Experimental Physiology

Effect of swimming on myostatin expression in white and red gastrocnemius muscle and in cardiac muscle of rats

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The aim of this study was to test the hypothesis that swimming training might impact differentially myostatin expression in skeletal muscles, depending on fibre type composition, and in cardiac muscle of rats. Myostatin expression was analysed by real time reverse transcriptase-polymerase chain reaction, Western blot and immunohistochemistry of the red deep portion (mainly composed of slow and type II A fibres) and in the superficial, white portion (composed of fast type II X and II B fibres) of the gastrocnemius muscle in adult male Wistar rats: (i) subjected to two consecutive swimming bouts for 3 h; (ii) subjected to intensive swimming training for 4 weeks; and (iii) sedentary control rats. Myostatin mRNA content was in all cases higher in white than in red muscles. Two bouts of swimming did not alter myostatin expression, whereas swimming training for 4 weeks resulted in a significant reduction of myostatin mRNA contents, significant both in white and red muscles but more pronounced in white muscles. Western blot did not detect any change in the amount of myostatin protein. Immunohistochemistry showed that, in control rats, myostatin was localized in presumptive satellite cells of a few muscle fibres. After training, the number of myostatin-positive spots decreased significantly. Myostatin mRNA content in cardiac muscle was lower than in skeletal muscle and was significantly increased by swimming training. In conclusion, the results obtained showed that intense training caused a decreased expression of myostatin mRNA in white and red skeletal muscles but an increase in cardiac muscle.

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Myostatin belongs to the transforming growth factor-β superfamily and is known to play an essential role in the regulation of skeletal muscle mass. In particular, it has been characterized as a potent negative skeletal muscle growth factor (McPherron *et al.* 1997), which inhibits satellite cell activation (McCroskery *et al.* 2003), myoblast proliferation (Thomas *et al.* 2000) and myogenic differentiation *in vitro* (Rios *et al.* 2002). The predicted human, rat, mouse, porcine, chicken, turkey and fish myostatin proteins are identical in the biologically active carboxy-terminal region, suggesting that the myostatin gene is highly conserved throughout evolution (McPherron *et al.* 1997; Radaelli *et al.* 2003). Myostatin transcript and/or protein expression have been shown to be regulated during different physiological and pathological situations which affect muscle mass, such as muscle atrophy, heart infarct, muscle unloading, HIV infection, microgravity exposure, chemical muscle damage, muscle regeneration and muscle reloading (reviewed by Matsakas & Diel, 2005). These findings have raised the possibility that myostatin also plays an important role in muscle growth during postnatal life (Kambadur *et al.* 2004).

Recent evidence points to a decreased myostatin mRNA expression in the muscles of rodents exposed to exercise or training. We and others have previously found lower muscle RNA transcripts of myostatin in response to muscle exercise through different types of muscle loading, e.g. acute and short-term swimming training (Matsakas *et al.* 2005); chronic wheel running (Matsakas A. & Patruno M. unpublished observations); and muscle loading through treadmill running (Wehling *et al.* 2000) or isometric resistance training after atrophy induced by hindlimb unloading (Haddad *et al.* 2006). However, although there have been several studies addressing the

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effect of muscle loading on myostatin expression, there is still some controversy among researchers. In humans, for example, both decreasing and increasing levels of myostatin mRNA and protein have been observed, since one bout of resistance exercise as well as heavy resistance training for several weeks have been reported to be followed by a reduced myostatin mRNA expression (Roth *et al.* 2003; Kim *et al.* 2005) and a decreased concentration of circulating myostatin (Walker *et al.* 2004). In contrast, other studies have shown that a heavy resistance training in healthy individuals for 12 weeks was accompanied by an increase of myostatin mRNA and protein expression and subsequent increase in serum level (Willoughby, 2004). An increased expression has recently been reported in rat gastrocnemius after sciatic nerve resection (Zhang *et al.* 2006). Such observations indicate that the up- and/or downregulation of the myostatin molecule is surely influenced by several external factors, including training or disuse, although only a single study (Walker *et al.* 2004) produced evidence for a link between changes of myostatin expression pattern and muscle mass increase. In addition, myostatin is expressed in other tissues, such as heart muscle (Sharma *et al.* 1999), and recent data suggest that stretch of cardiomyocytes induces an increased myostatin expression (Shyu *et al.* 2005). At present it is not known whether exercise training may affect cardiac myostatin expression.

The aim of this study was to assess whether heavy endurance training might impact myostatin expression and whether distinct changes might occur in relation to fibre type composition. To this end, we chose swimming with weights as an exercise protocol in the rat, and we used as sources of fast-glycolytic and slow-oxidative fibres the superficial and deep portions of the gastrocnemius muscle, which is heavily involved in swimming (Laughlin *et al.* 1984; Nakao *et al.* 2001). Previous findings suggest that short-term swimming exercise in rats is followed by reduced myostatin (MSTN) mRNA levels only in gastrocnemius but not in soleus muscle, suggesting a fibre type-dependent transcriptional regulation (Matsakas *et al.* 2005). On the basis of our previous experience (Matsakas *et al.* 2005), we expected a significant decrease of myostatin mRNA expression, especially after intense and long-lasting training. We hypothesized that the changes in myostatin mRNA expression induced by training would be different in fast and slow muscle fibres, since the basal level of this molecule is different in the two muscle types (Carlson *et al.* 1999; Wehling *et al.* 2000), and the muscle response to both strength and endurance training protocols is dependent on fibre type (Booth & Thomason, 1987; Aagaard *et al.* 2001; D'Antona *et al.* 2006). Real time reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry were used to follow myostatin expression at mRNA and protein levels. We also explored the possible effects of swimming training on myostatin mRNA levels in the cardiac muscle.

Methods

Animal maintainance

Specific pathogen-free male Wistar rats, weighing 120–150 g, born and maintained at the animal facilities of the Department of Anatomy and Physiology, University of Padova were used for this study. Rats were housed under controlled environmental conditions (20–22°C, 12 h–12 h light–dark cycle) and were allowed free access to standard rodent chow (ALTROMIN-R, A. Rieper Spa, Vandoies, BZ, Italy) and tap water. The animals were maintained according to the European Union guidelines for the care and use of laboratory animals.

Exercise and training protocols

Exercise: acute swimming. Ten animals were randomly assigned to a swimming group (acute swim, $n = 5$) and a sedentary group (control, $n = 5$). Rats of the swimming group were acclimated to swimming for 10 min day^{-1} for 2 days and subsequently swam together in a large water tank at a water temperature of 35 ± 1°C by using conditions similar to those previously described (Matsakas *et al.* 2005). Following the protocol of Reynolds *et al.* (2000), rats performed two bouts of swimming, each lasting 3 h, once in the light and once in the dark phase of their diurnal cycle, separated by a 6 h rest period. During the rest period, animals were towel dried, kept warm, and given food and water. Animals were killed and muscle tissues dissected out immediately after completion of the second swimming bout.

Training: chronic swimming. Animals were randomly assigned to a swimming training group (trained, $n = 6$) and a sedentary group (untrained, $n = 6$). Rats of the swimming training group were subjected to an intensive daily swimming protocol which lasted for 4 weeks, 5 days week^{-1}, with increasing duration and intensity of the training stimulus gradually as follows. Rats swam together 60–90 min day^{-1} during the first week. Group swimming was chosen because rats usually climb over each other, and in this way more vigorous muscle activity is achieved than when animals are allowed to swim alone. Initial water exposure was accompanied by vigorous and hectic movements during the first few minutes, and rats spent most of the time underwater looking for an escape option. However, rats soon relaxed, and they balanced on their tails and rotated on their bellies, in this way reducing their swimming activity. For this reason, at the beginning of the second week an external weight corresponding to 1.5% of the animal's body mass was attached to the base of the tail, and rats swam for 90 min day^{-1}. During the third and fourth week, rats swam for 90 min day^{-1} by re-adjusting the tail-weight weekly in order to reach to 3 and 6% of animal's body mass, respectively. In parallel,

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untrained rats were placed in the water for 1–3 min every other day during the fourth week of the experimental period, in order to simulate the stress of water exposure. Animals were killed and muscles dissected out 24 h after the completion of the last swimming bout. Indeed, the use of external weights has been reported to prevent passive floating of rats and standardize the swimming activity (Lynch *et al.* 1991), as well as to increase the intensity of exercise (Nakao *et al.* 2000; Peijie *et al.* 2004). Similar animal treatments have been reported in the literature (e.g. Laughlin *et al.* 1984; Lennon & Mance, 1986; Lynch *et al.* 1991; Peijie *et al.* 2004).

Muscle dissection

Animals were killed by cervical dislocation under anaesthesia induced with an intraperitoneal injection of tiletamine and zolazepam (50 mg (kg body mass)$^{-1}$) as well as xylazine (15 mg (kg body mass)$^{-1}$) at approximately the same time of day (11.00–13.00 h). Samples of slow-oxidative and fast-glycolytic muscles were obtained by dissection of gastrocnemius muscles, which were quickly excised from both hindlimbs of the animals and dissected according to the muscle colour (corresponding to myoglobin concentration) into a red portion (derived from the deep part of the lateral head) and a white portion (derived from the superficial part of both lateral and medial head). The difference between the two portions was confirmed *a posteriori* by analysing the myosin heavy chain isoform composition (see Fig. 1) of the samples as described below. Gastrocnemius muscle has been shown to be recruited during swimming exercise, as evidenced by glycogen depletion (Nakao *et al.* 2001), increased blood flow (Laughlin *et al.* 1984) and increased electromyographic activity (Roy *et al.* 1985; Roy *et al.* 1991). The specimens were immediately immersed in melting isopentane, cooled in liquid nitrogen and stored at −80°C for subsequent analyses. The heart muscle was also removed, weighed and stored for RNA extractions.

RNA extraction and real time RT-PCR

Total RNA was extracted from 100 mg of muscle tissue by using the TRIzol® Reagent from Invitrogen (Paisley, UK) as previously described (Matsakas *et al.* 2005). The integrity of RNA was checked on 2% agarose gels, and total RNA concentration was estimated by a spectrophotometer (Pharmacia). Two micrograms of total RNA was reverse-transcribed to synthesize cDNA by using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen) after treatment with DNAse I (Invitrogen) to remove contaminating genomic DNA. Real time PCR amplification reactions were carried out on 30 μl aliquots (containing 3 μl cDNA at a 1:8 dilution) on an ABI 7500 Real Time PCR System (Applied Biosystems, Milan, Italy) by SYBR Green I dye chemistry detection under amplification conditions reported elsewhere (Patruno *et al.* 2006). Myostatin mRNA levels were analysed against cyclophilin (CYP) and hypoxanthine–guanine phosphorybosyl transferase (HPRT), both of which served as reference genes. MSTN and CYP primer sequences have been published previously (Matsakas *et al.* 2005), and HPRT primer has been adopted from the work of (Peinnequin *et al.* 2004). Quantification of the mRNA data was done by using the comparative threshold cycle (CT) method as previously described (Matsakas *et al.* 2005), with the modification that the relative efficiency of each primer was included in the calculation. The specificity of the PCR amplification was always verified with melting curve analysis, while the mean CT values of both CYP and HPRT were not different among groups.

Morphometry and immunohistochemistry

Samples from the mid-belly of both the lateral and medial heads of gastrocnemius muscle from trained ($n = 6$) and untrained rats ($n = 6$), used to determine the fibre cross-sectional area (CSA) and for immunohistochemistry purposes, were set into composite blocks and frozen in isopentane cooled with fluid nitrogen, and serial sections (10 μm) were cut in a cryostat as described by Toniolo *et al.* (2005). To determine the CSA, sections were stained with the standard Haematoxylin and Eosin method. A specific staining to detect adipose tissue (Oil Red O method for lipids; Karunaratne *et al.* 2005) was also performed in sections of muscle tissue. The cross-sections were analysed using DP SOFT software (Olympus, version 3, Italy). Digital pictures of random areas of every sample (both red and white portion of the muscle) were obtained, and the CSA of approximately 500 muscle fibres in total was determined for each sample. For immunohistochemistry, sections were blocked in 10% rabbit serum, 1% BSA for 30 min, and incubated overnight with or without goat antirat myostatin C-terminal antibody (1:50 dilution, GDF-8 (C-20); sc-6884, Santa Cruz Biotech., Santa Cruz, CA, USA) or rabbit antihuman myostatin antibody (GDF8-BL891; Bethyl Laboratories Inc., Montgomery, TX, USA) as suggested by Gonzalez-Cadavid *et al.* (1998). The rabbit antimouse laminin (AB2034; Chemicon Int., Milan, Italy) was used at 1:80 dilution as suggested by the supplier; the monoclonal antidistrophin antibody (MANDRA 1, Sigma, Milan, Italy) was used at 1:80 dilution, while the monoclonal anti-Pax7 antibody (Mab1675 R&D Systems Inc., Minneapolis, MN, USA) was used at 10 μg ml^{-1}.

Standard immunofluorescence protocols followed the primary incubation; secondary antibodies used were antigoat or antirabbit and/or antimouse conjugates with fluorescein (FITC; Vector Laboratories, Milan, Italy) and antimouse coupled with Texas Red (Vector Laboratories)

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at a dilution of 1:200, while nuclear staining was performed with TO-PRO®-3 iodide purchased from Molecular Probes (Invitrogen, S.R.L., San Giuliano Milanesé, Milan, Italy). All antibodies were diluted in PBS. Images were obtained with a Leica TCS-SP2 confocal laser scanning microscope (CLSM).

The immunohistochemical reactions were also performed using the Envision method (goat antirabbit immunoglobulins conjugated to peroxidase-labelled complex; Dako, Milan, Italy). After washes in PBS, the immunoreactive sites were visualized using a freshly prepared solution of 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 15 ml of a 0.05 M Tris buffer at pH 7.6, containing 1.5 ml of 0.03% H_2O_2. Sections were mounted in Eukitt and examined under an Olympus BX50 photomicroscope. Digital pictures of random areas of every sample were taken, and the spots positive for the myostatin antibody were counted in areas of approximately 200 muscle fibres per sample.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Muscle samples from the two portions of gastrocnemius of untrained ($n = 6$ for each portion) and trained rats ($n = 6$ for each portion) were reduced to powder by manual mortar and pestle grinding, cooled with liquid nitrogen and homogenized in a solution of 62.5 mM Tris, pH 6.8, 10% glycerol and 10% SDS. The homogenate was boiled for 3 min and centrifuged at 12 000*g* for 20 min. Protein concentration was determined on the supernatant by a protein assay kit (Dc Protein Assay, Bio-Rad, Munich, Germany) and samples were dissolved in Laemmli solution for SDS-PAGE. Myostatin protein expression was determined by SDS-PAGE on a 10–20% gradient separating gel. Between 150 and 300 μg of total protein were loaded onto gels, and electrophoresis was performed for ∼8 h at 4°C with a current of 10 mA per gel. Gel proteins were next transferred to a nitrocellulose membrane (0.2 μm) in order to identify the myostatin signal by immunostaining. Transfer was obtained by a full immersion transfer procedure at an intensity of 60 V for 4 h. The quality of protein transfer and protein loading was validated using actin as a loading control. The amount of actin transferred was determined on nitrocellulose membranes stained with Ponceau Red. The Coomassie Blue staining of the residual actin on the gels confirmed optimal protein transfer.

Nitrocellulose membranes were incubated overnight at 4°C with a primary polyclonal antibody directed against myostatin (GDF-8 (C-20), sc-6884; Santa Cruz Biotechnology) at a dilution of 1:200 with PBS, 0.1% TWEEN 20 (TBST) and then with a peroxidase-conjugated (rabbit antigoat) secondary antibody (P 260; Dako, Copenhagen Denmark) for 1 h at a dilution 1:10 000 with TBST. Bands were visualized by using the enhanced chemiluminescent method, in which luminol was excited by peroxidase in the presence of H_2O_2 (ECL Amersham Products, Milano, Italy). Identification of the myostatin band was based both on the migration of purified human myostatin (Human Myostatin, His tagged Fusion Protein) from Bio Vendor (Heidelberg, Germany), which served as a positive control and could be recognized by the myostatin antibody, and on low molecular weight markers (no. M3913; Sigma).

Myosin heavy chain isoforms were separated on 8% SDS-PAGE by loading 5 μg of total proteins and by silver-staining the gels (Bio-Rad Silver Stain Plus) as previously described (Toniolo *et al.* 2004, 2005).

Densitometric analyses were performed on SDS-PAGE gels and Western blots by means of Power look III, colour scanner, UMAX (Amersham Pharmacia Biotechnology, Milan, Italy).

Statistical analysis

All values were expressed as the means ± S.E.M. Normality of the data was checked with a Kolmogorov–Smirnov test ($\alpha = 10\%$). Significant differences in body mass, heart mass, heart mass relative to body mass, cross-sectional area and myostatin-positive spots between groups were determined by performing Student's unpaired *t* test. The statistical analysis of MSTN mRNA levels was accomplished with a General Linear Models two-way (training status × muscle fibre type) ANOVA by using SPSS (version 11.0). The level of statistical significance was set at $P < 0.05$ for all analyses.

Results

Basal difference in myostatin expression between white and red skeletal muscles in control rats

The dissection of the gastrocnemius was guided as described in the Methods by the colour and the anatomical localization, and this resulted in samples which showed a clearly different distribution of myosin heavy chain (MHC) isoforms as shown by SDS-PAGE (see Fig. 1).

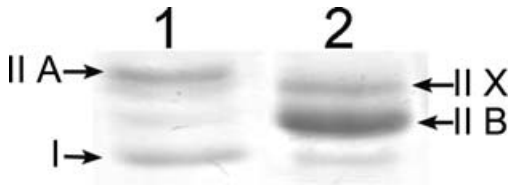

Figure 1. Representative SDS-polyacrylamide gel stained with Silver Stain-Plus
The figure shows separation of MHC isoforms in deep, red (lane 1) and superficial, white gastrocnemius (lane 2).

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The deeper and red portion was mainly composed of type I (∼50%) and II A (∼40%) muscle fibres, while the superficial and white layer was mainly composed of II B and II X fibres (> 90%, together), in full agreement with previous studies (Delp & Duan, 1996).

Real time RT-PCR showed that myostatin expression was significantly related to muscle fibre type distribution ($P = 0.001$). In particular, the comparison between fast-glycolytic and slow-oxidative portions of the gastrocnemius in control rats showed that myostatin transcript expression was four to five times greater in the fast-glycolytic than in the slow-oxidative portions (see Figs 2 and 3, left panels).

Western blot analysis using a polyclonal antimyostatin antibody (GDF-8 (C-20), sc-6884) revealed a slight band corresponding to ∼19 kDa in the total homogenate of the gastrocnemius muscle (Fig. 4). No differences were detected between the two portions of the gastrocnemius. A monomer of purified human myostatin of about 12.5 kDa was also loaded into the gel as positive control and strongly reacted with the polyclonal antibody, thus confirming the specificity of the reaction (Fig. 4*B*, lane 1).

Acute swimming exercise

Although approximately 45% lower MSTN mRNA levels were observed in the fast-glycolytic portion of the gastrocnemius of the swimmers compared to the sedentary rats, the effect of swimming bouts on myostatin expression failed to be significant ($P = 0.06$, Fig. 2), nor was the interaction between training status and muscle fibre type significant.

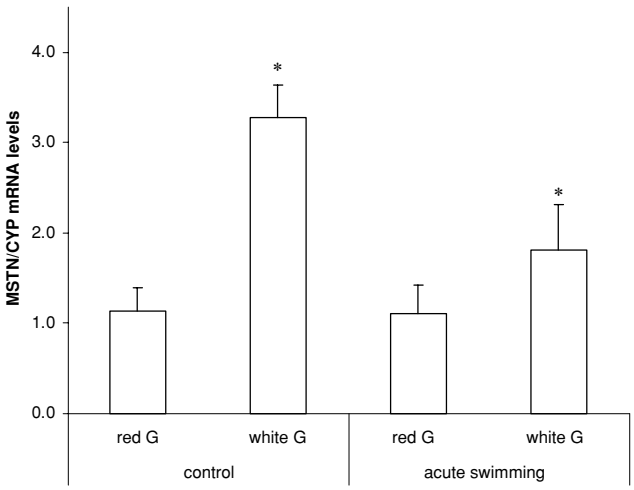

Figure 2. Myostatin (MSTN) mRNA contents in deep, red and superficial, white portions of gastrocnemius (G) of sedentary (control) and active rats (acute swimming protocol), normalized to cyclophilin (CYP), expressed in arbitrary units (*y* axis)
Data are means ± S.E.M., $n = 5$ in each group. * Significantly different from deep, red gastrocnemius of the same group ($P < 0.001$; analysed by two -way ANOVA).

Long-term swimming training

After an intensive training based on 4 weeks with daily swimming sessions, body mass was lower in trained compared to sedentary control rats, whereas heart-mass-to-body-mass ratio was greater (see Table 1). The decrease in body mass probably resulted from a decrease of the body fat component, as suggested by the observation that an abdominal mid-line incision showed lower amounts of

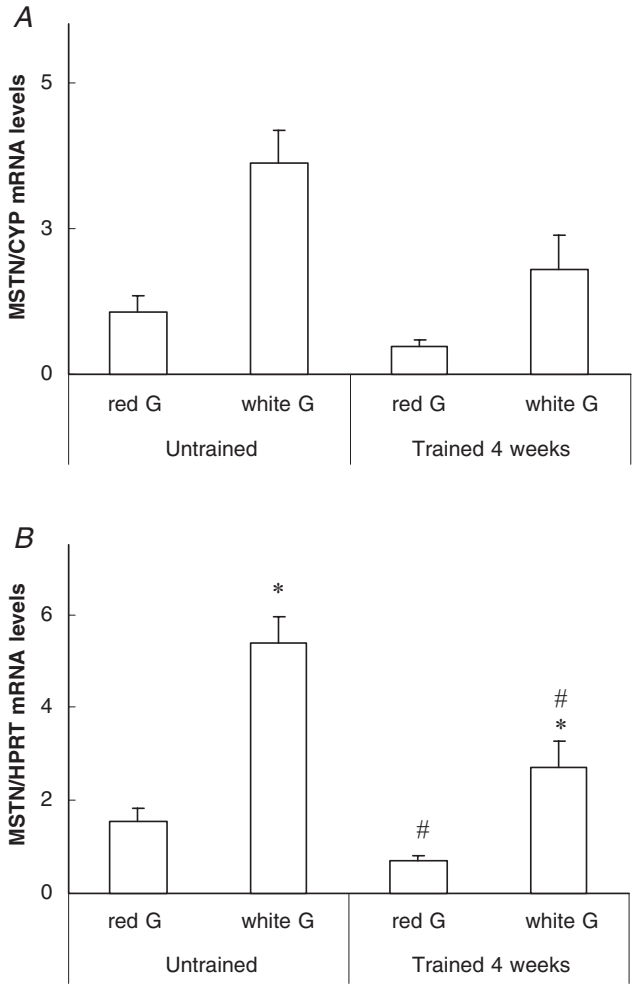

Figure 3. Myostatin (MSTN) mRNA contents in deep, red and superficial, white gastrocnemius (G) of untrained and rats swimming trained for 4 weeks normalized against cyclophilin (CYP; *A*) and hypoxanthine–guanine phosphorybosyl transferase (HPRT; *B*), expressed in arbitrary units (*y* axis)
The '/' symbol on the Y axis of the panels indicates the endogenous gene used in each case and does not denote any ratio. Data are means ± S.E.M., $n = 6$ in each group. * Significantly different from deep, red gastrocnemius of the same group ($P < 0.001$); # significantly different from the corresponding portion of gastrocnemius of the untrained group ($P < 0.001$; analysed by two-way ANOVA).

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Table 1. Effects of 4 weeks swimming training on rat body mass and heart mass

	Untrained	Trained 4 weeks	*P* value
Body mass (g)	296 ± 12	265 ± 6	$P = 0.041$
Heart mass (mg)	840 ± 8	880 ± 26	n.s.
Heart mass relative to body mass (mg g^{-1})	2.85 ± 0.1	3.31 ± 0.1	$P = 0.008$

Data are means ± S.E.M.; n.s., not statistically significant.

intraperitoneal body fat in trained than in sedentary rats. However, a specific adipose tissue staining (Oil Red O) did not reveal any obvious change of intramuscular fat between untrained and trained animals (data not shown). Skeletal muscles showed signs of hypertrophy, with the average cross-sectional area of gastrocnemius fibres being significantly greater, corresponding to an approximate change 40–50% increase, in the group trained for 4 weeks compared to the untrained rats (see Table 2). As can be seen by comparing Fig. 5*A* and *B*, fibres showed a size increase and were surrounded by thinner layers of perimysium in trained rats. Moreover, a number of enlarged myofibres with a typical round swollen shape were found in trained rats (Fig. 5*B*, inset).

Myostatin expression at the level of mRNA was markedly affected by training, since two-way ANOVA revealed significant effects of both training status (chronic trained *versus* untrained, $P = 0.001$) and muscle fibres type (deep, red *versus* superficial, white, $P < 0.001$) on myostatin mRNA, owing to a significant reduction of myostatin mRNA in both slow-oxidative and fast-glycolytic regions of the gastrocnemius of trained rats compared to untrained rats (Fig. 3). Accordingly, the expression was reduced in such a way that a statistically significant difference between the two fibre populations was also present in the gastrocnemius of trained animals (Fig. 3). No interaction was observed between training status and muscle fibre types. In order to strengthen the results of the analysis on myostatin expression in untrained and trained rats, the myostatin mRNA quantification was performed against two reference genes (cyclophilin and hypoxanthine–guanine phosphorybosyl transferase), and the results obtained were in full agreement.

Western blot analysis using a polyclonal antimyostatin antibody revealed a slight band corresponding to ∼19 kDa in both deep, red and superficial, white regions of the gastrocnemius muscle of the trained and the untrained rats (see Fig. 4*B*), and no changes were detected by densitometry analysis (data not shown). Immunohistochemical staining with antimyostatin antibody on cryosections of gastrocnemius of untrained rats showed a moderate reactivity for myostatin in some scattered spots localized at the periphery of muscle fibres and probably corresponding to cell nuclei (Fig. 5*C*), whereas virtually no immunoreactivity was observed in the swimming-trained rats (Fig. 5*D*).

In order to determine the localization of the spots positive to antimyostatin antibodies, longitudinal and transverse sections of gastrocnemius muscle were stained with the antimyostatin antibody and counterstained with TO-PRO®-3 iodide, which detects nuclei of muscle fibres. The results showed that the signal was located at the periphery of a few muscle fibres, close to the sarcolemma in perinuclear areas; this was evident especially in transverse sections of the muscle at high magnification (Fig. 6*C*) but not in longitudinal sections (Fig. 6*A*). Differences in myostatin expression in the fast superficial region of the gastrocnemius between untrained and trained rats were detected by direct inspection and spot counting on transverse cryosections using confocal images of various fields (Fig. 6*A* and *B* and Table 2). A double immunofluorescence with antidystrophin antibody, which detects the plasma membrane, and antimyostatin antibody showed that the spots stained by the latter were outside the plasma membrane of the muscle fibre (Fig. 6*D* and *E*). A double immunofluorescence with antimyostatin and antilaminin antibody (data not shown) pointed to a localization of the spots reactive to antimyostatin within the basal lamina. A double immunofluorescence with anti-Pax7 antibody and antimyostatin antibody showed a colocalization, confirming the localization of the myostatin-positive spots in satellite cells (Fig. 6*F*).

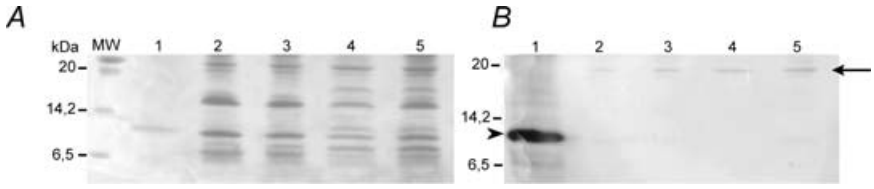

Figure 4. Myostatin immunoblotting on gastrocnemius muscle
A, the Ponceau Red staining shows the migration of the low molecular weight marker (MW) and samples of myostatin-positive control (lane 1, about 12.5 kDa) and gastrocnemius muscle. *B*, Western blot shows a slight myostatin band at 19 kDa (arrow on the right). Lane 1, myostatin-positive control (arrowhead); lane 2, untrained red portion of gastrocnemius; lane 3; trained red portion of gastrocnemius; lane 4, untrained white gastrocnemius; and lane 5, trained white gastrocnemius.

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Table 2. Effects of 4 weeks swimming training on morphometric parameters of rat gastrocnemius

	Untrained	Trained 4 weeks	*P* value
Fibre cross-sectional area (?m2)	1796 ± 75	2705 ± 109	$P < 0.001$
Myostatin-positive spots (number per field of 200 muscle fibres)	4.7 ± 1.7	0.9 ± 0.59	$P < 0.001$

Data are means ± S.E.M.; n.s., not statistically significant.

In view of the greater heart-mass-to-body-mass ratio of trained compared to sedentary rats (see Table 1), which suggested that swimming training caused an increased load on the heart, the possible changes in myostatin expression in cardiac muscle were also investigated. Myostatin mRNA content in ventricular myocardium of control rats was many-fold lower than in gastrocnemius (see Fig. 7) and, interestingly, showed an increase of approximately 74% ($P < 0.05$) in trained rats compared to untrained sedentary controls.

Discussion

The aim of this study was to test whether intense training influences myostatin expression and whether changes in myostatin expression are dependent on muscle fibre type. Analysis of myosin heavy chain isoforms by

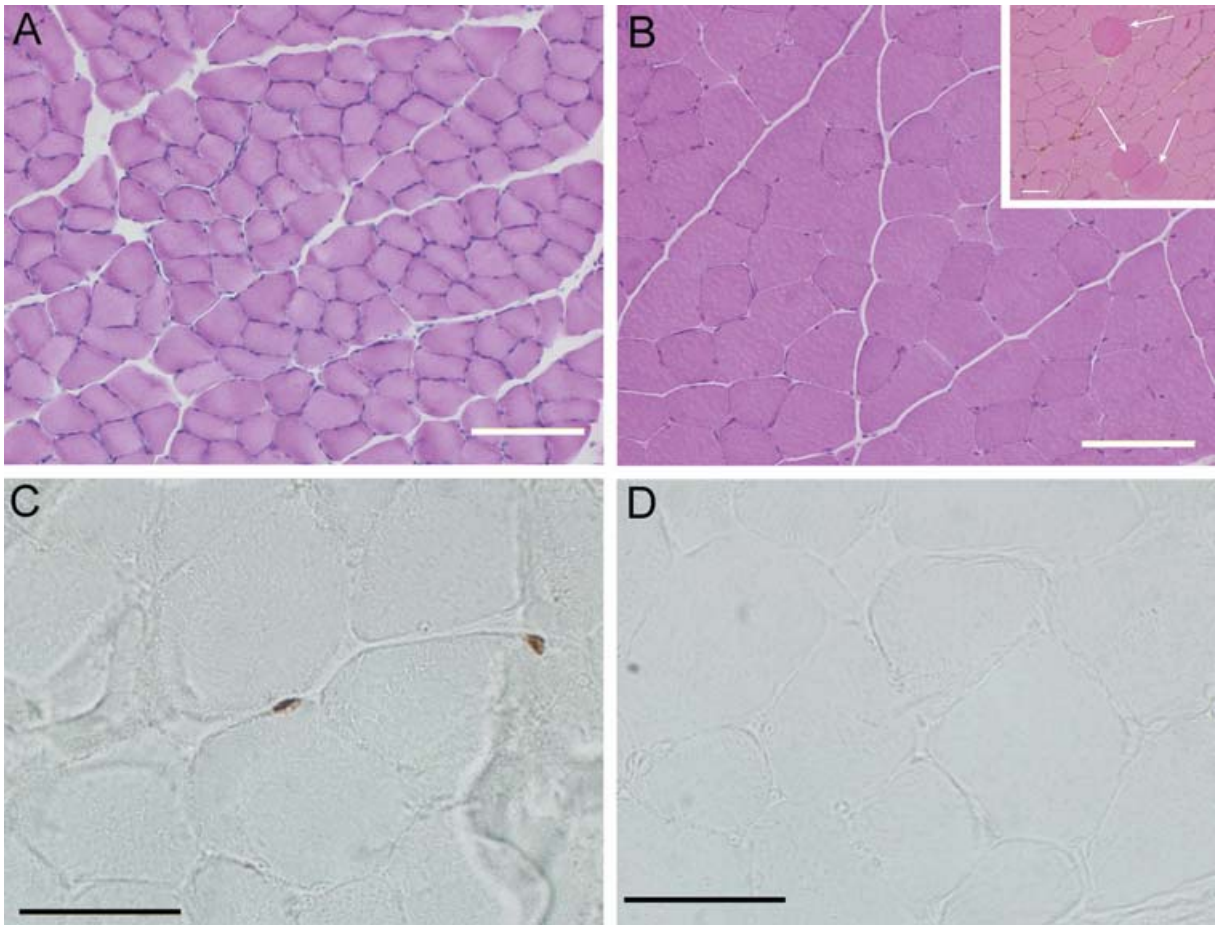

Figure 5. Representative Haematoxylin-and-Eosin-stained sections showing the morphological phenotype of untrained (*A*) and trained gastrocnemius (*B*) and the relative immunolocalization of myostatin (*C* and *D*)

Scale bars in *A* and *B* indicate 200 μm. The inset in *B* indicates the presence of some 'giant fibres' (arrows); scale bar, 200 μm. *C*, two presumptive satellite cells of large fibres from an untrained rat that were positive to myostatin staining (GDF8-BL891 antibody); scale bar, 100 μm. *D*, similar areas from a trained rat did not show myostatin immunoreactivity; scale bar, 100 μm.

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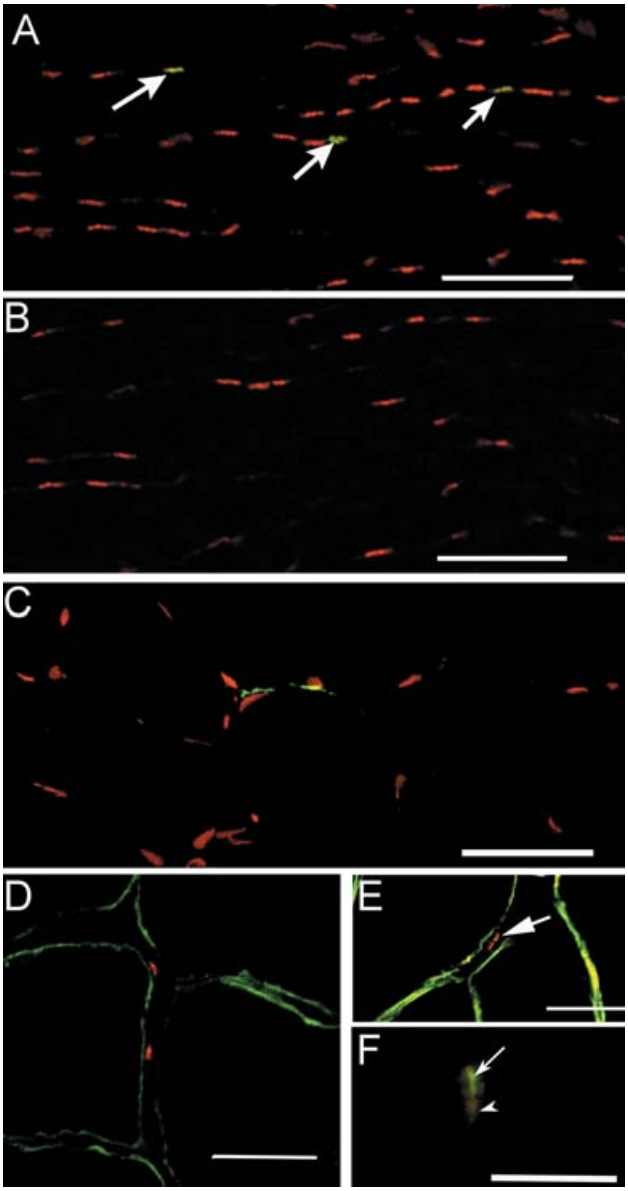

Figure 6. Immunofluorescence staining for myostatin (GDF-8 (C-20): sc-6884 antibody) and dystrophin
A and *B* show longitudinal sections of the gastrocnemius muscle of untrained rats (*A*) and trained rats (*B*) in which the myostatin immunoreactivity (FITC staining; arrows) is counterstained with the red nuclear staining with TO-PRO®-3 iodide. Scale bars, 90 μm. Note the absence of myostatin-immunopositive nuclei in *B*. *C*, transverse section of the gastrocnemius muscle of untrained rats; myostatin staining (green) is localized in the perinuclear area of two presumptive satellite cells. The red nuclear staining is performed with TO-PRO®-3 iodide. Scale bar, 45 μm. *D* and *E*, transverse and longitudinal sections of the gastrocnemius muscle; green colour shows dystrophin staining, while red spots indicate myostatin immunoreactivity. In *E*, myostatin staining is clearly outside the sarcolemma (arrow), stained by antidystrophin antibody (green). Scale bar, 24 μm. *F*, double immunofluorescence with antimyostatin (red) and anti-Pax7 (green) antibodies showing that, at high magnification, the colocalization is well evident, although the myostatin staining (red; arrowhead) surrounds the central core (green; arrow) of the Pax7 positivity. Scale bar, 12 μm.

SDS-PAGE confirmed the different composition of the samples dissected from the deep, red and from the superficial, white part of the gastrocnemius muscle.

The results obtained showed that: (i) in control conditions, myostatin expression was higher in white than in red fibres; and (ii) myostatin expression was significantly depressed by intense and long-lasting training, although acute swimming exercise did not significantly modify its mRNA level.

Suitability of the swimming model

Swimming and gastrocnemius were chosen as exercise and muscle models, respectively. The gastrocnemius muscle shows a heterogeneous composition in slow and fast fibres (Delp & Duan, 1996) and it is intensively involved in swimming. In general terms, the swimming model has been extensively used for training rodents. Two bouts of 3 h swimming are considered as low-intensity prolonged exercise (Terada *et al.* 2004), while swimming with an external weight equal to 6% of body mass has been reported to correspond to a high-intensity activity (Peijie *et al.* 2004). As explained in the Methods, swimming is a physiological activity for rats, and swimming training performed under well-controlled

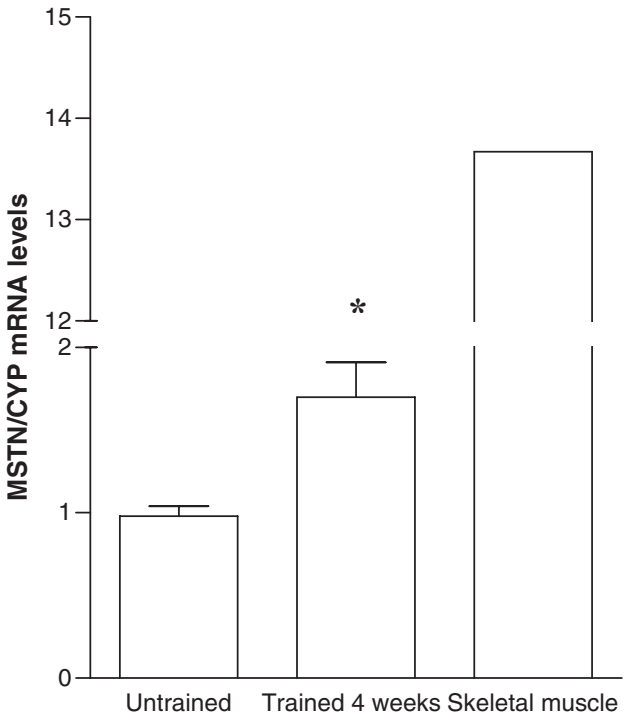

Figure 7. Myostatin (MSTN) mRNA contents in cardiac muscle of untrained rats and those trained for 4 weeks, as well as of a skeletal muscle sample (red gastrocnemius), normalized to cyclophilin (CYP) and expressed in arbitrary units
Data are mean ± S.E.M., $n = 6$ in each group. * Significantly different from untrained rats ($P < 0.05$; Student's unpaired t test).

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conditions does not cause psychological stress to the animals (Nakao *et al.* 2000). Data in the literature suggest that the amount of stress is equal to or even lower in swimming than in treadmill running (Dimauro *et al.* 1992; Terada & Tabata, 2004). The possibility that untrained animals (which had normal cage activity) exhibited muscle atrophy to an extent sufficient to influence the parameters measured (e.g. Wehling *et al.* 2000) does not seem plausible. Supporting evidence for this view derives from studies focused on immobilization-induced muscle atrophy (e.g. Kannus *et al.* 1998; Sakakima *et al.* 2004).

Chronic swimming in rats has been reported to induce an increase in muscle CSA (Klitgaard *et al.* 1989), although other studies did not confirm this effect (Dimauro *et al.* 1992). In the present study, we found significantly higher fibre CSA in muscle fibres from gastrocnemius of swimming trained rats compared to untrained animals (Table 2 and Fig. 5). The presence of extremely large fibres probably reflects the intense swimming protocol. These large fibres have been considered as giant fibres arising from hypercontraction of muscle fibres that are not able to undergo normal relaxation (Rehfeldt *et al.* 2004). It has been suggested that giant fibres might represent a morphological indicator of muscle fibre dysfunction, and their increased occurrence has been related to accelerated glycolysis. Therefore, the fact that trained rats showed these swollen round-shaped fibres might be linked to a metabolic and/or oxidative stress caused by intense swimming.

Myostatin mRNA transcript

Two groups of sedentary rats were examined and used as control animals in two independent experiments (acute swimming and chronic swimming training). In both experiments, a basal different myostatin expression pattern was found between the two muscle portions. Myostatin expression was significantly higher in the superficial, white portion of the gastrocnemius compared to the deep, red portion. In previous studies, basal differences of myostatin mRNA levels between fast- and slow-twitch muscles have been reported (Carlson *et al.* 1999; Wehling *et al.* 2000), with myostatin mRNA expression being more abundant in fast muscle fibres.

Although myostatin mRNA levels in the superficial, fast portion of the gastrocnemius of rats exposed to two bouts of swimming were approximately 45% lower than in the sedentary control animals, the effect of acute swimming on myostatin mRNA levels failed to be statistically significant. Acute (one-bout) and short-term (3–5 days) swimming training have previously been found to be accompanied by lower myostatin mRNA contents in rat gastrocnemius muscle 7 and 24 h postexercise, respectively (Matsakas *et al.* 2005). The present study confirmed and extended previous findings demonstrating that intensive long-term swimming training resulted in significantly reduced myostatin mRNA contents in active animals compared with respective controls. Since tissues from the chronic swimming experiment were excised 24 h after the last bout of exercise, myostatin RNA transcript changes are likely to reflect stable adaptation of muscle fibres more than direct acute effects of the last exercise session (Matsakas *et al.* 2005). However, it is not known whether myostatin mRNA changes are detectable at time points longer after the completion of an exercise session, e.g. 48 h. Comparison with available studies suggests that the type of exercise is not a critical factor affecting myostatin RNA transcript abundance, since changes have been described in response to acute and chronic resistance training in humans (Roth *et al.* 2003; Walker *et al.* 2004; Kim *et al.* 2005), as well as low-to-high intensity swimming (Matsakas *et al.* 2005; present study) and wheel-running in rats (Matsakas A & Patriuno M. unpublished observations).

Cardiac muscle and myostatin

Chronic conditioning by swimming for 4 weeks is known to increase heart mass and heart-mass-to-body-mass ratio in mice (Kaplan *et al.* 1994). In our study, 4 weeks of intensive swimming training in rats resulted in a higher heart-mass-to-body-mass ratio in the trained group compared to untrained rats, although the increase in absolute heart mass was below statistical significance (Table 1). Expression of myostatin in heart muscle has been documented by Sharma *et al.* (1999), who found an upregulation after heart infarction. By employing real time RT-PCR we were able to detect myostatin RNA transcript in heart muscle of rats, but at much lower levels than in skeletal muscles (Fig. 7), as already reported (Sharma *et al.* 1999). To our knowledge, this is the first study to describe the effect of exercise training on myostatin mRNA contents of the cardiac muscle. Compared to untrained rats, trained rats showed increased myostatin mRNA levels after 4 weeks of intensive swimming training. This is in general accordance with the data of Cook *et al.* (2002), who reported an upregulation of myostatin in hypertrophied hearts of transgenic mice, although the biological significance of this finding is not fully understood and remains to be elucidated. However, this fact suggests that myostatin might be important in heart adaptation to exercise by playing a role in the long-term homeostatic control and maintenance of cardiac muscle. Along this line, it is worth mentioning an intriguing hypothesis (Shyu *et al.* 2005) which proposes that myostatin represents a chalone of the insulin-like growth factor I pathway in the cardiac muscle. An alternative explanation is that higher myostatin mRNA levels in the heart of trained rats might be linked to cardiac tissue damage, such as stress-induced infarctions (Sharma

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et al. 1999). This explanation deserves attention in future studies, and histological examination of the myocardium might give a clear answer.

Myostatin protein

Although the present study was mainly aimed at examining changes in myostatin mRNA expression, attempts were made to quantify changes in myostatin protein levels in gastrocnemius muscle by means of Western blotting and immunohistochemistry. Western blotting was performed using a polyclonal antimyostatin antibody, which detected one main band at about 18–19 kDa. Mendler *et al.* (2000) have reported that this antibody recognizes a band at 19 kDa and proposed that this is the biologically active form of the protein. The specificity of the antibody was also tested by means of a positive control, such as the purified human myostatin with a molecular weight of 12.5 kDa. The fact that rat myostatin exhibits a higher molecular weight confirms that some discrepancy still exists concerning the precise molecular size of mature myostatin, probably because of interspecies differences or postranscriptional and/or postranslational modifications, such as glycosylation of the myostatin peptide (Mendler *et al.* 2000; Kambadur *et al.* 2004). Wehling *et al.* (2000) found a 2 kDa difference even in the same species when they detected the latent form of myostatin in two different types of muscle. Western blot, however, did not resolve the matter of quantifying possible differences in the content of myostatin protein between the experimental groups, probably because of the antibody used in this study.

Immunohistochemistry data showed some intriguing changes in myostatin immunostaining in trained rats. As observed by other authors, the basal level of myostatin immunoreactivity is very low in rats (Mendler *et al.* 2000), and the present results showed that it became virtually absent in trained animals. Indeed, a semi-quantitative analysis based on counting of positive spots on immunostained sections showed a significant decrease of myostatin protein in trained rats. This result is in agreement with the observations of Martin & Johnston (2005) which, in a fish model, showed a decrease of myostatin protein in fast muscles after chronic exercise training. Furthermore, Walker *et al.* (2004) showed a similar trend for reduction of plasma myostatin in healthy men after high-intensity resistance exercise of the elbow flexor muscles. There are, however, two separate studies in human vastus lateralis muscle, following a heavy resistance training, which reached opposite conclusions: Roth *et al.* (2003) showed a decrease of myostatin mRNA in response to training, while Willoughby (2004) observed an increase of myostatin mRNA and protein. Recent studies suggest an autoregulatory feedback loop that determines an inverse relationship between myostatin protein activity and myostatin mRNA transcription (Forbes *et al.* 2006). The present study showed that real time RT-PCR and immunohistochemistry results seemed to converge, since the decreased level of mRNA corresponded to a reduction of the antimyostatin antibody-positive spots in the gastrocnemius of the swimming trained rats.

A controversy exists about the cellular localization and the mechanism by which myostatin can produce its effects on muscle cell growth and proliferation. The active portion of myostatin, when bound to membrane receptors, might activate intracellular pathways that eventually control transcription. Mendler *et al.* (2000) found that the presence of the 18–19 kDa band was evident particularly in the nuclear pellet of the muscle homogenate, although the same authors, by means of immunohistochemistry, did not observe a clear nuclear localization. Again, Artaza *et al.* (2002) stated that the myostatin nuclear localization found in C2C12 myotubes appears to be cell specific because other cell types, investigated in the same study, showed cytoplasmic staining. However, in a recent study carried out on the same cell type, but with the use of a specific antibody for recombinant myostatin (Budasz-Rwiderska *et al.* 2005), only cytoplasmic staining was observed. In the present study, the combination of the nuclear staining (TO-PRO®-3 iodide) and the use of antimyostatin, antilaminin, antidistrophin and anti-Pax7 (a marker of satellite cells) antibodies strongly suggests that myostatin immunoreactivity is localized particularly in the nuclear area of satellite cells, which are 'wedged' between the plasma membrane and the basement membrane of muscle fibres (Mauro, 1961). Myostatin immunoreactivity seems to be localized around the nuclei of satellite cells, which are positive to anti-Pax7 staining. Satellite cells are essential in postnatal growth and reparative/regenerative mechanisms, and it has been shown that myostatin is expressed in satellite cells, is a marker of their quiescence, and negatively regulates their self-renewal (McCroskery *et al.* 2003). Therefore, it can be suggested that swimming training induced myostatin suppression in order to increase satellite cell turnover, providing nuclei to enlarging fibres or to repairing damaged fibres. In support of this hypothesis, we observed that numbers of Pax7-positive cells are increased after an intensive period of training (Patruno M, Matsakas A, Reggiani C, unpublished results).

In conclusion, the present results confirmed that myostatin expression is greater in fast-glycolytic (white) than in slow-oxidative (red) muscles and showed that 4 weeks of swimming training, but not single bouts of exercise, are followed by lower myostatin mRNA levels. Interestingly, this study provided the first evidence for an increase in myostatin mRNA in the heart after training, providing an argument in favour of the role of myostatin in regulation of cardiac muscle.

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