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RESEARCH ARTICLE

Fatiguing stimulation of one skeletal muscle triggers heat shock protein activation in several rat organs: the role of muscle innervation

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SUMMARY

We hypothesised that activation of muscle afferents by fatigue triggers a widespread activation of heat shock proteins (HSPs) in resting muscles and different organs. In anaesthetised rats, HSP25 and HSP70 levels were determined in both tibialis anterior (TA) and extensor digitorum longus (EDL) muscles and in the diaphragm, kidney and brain by ELISA, which mostly identifies phosphorylated HSP, and western blotting. One TA muscle was electrically stimulated and tissues were sampled 10 or 60 min after the stimulation had ended. The nerve supply to the stimulated TA or its counterpart in the contralateral limb was left intact or suppressed. In control rats, no muscle stimulation was performed and tissues were sampled at the same time points (10 or 60 min). After TA stimulation, ELISA showed an increased HSP25 content in the contralateral TA, EDL and diaphragm at 10 min but not at 60 min, and HSP70 increased in all sampled tissues at 60 min. Western blotting did not show any changes in HSP25 and HSP70 at 10 min, while at 60 min HSP25 increased in all sampled tissues except the brain and HSP70 was elevated in all tissues. Denervation of the contralateral non-stimulated limb suppressed HSP changes in TA and after denervation of the stimulated TA the widespread activation of HSPs in other organs was absent. Our data suggest that fatigue-induced activation of skeletal muscle afferents triggers an early increase in phosphorylated HSP25 in muscles and a delayed elevation of non-phosphorylated HSP25 and HSP70 in skeletal and respiratory muscles, kidney and brain.

Key words: muscle fatigue, heat shock protein, muscle afferent, oxidative stress.

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INTRODUCTION

The exercise-induced expression of heat shock proteins (HSPs) in skeletal muscle is well documented in rodents (Huey et al., 2010; Locke et al., 1990; McArdle et al., 2001; Skidmore et al., 1995) and also humans (Morton et al., 2006; Paulsen et al., 2007; Tupling et al., 2007). Plasma HSP levels also increase after static (Brerro-Saby et al., 2010) and dynamic exercise (Jammes et al., 2009) in healthy subjects. HSPs function to protect the cell against oxidative stress and provide protection against future insults (McArdle and Jackson, 2002).

Human studies suggest an extra-muscular origin of the exercise-induced increase in plasma HSP level. Walsh and colleagues have reported that the elevated plasma HSP72 level after strenuous exercise precedes the increase in HSP72 gene and protein expression in sampled working muscle (Walsh et al., 2001). A possible release of HSP72 from the brain following 180 min of cycling at 60% $\dot{V}_{O_2,max}$ has also been suggested (Lancaster et al., 2004).

Current data support the hypothesis of a role for the nervous system in HSP activation. Indeed, glutamate mediates HSP70 expression in the hippocampus (Ayala and Tapia, 2003) and NMDA receptor antagonists cause HSP70 expression in the cortex (Hashimot et al., 1997). Also, in the peripheral nervous system, noradrenaline (norepinephrine) stimulates the release of exomes containing HSP72 through the activation of adrenergic receptors (Johnson et al., 2005). Other studies have shown that the muscle nerve supply modulates HSP expression in resting muscle (Huey et al., 2005; Kato et al., 2002).

Possible candidates for the widespread activation of HSPs are the group III and IV muscle nerve afferents, also called the ‘metaboreceptors’, because they are activated by stressors responsible for HSP expression, i.e. muscle contraction at a high strength, hypoxia, ischaemia and the reactive oxygen species (Darques and Jammes, 1997; Decherchi et al., 1998; Delliaux et al., 2009; Rotto and Kaufman, 1988). The sensory pathways carried by the group III and IV muscle afferents control the circulatory response to fatiguing muscle contractions through a general sympathetic activation (Kaufman and Hayes, 2002) and modulate the motor drive not only of the working muscles but also of resting ones through their spinal and supraspinal projections (Degtyarenko and Kaufman MP, 2002; Ling et al., 2003). In a recent review (Whitham and Fortes, 2008), it was hypothesised that the muscle metaboreflex might participate in general HSP production but the role of muscle afferents in triggering this production has never been investigated.

We speculated that the activation of muscle afferents by fatiguing exercise may trigger a HSP response in non-contracting skeletal and respiratory muscles and major organs such as the kidney and brain. The presence of a widespread activation of chaperone proteins after leg exercise might have high clinical significance in sport and rehabilitation medicine. Thus, the benefits of exercise would not be limited to the working muscles and might concern the whole body.

In the present study, we tested the hypothesis that rhythmic electrical stimulation of one skeletal muscle, eliciting a fatiguing exercise, might produce a nerve signal able to trigger activation of

HSPs in contralateral resting muscles (tibialis anterior, TA; and extensor digitorum longus, EDL), a respiratory muscle (diaphragm) and major organs (kidney and brain). We explored different situations in which the nerves supplying the stimulated or the contralateral resting muscles were left intact or sectioned. We also verified that our protocol of electrically induced muscle fatigue elicited an oxidative stress, a potent stimulus for the group IV muscle afferents (Delliaux et al., 2009). The HSP25 and HSP70 content in the different tissues was measured using western blotting and ELISA, which explored the activation of non-phosphorylated and phosphorylated HSPs, respectively.

MATERIALS AND METHODS

Ethical approval

Animal experiments were performed in 114 adult (8–10 weeks old, 300–405 g) Sprague–Dawley rats (Iffa-Credo, Les Oncins, France). The protocol was approved by the Jean Roche Research Institute ethics committee of our faculty under licence no. C 13-055-8. All experiments, including surgery, monitoring and euthanasia, were conducted by physiologists authorised to perform animal surgery.

Animal care and general preparation

The rats were anaesthetised by an intra-peritoneal injection of sodium pentobarbital (Nembutal, 40 mg kg^{-1}, Sanofi-Aventis, Paris, France). The common carotid artery was cannulated to continuously measure arterial blood pressure with an electromanometer (Gould Statham P23 Db, Hato Rey, Puerto Rico, USA). The animals were ventilated at constant volume (10 ml kg^{-1}) and frequency (50 min^{-1}) with a Harvard volumetric pump. The inhaled gas mixture was 30% O_2 and 70% N_2. End-tidal O_2 and CO_2 fractions were measured with rapid pyrolytic (Gauthier, Paris, France) and infra-red gas analysers (Godart, The Netherlands), respectively. A heating pad maintained the rectal temperature in the range 37–38°C. Both during and after the operative procedure, the adequacy of the level of anaesthesia was judged from the changes in blood pressure and heart rate, and the absence of the corneal reflex and response to pain stimuli applied on the adipose pad of the animal's paw. The changes in circulatory variables and the re-appearance of reflex responses governed the injection of supplementary doses of sodium pentobarbital. At rest, the mean blood pressure remained stable (at 10 min, 128±8 mmHg; at 60 min, 118±5 mmHg). At the end of the experiments, the muscles and kidney were sampled and the rats were killed by an intra-arterial injection of a hyperosmolar potassium chloride solution. Then, the brain was sampled as a whole.

Experimental design

Four groups of rats were studied. In the three first groups, both TA muscles, the contralateral EDL muscle, the diaphragm, kidney and brain were sampled 10 or 60 min after the blood pressure had stabilised (Controls) and after the electrical stimulation had stopped (Tests).

Controls (*N*=48)

Resting oxidant/antioxidant status and HSP25 and HSP70 levels were determined in anaesthetised rats whose TA muscle was not stimulated. In an equal proportion of rats, the nerve supply to both TA muscles was left intact or a unilateral transection of the peroneal and sciatic nerves was performed. In each series (innervated or denervated TA), all tissues were sampled after 10 or 60 min. These control series served to determine whether: (1) the prolongation of anaesthesia constituted a stress influencing the HSP level and (2) the unilateral nerve section affected the HSP content.

Tests

There were three different test situations. (1) Stimulation of an innervated TA muscle (*N*=28), for which the peroneal and sciatic nerves were left intact. (2) Stimulation of a denervated TA muscle (*N*=28), for which both the peroneal and sciatic nerves were dissected then sectioned, and after a 10 min rest period the homolateral TA muscle was stimulated. (3) Stimulation of an innervated TA muscle while the contralateral TA muscle was denervated (*N*=10), and sampling concerned both TA muscles.

In all rats, HSP content was determined by ELISA. Western blotting was used to confirm the HSP changes in 7 animals in each group. Samples of TA, EDL, diaphragm, kidney and brain were obtained 10 and 60 min after TA stimulation.

Electrically induced muscle fatigue

As in our previous animal studies (Darques and Jammes, 1997; Decherchi et al., 1998; Delliaux et al., 2009), muscle stimulation consisted of rhythmic contractions of the TA muscle with the blood vessels intact. Two steel hook electrodes (inter-electrode distance 4 mm) were fixed in the belly of the muscle and contractions were produced by a neurostimulator through an isolation unit (Grass S8800, Quincy, MA, USA). Trains of rectangular pulses were delivered for a 10 min period (1 ms pulse duration, 10 Hz stimulation frequency, 500 ms train duration, 1000 ms rest period between two successive trains). In all cases, the voltage was twice that evoking the maximum force (maximum voltage 18 V). This pattern of stimulation frequency was chosen because it was known to elicit the highest metabolic changes (Darques et al., 2003), the strongest activation of the group IV muscle afferents (Darques and Jammes, 1997), and significant circulatory and respiratory responses (Decherchi et al., 2007). To measure the force output, the distal tendon was attached to an isometric strain gauge (Myograph F-60, Narco Bio-systems, Houston, TX, USA). Muscle fatigue always occurred during the 10 min muscle stimulation and the maximum fall of peak force (0.53±0.04 N) was 65±8%. To ensure that the unilateral electrical muscle stimulation did not elicit contraction of the contralateral muscles, in 7 animals we simultaneously recorded muscle force in both TA muscles. In these rats, the 'pressor reflex' was measured from the arterial blood pressure recording. Fig. 1 shows the changes in muscle force and arterial blood pressure during a 10 min muscle stimulation of an intact (Fig. 1A) and a denervated TA muscle (Fig. 1B).

Organ sampling and biochemical analyses

Immediately after sample collection, both TA muscles, the contralateral EDL, the diaphragm, kidneys and brain were divided between several aliquots (each aliquot corresponding to a specific assay), which were frozen in liquid nitrogen and then stored at –80°C until biochemical analysis. As in a previous rat study (Steinberg et al., 2004), the muscle concentration of two indicators of oxidant/antioxidant status were measured: thiobarbituric acid reactive substances (TBARS) and an endogenous antioxidant, the reduced ascorbic acid (RAA) present in rat hindlimb muscles (Graf et al., 1965; Wilson et al., 1996). Muscle extracts were homogenised in 5% trichloroacetic acid (TCA) according to a 1/4 mass/volume ratio with Ultra-Turrax T25 basic disperser (Ika-Werke, Staufen, Germany) at 24,000 r.p.m. The resulting mixtures were then centrifuged (10,000 ***g***) at 4°C for 15 min, and TBARS and RAA levels were measured in the supernatants. The muscle TBARS level was assessed according to a spectrofluorometric method (see Uchiyama and Mihara, 1978). In test tubes containing 200 µl of 5% TCA supernatants, we successively added 200 µl of 8.1% SDS,

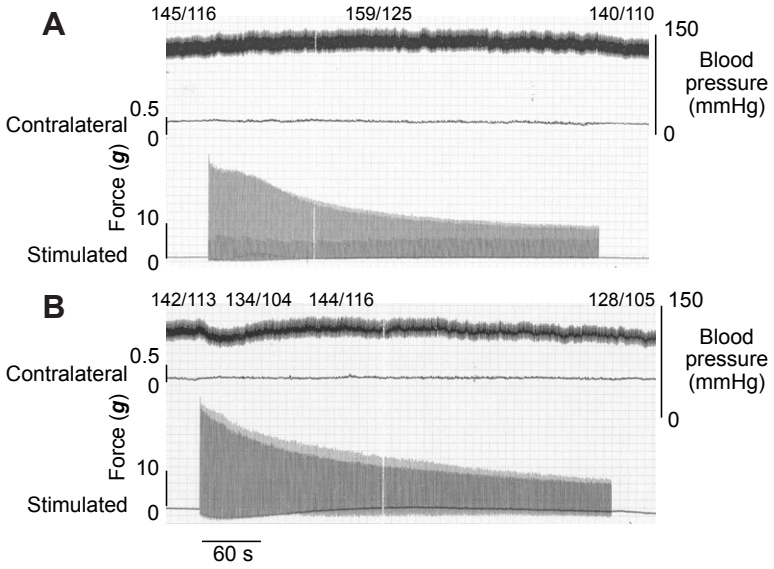

Fig. 1. Muscle force recorded in both tibialis anterior (TA) muscles during the 10 min muscle stimulation of one TA, and blood pressure measured in a carotid artery. Responses to the stimulation of an innervated (A) or denervated (B) TA muscle are shown as well as corresponding responses in the contralateral muscle. Mean values of systolic and diastolic blood pressure measured before the stimulation bout, at the instant of maximal change, and 1 min after the stimulation ended are indicated.

1.5 ml of 20% acetate buffer (pH 3.5), 1.5 ml of freshly prepared 0.8% thiobarbituric acid and 400 µl of an ethanolic solution of 0.1% butylated hydroxytoluene. Glass beads were added and the test tubes were then heated at 100°C for 60 min, then cooled in tap water at room temperature. We added 4 ml of *n*-butanol and 1 ml of water to each tube; the mixture was vortexed for 5 min, then centrifuged (2000 ***g***) for 3 min to obtain a rapid separation between organic and aqueous phases. The upper organic phase was pipetted off and the pink pigment was measured using a spectrofluorometer at an excitation wavelength of 515 nm and an emission wavelength of 553 nm (Shimadzu model RF-5031 PC, Kyoto, Japan). A standard curve of TBARS was obtained after overnight hydrolysis at room temperature of a solution containing 1 mmol tetraethoxypropane (Sigma-Aldrich, Saint Quentin Fallavier, France) in 100 ml of 0.1 mol l^{-1} HCl. The muscle RAA levels were estimated by spectrophotometry using a method based on the reduction of iron by ascorbic acid in the presence of orthophosphoric acid and α-α′-dipyridyl (Maickel, 1960). In a test tube containing 200 µl of muscle extract in 5% TCA, we added 60 µl of 0.5% orthophosphoric acid, 1 ml of 0.5% α-α′-dipyridyl and 200 µl of 1% ferric chloride. The mixture was vortexed then left for 10 min at room temperature in the dark. The optical density was measured at 525 nm on a spectrophotometer (Helios γ, Milton Roy Company, Rochester, NY, USA). A new standard curve was obtained with ascorbic acid in 5% TCA.

ELISAs

The HSP content in cell lysates of sampled muscles was related to the total protein content. Muscle aliquots were homogenised on ice in cold phosphate-buffered saline, pH 7.3, containing 0.5% Triton X-100, 18.6 g l^{-1} EDTA, 0.1 mmol l^{-1} PMSF and 1 µg ml^{-1} of complete mini protease inhibitor cocktail tablets with leupeptin, aprotinin and pepstatin (Roche Diagnostics, Meylan, France) according to a 1/4 mass/volume ratio with an Ultra-Turrax T25 basic disperser (Ika-Werke) at 24,000 r.p.m. The resulting mixture was then centrifuged (10,000 ***g***) at 4°C for 15 min and the total protein content and the phosposphorylated HSP25 and HSP70 levels were measured in the supernatants. The total muscle protein content was estimated by spectrophotometry using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). This method is based on the reduction of Cu^{2+} to Cu^{+} in the presence of bicinchoninic acid and proteins in alkaline medium. The resulting chromophore exhibits a characteristic purple colour at 562 nm. BSA allows the measurement of total protein content (mg protein g^{-1} of wet tissue). Phosphorylated HSP25 and HSP70 levels were determined with high-sensitivity ELISA kits dedicated to HSP measurement in rat tissues using polyconal IgG against HSP25 phosphorylated at Ser82 (Genway GWB-E2BCBF, San Diego, CA, USA) and against HSP70 phosphorylated at Tyr525 (Biorbyt orb5488, Cambridge, UK). All measurements were made in duplicate by spectrophotometry on a Statfax 3200 microplate reader (Awareness Technology, Inc., Palm City, FL, USA) using a point-by-point method, which allows a better estimation of HSP levels. The limits of detection of the assay were 0.44 and 0.78 ng ml^{-1} for HSP25 and HSP70, respectively. The HSP25 and the HSP70 levels are expressed in ng ml^{-1} of protein content.

Antibodies and western blotting

Primary mouse monoclonal antibodies specific for rat HSP25 (ADI-SPA-800) and HSP70 (ADI-SPA-810) were obtained from Enzo Life Sciences (supplied by Covalab S.A.S., Villeurbanne, France). The western blotting procedure was as previously described (By et al., 2010). Tissues from rat were immediately frozen at –20°C, lyophilised, crushed, and solubilised with 4% SDS aqueous solution. This was centrifuged (10,000 ***g***) for 10 min at room temperature. The pellet was discarded and the protein content of the solubilisate was tested by Pierce BCA protein assay kit; 60 µg of muscle solubilisate was diluted in 62.5 mmol l^{-1} Tris HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% Bromophenol Blue and 5% mercaptoethanol, sonicated for 10 min at 47 kHz and subjected to a standard electrophoresis procedure in a Mini Protean II system (Bio-Rad, Hercules, CA, USA) on a 12% acrylamide minigel. Proteins were transferred onto a PVDF membrane; the blotted membrane was placed into the blot holder of a protein detection system (Millipore, Billerica, MA, USA), saturated with non-fat dried milk and incubated for 20 min with 1/1000 dilution of primary mouse monoclonal antibody (anti-HSP25 and anti-HSP70). Blots were visualised by horseradish peroxidase-labelled anti-mouse IgG Fab-specific antibodies and enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce Biotechnology) using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY, USA). The staining intensities of

Table 1. Control HSP25 and HSP70 content measured by ELISA at 10 or 60 min when one limb was innervated or denervated

			10 min		60 min	
	Nerve	Rat no.	HSP25	HSP70	HSP25	HSP70
Tibialis anterior						
	Intact	16	13±1	54±4	16±2†	70±5†
	Severed	16	24±3*	85±4**	17±2†	68±4†
Extensor digitorum longus						
	Intact	8	40±4	62±9	46±3†	80±11†
	Severed	8	49±4*	75±9*	51±3†	92±10†
Diaphragm						
	Intact	8	37±9	41±6	48±7	45±6
	Severed	8	42±6	45±6	52±10	43±8
Kidney						
	Intact	8	65±3	57±7	56±9	51±7
	Severed	8	68±5	51±7	58±3	49±11
Brain						
	Intact	8	43±3	79±16	44±6	70±5
	Severed	8	40±5	70±15	46±6	65±10

HSP25 and the HSP70 levels are expressed in ng ml^{-1} of protein content.
Asterisks indicate a significant effect of denervation compared with the intact preparation (*P<0.05, **P<0.01).
Daggers indicate a significant difference in heat shock protein (HSP) values measured at 60 min compared with 10 min (†P<0.05.
Values are means ± s.e.m.

the bands were densitometrically measured with the public domain NIH Image software developed at the US National Institutes of Health.

Data analysis

Values are expressed as means ± 1 s.e.m. We used a two-way analysis of variance to evaluate, at the same time (10 or 60 min) in each situation, the changes in HSP levels elicited in the stimulated TA muscle and the contralateral resting muscles (TA, EDL) and other tissues (diaphragm, kidney, brain) compared with the corresponding control levels. The first factor was the muscle status (stimulated *versus* contralateral) and the second factor was the presence or absence of a unilateral muscle nerve section. A *post hoc* Student–Newman–Keuls test indicated the direction and the magnitude of the changes for the different conditions. Data processing was realised on absolute HSP levels with SigmaStat software (Jandel, Chicago, IL, USA). A difference was accepted as significant if P<0.05. Regression analysis was used to search for relationships between the HSP changes in the contralateral TA and the magnitude of force decay (i.e. fatigue) in the stimulated TA.

RESULTS

Control HSPs levels

Table 1 shows control values of HSP25 and HSP70 measured by ELISA in resting TA muscles and the different sampled tissues. We noted significant elevation of both HSP25 and HSP70 in TA and EDL sampled at 60 min but this effect of time was not found in the other tissues. We noted that TA had the lowest HSP25 content. Section of the TA nerve of one limb significantly increased the HSP25 and HSP70 levels in TA and EDL sampled at 10 min but these differences were no longer present at 60 min. Nerve section had no effect on the HSP content in the diaphragm, kidney and brain. Western blotting indicated that TA denervation significantly reduced total HSP25 (5.10±0.35% *versus* 7.90±0.38%; P<0.01) and HSP70 content (5.95±1.0% *versus* 7.71±1.02%; P<0.05) at 10 min only and had no effect on HSP content in the other tissues. It was noted that the unilateral hindlimb denervation elicited a transient (10 min), significant increase in mean blood pressure (+12±5%; P<0.05).

Response to unilateral muscle stimulation

Accompanying changes in physiological variables

During muscle stimulation, no change in force was measured in the contralateral TA muscle (Fig. 1). A modest but significant elevation of the mean arterial blood pressure (+18±4%; P<0.01) was noted in the innervated TA preparation but not when the denervated TA was stimulated.

Fatigue-induced oxidative stress

In both the innervated and denervated stimulated TA, oxidative stress, combining increased TBARS level and reduced RAA content, was measured 10 min after the stimulation bout had ended while at 60 min only an increase in TBARS was noted (Fig. 2). As shown in Fig. 2, no changes in TBARS and RAA level were measured in contralateral resting TA muscles.

Fatigue-induced HSP changes in TA muscles

In the innervated (Fig. 3A,E) or denervated (Fig. 3C) stimulated TA, the HSP content significantly increased but the time course of HSP25 and HSP70 changes differed: HSP25 peaked at 10 min, then disappeared, while the HSP70 response increased up to 60 min (Fig. 3A,E). Stimulation of the innervated TA also increased the HSP levels in the contralateral resting TA and the time course of the change in HSP followed that described in the stimulated muscle (Fig. 3B). After denervation of the stimulated TA (Fig. 3D) or of the contralateral hindlimb only (Fig. 3F), no significant changes in HSP level were noted in the contralateral resting TA. The HSP25 content in the contralateral innervated TA sampled at 10 min increased in proportion to the force decay measured in the stimulated muscle (Fig. 4). We did not find any correlation between HSP70 content and the force decay.

Fatigue-induced HSP changes in other tissues

Fig. 5 shows that stimulation of an innervated TA induced a significant early and transient increase in phosphorylated HSP25 content, which is the active form, in the contralateral EDL muscle and the diaphragm. The HSP70 content was significantly elevated at 60 min in all tissues (EDL, diaphragm, kidney and brain). After limb denervation, TA stimulation no longer induced HSP changes in all sampled tissues.

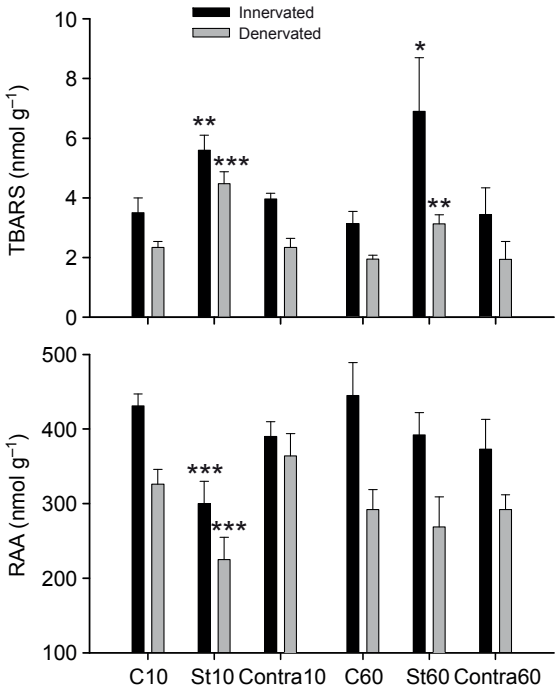

Fig. 2. Intramuscular concentrations of thiobarbituric acid reactive substances (TBARS) and reduced ascorbic acid (RAA) measured at 10 and 60 min in resting TA muscles (C10 and C60) and after the stimulation bout in the stimulated (St10 and St60) and contralateral TA muscles (Contra10 and Contra60) in a series in which the nerve supply to the stimulated muscle was left intact or suppressed. Asterisks denote significant differences from controls (*P<0.05, **P<0.01, ***P<0.001).

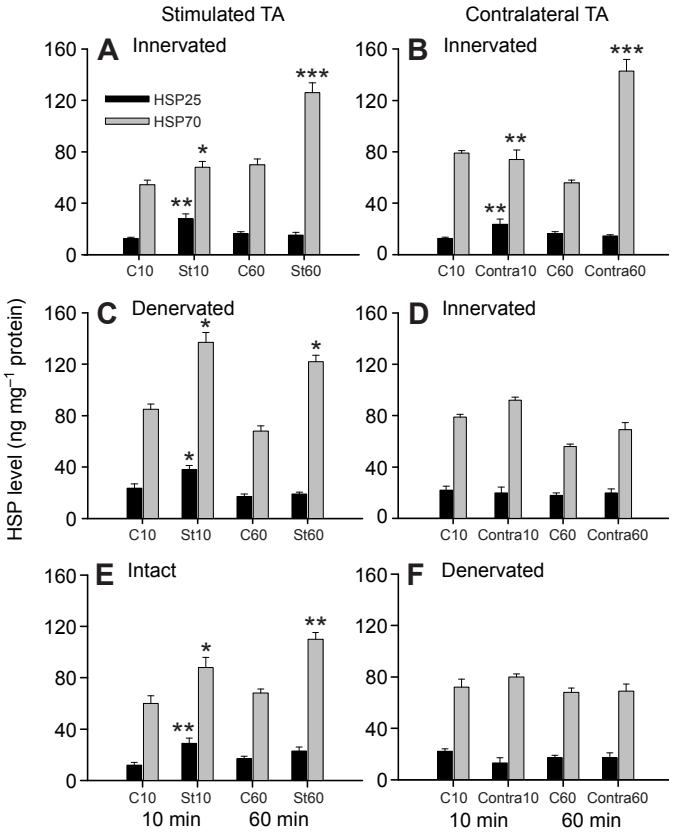

Fig. 3. Absolute values of HSP25 and HSP70 protein level given by ELISA in the three experimental conditions: (1) stimulation of innervated TA/innervated contralateral resting TA (A and B), (2) stimulation of denervated TA/innervated contralateral resting TA (C and D), (3) stimulation of innervated TA/denervated contralateral resting TA (E and F). In each panel, we report control HSP levels at 10 and 60 min in resting TA muscles (C10, C60) and HSP levels measured 10 and 60 min after the stimulation bout in the stimulated (St10 and St60) and contralateral TA muscles (Contra10 and Contra60). Asterisks denote significant differences between stimulation-induced HSP changes in stimulated or contralateral muscles and the corresponding control values (*P<0.05, **P<0.01, ***P<0.001).

Western blotting confirmed the suppression of HSP25 and HSP70 responses to TA stimulation when the stimulated TA was denervated (Figs 6, 7). However, western blotting showed that the HSP25 response to the stimulated innervated TA muscle slightly differed from that found by ELISA. Indeed, Fig. 7 shows that western blotting did not reveal an early HSP25 increase at 10 min in the different tissues, only a significant HSP25 elevation in the contralateral TA, EDL, diaphragm and kidney sampled at 60 min. As for ELISA, western blotting revealed a significant HSP70 increase in all tissues sampled at 60 min but not at 10 min.

DISCUSSION

The present study shows that with an intact nerve supply to both TA muscles, a 10 min fatiguing stimulation to one TA efficiently elicits a 'pressor' reflex (Fig. 1) and an oxidative stress in that muscle (Fig. 2), and increases the total HSP25 and HSP70 protein level in both TA muscles (Fig. 3), the contralateral EDL muscle, diaphragm, kidney and brain (Fig. 5). ELISA revealed that the phosphorylated HSP25 transiently increased in the contralateral TA, EDL and diaphragm 10 min after stimulation had stopped and both ELISA (Fig. 5) and western blotting (Fig. 7) showed elevated levels of HSP25 and HSP70 in all tissues sampled at 60 min. After denervation of the contralateral hindlimb, HSP levels continued to increase in the stimulated TA but the HSP response to TA stimulation no longer occurred in the contralateral resting TA (Figs 3 and 7). Denervation of the stimulated TA suppressed the HSP changes in all sampled tissues (Figs 5, 7). Together, these data suggest that the activation of muscle nerve afferents by a fatiguing stimulation elicits a HSP response in skeletal and respiratory muscles and also in the kidney and brain. In resting preparations, the denervation of hindlimb muscles transiently increased phosphorylated HSP25 and HSP70 content in TA and EDL but not in the diaphragm, kidney and brain (Table 1). In contrast, western blotting indicated a significant decrease in total HSP25 and HSP70 levels in the denervated TA. This observation strongly suggests that muscle nerve pathways might play a key role in baseline HSP regulation. The very early increase in HSP25 level cannot result from an increase in protein synthesis, which takes at least 1 h, but must be secondary to HSP activation by phosphorylation. Our ELISA results, which evaluate the phosphorylated (activated) form of HSP, indicate that this activation occurs a few minutes following muscle stimulation.

The purpose of our study was only to demonstrate the existence of fatigue-induced HSP activation in non-stimulated tissues and the involvement of afferent nerve pathways in this response. We did not focus our study on HSP translocation between the different cell compartments (cytosol, cytoskeleton) and/or mRNA synthesis (translational response), which takes several hours to be significant. Also, we did not examine the potential sources for HSP activation – the nerve terminals, blood vessels and muscle fibres themselves.

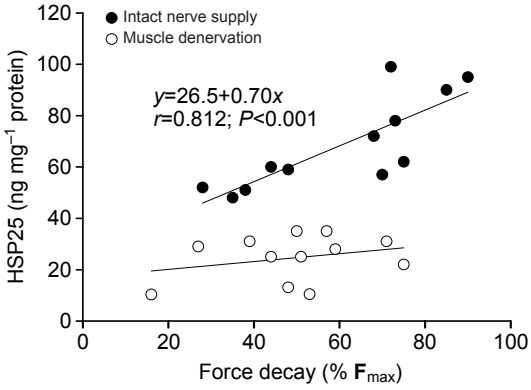

Fig. 4. HSP25 content from ELISAs in innervated contralateral resting TA muscles, sampled 10 min after electrical stimulation of the corresponding TA, plotted against the force decay in the fatigued muscle. No significant correlation was found when the nerve supply to the stimulated muscle was suppressed.

As in previous rat (Skidmore et al., 1995) and human studies (Bai et al., 2002; Huang et al., 2007; Matt et al., 2007), we used ELISA kits to measure the phosphorylated HSP contents in tissues. Our study was complemented by measuring the changes in non-phosphorylated HSP content using western blotting. HSP phosphorylation occurs rapidly in response to a variety of stresses, including hydrogen peroxide and other oxidants, and the phosphorylation status determines the capacity of HSPs to interact with different apoptotic proteins (Schmitt et al., 2007). In the present study, ELISA and western blotting revealed different responses to TA stimulation of HSP25 and HSP70. ELISA indicated that the phosphorylated HSP25 transiently increased at 10 min in all the sampled tissues whereas the phosphorylated HSP70 content increased at 60 min. In contrast, western blotting showed a delayed elevation of non-phosphorylated HSP25 and HSP70 in all the sampled tissues 1 h after the fatiguing TA stimulation bout. Thus, muscle fatigue seems to trigger a rapid and transient increase in phosphorylated HSP25 in all the sampled tissues and a delayed increase in non-phosphorylated HSPs. We did not find data in the literature on simultaneous measurement of phosphorylated and non-phosphorylated HSPs in muscles. Indeed, most previous studies used western blotting to measure the HSP content in muscle biopsies and reported a significant HSP70 elevation in working muscle 20 min (Locke et al., 1990) and 30 min (Skidmore et al., 1995) after a treadmill bout in rats and 30 min after an eccentric exercise in humans (Paulsen et al., 2007). ELISA measurements of plasma HSP27 and HSP70 content were performed in healthy humans and reported an early elevation of the phosphorylated HSP27 10 min after maximal static handgrip (Brerro-Saby et al., 2010) and maximal cycling exercise (Jammes et al., 2009) while the phosphorylated HSP70 response began at 60 min (Jammes et al., 2009). The early increase in phosphorylated HSP content in tissues might result from their damage. This could occur in the stimulated TA muscle and could also concern the contralateral muscles if the electrical stimulation spread over the contralateral limb. Because we measured no increase in muscle force and no oxidative stress in the contralateral TA during fatiguing stimulation of the corresponding muscle, any damage to the resting TA (and also probably EDL) can be disregarded. Moreover, we also measured a significant increase in phosphorylated HSP levels in the diaphragm, kidney and brain after TA stimulation, and there was no reason for these organs to become damaged in the stable anaesthetised animal preparation. An increase in HSP content could occur in hypoxic tissues during prolonged anaesthesia. This would partly explain the increased HSP content in all sampled tissues at 60 min. However, hypoxia-induced HSP changes should also occur in our rats where the stimulated TA muscle was denervated but no significant changes in HSPs were measured. Thus, at the present time we cannot propose any satisfactory explanation for the differences between the results obtained using ELISA and western blotting techniques.

Because the denervation of the stimulated TA abolished the HSP25 and HSP70 changes in resting TA and other tissues, whatever the method used to measure the HSP content, we concluded that the activation of sensory nervous pathways arising from the working muscle was solely responsible for the HSP response to muscle stimulation. Moreover, the absence of a HSP response in the contralateral denervated TA muscle demonstrated

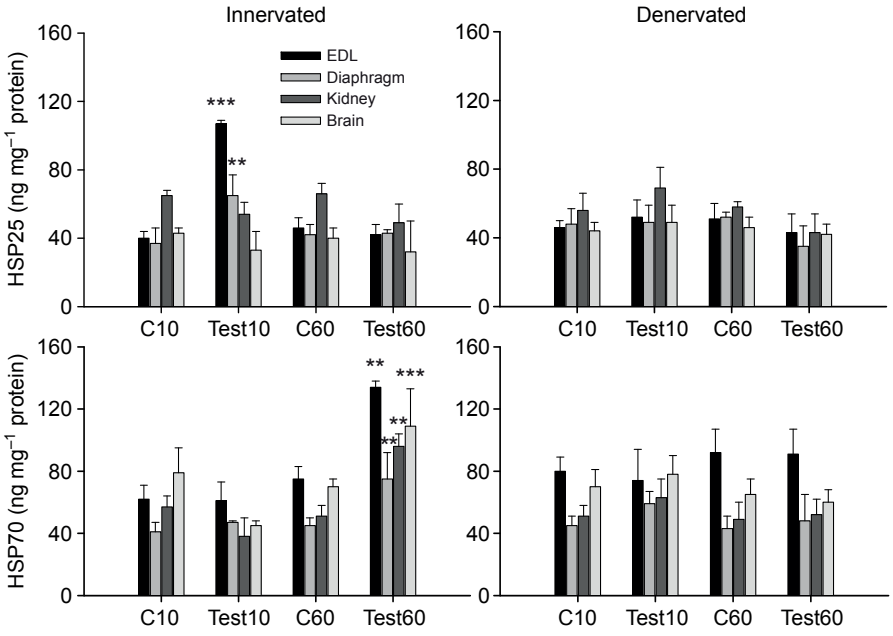

Fig. 5. ELISA measurement of HSP25 and HSP70 content in different tissues [extensor digitorum longus (EDL), diaphragm, kidney, brain] sampled 10 or 60 min after electrical stimulation of one innervated (left) or denervated (right) TA muscle. Asterisks denote significant differences between stimulation-induced HSP changes (Test10 or Test60) and the corresponding control values (C10 or C60) (**P<0.01, ***P<0.001).

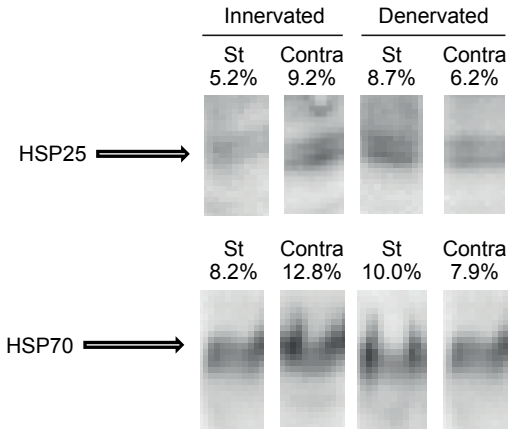

Fig. 6. Example of HSP25 and HSP70 content using western blotting in one stimulated innervated or denervated TA muscle (St) and the resting contralateral muscle (Contra). Sampling was performed 10 min (HSP25) or 60 min (HSP70) after the stimulation bout had ended, i.e. at the time of major changes in these protein levels. The relative HSP levels after TA stimulation are expressed as a percentage of baseline values measured in resting TA.

the existence of a motor arm of this reflex loop. In the absence of a rat series demonstrating the key role played by muscle innervation in the HSP response to exercise, it would be tempting to speculate that the circulating catecholamines explain the increase in HSP70 level in the different tissues. Indeed, it has previously been shown that the activation of α1-adrenoceptors results in a rapid increase in circulating HSP72 (Johnson et al., 2005). It must be noted that our protocol of muscle stimulation elicited an increase in HSP70 level in all sampled tissues but an elevated HSP25 content in just skeletal (TA, EDL) and respiratory muscle and the kidney, but not in the brain. This suggests the existence of different mechanisms for the induction of HSP25 and HSP70 responses.

Electrophysiological studies in animals have documented that fatigue (Darques and Jammes, 1997; Decherchi et al., 1998) and also the reactive oxygen species (Delliaux et al., 2009) activate the muscle nerve endings connected to the group III and IV afferents. The activation of these muscle afferents modifies the motor control to muscles (Garland and Kaufman, 1995) and activates the sympathetic pathways to peripheral vessels (Kaufman and Hayes, 2002; Rowell and O'Leary, 1990). In the present study, the stimulation of an innervated TA muscle elicited a modest but significant increase in the mean arterial blood pressure, an observation already reported in the same anaesthetised rat model (Decherchi et al., 2007). The exercise-induced 'pressor' reflex is not limited to limb muscles but also concerns the renal circulation (Koba et al., 2006) and this might explain our observation of a HSP response in muscles and kidney. We also report here that unilateral transection of the peroneal and sciatic nerves, which must transiently activate all nerve fibres, elicited a modest but significant increase in the mean arterial blood pressure (+12±5%). Thus, together these observations reveal that muscle nerve stimulation activates the sympathetic system in anaesthetised rats.

The present study also demonstrates that muscle innervation plays a key role in the baseline HSP levels in resting muscles. Indeed, unilateral nerve transection transiently increased the phosphorylated HSP25 and HSP70 levels but reduced the non-phosphorylated HSP content in resting limb muscles (TA and EDL). It must be emphasised that the effects of muscle denervation were localised to the hindlimbs and were absent in the other sampled tissues. Nerve section, which transiently increased the blood pressure, must activate all the afferent pathways in the severed nerve, including those involved in reflex sympathetic activation. Nerve section also stimulates the axons contained in the distal portion of the nerve trunk, eliciting the release of different neurotransmitters including noradrenaline. Only long-term effects of muscle denervation on HSP levels have been reported in the literature. Huey and colleagues found a significant decrease in phosphorylated HSP24 in denervated resting rat plantaris muscles sampled 1–28 days after nerve transection (Huey et al., 2005). Kato and co-workers also found that transection of the sciatic nerve trunk abolished the HSP27 response in disused muscles (Kato et al., 2002). The aforementioned studies, including ours, clearly show the existence of nerve activity-dependent processes for optimal HSP expression.

Our study shows that nerve pathways arising from an electrically stimulated muscle trigger HSP changes in contralateral resting skeletal muscles (TA, EDL), respiratory muscle and also the kidney and brain. A very recent study in rat pups (Daniels et al., 2012), using proteomic analysis, reports that exercise training in

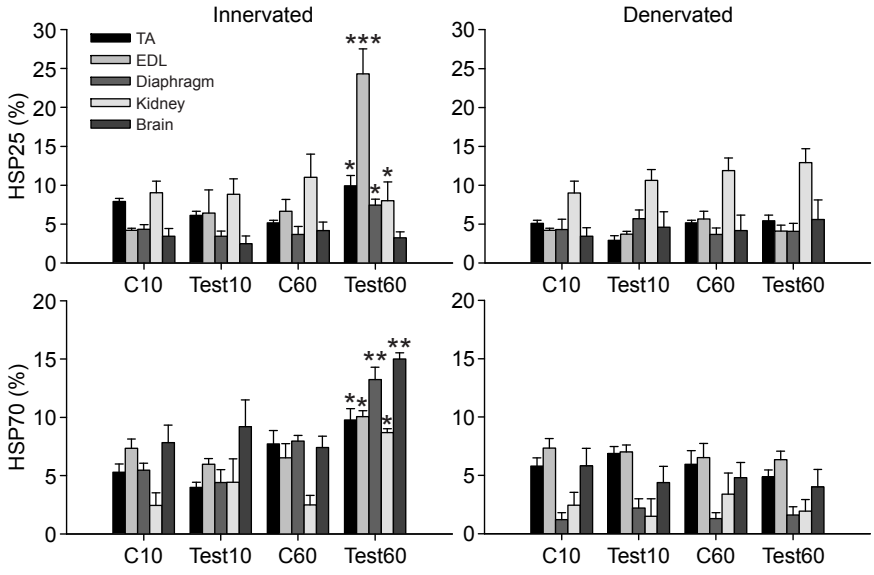

Fig. 7. Measurement of HSP25 and HSP70 using western blotting in contralateral TA, EDL, diaphragm, kidney and brain, 10 min (Test10) and 60 min (Test60) after unilateral stimulation of an innervated (left) or denervated (right) TA muscle. Asterisks denote significant differences between stimulation-induced HSP changes (Test10 and Test60) and the corresponding control values measured at 10 or 60 min (C10 and C60) (*P<0.05, **P<0.01, ***P<0.001).

adolescence counteracts the detrimental effects of maternal separation on the expression of chaperone proteins, which protect the brain against the oxidative stress. Further studies are needed to precisely identify the role played by the different muscle afferents and to verify the hypothesis of a role for sympathetic activation in the HSP response to fatiguing muscle contraction. Nevertheless, the presence of widespread activation of chaperone proteins after leg exercise might have high clinical significance in sport and rehabilitation medicine. It must be highlighted that the exercise-induced phosphorylation of HSPs, reported in the present study, might constitute a potential mechanism of controlling cytoskeletal assembly and remodelling (Ganote and Armstrong, 1993).

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