

Cellular distribution of Hsp70 expression in rat skeletal muscles. Effects of moderate exercise training and chronic hypoxia

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Abstract Rat hindlimb muscles constitutively express the inducible heat shock protein 72 (Hsp70), apparently in proportion to the slow myosin content. Since it remains controversial whether chronic Hsp70 expression reflects the overimposed stress, we investigated Hsp70 cellular distribution in fast muscles of the posterior rat hindlimb after (1) mild exercise training (up to 30 m/min treadmill run for 1 h/day), which induces a remodeling in fast fiber com-

position, or (2) prolonged exposure to normobaric hypoxia (10%O₂), which does not affect fiber-type composition. Both conditions increased significantly protein Hsp70 levels in the skeletal muscle. Immunohistochemistry showed the labeling for Hsp70 in subsets of both slow/type 1 and fast/type 2A myofibers of control, sedentary, and normoxic rats. Endurance training increased about threefold the percentage of Hsp70-positive myofibers ($P < 0.001$), and changed the distribution of Hsp70 immunoreactivity, which involved a larger subset of both type 2A and intermediate type 2A/2X myofibers ($P < 0.001$) and vascular smooth muscle cells. Hypoxia induced Hsp70 immunoreactivity in smooth muscle cells of veins and did not increase the percentage of Hsp70-positive myofibers; however, sustained exposure to hypoxia affected the distribution of Hsp70 immunoreactivity, which appeared detectable in a very small subset of type 2A fibers, whereas it concentrated in type 1 myofibers ($P < 0.05$) together with the labeling for heme-oxygenase isoform 1, a marker of oxidative stress. Therefore, the chronic induction of Hsp70 expression in rat skeletal muscles is not obligatory related to the slow fiber phenotype but reveals the occurrence of a stress response.

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Abbreviations

Hsp70 heat shock protein 72
BR blocking reagent
TBST tris buffered saline-Tween 20
RT room temperature

PBS	phosphate buffered saline
MDA	malondialdehyde
n	number
SEM	standard error of mean
SD	standard deviation
HO-1	heme-oxygenase isoform 1
ROS	reactive oxygen species
COX	cytochrome c oxidase
MHC	myosin heavy chain

Introduction

Despite being inducible, the 72-kDa isoform of the heat shock protein 70 (Hsp70) is constitutively expressed in certain rat skeletal muscles. It is highly abundant in slow-twitch muscles, such as the soleus, compared to fast-twitch muscles (Locke et al. 1991, 1994), such as the gastrocnemius and plantaris. In mixed muscles, the amount of Hsp70 is apparently in proportion with the percentage of type 1 myofibers, which contain slow myosin heavy chains (MHCs; Locke et al. 1991, 1994; Kelly et al. 1996).

The correlation between Hsp70 amount and slow myosin content in the skeletal muscle is apparently disrupted after acute exposure to stresses, such as heat, ischemia, exercise, increased contractile activity, or mechanical load, which, however, upregulate Hsp70 expression also in skeletal muscles lacking type 1 myofibers (reviewed by Liu et al. 2006). Hsp70 expression increases after sustained exposure to stress too; however, since exercise, increased contractile activity, or mechanical load do affect muscle fiber type composition, it remains still unsolved whether the increase in Hsp70 levels observed in these conditions simply reflects the changes in fiber-type populations (Liu et al. 2006). This possibility has been raised by investigations performed using experimental models, which induced a fast-to-slow fiber-type transition (reviewed by Liu et al. 2006). In that context, the persistent increase in Hsp70 levels was concomitant with the muscle shift from a fast-twitch to a slow-twitch phenotype, suggesting that a “constitutive” Hsp70 expression was replacing the inducible one (O’Neill et al. 2006).

A useful approach to investigate whether inducible Hsp70 expression modifies or overlaps to the distribution of the “constitutive” Hsp70 within muscle fiber populations is provided by immunohistochemistry. The studies so far available are scanty and concern mainly Hsp70 expression accompanying fast-to-slow fiber type transition, induced either in rabbit tibialis anterior muscle, by chronic low-frequency stimulation (Neufer et al. 1996), or in rat plantaris muscle, by compensatory overload (O’Neill et al. 2006). Both studies showed that Hsp70 immunoreactivity was

detectable only in a subset of type 1 fibers and fast-oxidative type 2A ones, but, rather surprisingly in the case of the rat, which expresses constitutively Hsp70, the pattern of such a distribution was not changed by the twofold increase in protein levels induced by muscle overload (O’Neill et al. 2006). Furthermore, the latter investigation demonstrated that hampering slow myosin expression, by means of thyroid hormone administration, also blocked the increase in Hsp70. Therefore, authors suggested that chronic changes in Hsp70 expression are related to muscle fiber remodeling toward a slow-twitch phenotype and, hence, to the peculiar yet unknown role played constitutively by Hsp70 in rat type 1 and 2A myofibers (O’Neill et al. 2006).

Nevertheless, it remains still undetermined which muscle fiber population is responsible for increased Hsp70 expression, when sustained exposure to stress induces the redistribution of fast muscle fibers or, alternatively, no remodeling of muscle fiber populations at all. The present study addressed this question analyzing the distribution of Hsp70 in rat hindlimb fast muscles, by means of immunohistochemistry, in two models of sustained exposure to stress. Moderate endurance training (treadmill running) is known to induce both Hsp70 upregulation (reviewed by Liu et al. 2006) and redistribution of fast muscle fiber populations (Abdelmalki et al. 1996; Kelly et al. 1996; Desplanches et al. 2004). By contrast, the exposure to hypoxia upregulates Hsp70 expression (Benjamin et al. 1990) but apparently does not affect muscle fiber type composition (Abdelmalki et al. 1996). Therefore, the present investigation tested the hypothesis that chronic exposure to stressors, such as exercise or hypoxia, stimulates the expression of Hsp70 in skeletal muscles of the rat hindlimb and changes its distribution among skeletal muscle cells in a stressor-specific way.

Materials and methods

Animals and experimental procedures

Adult male Sprague–Dawley rats were used in this study.

Fifteen 2-month-old rats were included in the aerobic exercise training group: seven rats run on a motorized treadmill 1 h/day, three times a week, for 14 weeks, whereas the remaining ones were used as sedentary controls (Marini et al. 2007). Training was accomplished using a six-lane rodent treadmill (Tecmachine, Medical Development, France). Rats trained 1 h a day (at the same time of the day), 3 days in a week (on the same days of the week). During the first training session, rats familiarized with the treadmill. After few minutes, they ran at 10 m/min, with 0% incline. The running speed and exercise duration were then gradually increased during the following weeks to reach

about 55–60% of VO_{2max} (Armstrong et al. 1983) in 11 weeks. At the end of this period, the speed was 30 m/min, with 0% incline. This workload was thereafter maintained for three more weeks. Untrained rats were placed on the switched-off treadmill immediately after the training sessions of the other animals.

Animals were killed after 48 h from the last exercise session under anesthesia.

Twenty-seven 5-week-old male rats were divided into three different groups (Milano et al. 2002). Normoxic rats ($n=8$) breathed room air ($FiO_2=0.21$), whereas 19 rats were exposed for 2 weeks to a normobaric hypoxic atmosphere ($FiO_2=0.10$). A subgroup of eight normobaric hypoxic rats was additionally exposed to room air for 1 h/day. For killing, the animals were first transferred to the compensation chamber, which was continuously flushed with the hypoxic gas; the animals were then anesthetized with an intraperitoneal injection of sodium thiopental (10 mg/100 g body weight).

A sample of blood was withdrawn by cardiac puncture into a heparinized syringe.

Posterior and anterior hindlimb muscles were excised and immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$ for subsequent use. Both anterior and posterior hindlimb muscles were considered in the study for trained and untrained rats, insofar they might reflect their different recruitment during running; conversely, the investigation was limited to the gastrocnemius muscle for normoxic and hypoxic rats, which were all sedentary.

Gel electrophoresis and immunoblotting

Cryosections obtained from frozen samples were homogenized in the presence of sodium dodecyl sulfate and in the absence of bromophenol blue and β -mercaptoethanol and used for protein determination, as previously described (Vitadello et al. 2001). About 30 μ g of lysate were separated on 10% linear polyacrylamide gels together with commercially available molecular weight standards (Broad Range, Bio-Rad). After transfer to nitrocellulose and saturation with 1% blocking reagent (BR, Roche Diagnostics) in Tris-buffered saline–Tween 20 (TBST; 150 mM NaCl; 10 mM Tris–HCl pH 8.0; 0.05% Tween-20) for 1 h at room temperature (RT), blots were sequentially incubated with monoclonal anti-Hsp70 antibody (SPA-810, Stressgen) 1:50,000 and monoclonal anti- α -actinin antibody (clone EA-53; Sigma) 1:20,000. After extensive rinses with TBST, filters were incubated with appropriate dilution of anti-mouse immunoglobulins conjugated with peroxidase (sc-2005; Santa Cruz Biotech) 1:4,000 in BR 0.5% in TBST. Peroxidase activity was revealed using chemiluminescence (ECL, GE Healthcare). About 80 μ g of lysate were separated on 10% linear polyacrylamide gels for the

demonstration of heme-oxygenase isoform 1 (HO-1) immunoreactivity. After saturation with 5% nonfat dry milk, blots were incubated in TBST added with 1% BR and 1:5,000 dilution of the mouse monoclonal anti-HO-1 antibody (OSA-111; Stressgen). Filters were then processed as described above. Quantitative densitometry was performed on Western blots of the samples analyzed. Autoradiographic-positive bands were analyzed using a Shimadzu chromatoscanner CS-930 at a wavelength of 600 nm. Values were normalized with the corresponding densitometric value of α -actinin and expressed either as mean and SEM or as percentage of the control mean value.

Immunocytochemistry

Serial consecutive 12- μ m cryosections of whole-muscle cross-sections were prepared from frozen muscle samples, collected on gelatin-coated glasses, and assayed for indirect peroxidase immunohistochemistry, following previously described protocols (Gorza 1990).

In particular, for Hsp70 immunolocalization, sections were fixed for 10 min at RT with 4% freshly buffered paraformaldehyde and, after two rinses with phosphate-buffered saline (PBS; NaCl 136 mM, KCl 2.68 mM, $NaHPO_4$ 8 mM, KH_2PO_4 1.4 mM, pH 7.4), incubated in methanol added with 0.3% H_2O_2 for 30 min at RT to inhibit endogenous peroxidase activity. Sections were incubated overnight at $4^{\circ}C$ with anti-Hsp70 monoclonal antibody SPA-810 diluted 1:100. After adequate rinses in PBS, section were sequentially incubated for 2 h at RT with rabbit anti-mouse immunoglobulins conjugated with peroxidase (P0260; Dakocytomation), diluted 1:100 and preabsorbed with rat serum to eliminate crossreactive immunoglobulins and then with anti-rabbit immunoglobulins conjugated with peroxidase (P-0399; Dakocytomation), diluted 1:300. Peroxidase activity was revealed using diaminobenzidine as a substrate. A minimum of 1,000 muscle fibers from representative fields of each whole-muscle cross-section was evaluated for the presence of Hsp70 immunoreactivity. Consistency of the immunostaining was validated by independent analysis of adjacent cryosections.

Specificity of the staining was checked by processing adjacent sections with the same protocol, except for the use of nonimmune mouse immunoglobulins (1 μ g/ml; Sigma) as the primary antibody.

Fiber typing was achieved by means of labeling of serial sections with anti-MHC antibodies. Sections were fixed for 10 min with cold acetone and incubated overnight at $4^{\circ}C$ with appropriate dilutions of the following mouse monoclonal antibodies (Gorza 1990): BA-D5, which specifically recognizes type 1 MHC; SC-71, which labels type 2A MHC; BF-35, which reacts with all the MHC types, except

the 2X one, and, therefore, characterizes through the absence of staining a fast fiber type populations with intermediate oxidative ability, dubbed also 2D (Pette and Staron 2000). Intermediate type 1/2A fibers were identified by the presence of immunostaining with both BA-D5 and SC-71. Intermediate type 2A/2X fibers were identified by the presence of weaker staining with SC-71 antibody, than that displayed by type 2A fibers, and the absence of staining with BA-D5 antibody. Type 2B fibers were identified by the presence of staining with BF-35 and the absence of staining with BA-D5 and SC-71 antibodies. After adequate rinses with PBS, sections were incubated with appropriate dilutions of goat anti-mouse immunoglobulins, conjugated with peroxidase and preadsorbed with rat serum (sc-2005; Santa Cruz Biotech), and were then revealed with diaminobenzidine.

Vasculature was identified using α -smooth actin as a marker. Sections were fixed for 10 min at RT with 4% freshly buffered paraformaldehyde and permeabilized with cold 0.2% Triton X-100 for 10 min, before overnight incubation at 4°C with a 1:2,000 dilution of the anti- α -smooth actin mouse monoclonal antibody (clone 1A4; Sigma). Sections were then processed with the secondary antibody, as described for anti-MHC1 antibodies.

Immunolocalization of HO-1 was achieved by fixing sections as described for Hsp70 and incubating them overnight at 4°C with a 1:20 dilution of anti-HO-1 goat polyclonal antibody (sc-1797; Santa Cruz Biotech). Sections were then processed as described above, using anti-goat immunoglobulins conjugated with peroxidase (sc-2056; Santa Cruz Biotech) as secondary antibodies.

Cross-sectional areas of muscle fibers were measured using the Image J software. Mean fiber area was obtained considering at least 80 fibers within each fiber type population.

Malondialdehyde

Blood was centrifuged, and the plasma was stored at -80°C for the determination of malondialdehyde (MDA) concentration, which was determined by high-performance liquid chromatography according to Kawai et al. (1989). To allow comparison among different experimental protocols (training vs. chronic and intermittent hypoxia), each value was expressed as a percentage of the mean of the respective control values.

Statistical analyses

Statistical analysis was performed utilizing one-way analysis of variance and the Student's *t* test ($P \leq 0.05$). All statistical analyses were performed using Sigma Stat version 2.0 (Jandel Europe, Germany).

Results

Changes in muscle Hsp70 levels and cellular distribution after moderate training

Our protocol of aerobic (nonexhaustive) treadmill running was not followed by the appearance of hindlimb muscle hypertrophy, as shown by the muscle weight/body weight ratio at the moment of the killing (Table 1). Consistently with literature data (Kelly et al. 1996), Western blot analyses revealed significant differences in the total content of Hsp70 among hindlimb muscles of sedentary rats (Fig. 1). Increased signals for Hsp70 occurred in posterior hindlimb muscles, such as the plantaris and soleus, of trained rats, compared to those of sedentary ones, whereas no parallel change in Hsp70 levels was detectable in anterior hindlimb muscles, such as tibialis anterior, of trained animals (Fig. 1a). Densitometric analysis and subsequent normalization to α -actinin levels confirmed the presence of a significant fourfold increase in Hsp70 levels in the trained plantaris and of a twofold increase in trained soleus muscles ($P < 0.01$) (Fig. 1b).

We then evaluated by immunohistochemistry the distribution of Hsp70 immunoreactivity in a posterior fast hindlimb muscle, such as the plantaris muscle, of sedentary and trained rats. In sedentary muscles, type 1 and type 2A fibers, identified by means of immunostaining with specific anti-MHC antibodies, represented about one third of muscle fibers (mean percentage and SEM of type 1 and type 2A fibers, 4.18%±1.35 and 20.17%±2.87, respectively, average *n* of fibers evaluated in representative fields for each muscle=1,500). Hsp70 immunoreactivity appeared weak and involved about 10% of the muscle fibers. The large majority of type 1 fibers and about one third of type 2A fibers were labeled by Hsp70 antibodies (Fig. 2). Consistently with literature data (Abdelmalki et al. 1996; Desplanches et al. 2004), trained muscles showed a significant increase in the percentage of type 2A fibers (mean percentage and SEM of type 1 and type 2A fibers, 4.78%±0.67 and 33.06%±1.95, respectively; average *n* of fibers evaluated in representative fields for each muscle=1,500; $P < 0.005$ for the percentage of type 2A fibers in trained muscles compared to the value observed in sedentary ones). Exercise training increased about threefold the percentage of fibers displaying immunoreactivity for Hsp70, compared to sedentary muscles (average *n* of fibers evaluated in representative fields for each muscle=1,000; $P < 0.001$; Fig. 2e,i). Similarly to sedentary rats, reactivity for Hsp70 was detected within the large majority of type 1 fibers, whereas, differently from the sedentary muscles, Hsp70 immunoreactivity involved a larger proportion of type 2 fibers (Fig. 2e-h,j). Training almost doubled the percentage of type 2A fibers displaying Hsp70 immunore-

Table 1 Mean and SE values of body and hindlimb muscle weights of sedentary and trained rats

	Sedentary rats (<i>n</i> 7)	Trained rats (<i>n</i> 7)
Body weight (g)	562.8±27.0	477.4±14.1
Soleus		
Muscle weight (g)	0.255±0.007	0.218±0.010
MW/BW ratio × 10 ⁻³ (g)	0.45±0.07	0.45±0.04
Plantaris		
Muscle weight (g)	0.554±0.050	0.468±0.055
MW/BW ratio × 10 ⁻³ (g)	0.98±0.20	0.98±0.28
Tibialis anterior		
Muscle weight (g)	0.740±0.053	0.714±0.022
MW/BW ratio × 10 ⁻³ (g)	1.31±0.02	1.49±0.15

activity and dramatically increased Hsp70-positive staining among intermediate type 2A/2X fibers ($P<0.001$); furthermore, it significantly increased the number of Hsp70-positive fibers among type 2X and 2B fibers, compared to sedentary muscles ($P<0.01$).

Immunohistochemical analyses revealed other interesting findings concerning stress-induced redistribution of Hsp70 immunoreactivity in trained muscles. Differently from muscle of sedentary rats, both slow and fast hindlimb muscles of trained rats showed increased immunoreactivity in smooth muscle cells and myofibroblasts of muscle vessels, as deduced by comparison with immunostaining for the smooth muscle α -actinin isoform (Fig. 3).

Changes in muscle Hsp70 levels and cellular distribution after chronic hypoxia

Since rats exposed to hypoxia were sedentary, we excluded from our study the anterior hindlimb muscle and focused on the fast posterior hindlimb muscles, among which we chose

the gastrocnemius for our analysis because of its relative higher abundance of type 1, 2X, and 2B fibers, compared to the plantaris muscle. Western blot analysis of homogenates from cross-sections of whole medial or lateral gastrocnemius muscles, obtained from rats exposed to either normoxia or chronic or intermittent normobaric hypoxia, showed increased immunoreactivity for Hsp70 in samples obtained after chronic permanence in hypoxia, compared to those obtained after exposure to normoxia or to intermittent hypoxia (Fig. 4a). Densitometric analysis and subsequent normalization to α -actinin levels confirmed the presence of a significant threefold increase in Hsp70 levels in muscles exposed to chronic hypoxia, compared to those maintained in normoxia ($P=0.02$). A small but significant increase in Hsp70 amount was observed also in samples exposed to intermittent hypoxia ($P=0.01$; Fig. 4b).

We then evaluated the distribution of Hsp70 immunoreactivity in cross-sections of whole muscles of rats exposed either to normoxia or chronic or intermittent hypoxia. In the normoxic muscle, mean percentage and SEM of type 1 and type 2A fibers, identified by means of immunostaining with specific anti-MHC antibodies, was $8.71\pm 2.71\%$ and $11.99\pm 3.15\%$, respectively (average *n* of fibers evaluated in representative fields for each muscle=1,500). Normoxic muscles displayed a weak Hsp70 immunoreactivity, which involved about 10% of total fibers and was distributed within a number of type 1 and type 2A fibers (average *n* of muscle fibers evaluated in representative fields for each muscle=1,000, Fig. 5). The exposure to either chronic or intermittent hypoxia did not change significantly the relative amount of either type 1 or type 2A fibers compared to normoxic muscles, consistently with previous reports on fiber type composition of fast hindlimb muscles of hypoxic rats (Abdelmalki et al. 1996). Chronically hypoxic muscles showed a mean percentage and SEM of type 1 and type 2A

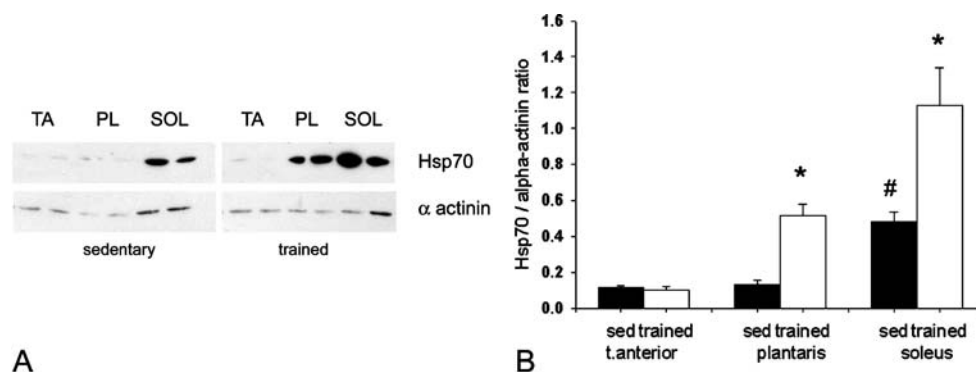


Fig. 1 Hsp70 protein levels in rat hindlimb muscles after moderate training. **a** Representative Western blot of different soleus (SOL), tibialis anterior (TA), and plantaris (PL) muscles, obtained from sedentary and trained rats, stained with antibodies for Hsp70. Labeling for α -actinin was shown as a reference for sample loading. **b** Histogram shows mean and SEM of Hsp70/ α -actinin ratio of muscles

from trained rats ($n=7$) relative to those of sedentary (sed) ones ($n=8$). Asterisks indicate significant difference vs. control muscles, whereas gate symbol (#) indicates significant difference of sedentary soleus muscle vs. the other sedentary muscles ($P<0.01$). Note the absence of a significant increase in Hsp70 levels in trained tibialis anterior muscle

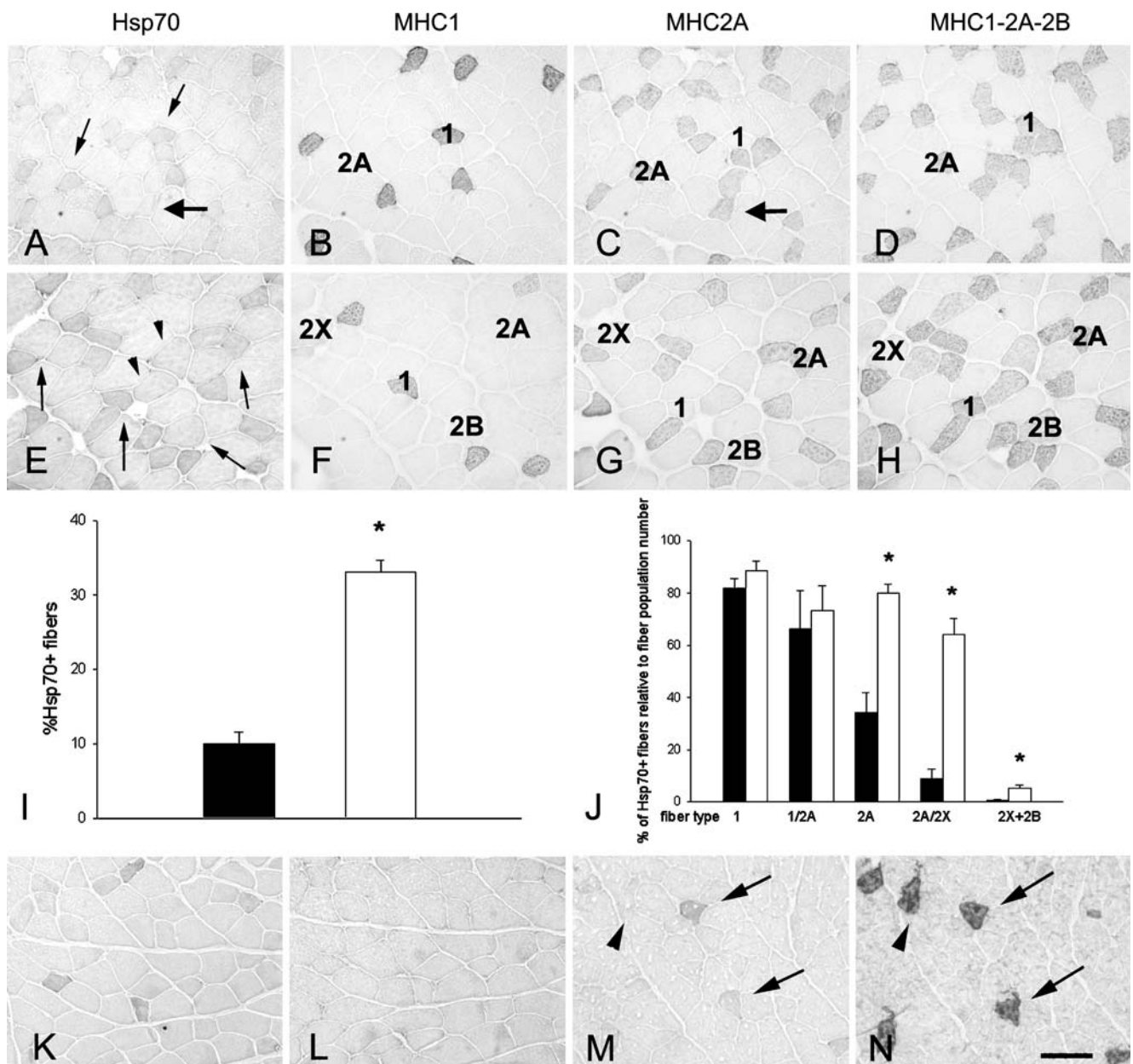


Fig. 2 Distribution of Hsp70 immunoreactivity among fiber type populations of sedentary and trained muscles. Indirect immunoperoxidase staining of serial adjacent cryostat sections from sedentary (**a–d**) and trained (**e–h**) plantaris muscle with the following antibodies: anti-Hsp70 (**a, e**); BA-D5 for slow myosin heavy chain (*MHC1*; **b, f**); SC-71 for fast 2A myosin heavy chain (*MHC2A*; **c, g**); BF-35 for staining of MHC1-2A-2B and, thus, negative visualization of fast 2X myosin heavy chain (**d, h**). Hsp70 immunoreactivity, albeit at variable levels of intensity, is detectable in both sedentary and trained muscles in type 1 and 2A fibers and in trained muscle also in a few type 2X and 2B fibers (*thin arrows* in **a** and **e** indicate representative fibers). *Large arrows* in **a** and **c** indicate a representative Hsp70 negative/type 2A fiber. *Arrowheads* in **e** indicate the representative staining for Hsp70 in fibers of trained muscles, which were classified as intermediate type 2A/2X fibers (*arrowheads* in **g, h**), because of their intermediate level of staining with SC-71 and BF-35 antibodies and lack of staining with anti-MHC1 antibody. **i** The histogram shows the absolute percentage

of Hsp70-positive fibers, evaluated on whole cross-sections of sedentary (*black bar*) and trained muscles (*white bar*). **j** The histogram illustrates the percentage of Hsp70-labeled fibers relative to the fiber number of each fiber type population indicated in abscissa, of sedentary (*black bar*) and trained muscles (*white bar*). Values correspond to mean and SEM (*n* of sedentary and trained muscles considered=5 and 6, respectively; average *n* of muscle fibers evaluated in representative fields for each muscle=1,000). *Asterisks* indicate significant difference vs. the value observed in sedentary muscles ($P<0.01$). **k–l** Indirect immunoperoxidase staining of serial adjacent cryostat sections from the sedentary plantaris muscle with anti-Hsp70 (**k**) and nonimmune mouse immunoglobulins (**l**). **m–n** Indirect immunoperoxidase staining of serial adjacent cryostat sections from sedentary plantaris muscle with anti-Hsp70 (**m**) and anti-MHC1 antibody (**n**). *Thin arrows* indicate Hsp70-positive/type 1 fibers, whereas *arrowhead* indicates Hsp70-negative /type 1 fibers. *Bar*=50 μ m

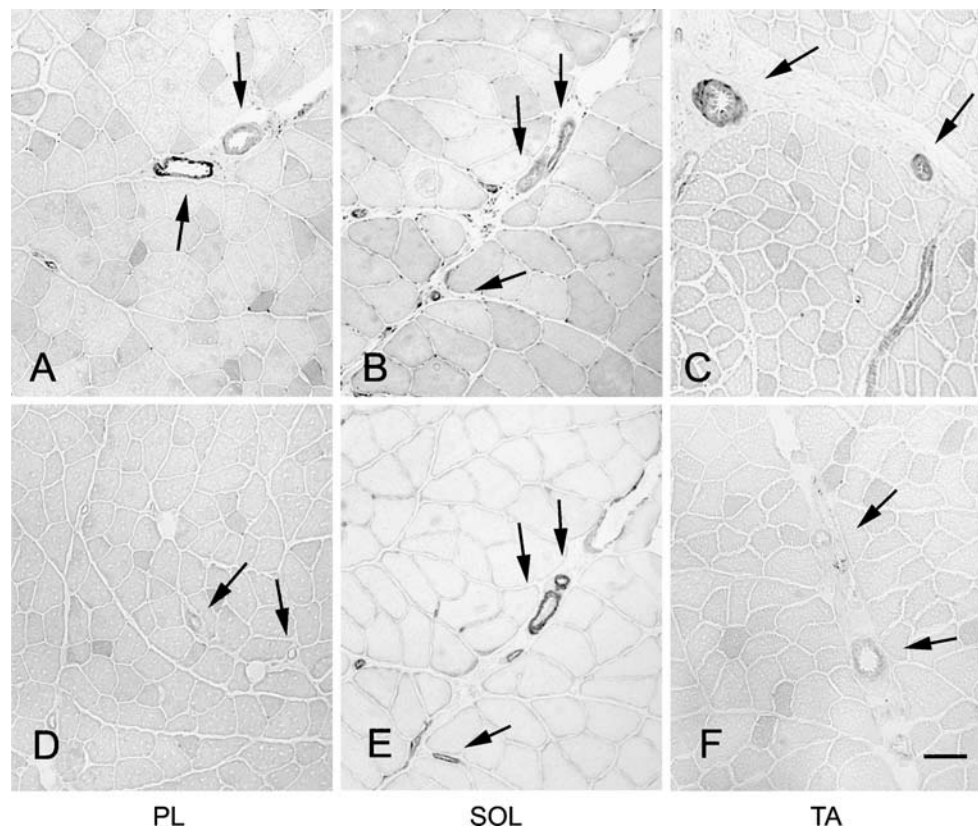


Fig. 3 Moderate intensity training induces Hsp70 immunoreactivity in muscle vessel wall. Indirect immunoperoxidase staining of cryostat sections obtained from different trained (a–c, e) and sedentary hindlimb muscles (d, f) with anti-Hsp70 antibodies. a and d illustrate trained and sedentary plantaris muscle (PL). b and e correspond to

serial adjacent sections of trained soleus muscle (SOL); e shows labeling for anti- α -smooth actin. c and d illustrate trained and sedentary tibialis anterior muscle (TA). Arrows indicate skeletal muscle vessels. Bar=50 μ m

fibers, corresponding to $12.98 \pm 1.60\%$ and $16.36 \pm 3.50\%$, respectively (average n of fibers evaluated in representative fields for each muscle=1,500); intermittently hypoxic muscles showed a mean percentage and SEM of type 1 and type 2A fibers, corresponding to $6.38 \pm 1.57\%$ and $15.65 \pm 3.03\%$, respectively (average n of fibers evaluated in representative fields for each muscle=2,000).

Differently from trained muscles, the increase in Hsp70 levels observed in Western blot after exposure to hypoxia was not accompanied by an increase in the percentage of Hsp70-positive fibers, whose value appeared comparable to that one observed in normoxic muscles (Fig. 5). The evaluation of the myofiber cross-sectional area of chronically hypoxic muscles showed an 8.52% decrease in type 2X and 2B myofiber populations (mean and SD values $2,016.46 \pm 526.04 \mu\text{m}^2$), compared to normoxic ones ($2,204.24 \pm 644.41 \mu\text{m}^2$; $P < 0.01$). When we considered the cross-sectional area of the myofiber populations, which displayed the large majority of Hsp70 immunoreactivity in normoxic muscles namely, type 2A and type 1 fibers, we observed a slight decrease in mean area of type 2A fibers of chronically hypoxic muscles ($1,185.72 \pm 359.3 \mu\text{m}^2$ SD),

compared to that of normoxic type 2A fibers ($1,288.65 \pm 306.3 \mu\text{m}^2$ SD; $P = 0.053$), whereas no change was observed for type 1 fiber areas, whose mean and SD values were $1,393.33 \pm 521.3$ and $1458.45 \pm 408.9 \mu\text{m}^2$, for chronically hypoxic muscles and normoxic ones, respectively ($P = 0.40$). The reduction in the muscle cross-sectional area, observed in chronically hypoxic muscles, increased the relative value of the area occupied by Hsp70-immunoreactive fibers in normoxic muscles from 6.6% to 7.4%.

Nevertheless, chronic hypoxia significantly affected the distribution of Hsp70 immunoreactivity: at variance with normoxic muscles, the percentage of type 2A fibers displaying Hsp70 immunoreactivity showed a sixfold reduction ($P = 0.01$), whereas that of Hsp70-positive type 1 fibers increased ($P < 0.05$). Conversely, such a redistribution was not observed in muscles exposed to intermittent hypoxia, which displayed a pattern of Hsp70 immunoreactivity comparable to that observed in normoxic muscles (Fig. 5).

At variance with muscle fibers, a stronger Hsp70 immunoreactivity was detected in the muscle vasculature of hypoxic rats, compared to normoxic rats. Immunostain-

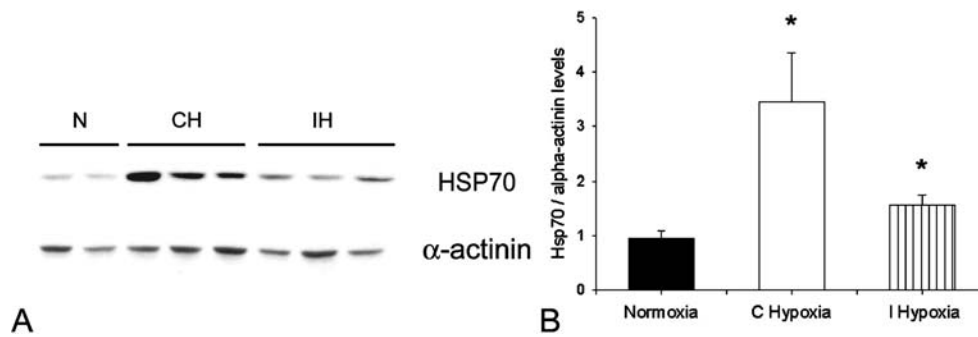


Fig. 4 Hsp70 protein levels in rat hindlimb muscles after exposure to chronic hypoxia or intermittent hypoxia. **a** Representative Western blot of different gastrocnemius muscles obtained from rats exposed to normoxia (N), chronic hypoxia (CH), and intermittent hypoxia (IH), labeled with anti-Hsp70 and α -actinin antibodies. **d** Histogram shows

mean and SEM of Hsp70/ α -actinin ratio of muscles exposed either to chronic hypoxia ($n=10$) or to intermittent hypoxia ($n=8$), relative to normoxic ones ($n=8$). Asterisks indicate significant difference vs. the respective control muscle ($P \leq 0.02$)

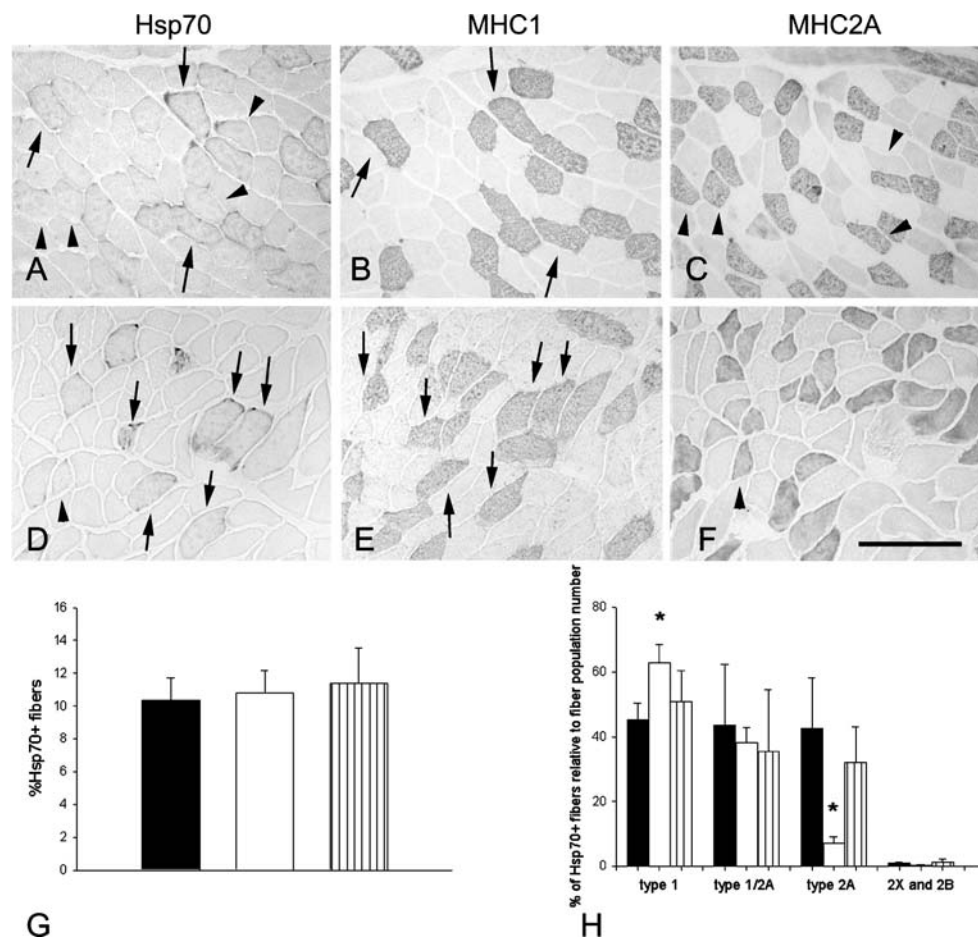


Fig. 5 Distribution of Hsp70 immunoreactivity among muscle fiber type populations after exposure to chronic hypoxia or intermittent hypoxia. Representative indirect immunoperoxidase staining of adjacent serial cryosections from the deep portion of gastrocnemius muscles exposed to normoxia (**a–c**) or to chronic hypoxia (**d–f**). **a** and **d** were stained for Hsp70; **b** and **e** were labeled with BA-D5 antibody to visualize slow myosin heavy chain type 1 distribution (MHC1). **c** and **f** were labeled with SC-71 antibody to visualize type 2A myosin heavy chain distribution (MHC2A). Arrows indicate Hsp70 immunoreactivity in type 1 myofibers, whereas arrowheads show labeling for Hsp70 in type 2A myofibers. Bar=100 μ m. **g** The histogram shows the absolute percentage of Hsp70-positive fibers, evaluated on whole

cross-sections of muscles exposed to normoxia (black bar), to chronic hypoxia (white bar), or to intermittent hypoxia (striped bar). **h** The histogram illustrates the percentage of Hsp70-labeled fibers relative to the fiber number of each fiber type population indicated in the abscissa, of muscles exposed to normoxia (black bar), to chronic hypoxia (white bar), or to intermittent hypoxia (striped bar). Values correspond to the mean and SEM (n of normoxic, chronically hypoxic, and intermittently hypoxic muscles considered=7, 8, and 8, respectively; average n of muscle fibers evaluated in representative fields for each muscle=1,000). Asterisks indicate significant difference vs. the value observed in normoxic muscles ($P < 0.05$)

ing involved preferentially large venous vessels and was detectable also in the superficial “white” regions of the gastrocnemius muscle, where Hsp70 immunolabeling was apparently undetectable in myofibers, which belonged to type 2B and 2X populations (Fig. 6).

Hsp70 expression and oxidative stress markers

The differences in Hsp70 expression and distribution, observed between control and experimental muscles, could be due to different factors, among which one is oxidative stress. Plasma MDA increased significantly in rats exposed either to moderate exercise training, to chronic hypoxia or to intermittent hypoxia, compared to their corresponding controls (Fig. 7). No significant difference in plasma MDA levels was, however, detected between the two latter conditions.

We then determined whether type 1 myofibers of hypoxic muscles, in the absence of shifts in fiber composition or increase in fiber size, displayed other signs of an active stress response, in addition to Hsp70 expression. We thus analyzed the distribution of the immunohistochemical reactivity for HO-1, a gene upregulated by hypoxia (Morita et al. 1995). In normoxic muscles, immunoreactivity for HO-1 was predominantly detectable within type 2A fibers, whereas about half of type 1 myofibers (mean and SEM $44.36 \pm 3.44\%$; average n of type 1 fibers evaluated for each sample = 150) were apparently unreactive (Fig. 8a–d). A comparable distribution of HO-1 immunoreactivity was observed in intermittently hypoxic muscles; conversely, a significantly higher percentage of type 1 fibers (mean and SEM = $76.28 \pm 6.64\%$, $P = 0.005$ compared to normoxic muscles) appeared labeled by anti-HO-1 antibodies in chronically hypoxic muscles (Fig. 8e–h). Western blot analysis indeed confirmed the presence of a significant increase in HO-1 protein levels in

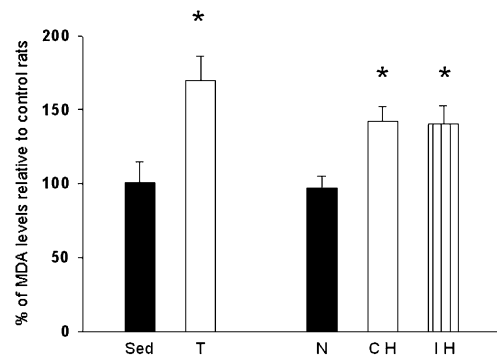


Fig. 7 Training and exposure to hypoxia increase plasma malondialdehyde (MDA) levels. Histogram represents the percentage of plasma MDA levels relative to control samples, evaluated in the two experimental models. Values correspond to mean and SEM. Abbreviations: *Sed* sedentary muscles, *T* trained muscles, *N* normoxic muscles, *CH* chronically hypoxic muscles, *IH* intermittently hypoxic muscles. Asterisks indicate significant difference vs. control muscles ($P < 0.01$)

the muscles of rats exposed to chronic hypoxia, compared to those maintained in normoxia or intermittently exposed to hypoxia (Fig. 8i–j; $P < 0.05$).

Discussion

This study investigated chronic Hsp70 expression and distribution in skeletal muscles of rats exposed to different stressing conditions, such as moderate exercise training and normobaric hypoxia. Although both conditions significantly increased the relative protein level in posterior hindlimb muscles, results showed that Hsp70 distribution in muscle fibers and vasculature differed among each experimental context, confirming our hypothesis that the chronic expression of Hsp70 in rat skeletal muscle reflected the continuative exposure to and the specificity of the stressing stimulus. In trained muscles, Hsp70 immunoreactivity was

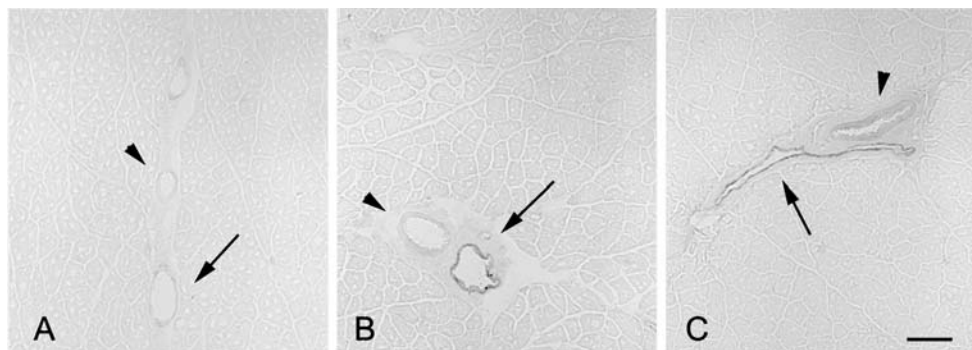


Fig. 6 Exposure to hypoxia induces Hsp70 immunoreactivity in muscle vein wall. Indirect immunoperoxidase staining of cryosections of superficial white regions of the gastrocnemius muscles from rats exposed to normoxia (a), chronic hypoxia (b), and intermittent hypoxia (c), labeled with anti-Hsp70 antibody. Arrows indicate

skeletal muscle veins, whereas arrowheads indicate muscle arteries. Note the absence of Hsp70 immunostaining from the skeletal muscle fibers of the superficial muscle region, which is entirely composed of type 2X and type 2B muscle fibers. Bar = 100 μm

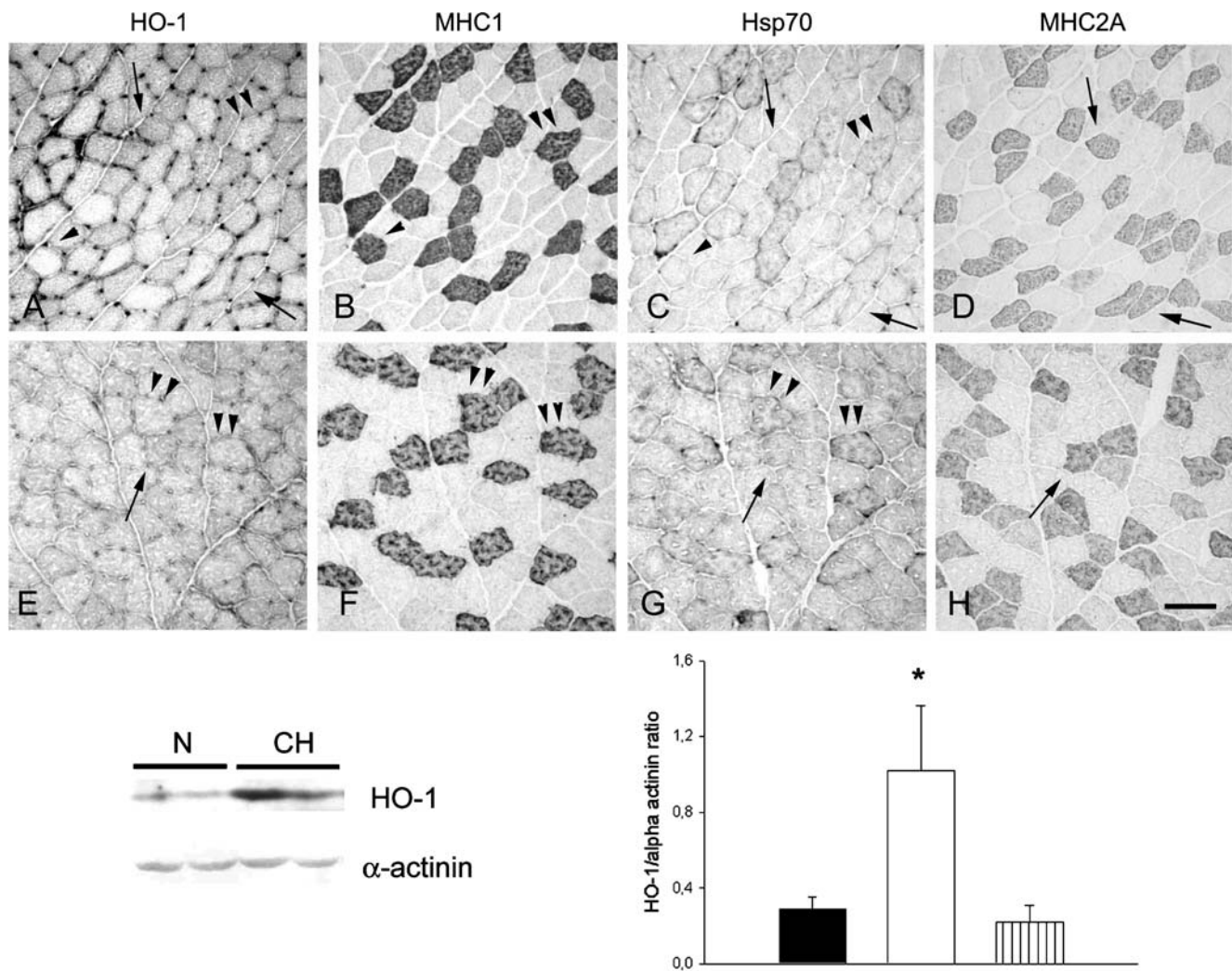


Fig. 8 Type 1 fibers of chronically hypoxic muscles display increased immunoreactivity for heme-oxygenase 1 (*HO-1*). Indirect immunoperoxidase staining of adjacent serial cryosections from the deep portion of the gastrocnemius muscles exposed to normoxia (**a–d**) or to chronic hypoxia (**e–h**). **a** and **e** were stained for *HO-1*; **b** and **f** were labeled with BA-D5 antibody to visualize slow type 1 MHC; **c** and **g** were stained for Hsp70; **d** and **h** were labeled with SC-71 antibody to visualize type 2A MHC. Double arrowheads indicate *HO-1* immunoreactivity in type 1 myofibers, which also stain for Hsp70; single arrowhead shows a representative *HO-1* negative fiber, which belong

detectable in smooth muscle cells of vessels and in a larger population of fast myofibers compared to sedentary muscles. In hypoxic muscles, Hsp70 immunoreactivity was detectable in vein walls and in muscle myofibers, although their relative number did not increase compared to normoxic muscles. However, only the exposure to chronic hypoxia affected myofiber distribution of Hsp70 immunoreactivity, which was predominantly observed within type 1 fibers, at variance with normoxic and intermittently hypoxic muscles. Therefore, Hsp70 expression in skeletal muscle fibers appeared to occur consequentially to the specific

to type 1 fiber population; arrows indicate representative type 2A fibers, which also stain for *HO-1*. **i** Representative Western blot of different gastrocnemius muscles obtained from rats exposed to normoxia (*N*) and chronic hypoxia (*CH*), labeled with anti-*HO-1* and α -actinin antibodies. **j** Histogram shows mean and SEM of *HO-1*/ α -actinin ratio of muscles exposed either to chronic hypoxia (white bar) or to intermittent hypoxia (striped bar), relative to normoxic ones (black bar). Asterisk indicates significant difference vs. normoxic muscles ($P \leq 0.05$)

stressing stimulus, which determined a redistribution within the fast fiber population, in the case of moderate exercise training, whereas it evoked a stronger stress response in the slow fibers compared to the fast ones, in the case of chronic exposure to normobaric hypoxia, as suggested by the parallel staining for the stress protein *HO-1*.

The 72-kDa inducible isoform of Hsp70 is constitutively expressed in rat skeletal muscles apparently in proportion with the percentage of type 1 myofibers, which contain slow MHCs (Locke et al. 1991, 1994; Kelly et al. 1996). Our study, which focused on mixed fast-twitch muscles of

the posterior hindlimb, such as the plantaris and gastrocnemius, showed that Hsp70 immunoreactivity of control, sedentary, and normoxic rats was distributed among type 1 and type 2A myofibers (Figs. 2 and 5). This result is quite consistent with that reported by O'Neill et al. (2006), for the control rat plantaris muscle. It has to be mentioned, however, that the percentage of Hsp70-immunoreactive fibers in sedentary and normoxic muscles did not correspond to the whole type 1 and type 2A fiber populations; at variance, only a subset of these fibers were labeled by anti-Hsp70 antibodies. Our body of evidence that Hsp70 immunoreactivity distributed within a proportion of type 2A fibers of gastrocnemius muscles, being at the same time absent from a part of type 1 fibers, argues against a constitutive role of Hsp70 in the slower fiber phenotype.

To verify the hypothesis linking Hsp70 expression to muscle fiber transitions, we examined two experimental stressing conditions in the rat, which oppositely affected the muscle fiber phenotype. Since a large body of evidence already exists concerning Hsp70 immunoreactivity in the presence of fast-to-slow fiber transitions, we investigated a well-characterized model that primarily involves changes within fast fiber populations, i.e., that one induced by exercise training (Kelly et al. 1996; Abdelmalki et al. 1996; Desplanches et al. 2004); in addition, we investigated a condition that did not apparently affect muscle fiber composition (Abdelmalki et al. 1996) but represented a candidate stimulus for Hsp70 upregulation in skeletal muscle cells, i.e., chronic exposure to hypoxia (Benjamin et al. 1990). Consistently with that reported by the literature (Kelly et al. 1996; Samelman 2000; Desplanches et al. 2004; Noble et al. 2006; Liu et al. 2006 as a review), exercise training of moderate intensity by treadmill running significantly increased Hsp70 protein levels. Our data further show that this occurred in the posterior hindlimb muscles, which play a dynamic role during running, whereas it was apparently absent in muscles, like the tibialis anterior, which exhibited a predominant tonic activity (Jasmin and Gisiger 1990). In addition, normobaric hypoxia significantly increased muscle levels of Hsp70, similarly to that described after exposure to chronic hypobaric hypoxia (Magalhaes et al. 2005a). Nevertheless, the extent of the Hsp70 response to either sustained or intermittent hypoxia was strikingly different, insofar chronic exposure to normobaric hypoxia induced a threefold increase in Hsp70 protein levels, whereas the intermittent one only a 50% increase. In addition to represent the first report describing the different effects of intermittent and chronic hypoxia on stress protein upregulation in the skeletal muscle, our data show that the threefold increase in Hsp70 levels, observed after 2 weeks of sustained hypoxia, cannot be explained as the bare consequence of the 8.52% decrease in fiber cross-sectional area of type 2X

and 2B fibers because it largely exceeded the increase in the relative cross-sectional area of Hsp70-immunoreactive fibers.

Among the possible mechanisms responsible for Hsp70 redistribution within skeletal myofibers, the transition in fiber phenotype may play a prominent role. Consistently with literature data (Abdelmaki et al. 1996), our exercise training protocol induced a significant increase in the type 2A fiber population, indicating that a fiber transition was taking place among fast fiber populations. Differently to that described for a fast-to-slow fiber transition in an overloaded rat plantaris (O'Neill et al. 2006), the absolute percentage of myofibers displaying Hsp70 immunoreactivity significantly increased after exercise training and redistributed within distinct fiber populations. In particular, we observed that the relative percentage of Hsp70-positive fibers detected within either type 2A or the intermediate type 2A/2X or the type 2X and 2B subpopulation significantly increased in trained muscles, compared to sedentary muscles. A comparable redistribution was observed in rabbit tibialis anterior after chronic low frequency stimulation (Neufer et al. 1996), suggesting that the chronic expression of stress proteins might be related to the increased requirements of stimulated myofibers in the synthesis and translocations of proteins. Nascent myosin myofibrils require the constitutive chaperone complex Hsc70–Hsp90 for myofibril assembly and maturation in skeletal muscle myoblasts and myotubes (Srikakulam and Wilkenmann 2004); it is then possible to speculate that the stress-inducible isoform Hsp70 might be also recruited in this process. A study investigating fast-to-slow fiber transition in an overloaded rat plantaris showed that the inhibition of the transcription of type 1 MHC gene, by exposure to high levels of the thyroid hormone, hampered Hsp70 upregulation (O'Neill et al. 2006); however, the proposed specificity of Hsp70 expression for type 1 MHC was questioned by the evidence that, in the overloaded rat plantaris model, Hsp70 levels increased despite of either the inhibition of calcineurin, another positive regulator of the type 1 fiber phenotype (Oishi et al. 2005), or the inhibitory effects of prior heat stress on type 1 MHC accumulation (Frier and Locke 2007). Our present results, obtained studying a model of fiber transition among fast types, confirm the above mentioned observations.

At variance with exercise, exposure to hypoxia did not modify the relative proportions of fiber types (Abdelmaki et al. 1996) nor increased the absolute percentage of myofibers labeled with Hsp70. The young age of the animals used in the hypoxic protocol (7 weeks at the moment of killing) argues against possible derangements of Hsp70 upregulation in fast-twitch muscles of the posterior hindlimb, which, conversely, was reported for old rats (Naito et al. 2001). However, the percentage of Hsp70-positive fibers detected within the type 1 fiber population was

significantly increased, and that one belonging to type 2A was drastically reduced in muscles of rats exposed to chronic hypoxia, compared to those exposed either to normoxia or to intermittent hypoxia. Therefore, we searched for other stress markers, which might characterize chronic sustained hypoxia from the other experimental conditions. Consistently with current knowledge (Askew 2002), plasma MDA, a systemic marker of oxidative stress, was significantly higher under all the three experimental conditions studied, demonstrating the presence of derangements in mitochondrial metabolism in both exercise training and chronic and intermittent hypoxia (Sen 2001; Magalhaes et al. 2005b; Guzy and Shumacher 2006). An antioxidant protein highly induced by hypoxia and by ROS is HO-1, a member of the HSP family (Morita et al. 1995; Ryter et al. 2006; Borger and Essig 1998). Although inducible, low levels of HO-1 are constitutively detectable in the skeletal muscle (Hunter et al. 2001; Ryter et al. 2006). This report shows for the first time that HO-1 protein levels were significantly increased in chronically hypoxic muscle, compared either to normoxic or to intermittently hypoxic ones. Furthermore, exposure to chronic hypoxia redistributed HO-1 immunoreactivity, insofar it increased the percentage of type 1 fibers positive for HO-1 immunolabeling. Such a result adds further evidence in support of a major involvement of type 1 fibers in the stress response evoked by exposure to chronic hypoxia. Like type 2A fibers, type 1 ones are rich in mitochondria. The activity of the mitochondrial enzyme cytochrome *c* oxidase (COX) appeared decreased after exposure to chronic hypoxia, probably due to a reduction in the mitochondrial number and despite of a significant increment in the mitochondrial transcripts of the I and II subunits, whereas none of these changes was detectable after exposure to intermittent hypoxia (Ripamonti et al. 2006). Since COX is a heme protein, we might speculate that its enhanced turnover and heme release represented an additional signal to upregulate HO-1, which also acts as controller of intracellular iron homeostasis (Ryter et al. 2006). Intracellular accumulation of heme in a large proportion of type 1 fibers, which are apparently unreactive for HO-1 in normoxic muscles, might increase further the oxidative challenge and, hence, enhance the HSP response, which could be visualized by increased immunoreactivity for both Hsp70 and HO-1 in the majority of type 1 myofibers of chronically hypoxic muscles.

An additional interesting finding of the present report concerns the differential Hsp70 expression in the skeletal muscle vasculature, observed in muscle cells of both arterial and venous vessels in trained muscles, whereas it occurred only in veins in hypoxic muscles. Increased Hsp70 expression in vascular muscle cells was described in rat hearts exposed to whole-body heat shock (Leger et al. 2000). Whereas the increase in body temperature may

accompany high-intensity exercise, exercise-induced Hsp70 expression can occur independently from changes in body temperature (Liu et al. 2006; Noble et al. 2006), and apparently, muscle heating to levels comparable to those occurring during exercise failed to induce a significant increase in Hsp70 levels in humans (Morton et al. 2007). We observed increased Hsp70 immunoreactivity of the vasculature also in muscles that apparently did not respond with an increase in total Hsp70 levels, such as the tibialis anterior of trained rats (see Fig. 4c). Although an analogous protocol of moderate intensity training (60 min run at 22 m/min treadmill speed, 10% slope) increased significantly the rectal temperature of about 2.8° ($P < 0.001$, our unpublished observations), we cannot exclude that oxidative stress, due to exercise-induced increased blood flux and supply, acted to upregulate Hsp70 expression in vascular cells. The increase in oxidative stress might also operate after exposure to hypoxia, despite of the compensatory increase in circulating hemoglobin (Ripamonti et al. 2006). The dramatic decrease in oxygen tension in the venous blood might be responsible for the selective increase in Hsp70 immunoreactivity displayed by the venous wall.

In conclusion, our results indicate that the persistent Hsp70 expression in fast skeletal muscles of the rat hindlimb, induced after chronic exposure to different stresses, reflects the type and the duration of the overimposed stress. Depending on the exposure to either training or chronic hypoxia or intermittent hypoxia, Hsp70 expression involves differently arterial and venous vessels and redistributes within type 1 and 2A fiber populations, in ways that appear related to the ability of the affected cells to respond to stress.

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