

Matrix metalloproteinase imbalance in muscle disuse atrophy

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Summary. Muscle atrophy commonly occurs as a consequence of prolonged muscle inactivity, as observed after cast immobilization, bed rest or space flights. The molecular mechanisms responsible for muscle atrophy are still unknown, but a role has been proposed for altered permeability of the sarcolemma and of the surrounding connective tissue. Matrix metalloproteinases (MMPs) are a family of enzymes with proteolytic activity toward a number of extracellular matrix (ECM) components; they are inhibited by tissue inhibitors of MMPs (TIMPs). In a rat tail-suspension experimental model, we show that after fourteen days of non-weight bearing there is increased expression of MMP-2 in the atrophic soleus and gastrocnemius and decreased expression of TIMP-2. In the same experimental model the expression of Collagen I and Collagen IV, two main ECM components present in the muscles, was reduced and unevenly distributed in unloaded animals. The difference was more evident in the soleus than in the gastrocnemius muscle. This suggests that muscle disuse induces a proteolytic imbalance, which could be responsible for the breakdown of basal lamina structures such as Collagen I and Collagen IV, and that this leads to an altered permeability with consequent atrophy. In conclusion, an MMP-2/TIMP-2 imbalance could have a role in the mechanism underlying muscle disuse atrophy; more studies are needed to expand our molecular knowledge on this issue and to explore the possibility of targeting the proteolytic imbalance with MMP inhibitors.

Key words: Unloading, Matrix metalloprotease, Atrophy, Muscle disuse

Introduction

The extracellular matrix (ECM) proteins are important constituents of the surrounding micro-

environment, which ensures correct tissue architecture and specialization and which maintains the structural integrity and homeostasis of the tissues. In skeletal muscles, an altered turn-over of the connective tissue ECM components can underlie structural and functional changes (Takala and Virtanen, 2000).

Muscle disuse atrophy occurs as a result of reduced muscular activity, as commonly observed after prolonged cast immobilization, bed rest or space flights (Booth, 1982; Fitts et al., 1986). This condition features a decrease in muscle functional capacity, likely because of a reduction in mass, in myofiber size and contractile protein content, but no reduction in the myofiber population (Tidball et al., 1998; Fitts et al., 2000). The molecular mechanisms involved in the pathogenesis of muscle disuse atrophy are not fully understood, but it has been suggested that an altered turn-over of the ECM proteins could play a key role in regulating the integrity of the myofibers and in triggering the subsequent myopathy (Frisdal et al., 2000; Reznick et al., 2003).

The matrix metalloproteases (MMPs) belong to a family of zinc proteolytic enzymes that number at least 23 members, classified according to their biochemical features and ECM substrate specificity (Nagase et al., 1999; Giannelli and Antonaci, 2002). Two members of this family, MMP-2 and MMP-9, are secreted in a latent form and become active after having been proteolytically processed at a cellular membrane level by a membrane type-1-MMP (MT1-MMP), together with the tissue inhibitor of MMP-2 (TIMP-2), as has been reported for MMP-2 (Strongin et al., 1993; Nagase, 1997). Both enzymes exert a wide spectrum of action toward a number of different ECM proteins including Collagen and Gelatin, which is why they were originally named gelatinases. MMP-2 and MMP-9 are widespread in the human body, including the skeletal muscles. Their proteolytic activity is inhibited by TIMP-2 and TIMP-1, respectively, so that a too extensive and uncontrolled degradation of the ECM component is avoided (Brew et al., 2000). Therefore, proteolysis occurs as a result of an imbalance between gelatinases and their inhibitors, TIMP-2 and TIMP-1 (Galateau-Salle et al., 2000; Giannelli et al., 2002).

The aim of this study was to investigate how

gelatinases and their inhibitors correlate with skeletal muscle atrophy in a rat hindlimb-suspended model.

Materials and Methods

Animals

The present study utilized 22 male Wistar rats (100 g body weight at the start of the experiment).

Experimental rats were suspended using the Morey-Holton tail casting procedures approved by the institutional Animal care and Use Committee (Morey-Holton and Globus, 1998). MMP-2 and TIMP-2 concentrations were determined in tissue extract of the soleus and gastrocnemius muscles obtained from the suspended rats. As control, animals of the same age and weight were used. Animals were randomly assigned to control or hindlimb suspension (HLS) groups (n=11 per group). Suspended and control rats were fed Purina chow and water ad libitum, and maintained on a 12:12 hr light-dark cycle.

On day 14 of the experiment the rats were weighed and sacrificed by decapitation after anesthesia. The gastrocnemius and soleus muscles were removed, weighed and frozen in liquid nitrogen. Tissues were stored at -80°C .

Gelatin zymography

Frozen muscles were ground by a micro-dismemberer (B-Braun, Melsengen Ag, Germany) in a medium of Tris HEPES, pH 7.5, and then homogenised. Tissue extracts were centrifuged at 2000 rpm for 5 min and the supernatant was harvested and used to measure the protein concentration and to determine the gelatinase activity. The protein concentration was determined by the bicinchonic acid method (Pierce Chemical Co., Rockford, IL), while gelatinase levels were quantified by zymography using the conditioned medium of the HT 1080 cells as standard control for the detection of the MMP-2 activity (Giannelli et al., 2002). 20 μg of the total protein concentration of each sample was loaded onto SDS-PAGE gel. Gels were incubated overnight, stained with Coomassie Blue and destained with a methanol acetic acid solution. The gelatinolytic areas were shown as bright bands on a blue stained gel. Gels were acquired and quantified by an image-analysis software system (Image Master 1D Prime, Pharmacia Biotech, UK), using a standard curve of the HT 1080 preparation which has a linear relationship between the sample dilutions and gelatinase activity (Giannelli et al., 2002).

Detection of TIMP-2 by ELISA

TIMP-2 concentrations were measured using an ELISA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), which is based on a double-sandwich method where the antigen binds to a primary antibody coated on the well and, after washes, a

secondary antibody conjugated with horseradish peroxidase (HRP) is used to block the complex. Positivity was shown by tetramethylbenzidine and the optical density (OD) was read at 450nm in a microtitre plate reader.

Immunofluorescence

The tissues were included in Optimal Cutting Temperature 4583 (OCT) embedding compound (Miles Laboratories, Inc, Naperville, IL) and serial sections 5 μm thick were cut by a microtome (Microtom, HM 505E, Carl Zeiss Oberkochen, Germany). The sections were then collected on appropriate glass slides (Sigma, St Louis, MO).

Sections were first of all fixed by a cold mixture of chloroform/acetone for 10 min, air-dried and then incubated with the following primary monoclonal antibodies: anti-MMP-2 and anti-TIMP-2 (Oncogene Res. Prod. Boston, MA) and Collagen I and Collagen IV (Biodesign International Saco, ME). All the primary antibodies were diluted in a solution consisting of RPMI medium with 10% fetal calf serum (FCS) and after gentle washing, sections were incubated with a secondary fluorescent antibody Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR) for 45 min in a dark, humid box. Finally, sections were mounted with glycerol and observed with a Nikon Eclipse photomicroscope (Nikon, Corp).

Immunofluorescence staining was quantified by an imaging analysis software (Lucia, Nikon, Inc.). The expression of Collagen I and Collagen IV was measured as μm^2 of positive stained area by microscopic field as previously reported (Giannelli et al., 2002c). In Fig. 7 and 8, bars represent the average plus and minus standard deviation of ten randomly chosen microscopic fields examined for each section and for all the animals.

Results

The percentage difference in weight of the animals, measured before the beginning of the experiment and after fourteen days, is reported in Fig. 1. Control animals gained $116\pm 23.5\%$ of their original weight while suspended rats gained only $76.4\pm 21.3\%$; this difference was statistically significant: ($p<0.001$). The wet weight of the soleus and gastrocnemius after the fourteen-day experiment was also measured in the suspended and control animals. As reported in Fig. 2, the weight of the soleus in suspended animals was 34.58 ± 14.15 mg whereas in control animals it was 85.07 ± 29.61 mg, this difference being statistically significant $p<0.001$. Similar results were also observed for the gastrocnemius, which weighed 771.00 ± 129.33 μg in suspended animals versus 1045.63 ± 194.08 mg in controls ($p=0.002$). The atrophy induced by muscle disuse was further confirmed by the measurement of the fiber diameter, which was significantly different between control and suspended rats as regards the soleus (36.7 ± 9.5 μm versus 24.1 ± 5.9 μm ; $p<0.002$), whereas

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no difference was observed between the two groups of animals as regards the gastrocnemius muscle ($34.9 \pm 6.5 \mu\text{m}$ versus $32.1 \pm 8.6 \mu\text{m}$, $p=0.2$), Fig.3. This is not surprisingly since in Morey-Holton tail casting model solus is more damaged than gastrocnemius (Morey-Holton and Globus, 1998).

In conclusion, atrophy was determined based on muscle weight and fiber diameter consistently with other studies.

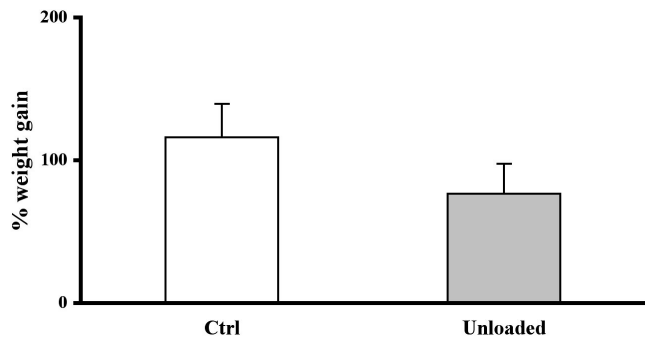


Fig. 1. Percentage of weight gain in control and suspended rats. After fourteen days the body weight increase is statistically different ($p < 0.001$) between control (empty bar) and unloaded animals (filled bar).

The soleus and gastrocnemius muscles were also investigated to determine the MMP-2 and TIMP-2 concentrations in control and suspended animals after measurement and normalization for the protein concentration. Gelatinase levels were quantified using a gelatin zymography assay, modified as previously described. As shown in Fig. 4, total MMP-2 levels were higher in the soleus of suspended rats than in controls ($5.06 \pm 1.48 \text{ pg/mg}$ total protein versus $3.98 \pm 0.64 \text{ pg/mg}$ total protein, $p=0.02$). The same trend was observed for pro-MMP-2 (respectively $4.06 \pm 1.15 \text{ pg/mg}$ total protein versus $3.28 \pm 0.63 \text{ pg/mg}$ total protein, $p=0.03$) and the active form of MMP-2 ($0.99 \pm 0.47 \text{ pg/mg}$ total protein versus $0.7 \pm 0.19 \text{ pg/mg}$ total protein, $p=0.003$). A similar situation was also observed in the gastrocnemius muscle, where total MMP-2 levels were more elevated in the muscle of suspended animals than controls ($5.66 \pm 2.4 \text{ pg/mg}$ total protein versus $4.35 \pm 0.92 \text{ pg/mg}$ total protein, $p=0.05$), while pro-MMP-2 was also more concentrated in the suspended animals ($4.81 \pm 1.74 \text{ pg/mg}$ total protein versus $3.42 \pm 0.45 \text{ pg/mg}$ total protein, $p=0.01$). No differences were observed as regards the active form of MMP-2 ($0.78 \pm 0.71 \text{ pg/mg}$ total protein versus $0.93 \pm 0.78 \text{ pg/mg}$ total protein, $p=0.28$). MMP-9 was not detectable in soleus and gastrocnemius tissue preparations of either suspended or control animals.

The same tissue extract preparations were used to

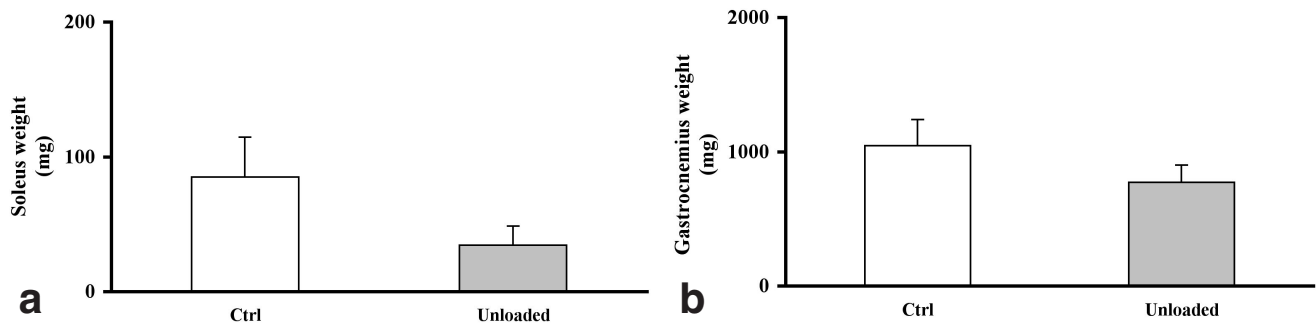


Fig. 2. Soleus and gastrocnemius muscle weight in control and suspended animals. After fourteen days the weight of the soleus and gastrocnemius is statistically different ($p < 0.001$ and $p = 0.002$, respectively) in unloaded (filled bar) as compared to control animals (empty bar).

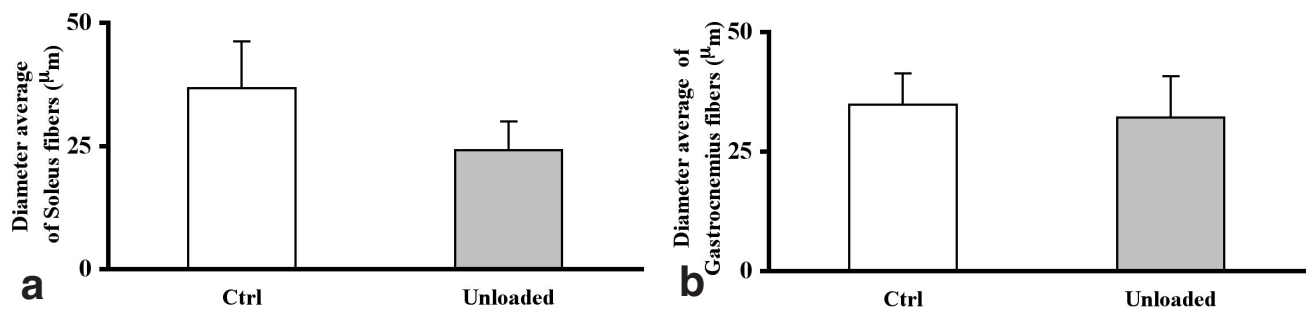


Fig. 3. Mean diameter of the soleus and gastrocnemius fibres. The mean diameter of the soleus fibers is significantly ($p < 0.002$) smaller in suspended animals (filled bar) than controls (empty bar), whereas no differences are observed in the gastrocnemius muscle in the two groups.

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determine TIMP-2 concentrations. As shown in Fig.5, TIMP-2 levels were decreased in both the soleus (0.53 ± 0.45 ng/mg total protein) and gastrocnemius (0.70 ± 0.31) muscle preparations of suspended rats as compared with controls (1.05 ± 0.7 ng/mg total protein and 0.92 ± 0.49 ng/mg total protein, respectively), the difference being statistically significant in soleus but not in gastrocnemius: $p=0.019$ and $p=0.097$, respectively.

In short, muscle atrophy was evident in suspended rats, especially in the soleus, while higher levels of MMP-2 and lower levels of TIMP-2 were evident in both the soleus and gastrocnemius muscles.

Finally, MMP-2 and TIMP-2 were immunolocalized in the soleus and gastrocnemius muscles of suspended and control animals by immunofluorescence. As shown in Fig. 6, MMP-2 expression was more abundant in both the soleus and gastrocnemius muscles of unloaded animals as compared with controls. On the contrary, TIMP-2 expression was reduced in the soleus and

gastrocnemius of the suspended rats. MMP-2 and TIMP-2 were mainly concentrated along the perimysium in both muscles, with a similar distribution in control and suspended rats.

In order to correlate the proteolytic imbalance with the muscle atrophy in the unloaded animals, we performed immunofluorescence staining of Collagen I and Collagen IV, two main components of the muscle ECM proteins mainly distributed in the perimysium and around the fibers. As shown in Fig. 7, the expression of Collagen I was strongly reduced in the soleus while this is not very evident in gastrocnemius unloaded muscles. To better quantify such a difference an appropriate software imaging analyzer was used, and as shown in Fig. 7 the positive stained area was 835.3 ± 214.5 in control soleus versus 349.1 ± 50.0 μm^2 in unloaded soleus, this difference being statistically significant ($p<0.02$). A similar trend was observed in the gastrocnemius, although the difference was not

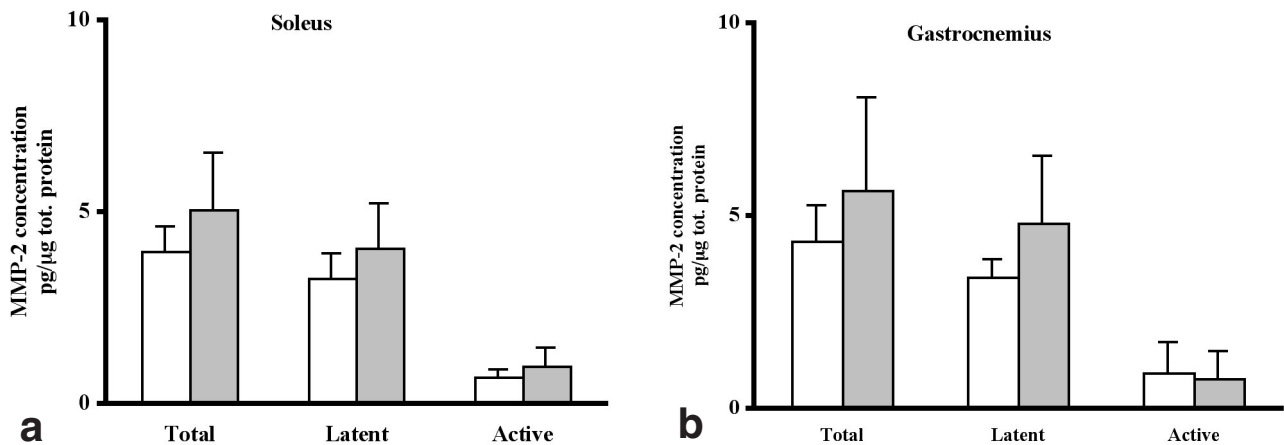


Fig. 4. MMP-2 levels in the soleus and gastrocnemius muscles. Latent and total MMP-2 levels (indicated as mean plus/minus standard deviation) are higher in the soleus and gastrocnemius of suspended (filled bar) animals than controls (empty bar).

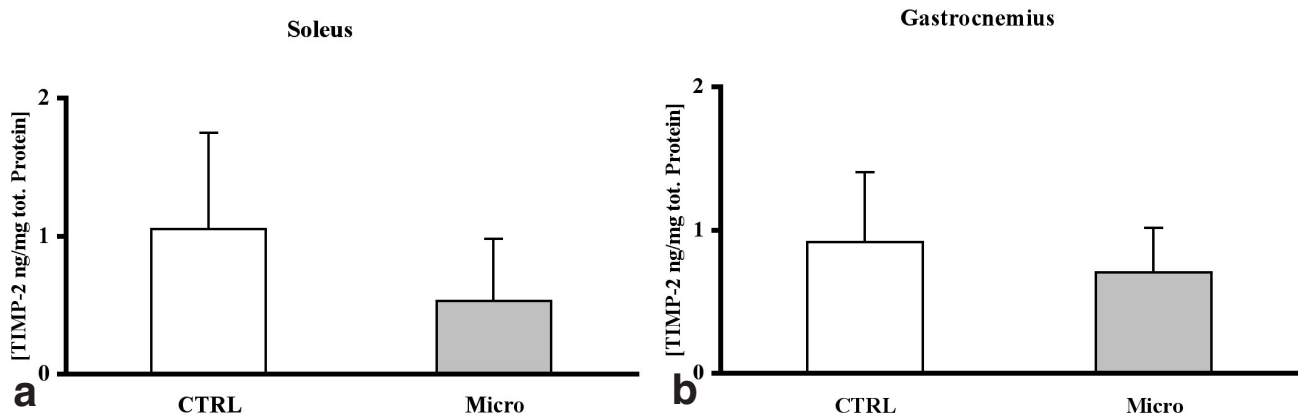


Fig. 5. TIMP-2 levels in the soleus and gastrocnemius. TIMP-2 levels are higher in the soleus ($p<0.002$) and gastrocnemius ($p<0.01$) of control (empty bar) than unloaded animals (filled bar).

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statistically significant between control and unloaded muscle ($p>0.1$). As shown in Fig. 8, the expression of Collagen IV was also reduced in unloaded as compared with control muscles. In particular, such a difference was evident in soleus muscle where the positive-stained area was 3395.6 ± 243.4 in control versus $3076.4\pm310.6 \mu\text{m}^2$ ($p=0.02$) in unloaded muscle, while the difference was

not statistically significant in the gastrocnemius ($p<0.2$).

Discussion

Muscle atrophy is a common consequence of reduced muscle activity over a prolonged period, such as after limb immobilization or space flight (Booth, 1982;

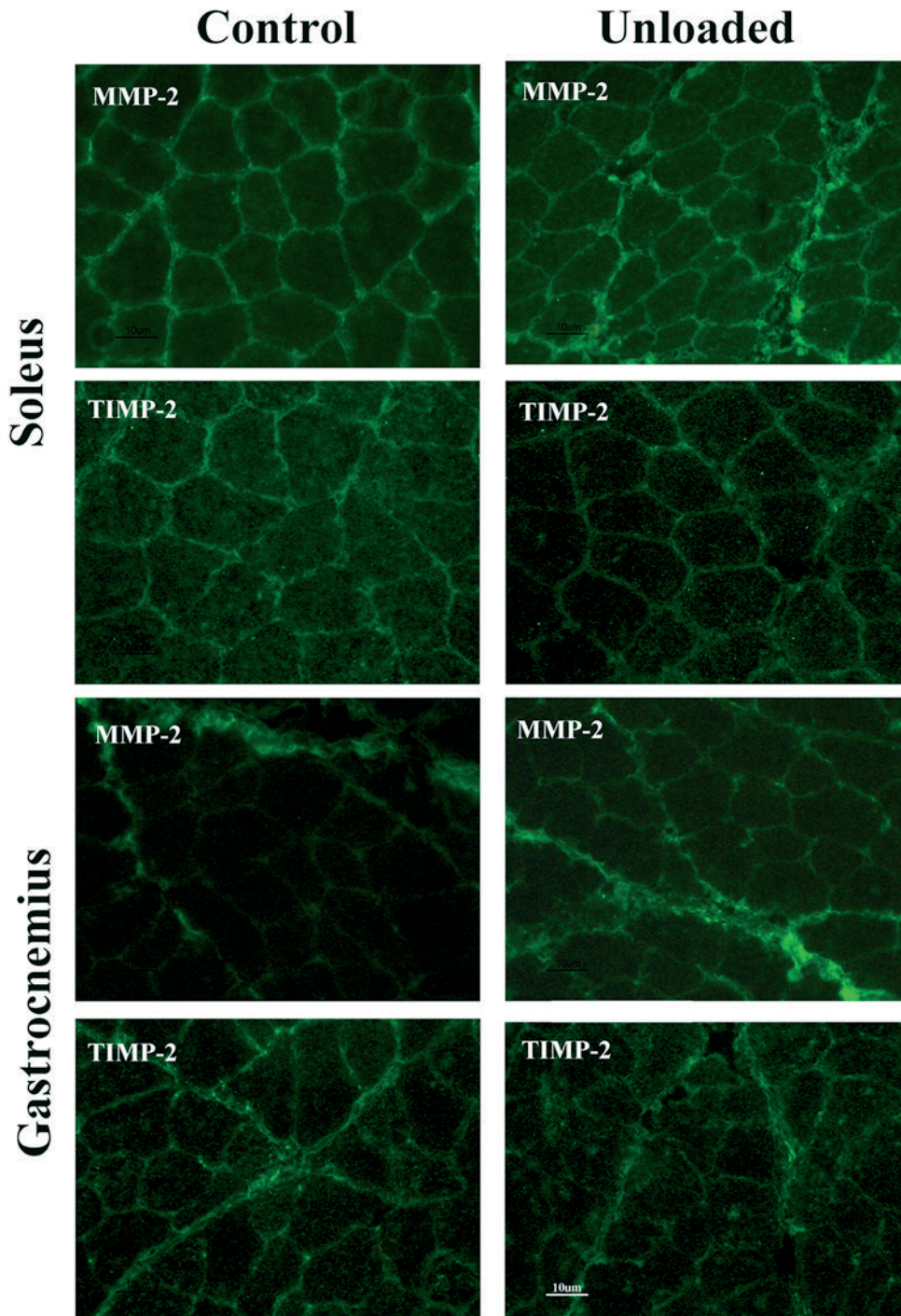


Fig. 6. MMP-2 and TIMP-2 localization in the soleus and gastrocnemius muscles of control and suspended rats. MMP-2 and TIMP-2 are localized mainly along the perimysium (at the sites where remodelling of ECM proteins is more evident) in both control and non-weight-bearing muscles. However, MMP-2 staining is more abundant in non-weight-bearing muscles, whereas TIMP-2 is more abundant in control muscles. Scale bar: 10 μm .

Caiozzo et al., 1994; Fitts et al., 2000). The molecular mechanisms underlying muscle atrophy are still unclear, but this issue is gaining more and more relevance for its clinical implications in the elderly.

In this study, we used rat tail-suspension as a well-established experimental model to investigate muscle atrophy induced by functional disuse (Morey-Holton and Globus, 1998). In agreement with the literature, our results confirm that fourteen days of hindlimb suspension induce reduced weight gain of the whole

animal and consistently, reduced weight of the soleus and gastrocnemius in non weight-bearing animals compared with controls (Mondon et al., 1992; Fitts et al., 2000). This is the first study to investigate MMPs and TIMPs in an “in vivo” model under unloading conditions. In our study, we quantified the total, latent and active MMP-2 concentrations in tissue muscle extract and our results show that all were increased in both the gastrocnemius and soleus muscles of suspended rats as compared with controls, although the results were more evident in the

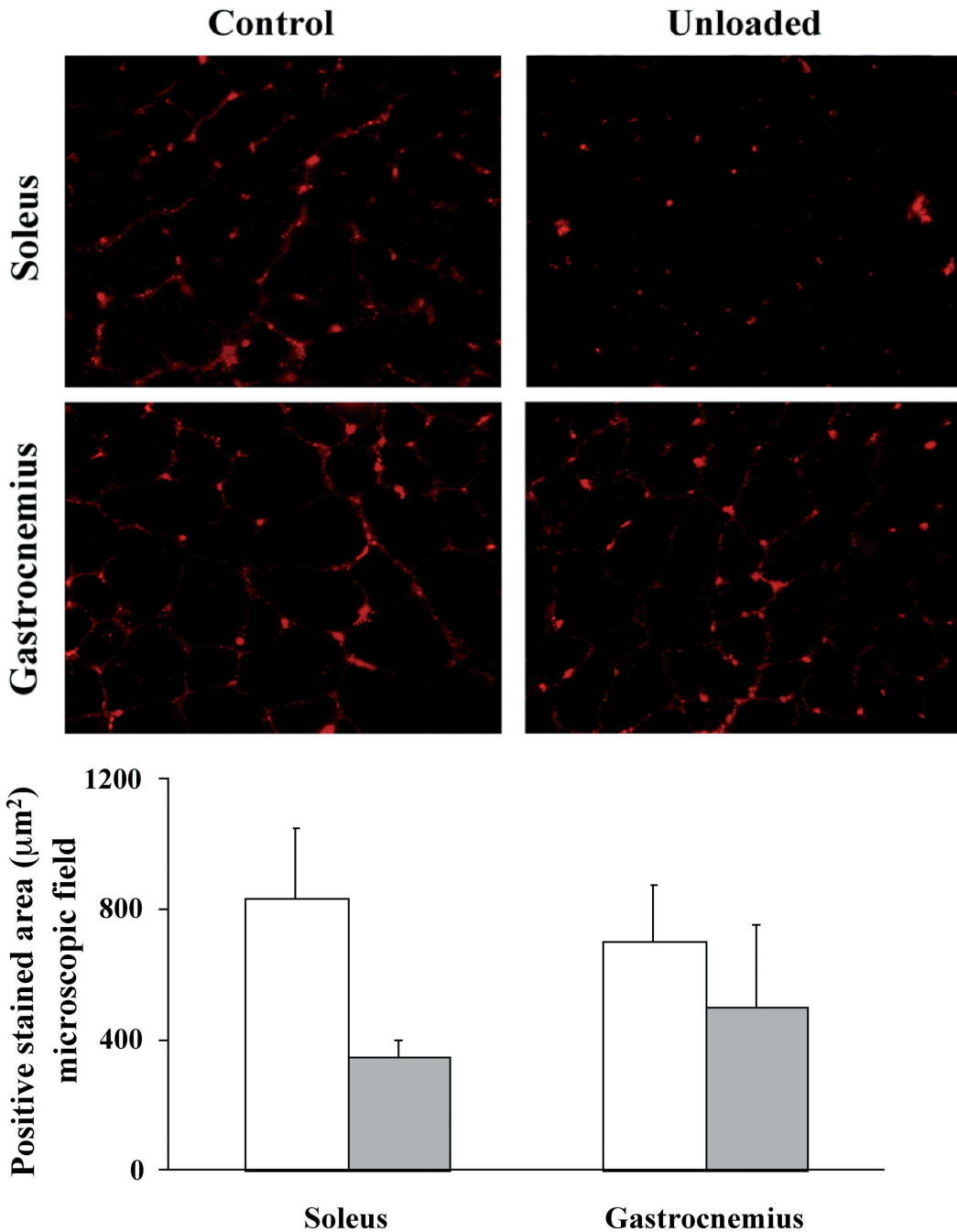


Fig. 7. Collagen I staining in the soleus and gastrocnemius muscles of control and suspended rats. Collagen I is localized around muscle fibers and along the perimysium in both the soleus and gastrocnemius. In suspended animals (filled bars) the expression of Collagen I is reduced, particularly in the soleus ($p < 0.02$). x 40

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soleus than the gastrocnemius. In addition, we measured the concentration of TIMP-2 in the same tissue samples, and found that TIMP-2 concentrations were lower in non weight bearing muscles compared with control muscles. Also in this case, the difference was more evident in the soleus muscle than the gastrocnemius. These results are consistent with those reported in the only other study present in the literature (Reznick et al., 2003), although in the present study for the first time the TIMP-2

concentrations were investigated and the MMP-2 levels quantified. Unlike in the previous study, in our case no MMP-9 was detectable in any muscle tissue preparation. This discrepancy could be related to the different weight (age) and species of the animals used in the two studies.

Our data suggest that increased proteolytic activity together with reduced collagen expression seem to play a role in inducing atrophy of the soleus and gastrocnemius muscles, probably because of an altered turn-over of the

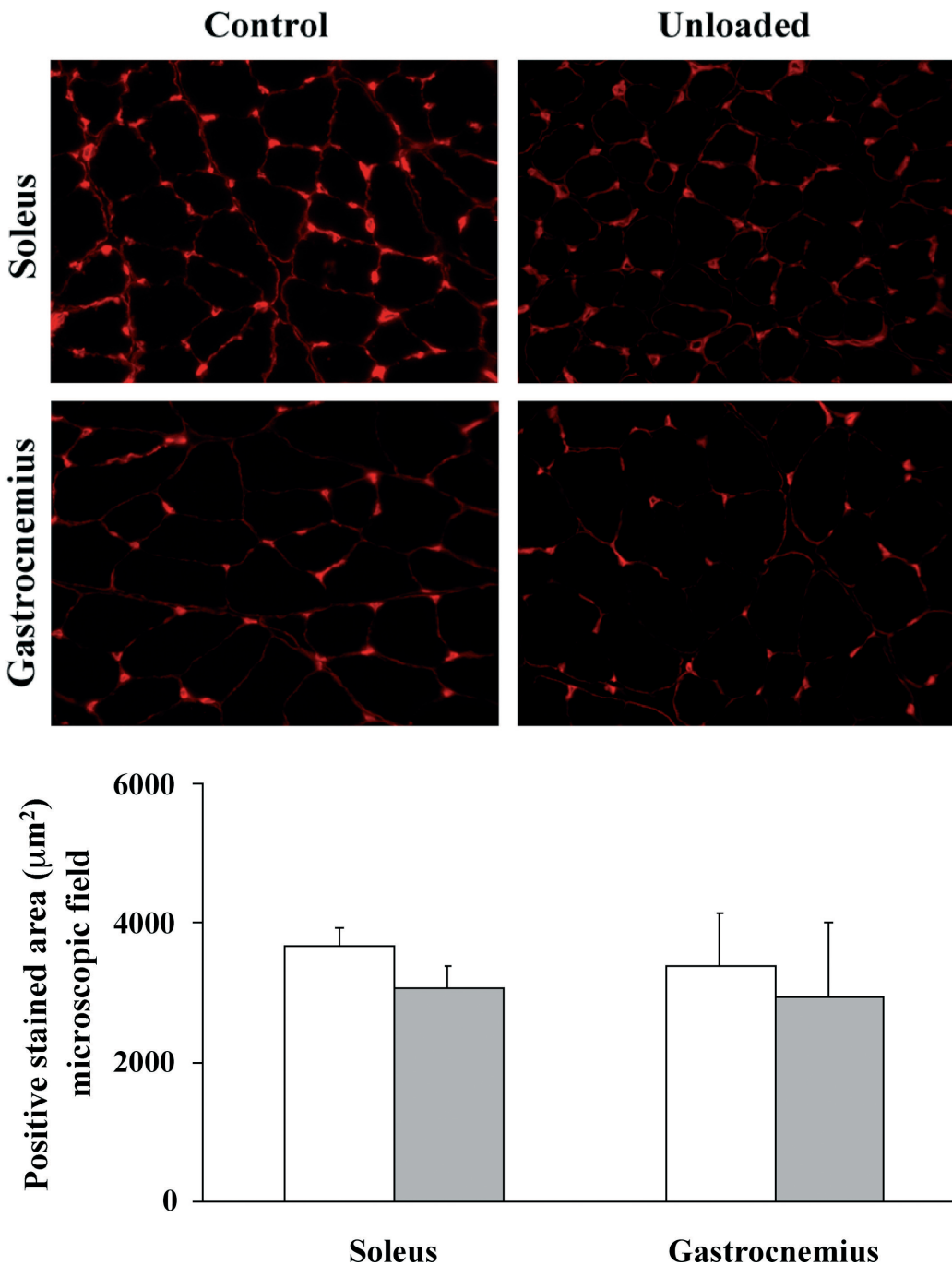


Fig. 8. Collagen IV staining in the soleus and gastrocnemius muscles of control and suspended rats. Collagen I is localized around muscle fibers and along the perimysium in both the soleus and gastrocnemius. In suspended animals (filled bars) the expression of Collagen IV is reduced, particularly in the soleus ($p=0.02$). x 40

ECM proteins present in the sarcolemma and in the connective tissue network (Takala and Virtanen, 2000). As a consequence, an increased permeability of the sarcolemma membrane occurs, which could explain the reduced diameter of the soleus fibers and the reduced protein concentrations observed in our study and reported in the literature (McDonald and Fitts, 1995; Thompson et al., 1998). In our study, in the soleus of unloaded animals an increased expression of MMP-2 and reduced expression of TIMP-2 along the perimysium where remodeling of the ECM components is most evident were observed (Kherif et al., 1999; Reznick et al., 2003). Collagen I and Collagen IV, two main components of the ECM present in the muscles (Takala et al., 2000) are well-known substrates of the MMP-2 proteolytic activity (Giannelli and Antonaci, 2002). In the soleus of unloaded animals, a reduced and uneven distribution of both Collagen I and Collagen IV might be the result of the increased proteolytic imbalance. Therefore, we propose an involvement of MMP-2 in the breaking-down of the ECM proteins such as Collagen I and Collagen IV, which could lead to altered permeability and consequent atrophy of the fibers at least in soleus muscle. The more evident tissue damage in the soleus rather than in the gastrocnemius is not surprising since it is more involved in the microgravity conditions (Fitts et al., 2000).

In conclusion, a proteolytic imbalance could play a role in muscle atrophy induced by muscle disuse, but more studies are necessary to expand our knowledge of this common event and to explore the hypothesis of targeting MMP inhibitors as potential therapeutic drugs.

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