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EFFECT OF CYCLOOXYGENASE-2 INHIBITION BY MELOXICAM, ON ATROGIN-1 AND MYOGENIC REGULATORY FACTORS IN SKELETAL MUSCLE OF RATS INJECTED WITH ENDOTOXIN

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Cyclooxygenase-2-induction by inflammatory stimuli has been proposed as a mediator of inflammatory cachexia. We analyse whether cyclooxygenase-2 inhibition by meloxicam administration is able to modify the response of skeletal muscle to inflammation induced by lipopolysaccharide endotoxin (LPS). Male rats were injected with 1 mg kg⁻¹ LPS at 17:00 h and at 10:00 h the following day, and euthanized 4, 24 or 72 hours later. Atrogin-1, MuRF1, myogenic regulatory factors and cyclooxygenase-2 in the gastrocnemius were determined by real time-PCR (mRNA) and Western blot (protein). In a second experiment the effect of meloxicam administration (1 mg kg⁻¹) was analyzed. Meloxicam was administered either in a preventive manner, 1 hour before each endotoxin injection, or in a therapeutic manner, starting 2 hours after the second LPS injection and at 24 and 48 hours afterwards. There was a marked increase in MuRF1 mRNA ($P<0.01$) 4 hours after LPS, and in atrogin-1 mRNA 4 hours ($P<0.01$) and 24 hours ($P<0.01$) after LPS. Cyclooxygenase-2 was increased, whereas MyoD was decreased at 4, 24 and 72 h. Both types of meloxicam treatment blocked LPS-induced increase in atrogin-1. Preventive, but not therapeutic, meloxicam decreased myostatin ($P<0.01$) and increased Pax7 ($P<0.01$) and MyoD ($P<0.05$). Therapeutic meloxicam treatment decreased gastrocnemius myogenin. These data suggest that cyclooxygenase-2 inhibition by meloxicam administration can prevent the increase in atrogin-1 and the decrease in MyoD induced by LPS administration. However, prolonged therapeutic meloxicam treatment seems to be less effective, since it can inhibit myogenic regulatory factors.

Key words: *cyclooxygenase-2, gastrocnemius muscle, lipopolysaccharide, MyoD, meloxicam, myostatin, myogenin, skeletal muscle, sepsis*

INTRODUCTION

Skeletal muscle is a plastic organ that can increase or decrease in volume in accordance with modifications in energy balance and environmental factors. Inflammatory illnesses such as sepsis, cancer and arthritis are associated with muscle wasting (1-3). Sepsis induces myopathy characterized by a reduction in muscle force-generating capacity and muscle wasting (3). Moreover, myopathy and its related syndromes are a major cause of mortality and long-term morbidity in critically ill patients (3). Critically ill patients with sepsis can have a 20% reduction in muscle mass within the first week (4). These patients have a normal muscle protein synthesis rate, whereas muscle proteolysis is increased up to 160% (5). Of the major proteolytic systems, ubiquitin-proteasome pathway is increased, whereas calpain and caspase activities are not changed in sepsis (5). The two E3 ubiquitin ligases, muscle RING-finger protein-1 (MuRF-1) and atrogin-1 are sensitive markers for muscular atrophy (6). The mechanisms by which chronic inflammatory diseases activate the ubiquitin-proteasome pathway are through the release of cytokines (2) or myostatin, a negative regulator of muscle mass (7).

Cyclooxygenases (COXs) are key enzymes in regulating the biosynthesis of prostaglandins. COX-2 isoform is inducible by pro-inflammatory cytokines and other stimuli, not only in immune cells, but also in other tissues such as skeletal muscle (8). COX-2 activation plays an important role in cachexia induced by experimental arthritis (3). Inhibition of COX-2, by meloxicam administration, ameliorates muscle wasting by preventing arthritis-induced upregulation of muscle atrogin-1 and MuRF1 (3). COX inhibitors are also able to improve cancer cachexia in both experimental animals and in humans (9, 10), by acting on the ubiquitin-proteasome pathway (11).

In spite of the anti-cachetic effect of anti-inflammatory therapy with COX-2 inhibitors, these drugs can have adverse effects on muscle regeneration and growth after muscle injury (12). COX-2 activation plays an important role in muscle regeneration after muscle injury (13). The role of COX-2 in muscle regeneration seems to be related to the fact that inflammatory response facilitates myogenesis (14). Although the role of COX-2 activation in sepsis is not well known, COX-2 inhibition prevents lipopolysaccharide-induced decrease in serum and liver IGF-I and decreases pro-inflammatory cytokines and liver injury in septic rats (15, 16).

The above data indicate that sepsis induces long-lasting myopathy, and COX-2 inhibition can prevent organ injury during sepsis, but COX-2 inhibition can have adverse effects on muscle regeneration after muscle injury. Therefore, the aim of this work was to study the effect of treatment with the COX-2 inhibitor meloxicam in two different approaches, preventive and therapeutic, on the response of skeletal muscle to inflammation induced by endotoxin. For this purpose, we analysed the effect of these treatments on atrogin-1, MuRF1, myostatin, myoblast determination protein-1 (MyoD) and myogenin expression in gastrocnemius after lipopolysaccharide (LPS) administration.

MATERIAL AND METHODS

Animals and experimental procedures

Male Wistar rats (200–225 g) were obtained from Charles River (Barcelona, Spain), and housed 3–4 per cage, at 22°C and with light on from 7:30 to 19:30 h. All procedures were carried out according to the guidelines recommended by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and were approved by the Complutense University Animal Care Committee.

Experiment I: The effect of LPS on the gastrocnemius muscle was studied at different periods after LPS injection. Twenty-seven rats were i.p. injected with 1 mg kg⁻¹ LPS (serotype 055:B5, Sigma Chemical Co, St. Louis, MO, USA) in 250 µL saline at 17:00 h and at 10:00 h the following day. This LPS administration protocol has been shown to increase TNF-α in the liver (17). Twelve rats were used as control group and were i.p. injected with 250 µL of saline. Rats were euthanized by decapitation 4, 24 or 72 hours after the second LPS or saline injection. Body weights were assessed daily. Left gastrocnemius were dissected and weighed, frozen in liquid nitrogen and stored at -80°C until RNA or protein extraction.

Experiment II: The effects of two types of meloxicam treatments on skeletal muscle response 48 hours after the second LPS administration were analysed. This time point was chosen since results in experiment I indicated that 72 hours after LPS administration, although MyoD was decreased, atrogin-1 was still not increased in the gastrocnemius. Thirty rats were i.p. injected with 1 mg kg⁻¹ LPS and were randomly divided into 3 different groups;

A) LPS group: this group received 250 µL saline at 9:00 h for two days after LPS treatment.

B) Meloxicam preventive treatment: these rats were injected s.c. with meloxicam (1 mg kg⁻¹, Sigma, Madrid, Spain) one hour before each LPS injection (at 16:00h and at 9:00 h), and they were injected s.c. with 250 µL saline for the following two days (Fig. 1). Meloxicam at this dosage prevented arthritis-induced increase in atrogin-1 and MuRF1 (3), as well as LPS-induced increase in liver TNF-α (15).

C) Meloxicam therapeutic treatment: this group was treated with 1 mg kg⁻¹ meloxicam s.c., starting 2 hours after the second LPS injection (at 12:00 h), and for the following two days at 9:00 h (Fig. 1). Another twenty rats were injected i.p. and s.c. with 250 µL saline for four days, and served as controls. This group was divided into: 1) control group fed *ad libitum* and 2) pair-fed rats. As LPS administration decreases food intake, the pair-fed group and all the groups of rats injected with LPS during the first day of the experiment were not fed between the two LPS injections. Meloxicam treatments did not modify food intake, since food intake in these groups was similar to that of the rats injected with LPS and saline. All rats were euthanized by decapitation, 3 hours after saline or meloxicam injection, on day 3 and 48 hours after the second LPS injection.

Left gastrocnemius was frozen in liquid nitrogen and stored at -80°C until RNA or protein extraction. For muscle morphology analysis, right gastrocnemius was placed in 4% paraformaldehyde for 48 hours at 4°C, and then in 30% sucrose. Twelve µm cryostat sections taken from the mid-belly region of the lateral gastrocnemius and stained with haematoxylin-eosin were used to determine the whole cross-sectional area. Digital images were acquired with a Leica DMI300 microscope. Sections were scanned (Epson scanner 4990) alongside with a transparent ruler, and the area was measured with ImageJ software. About 200 fibers from the deep portion of each section were used to measure fiber size with ImageJ software. All measurements were made by a blinded evaluator.

RNA extraction and real-time PCR

Total RNA was extracted from 100 mg gastrocnemius using UltraspecTM (Biotecx Laboratories Inc. Houston, Texas, USA). Concentration of RNA was determined with a BioPhotometer (Eppendorf, Germany), and the integrity of the RNA was confirmed by agarose gel electrophoresis. First-strand cDNA synthesis was performed using 1 µg of total RNA with a Quantiscript Reverse Transcription kit (Qiagen, Valencia, CA, USA).

Real-time PCR for quantification of mRNA was performed on a SmartCycler® (Cepheid, Sunnyvale, CA, USA) using a SYBR-Green protocol. Each real-time PCR reaction consisted of 10 ng total RNA equivalents, 1× Takara SYBR Green Premix Ex Taq (Takara BIO INC, Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25 µL. Primers for real-time PCR (Table 1) were purchased from Roche (Madrid, Spain). The thermal cycling profile consisted of a preincubation step at 95°C for 10 s followed by 40 cycles of 95°C denaturation steps for 15 s, 60°C annealing steps for 30 s, and 72°C extension steps for 30 s. The fluorescence signal was analysed and normalised against 18S RNA. Relative expression levels were obtained using cycle threshold 2(ΔΔCT) method. PCR products were separated using agarose gel electrophoresis to confirm the product presence and size.

Immunoblotting

Gastrocnemius (100 mg) was homogenised in 1 ml lysis buffer with protease inhibitor cocktail (Sigma-Aldrich, Madrid, Spain). Homogenate was centrifuged at 13,000 rpm at 4°C for 30 min to remove tissue debris, and protein concentration was determined using the Bradford protein assay. Extracts were boiled for 5 min with a 1:1 volume of Laemmli loading buffer (18). Proteins (100 µg) were resolved by electrophoresis on 14% polyacrylamide gels under reducing conditions, transferred onto nitrocellulose membranes and blocked with 5% non-fat dry milk, 0.1% Tween (Sigma-Aldrich, Madrid, Spain), in Tris-buffered saline. Membranes were probed (overnight at 4°C) sequentially with antibodies against COX-2 (Cayman Chemical Company, Ann Arbor, Michigan, USA), myostatin, myogenin and MyoD (Santa Cruz biotechnology, CA, USA), Pax7 (Abcam, Cambridge, UK) and α-tubulin (Sigma-Aldrich, Madrid, Spain). Membranes were incubated for 90 min with secondary antibodies conjugated to horseradish peroxidase (anti-mouse IgG Amersham Biosciences, Little Chalfont, UK; anti-rabbit IgG Biorad, Madrid, Spain), and peroxidase activity was detected using enhanced chemiluminescent reagent (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified using a PC-Image VGA24 program for Windows. The density of the protein band in each lane was expressed as the percentage of the mean density of control rats after load normalisation using either α-tubulin or Ponceau S at ~42 kDa.

Table 1. Primers for real-time PCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Product bp
18S	GGTGCATGGCCGTTCTTA	TCGTTTCGTTATCGGAATTAACC	60
atrogin-1	GAACAGCAAAACCAAACTC AGTA	GCTCCTTAGTACTCCCTTTGTGAA	74
MuRF1	TGTCTGGAGGTCGTTTCCG	ATGCCGGTCCATGATCACTT	58
myostatin	TGGGCATGATCTTGCTGTAA	TGTTACTTTGACTTCTAAAAAGG GATT	76
MyoD	GGAGACATCCTCAAGCGATGC	AGCACCTGGTAAATCGGATTG	104
myogenin	CCTTGCTCAGCTCCCTCA	TGGGAGTTGCATTCACTGG	94

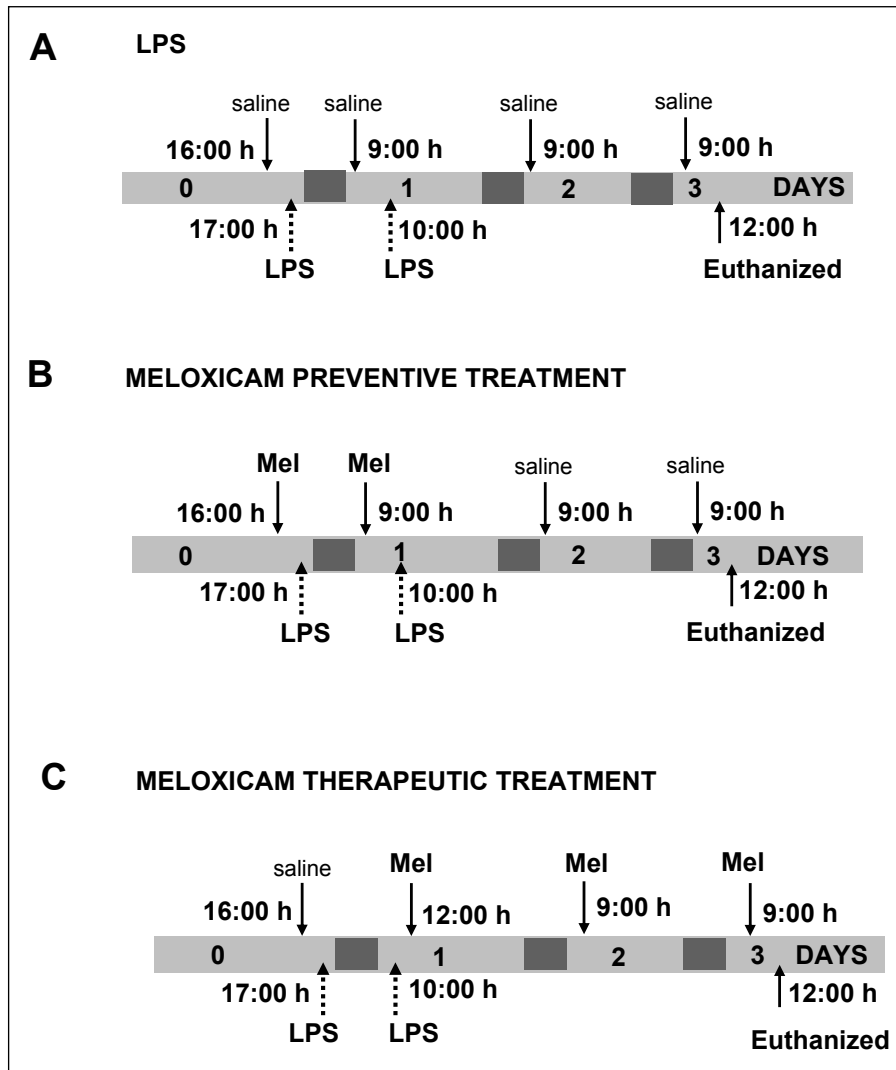


Fig. 1. Experimental paradigms for experiment II, (A) LPS group, (B) meloxicam preventive treatment and (C) meloxicam therapeutic treatment. Control and pair-fed group were only injected with saline.

Statistical analysis

Statistics were computed using the statistics program STATGRAPHICS plus for Windows. Data are presented as means \pm standard error of the mean and were tested with analysis of variance (ANOVA); post hoc comparisons were made using the LSD multiple range test. Because there were no significant differences in experiment I between data obtained in the control rats killed 4, 24 or 72 hours after saline injection, they were used as one control group. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of LPS administration

Two injections of LPS induced a transient decrease in body weight gain 4 and 24 hours after the second LPS injection (Fig. 2A). However, at 48 and 72 hours, rats injected with LPS had similar body weight gain to control rats. Gastrocnemius weight was decreased on 24 and 72 hours after LPS injection (Fig. 2B). This decrease in gastrocnemius weight can be secondary

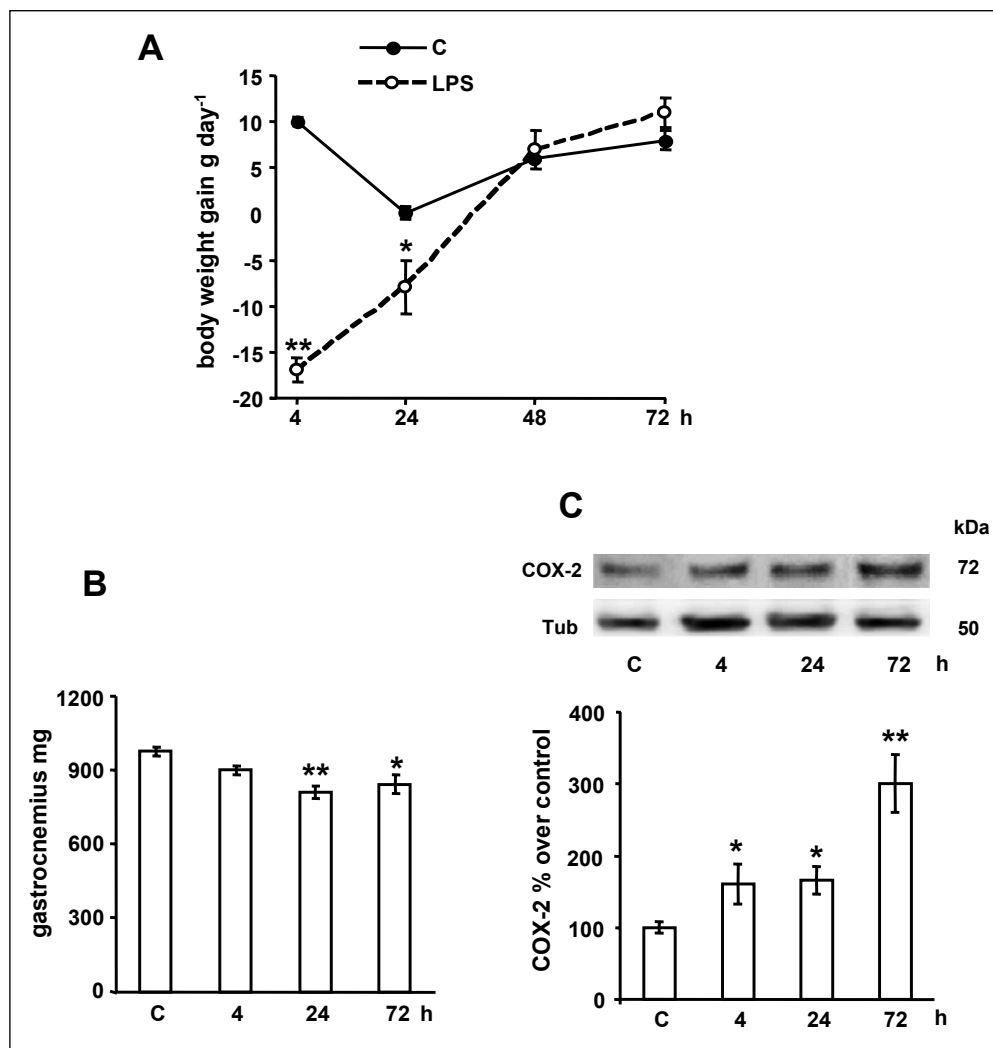


Fig. 2. Body weight gain (A), gastrocnemius weight (B) and COX-2 in the gastrocnemius 4, 24 and 72 hours after two injections of 1 mg kg⁻¹ LPS. COX-2 was measured by Western blot, normalised against α -tubulin and expressed as percentage of the control rats (C). Data represent mean \pm S.E.M. (n=6–10 rats). **P<0.01, *P<0.05 vs. control rats.

to the decrease in food intake, since in experiment II, there was no differences in gastrocnemius weight in the rats injected with LPS (1081 \pm 24 g) and pair-fed rats (1142 \pm 22 g). LPS increased gastrocnemius COX-2 at 4, 24 and 72 hours after injection (Fig. 2C).

MuRF1 and atrogin-1 expression in the gastrocnemius were increased 4 hours after the second LPS injection (Fig. 3A and 3B). Twenty-four hours after LPS, atrogin-1 level in the gastrocnemius was increased to a similar extent than after 4 hours, but MuRF1 mRNA returned to values similar to control rats. Myostatin mRNA and protein tended to increase in LPS-injected rats (Fig. 3C and 3D), but the increase was only significant for myostatin mRNA, 72 hours after LPS administration. There was a significant decrease in MyoD mRNA at 4 h, whereas MyoD protein was decreased, 4, 24 and 72 hours after the second LPS injection (Fig. 4A and 4B). LPS administration increased myogenin mRNA at 4 and 24 h, but myogenin protein level was not significantly increased at any time after LPS injection (Fig. 4C and 4D).

Effect of meloxicam treatment

Meloxicam treatments did not modify body weight gain or food intake, where the body weight and food intake in both groups of rats treated with meloxicam were similar to those of the rats injected only with LPS (data not shown). Basic

histological analysis of gastrocnemius muscle revealed a lack of massive or focal clusters of necrotic fibers. As shown in Fig. 5A, cross-section area of the gastrocnemius muscle in the rats injected with LPS was lower than the control (P<0.01) and pair-fed rats (P<0.05). Meloxicam had a different effect depending on the treatment. Therapeutic meloxicam treatment did not modify the inhibitory effect of LPS on cross-section area of the gastrocnemius. In contrast, preventive administration blocked LPS-induced decrease in gastrocnemius cross-section area, having similar values to pair-fed group. LPS injection also decreased the mean fiber area of the gastrocnemius as compared with control and pair-fed rats (Fig. 5B). Both types of meloxicam treatment prevented the inhibitory effect of LPS on mean fiber area (Fig. 5B).

Fig. 6 shows MuRF1 and atrogin-1 mRNA levels in the gastrocnemius of rats treated with saline or meloxicam, 48 hours after the second LPS injection. MuRF1 levels were similar in all groups of rats (Fig. 6A). In contrast, atrogin-1 mRNA was higher in the gastrocnemius of the rats injected with LPS than in control or pair-fed rats (P<0.01, Fig. 6B). Both types of meloxicam treatment, preventive and therapeutic, blocked LPS-induced increase in atrogin-1 expression in the gastrocnemius muscle. As found in experiment I, LPS administration did not modify myostatin mRNA or protein levels (Fig. 7A and 7B). Similarly, myostatin expression was not modified by meloxicam therapeutic treatment. However, in the rats that received

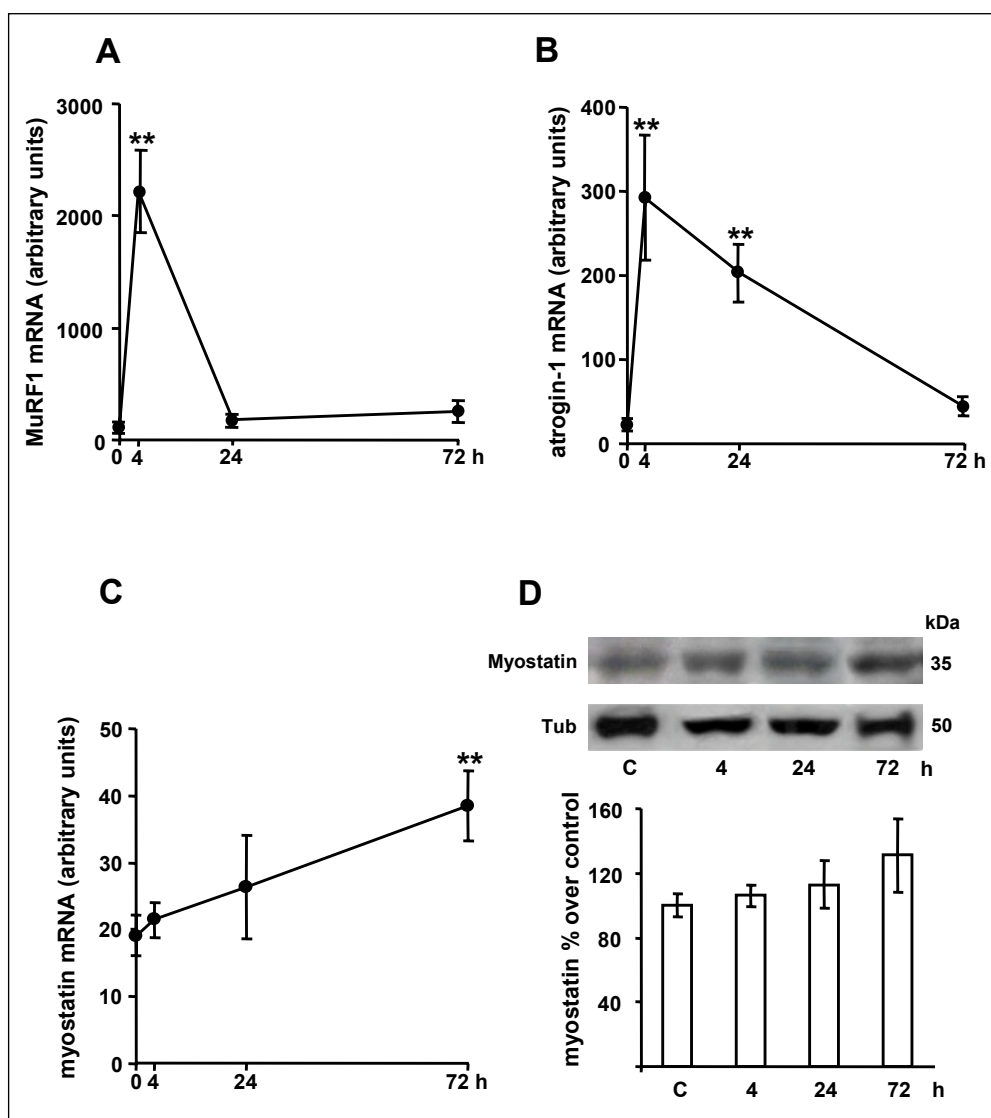


Fig. 3. Evolution of gastrocnemius MuRF1 mRNA (A), atrogen-1 mRNA (B), myostatin mRNA (C) and myostatin protein (D) at 4, 24 and 72 hours after two injections of 1 mg kg⁻¹ LPS. MuRF1, atrogen-1 and myostatin mRNA were quantified by real time PCR as described in material and methods and are presented in relation to the mean value in the control group. Myostatin was measured by Western blot, normalised against α -tubulin, value of control group was set at 100% and the protein expression was relative to this value. Data represent mean \pm S.E.M. (n=6–10 rats). **P<0.01 vs. control rats.

preventive meloxicam treatment, myostatin mRNA levels were lower than those of the rest of the groups. Preventive meloxicam treatment also decreased myostatin protein to levels lower than those of pair-fed rats.

The expression of paired box protein 7 (Pax7), an early myogenic regulatory factor, was also measured. As shown in Fig. 8, LPS induced a decreased in Pax7 protein levels (P<0.05). Meloxicam therapeutic treatment was not able to prevent the inhibitory effect of LPS on Pax7. In contrast, the rats that received preventive meloxicam treatment had similar Pax7 protein levels in the gastrocnemius to control rats. MyoD mRNA and protein levels were decreased in the LPS group, as well as in the rats injected with LPS that received meloxicam therapeutically, but the decrease was only significant in MyoD protein (Fig. 9A and 9B). However, in the rats injected with LPS that received preventive meloxicam treatment, MyoD mRNA and protein levels were similar to those of pair-fed rats, and higher than in the rats treated with LPS alone or with LPS and meloxicam therapeutically. LPS did not modify myogenin expression in the gastrocnemius muscle (Fig. 10A and 10B). Only meloxicam therapeutic treatment decreased myogenin mRNA with respect to the other two groups injected with LPS (Fig. 8A). Meloxicam therapeutic treatment also decreased myogenin protein levels in comparison with pair-fed rats (Fig. 10B).

DISCUSSION

Our data suggest that LPS has a detrimental effect on skeletal muscle, which can be observed by a transient increase in MuRF1 and atrogen-1 together with longer-lasting upregulation of COX-2 and downregulation of Pax7 and MyoD in the gastrocnemius. Inhibition of COX-2 activation by meloxicam administration prevents LPS-induced increase in atrogen-1 in the gastrocnemius muscle. However, meloxicam is more effective when administered preventively than therapeutically, since longer meloxicam treatment can decrease the expression of myogenic regulatory factors.

Two injections of LPS only decreased body weight gain and increased MuRF1 expression in the gastrocnemius in the first 4 hours after LPS injection, whereas the increase in gastrocnemius COX-2 and atrogen-1 was observed until at least 48 hours after LPS administration. Those changes are associated with a reduction of muscle cross-section area and fiber cross-sectional area. These data indicate that the catabolic state induced by LPS, although transient, is strong enough to induce morphological alterations in the skeletal muscle. A reduction in muscle mass and fiber area has been reported as early as two days after zymosan-induced sepsis (19). Upregulation of atrogen-1 and MuRF1 has been previously reported in different septic models

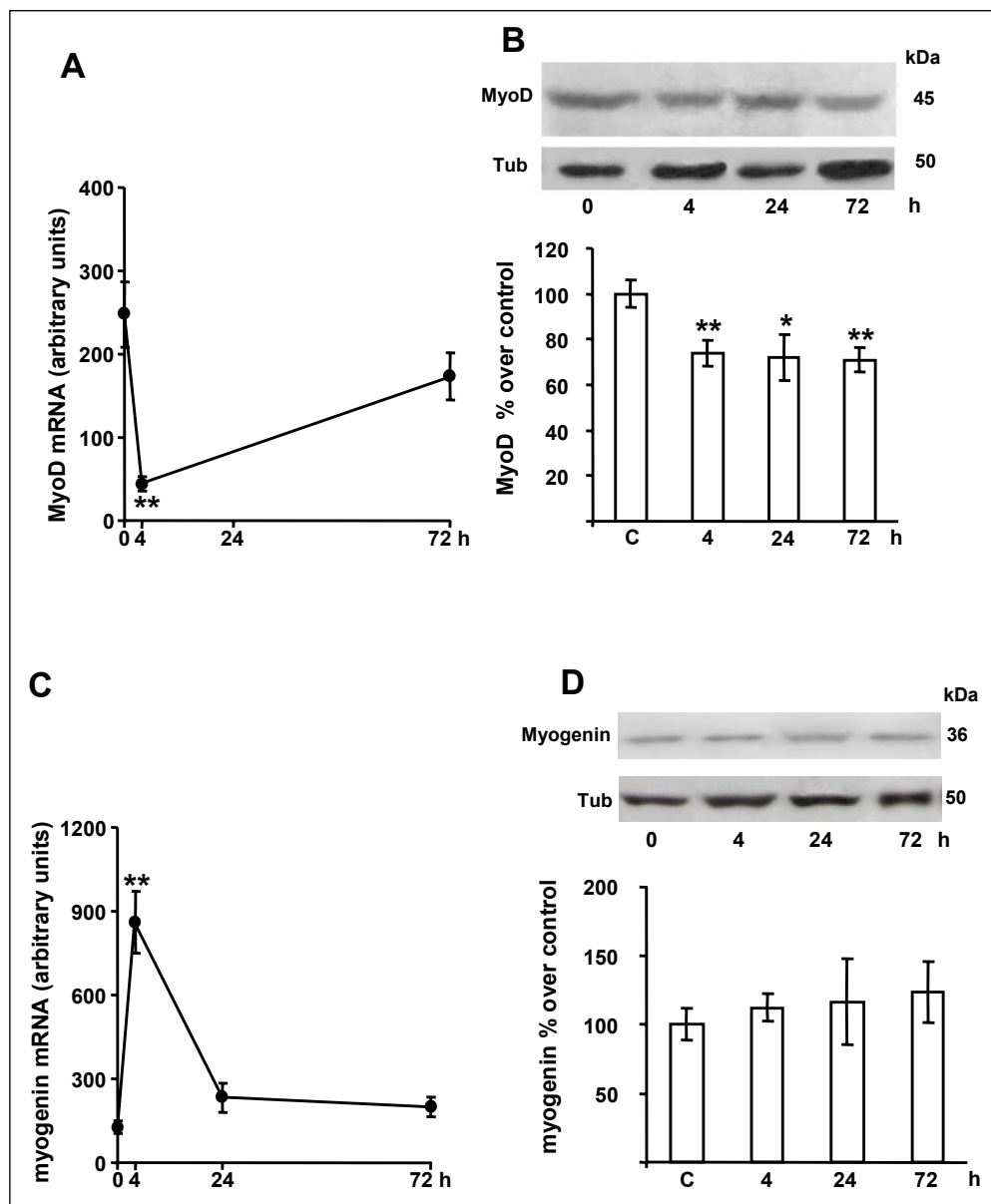


Fig. 4. Evolution of gastrocnemius MyoD mRNA (A), and protein (B), myogenin mRNA (C) and protein (D) at 4, 24 and 72 hours after two injections of 1 mg kg^{-1} LPS. mRNA was quantified by real time PCR as described in material and methods and is presented in relation to the mean value in the control group. Proteins were measured by Western blot, normalised against α -tubulin, value of control group was set at 100% and the protein expression was relative to this value. Data represent mean \pm S.E.M. ($n=6-10$ rats). ** $P<0.01$, * $P<0.05$ vs. control rats.

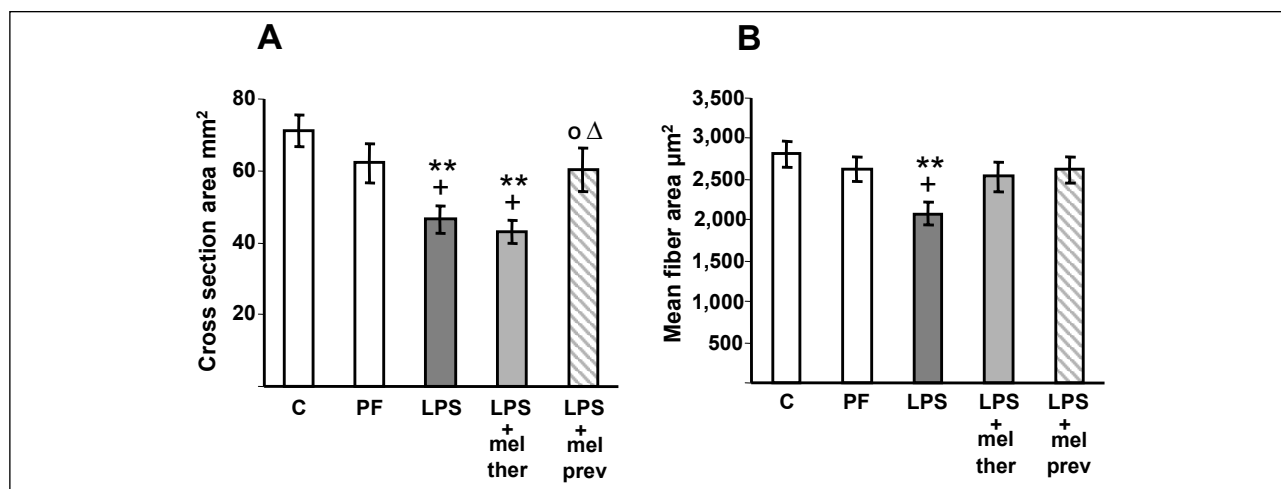
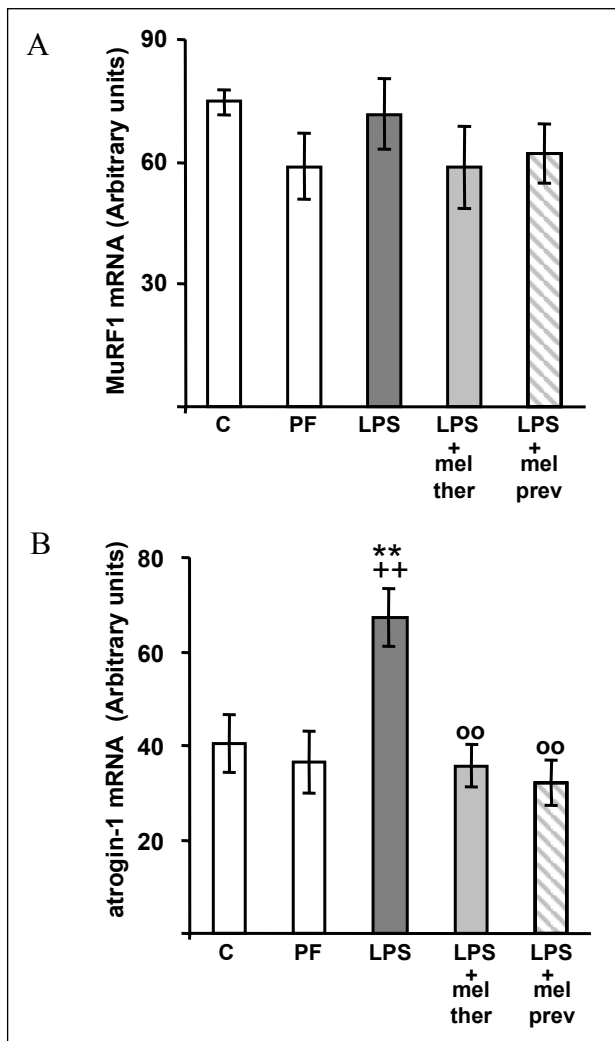


Fig. 5. Effect of 1 mg/kg meloxicam, administered therapeutically (mel ther) or preventively (mel prev), on LPS-induced decrease in gastrocnemius cross-section area (A) and on gastrocnemius mean fiber area (B). Preventive meloxicam treatment blocked the inhibitory effect of LPS on cross-section area, whereas both therapeutic and preventive meloxicam treatments blocked the decrease in mean fiber area. Data represent mean \pm S.E.M. ($n=9-10$). ** $P<0.01$ vs. control rats (C), * $P<0.05$ vs. pair-fed rats (PF), $^{\circ}\Delta P<0.05$ vs. LPS group, $^{\Delta}P<0.05$ vs. LPS + meloxicam therapeutic treatment.



(20, 21). In contrast to both atrogenes, myostatin protein was not significantly upregulated by LPS administration. Myostatin in muscle is increased in muscle wasting induced by cancer, muscle disuse or diabetes (7, 22, 23), but lack of changes in myostatin in sepsis has also been reported (24). These data suggest that muscle wasting in different conditions can be mediated by different mechanisms.

A decrease in MyoD was observed in all time points analysed after LPS, both in mRNA and protein. To our knowledge, there are no data on the effect of LPS administration on MyoD expression in muscle. However, a decrease in both MyoD mRNA and protein after TNF- α administration has been reported *in vitro* and *in vivo* (25, 26). Taking into account, that LPS injection increases TNF- α release, it is possible that LPS-induced decrease in MyoD expression is mediated by TNF- α . The decreased Pax7 in the gastrocnemius 48 hours after LPS administration can also be secondary to increased release of TNF- α . In this sense, Palacios *et al.* (27) reported that TNF- α -activated pathway decreases Pax7 expression in regenerating muscles. In adult skeletal muscle, Pax7 is expressed in quiescent satellite cells, and it is required for muscle growth and regeneration from injury (28). These cells, when activated, co-express Pax7 and MyoD (29). Similarly to our data, a decrease

Fig. 6. MuRF1 (A) and atrogin-1 mRNA (B) in the gastrocnemius of control rats (C), pair-fed rats (PF), LPS injected rats (LPS), as well as in LPS injected rats with therapeutic (LPS+mel ther) or preventive (LPS+mel prev) meloxicam treatments. Both types of meloxicam treatments blocked LPS-induced increase in atrogin-1 mRNA. MuRF1 and atrogin-1 mRNA were quantified by real time PCR as described in material and methods and are presented in relation to the mean value in the control group. Data represent mean \pm S.E.M. (n=9–10 rats). **P<0.01 vs. control rats, ++P<0.01 vs. pair-fed rats, °°P<0.01 vs. LPS group.

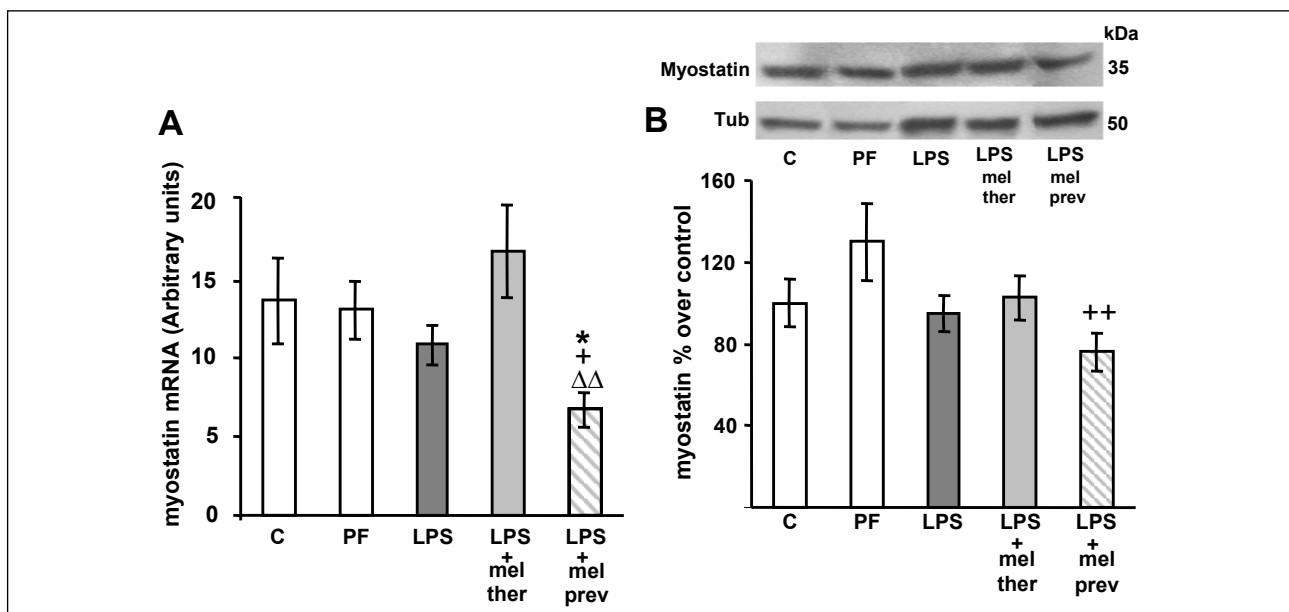


Fig. 7. Preventive meloxicam treatment decreased myostatin mRNA (A) and protein (B) in the gastrocnemius of control rats (C), pair-fed rats (PF), LPS injected rats (LPS), as well as in LPS injected rats with therapeutic (LPS+mel ther) or preventive (LPS+mel prev) meloxicam treatments. Myostatin mRNA was measured by real time PCR as described in material and methods and is presented in relation to the mean value in the control group. Myostatin protein was measured by Western blot, normalised against the α -tubulin, value of control group was set at 100% and the protein expression was relative to this value. Data represent mean \pm S.E.M. (n=9–10 rats). *P<0.05 vs. control rats, ++P<0.01, +P<0.05 vs. pair-fed rats, ΔΔP<0.01 vs. LPS + meloxicam therapeutic treatment.

in both MyoD and Pax7 in atrophic muscle from tumour-bearing rats has recently been reported (30). In contrast with these data, we have observed that arthritis did not only decrease MyoD expression in the muscle, but increased it (31). MyoD expression in muscle is also increased in muscle atrophy induced by cancer

or by chronic heart failure (32, 33). These results suggest that MyoD response can be different in acute and in chronic illnesses.

LPS administration induced a response on myogenin that was different to that induced on Pax7 and MyoD in the gastrocnemius, since LPS increased myogenin mRNA that peaked at 4 h and was also increased 24 h after injection. However, in spite of the upregulation of myogenin mRNA, the corresponding protein was not increased. It has been suggested that atrogen-1 overexpression in muscle fibres inhibits skeletal muscle protein translation (34). In addition to translation regulation, evidence that myogenin is an atrogen-1 substrate has also been reported (35). Therefore, LPS-induced atrogen-1 upregulation can be responsible for normal myogenin levels in spite of the increased myogenin mRNA. Disparity between myogenin mRNA and protein has also been reported in cultured myotubes, in which dexamethasone induced a rapid decline in myogenin protein levels, whereas the amounts of myogenin mRNA did not change (36). Taking into account that myogenin is an important initiator of the differentiation program, the lack of decrease in myogenin after LPS suggests

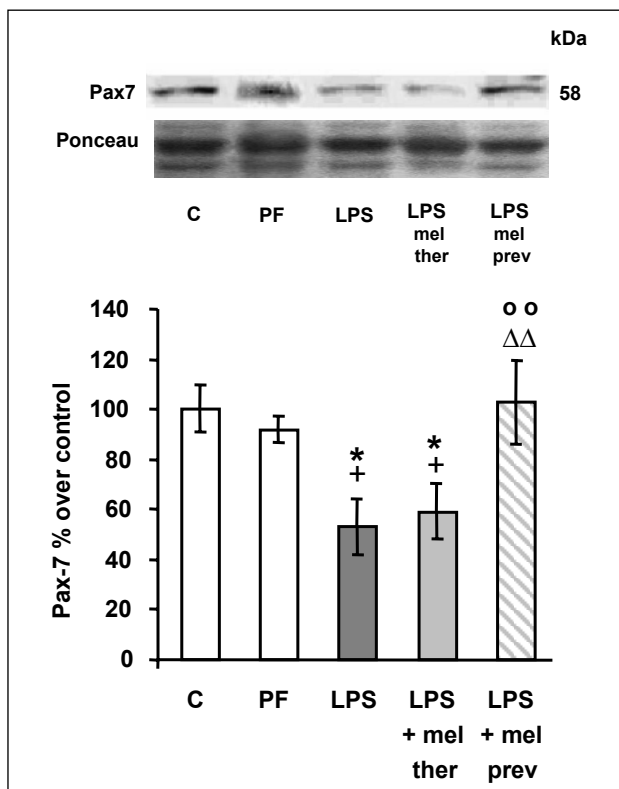


Fig. 8. Pax7 in gastrocnemius of control rats (C), pair-fed rats (PF), LPS injected rats (LPS), as well as LPS injected rats with therapeutic (LPS + mel ther) or preventive (LPS + mel prev) meloxicam treatments. LPS decreased Pax7 expression and preventive meloxicam treatment blocked this effect. Pax7 protein was measured by Western blot, normalised against Ponceau S at ~42 kDa, value of control group was set at 100% and the protein expression was relative to this value. Data represent mean ± S.E.M. (n=6–8 rats). *P<0.05 vs. control rats, +P<0.05 vs. pair-fed rats, °P<0.01 vs. LPS group, °°P<0.01 vs. LPS + meloxicam therapeutic treatment.

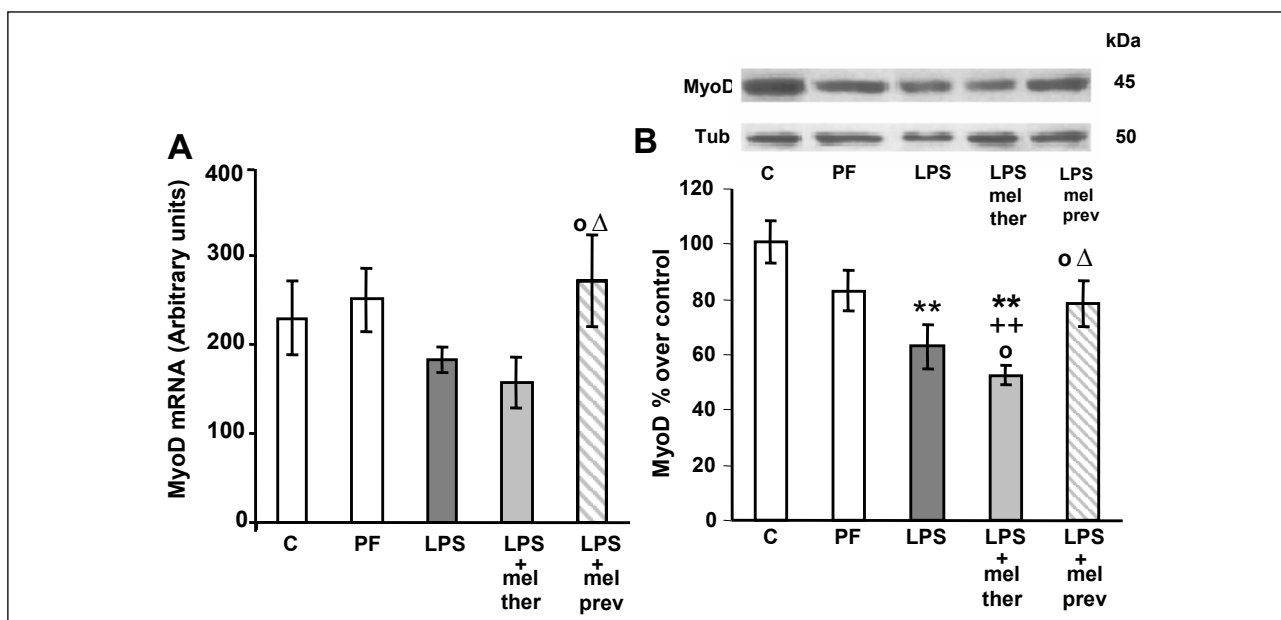


Fig. 9. MyoD mRNA (A) and protein (B) in the gastrocnemius of control rats (C), pair-fed rats (PF), LPS injected rats (LPS), as well as LPS injected rats with therapeutic (LPS + mel ther) or preventive (LPS + mel prev) meloxicam treatments. LPS decreased MyoD expression and preventive meloxicam treatment blocked this effect. MyoD mRNA was quantified by real time PCR as described in material and methods and is presented in relation to the mean value in control group. MyoD protein was measured by Western blot and normalised against α -tubulin. Value of control group was set at 100% and the protein expression was relative to this value. Data represent mean ± S.E.M. (n=8–10 rats). **P<0.01 vs. control rats, ++P<0.01 vs. pair-fed rats, °P<0.05 vs. LPS group, °°P<0.01 vs. LPS + meloxicam therapeutic treatment.

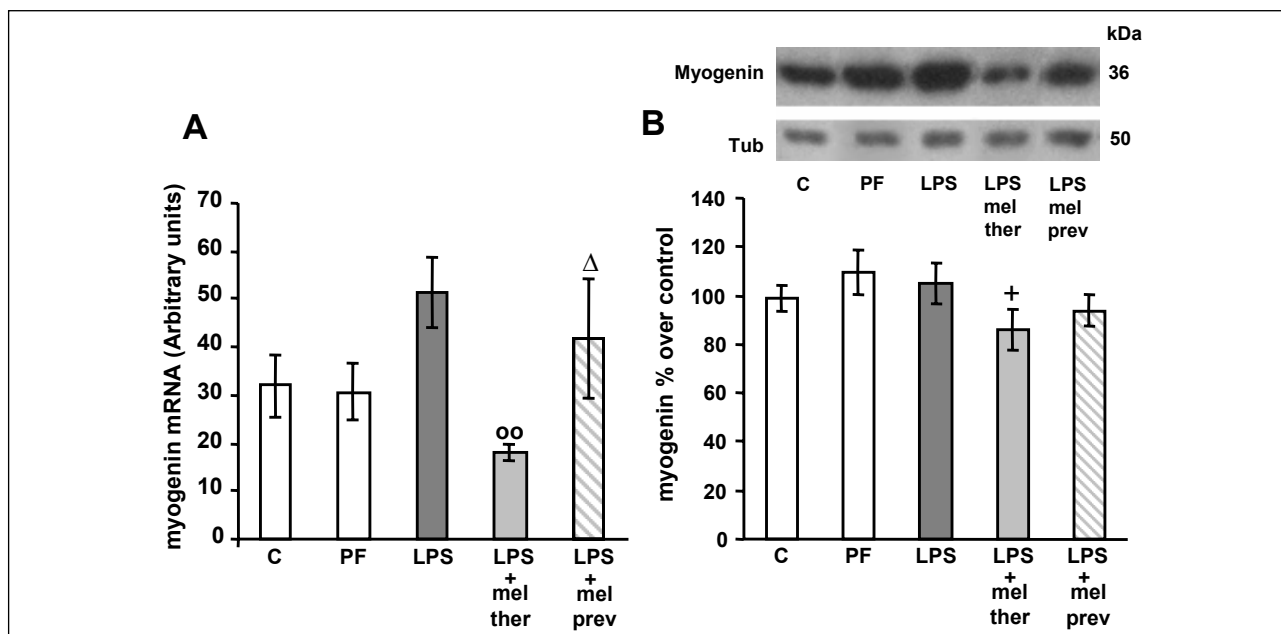


Fig. 10. Myogenin mRNA (A) and protein (B) in the gastrocnemius of control rats (C), pair-fed rats (PF), LPS injected rats (LPS), as well as in LPS injected rats with therapeutic (LPS + mel ther) or preventive (LPS + mel prev) meloxicam treatments. Meloxicam therapeutic treatment decreased myogenin expression. Myogenin mRNA was quantified by real time PCR as described in material and methods and is presented in relation to the mean value in the control group. Myogenin protein was measured by Western blot, normalized against the α -tubulin. Value of control group was set to a 100% and the protein expression was relative to this value. Data represent mean \pm S.E.M. (n=8–10 rats). ⁺P<0.05 vs. pair-fed rats, ^{oo}P<0.01 vs. LPS group, ^ΔP<0.05 vs. LPS + meloxicam therapeutic treatment.

that the inhibitory action of LPS on myogenesis is higher on proliferation than on differentiation.

Both types of meloxicam treatment prevented LPS-induced increase in atrogen-1. These data are in accordance with those that we have reported in arthritic rats, in which chronic meloxicam treatment is able to prevent arthritis-induced increase in atrogen-1 and MuRF1 in the gastrocnemius (3). However, both meloxicam treatments had different effects on myogenic regulatory factors. Preventive meloxicam administration blocked the inhibitory effect of LPS on muscle Pax7 and MyoD and decreased myostatin. In contrast, therapeutic meloxicam treatment did not prevent LPS-induced decrease in Pax7 and MyoD expression. Even more, therapeutic meloxicam treatment decreased myogenin. Blockade of LPS-induced decrease in Pax7 and MyoD expression in the rats receiving meloxicam preventively may be an indicator of satellite cell activity. It has been reported that increased MyoD mRNA and protein in muscle reflects increases in MyoD positive satellite cells, suggesting that satellite cells are in a proliferative phase (36). Since these cells in proliferation express MyoD, the preservation of gastrocnemius cross-sectional area in the groups that received preventive meloxicam treatment may be related to the recruitment and higher activity of the satellite cells. Increased expression of COX-2 in muscle fibers and/or myogenic precursor cells has been demonstrated during compensatory hypertrophy (12). In addition, a decrease in myogenin expression by COX-2 inhibitors has been reported in satellite cell cultures (37). However, there are conflicting data in relation to COX-2 on muscle regeneration and myogenesis. It has been reported that COX-2 inhibitors decrease myogenesis and muscle hypertrophy (12, 38, 39), and muscle adaptation to endurance training (40). However, it has been reported that the

COX-2 inhibitor celecoxib does not affect regeneration and adaptation processes after exercise-induced muscle damage in humans (41).

Two possibilities exist regarding the role of COX-2 in muscle recovery after sepsis. One possibility is that regeneration by the satellite cells involves prostanoids synthesised through the COX-2 pathway. In accordance with this hypothesis, COX-2 activity in satellite cells has been reported (37). Another possibility is that COX-2 inhibitors decrease the formation of lipid mediators that resolve the acute inflammatory response such as resolvins and protectins (42). In accordance with the last hypothesis, chronic meloxicam treatment can delay the resolution of the inflammatory response leading to a decrease in skeletal muscle recovery.

The present data therefore suggest that the anti-inflammatory action of COX-2 inhibition by meloxicam administration to septic rats has protective effects on the skeletal muscle decreasing atrogen-1 expression. However, the protective effect of meloxicam treatment is greater when administered only during the initial phases of inflammation, since prolonged treatment inhibits the myogenic regulatory factors involved in proliferation and differentiation.

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