhibit or enhance IGF-I activity (Schneider et al. 2002). The widespread growth retardation demonstrated by transgenic mice that overexpress IGFBP-5 suggests that its predominant effect is inhibitory (Salih et al. 2004). In arthritic rats, we found an increase in muscular IGFBP-5 gene expression, which has been previously reported in muscular atrophy (Awede et al. 1999) and in chondrocytes isolated from osteoarthritic cartilage (Olney et al. 1996). The increase in
IGFBP-5 in arthritic rats is probably not secondary to the increase in muscular IGF-I, since it has been recently observed in transgenic mice that overexpress IGF-I in muscle that IGFBP-5 mRNA expression was not affected (Musaro et al. 2001, Oliver et al. 2005). However, the increase in IGFBP-5 in arthritic rats could be secondary to the increase in TNF-α release, since TNF-α can stimulate IGFBP-5 in articular chondrocytes (Sunic et al. 1998). Moreover, PEG-sTNFRI administration did not modify TNF-α gene expression in gastrocnemius muscle as it did with the gene expression of IGFBP-5 in this muscle. These results suggest that the lack of effect of PEG-sTNFRI administration on muscular IGF-I gene expression could be due to the lack of effect of PEG-sTNFRI on muscular TNF-α and IGFBP-5.

In accordance with our results, the same dose of PEG-sTNFRI did not modify gastrocnemius IGF-I gene expression in rats with sepsis (Yumet et al. 2002) and in burned rats (Lang et al. 2002). This effect seems to be specific for muscular TNF, since the inhibition of TNF production by PEG-sTNFRI administration was able to attenuate the decrease in hepatic IGF-I mRNA in arthritic (Granado et al. 2006) and septic rats (Yumet et al. 2002). In addition, it was also able to attenuate the increase in hepatic IGF-I mRNA in burned rats (Lang et al. 2002).

The lack of effect of PEG-sTNFRI therapy on TNF-α mRNA in the skeletal muscle of arthritic rats is in agreement with the results observed on TNF-α mRNA in the synovium and in the inguinal lymph nodes after the administration of 10 mg/kg PEG-sTNFRI in adjuvant arthritis (Bush et al. 2002). In this study, PEG-sTNFRI therapy attenuated the illness, but it did not modify mRNA expression of synovial TNF.

Inhibition of TNF-α synthesis by xanthine derivatives prevents muscle protein breakdown in septic rats (Vary et al. 1999) and prevents cancer and sepsis-induced increase in muscular ubiquitin gene expression (Combaret et al. 2002). The discrepancy between these findings and our results might be due to the fact that xanthine derivatives inhibit TNF-α synthesis, thus decreasing local and circulating TNF; but in our study, PEG-sTNFRI administration only prevents the effect of circulating TNF. However, other authors have found that inhibiting TNF action by anti-TNF antibodies also reverted the increased muscle ubiquitin gene expression in tumour-bearing rats (Llovera et al. 1996). The differences in results could be explained by the fact that in tumour-bearing rats there is a substantial decrease in skeletal muscle TNF-α gene expression (Figuera et al. 2005), while in our study we found an increase in muscular TNF-α gene expression.

Although PEG-sTNFRI was effective as an anti-inflammatory agent, improving paw volume and arthritis index scores (Granado et al. 2006) as well as increasing body weight gain, it was not able to block the increase in the gene expression of TNF-α in the muscle of arthritic rats. It has been postulated that TNF-α exerts its catabolic effects in skeletal muscle in a paracrine/autocrine manner (Fernández-Çelemín et al. 2002). Therefore, the gene expression of E3 ubiquitin ligases MuRF-1 and MAFbx in the muscle of arthritic rats was not changed after PEG-sTNFRI administration probably as a consequence of the lack of effect of this protein complex on muscular TNF-α.

In conclusion, the administration of PEG-sTNFRI to arthritic rats does not modify the weight of gastrocnemius muscle and muscular MuRF1 and MAFbx gene expression, although it increases body weight and fat mass.

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