Revised

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

Yves Jammes[1], Jean Guillaume Steinberg[1], Youlet By[1], Christelle Brerro-Saby[2], Jocelyne Condo[1], Marine Olivier[2], Regis Guieu[1], and Stephane Delliaux[1].

1- UMR MD2, Faculty of Medicine, Aix-Marseille University, Marseille, France.

2 - Ecole de Podologie, Marseille, France

Corresponding author: Pr. Yves Jammes,

UMR MD2, Faculty of Medicine, Aix-Marseille University, Bd. Pierre Dramard, 13916 cedex 20 , Marseille, France

Email : yves.jammes@univ-amu.fr; Phone : 33 4 91 69 89 25 ; Fax : 33 4 91 69 89 27

Running title: Spread out HSP response to fatigue

Line number: 457 without references / 577 references included

Summary

We hypothesized that muscle afferents activation by fatigue may trigger a spread out activation of heat shock proteins (HSP) in resting muscles and different organs.

In anesthetized rats, HSP25 and HSP70 levels were determined in both *tibialis anterior* (TA) and *extensor digitorum longus* (EDL) muscles, diaphragm, kidney, and brain using ELISA kits, which mostly explore the phosphorylated HSP, and Western blotting (WB). One TA muscle was electrically stimulated and tissues sampled 10 (Test10) or 60 min (Test60) after the stimulation had ended. The nerve supply to the stimulated TA or its pair 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 (C10 and C60).

After TA stimulation, ELISA measured increased HSP25 content in contralateral TA, EDL, and diaphragm at Test 10 but not Test 60, and HSP70 increased in all sampled tissues at Test60. WB did not measure HSP25 and HSP70 variations at Test 10 while at Test 60 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 variations in TA and after denervation of the stimulated TA the spread out activation of HSPs in other organ 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 proteins – Muscle afferents – Oxidative stress

List of abbreviations:

EDL: *extensor digitorum longus* muscle

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

HSP: heat shock protein

Ig: immunoglobulin

NMDA: N-methyl D-aspartic acid

PMSF: phenylmethylsulfonyl fluoride

RAA: reduced ascorbic acid

TA: *tibialis anterior* muscle

TBARS: thiobarbituric acid reactive substances

TCA: trichloroacetic acid

VO_2max: maximal oxygen uptake

WB: western blotting

Introduction

The exercise-induced expression of heat shock proteins (HSP) 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). Increased plasma HSPs levels after static (Brerro-Saby et al., 2010) and dynamic exercise (Jammes et al., 2009) also occurs in healthy subjects. HSP functions to protect the cell against the oxidative stress and provide protection against future insults (McArdle et al., 2002).

Human observations suggest the existence of an extra-muscular origin of the exercise-induced increase in plasma HSP level. Walsh et al. (2001) have reported that the elevated plasma HSP72 level after a strenuous exercise preceded the increase in HSP72 gene and protein expression in sampled working muscle. Another observation by Lancaster and coworkers (2004) suggested a possible release of HSP72 from the brain following 180 min of cycling at 60% VO_2max.

Data support the hypothesis of a role played by the nervous system in HSPs activation. Indeed, in the central nervous system the glutamate mediates HSP70 expression in the hippocampus (Ayala et al., 2003) and NMDA-receptor antagonists cause HSP70 expression in the cortex (Hashimoto et al., 1997). Also in the peripheral nervous system, 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 the HSP expression in resting muscle (Huey et al., 2005; Kato et al., 2002).

The candidates for the spread out activation of HSPs might be the groups III and IV muscle nerve afferents, also called the “metaboreceptors”, because they are activated by stressors responsible for the HSPs expression, i.e., muscle contraction at a high strength,

hypoxia, ischemia, 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 groups 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 hypothesized that the muscle metaboreflex might participate to a general HSP production but the role of muscle afferents in triggering the HSP 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 as the kidney and brain. The presence of a spread out activation of chaperone proteins after leg exercise might have a 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 an activation of HSP 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 (WB) and

enzyme-linked immunosorbent assays (ELISA) which explored the activation of non phosphorylated and phosphorylated HSPs, respectively.

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 the licence number C 13-055-8. All experiments, including surgery, monitoring, and euthanasia, were conducted by physiologists authorized to perform animal surgery.

Animal care and general preparation.

The rats were anesthetized by an intra-peritoneal injection of sodium pentobarbital (Nembutal, 40mg.kg^{-1}, Sanofi-Aventis, France). The common carotid artery was cannulated to continuously measure arterial blood pressure with an electromanometer (Gould Statham P23 Db, Hato Rey, PR, 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 respectively measured with rapid pyrolytic (Gauthier, France) and infrared gas analysers (Godart, Netherlands). A heating pad maintained the rectal temperature in the range 37° to 38°C. Throughout and after the operative procedure, the adequacy of the level of anesthesia 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 pentobarbital sodium. At rest, the mean blood pressure remained stable (10th min: 128 + 8 mmHg; 60th min: 118 + 5 mmHg). At

the end of the experiments, after sampling the muscles and kidney 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 min or 60 min after the blood pressure had stabilized (Controls) and after the electrical stimulation had stopped (Tests).

- *Controls* (N = 48): resting oxidant/antioxidant status and HSP25 and HSP70 levels were determined in anesthetized rats whose the TA muscle was not stimulated. In an equal proportion of rats, the nerve supply to both TA muscles was left intact or an unilateral transection of the peroneal and sciatic nerves was performed. In each series (innervated or denervated TA), all tissues were sampled at the 10^{th} min or 60^{th} min (C10, C60). These control series served to determine if: 1) the prolongation of anesthesia might constitute a stress influencing the HSP level and 2) the unilateral nerve section might affect the HSP content.

- *Tests*

* *Stimulation of an innervated TA muscle (N = 28):* the peroneal and sciatic nerves were left intact.

* *Stimulation of a denervated TA muscle (N = 28):* both the peroneal and sciatic nerves were dissected then sectioned, and after a 10-min rest period, the homolateral TA muscle was stimulated.

* *Stimulation of an innervated TA muscle while the contralateral TA muscle was denervated (N = 10*). Sampling was limited to both TA muscles.

In all rats, HSPs content was determined by immunoassay (ELISA). Western blotting was used to confirm the HSP changes in 7 animals in each group.

Samples at the 10^{th} and 60^{th} min of stimulated TA were denominated St10 and St60, respectively, and of contralateral TA Contra 10 and Contra 60. Samples of other tissues (EDL, diaphragm, kidney, and brain) after TA stimulation were called Test 10 and Test 60.

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 in rhythmic contractions of the *tibialis anterior* 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; train duration: 500 ms; 1000-ms rest period between two successive trains). In all cases, the voltage was twice that evoking the maximal force (maximal 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, U.S.A). Muscle fatigue always occurred during the 10-min muscle stimulation and the maximal fall of peak force (0.53 + 0.04 N) was 65 + 8 %. To be ensured that the unilateral electrical muscle stimulation did not elicit a contraction of contralateral muscles, in seven animals we simultaneously recorded the muscle force in both TA muscles. In these rats, the “pressor reflex” was measured from the arterial blood pressure recording. Figure 1 shows the changes in muscle force and arterial blood pressure

during a 10-min muscle stimulation of an intact (upper part), then a denervated TA muscle (lower part).

Organ sampling and biochemical analyses

Immediately after sample collection, both TA muscles, the contralateral EDL, the diaphragm, kidneys, and brain were cut in several aliquots (each aliquot corresponding to a specific assay) which were frozen in liquid nitrogen and then stored at -80°C until biochemical analyses. As in a previous rat study (Steinberg et al., 2004), the muscle concentration of two indicators of the oxidant/antioxidant status were measured: the 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 homogeneized in 5% trichloroacetic acid (TCA) according to a ¼ weight/volume ratio with Ultra-Turrax T25 basic disperser (Ika-Werke, Staufen, Germany) at 24 000 rpm. The resultant mixtures were then centrifuged (10 000 g) at 4°C for 15 min, and the TBARS and RAA levels were measured in supernatants. The muscle TBARS level was assessed according to the spectrofluorometric method by Uchiyama and Mihara (1978). In test tubes containing 200 µl of 5% TCA supernatants, we added successively 200 µl of 8.1% sodium dodecylsulfate, 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. The test tubes containing glass beads were heated at 100°C for 60 min and then cooled in tap water at room temperature. Then, we added 4 ml of n-butanol and 1 ml of water in each tube. After vortexing for 5 min., the mixture was 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 of tetraethoxypropane (Sigma-Aldrich Co., Saint Quentin Fallavier, France) in 100 ml of 0.1N HCl. The muscle RAA levels were estimated by spectrophotometry using the 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. After vortexing, the mixture was 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, New York). A freshly-prepared standard curve was obtained with ascorbic acid in 5% trichloroacetic acid.

Immunoassays.

The HSP content in cell lysates of sampled muscles were reported to the total protein content. Muscle aliquots were homogeneized on ice in cold phosphate buffer saline, pH 7.3, containing 0.5% Triton X100, 18.6 g/l EDTA, 0.1 mM PMSF and 1 µg/ml of Complete Mini Protease Inhibitor Cocktail tablets with leupeptin, aprotinin and pepstatine (Roche Diagnostics, Meylan, France) according to a ¼ weight/volume ratio with an Ultra-Turrax T25 basic disperser (Ika-Werke, Staufen, Germany) at 24 000 rpm. The resultant mixtures were 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). This method is based on the reduction of Cu^{++} in Cu^{+} in the presence of bicinchoninic acid and proteins in alkaline medium. The resulting chromophore exhibits a characteristic purple color at 562 nm. A Bovine Serum Albumine (BSA) allows the measurement of total protein content (mg

protein.g^{-1} of wet tissue). Phosphorylated HSP25 and HSP70 levels were determined with high-sensitive enzyme-linked immunosorbent assay ELISA kits dedicated to HSP measurements in rat tissues (Using polyconal antibodies IgG against HSP25 phosphorylated at Ser 82 and anti phospho HSP 70, phosphorylated at Tyr 525; Genway GWB-E2BCBF). All measurements were made in duplicate by spectrophtometry on a Statfax 3200 micoplate reader (Awareness Technology, Inc., Palm City, FL, USA) using a point-by-point method which allows a better estimation of the HSPs levels. HSP25 levels were measured with the ImmunoSet HSP25 (rodent) ELISA development set from Enzo Life Sciences (supplied by Covalab S.A.S., Villeurbanne, France). The limits of detection of the assay were 0.44 ng.ml^{-1} and 0.78 ng.ml^{-1} for HSP 25 and HSP70, respectively. The HSP25 and the HSP70 levels were expressed in ng.mg^{-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 (Villeurbanne, France). Western blotting procedure as previously described (By et al., 2010). Tissues from rat were immediately freezed at -20°C, lyophilisated, crushed, and solubilised with 4% SDS aqueous solution. After centrifugation (10.000 x g) for 10 min at room temperature, the pellet was discarded and the protein content of solubilisate was tested by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). 60 µg of muscle solubilisate were diluted in 62.5 mM 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 standard electrophoresis procedure in Mini Protean II system (Bio-Rad, Hercules, CA). Separated proteins in 12% acrylamide minigel were transferred onto a PVDF membrane. Blotted membrane was placed into the blot holder of the protein detection system (Millipore, Billerica, MA), saturated with non fat dried milk and

incubated 20 min with 1/1000 dilution of primary mouse monoclonal antibodies, anti-HSP25 and anti-HSP70. Blots were visualized by horse-radish peroxydase labelled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak Image Station 440CF (Eastman Kodak Company, Rochester, NY). The staining intensities of the bands were densitometrically measured with the public domain NIH Image software developed at the US National Institutes of Health.

Data analyses.

Values are expressed as their mean + one SEM. 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 foreign tissues (diaphragm, kidney, brain) compared to the corresponding control levels. The first factor was the muscle status (stimulated vs. contralateral) and the second factor was the presence or absence of an unilateral muscle nerve section. A post-hoc Student-Newman-Keuls test indicated the direction and the magnitude of the variations between the different conditions. Data processing was realized on absolute HSP level values with software (SigmaStat, Jandel, Chicago, Illinois). 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 the 60th min but this effect of time was not found in the other tissues. We noted that TA had the lowest HSP25 content. The section of one limb muscle nerve significantly increased the HSP25 and HSP70 levels in TA and EDL sampled at the 10th min but these differences no longer persisted at the 60th min. The 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 % vs. 7.90 + 0.38 %; $p < 0.01$) and HSP70 contents (5.95 + 1.0 vs. 7.71 + 1.02; $p < 0.05$) at the 10th min only and had no effect on HSPs contents in the other tissues. It was noted that the unilateral hind limb denervation elicited a transient (10 min), significant increase in the mean blood pressure (+ 12 + 5%; $p < 0.05$).

Response to unilateral muscle stimulation.

- *Accompanying changes in physiological variables* (Fig. 1): during muscle stimulation, no change in force was measured in the contralateral TA muscle. A modest but significant elevation of the mean arterial blood pressure (+ 18 + 4%; $p < 0.01$) was present 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 an oxidative stress, combining increased TBARS level and reduced RAA content, was measured 10 min after the stimulation bout had ended while a TBARS increase was only noted at the 60th min (Fig. 2). As shown in Fig. 2, no changes in TBARS and RAA levels were measured in contralateral resting TA muscles.

- *Fatigue-induced HSPs variations in TA muscles*: in the innervated (Figs. 3A and 3E) or denervated (Fig. 3C) stimulated TA, the HSP content significantly increased but the time course of HSP25 and HSP70 changes differed: the peak HSP25 culminated at the 10th min, then disappeared, while the HSP70 response progressed at the 60th min (Figs. 3A and 3E). The stimulation of the innervated TA also increased the HSP levels in the contralateral

resting TA and the time course of HSP variations followed that described in the stimulated muscle (Fig. 3B). After denervation of the stimulated TA (Fig. 3D) or of the contralateral hind limb only (Fig. 3F) no significant changes in HSP levels were noted in the contralateral resting TA. The HSP25 content in the contralateral innervated TA sampled at the 10^{th} min increased in proportion of the force decay measured in the stimulated muscle (Fig. 4). We did not find any correlation between the HSP70 content and the force decay.

- *Fatigue-induced HSPs changes in other tissues:* Fig. 5 shows that the 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 the 60^{th} min in all tissues (EDL, diaphragm, kidney, and brain). After limb denervation, the TA stimulation no more induced HSP changes in all sampled tissues.

Western blotting showed that the HSP25 response to the stimulated innervated TA muscle slightly differed from those given by ELISA. Indeed, Figure 7 shows that Western blotting did not measure an early HSP25 increase at the 10^{th} min in the different tissues but only a significant HSP25 elevation in contralateral TA, EDL, and diaphragm sampled at the 60^{th} min. As ELISA, Western blotting measured a significant HSP70 increase in all tissues sampled at the 60^{th} min but not at the 10^{th} min. Despite these differences, Western blotting confirmed the suppression of HSP25 and HSP70 responses to TA stimulation when the stimulated TA was denervated (Figs. 6 and 7).

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), increases the total HSP25 and HSP70 proteins

levels in both TA muscles (Fig. 3), contralateral EDL muscle, diaphragm, kidney and brain (Fig. 5). ELISA revealed that the phosphorylated HSP25 transiently increased in contralateral TA, EDL, and diaphragm 10 min after the 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 the 60^{th} min. After denervation of the contralateral hind limb, HSP levels continued to increase in the stimulated TA but the HSP response to TA stimulation no more 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 and 7). Together, these data suggest that the activation of muscle nerve afferents by a fatiguing stimulation participates to a HSP response in skeletal and respiratory muscles and also in the kidney and brain. In resting preparations, the denervation of hind limb muscles transiently increased the phosphorylated HSP25 and HSP70 content in TA and EDL but not in the diaphragm, kidney, and brain (Table 1). On the opposite, Western blotting indicated significant decrease in total HSP25 and HSP70 levels in the denervated TA. This observation strongly suggests that muscle nerve pathways might play key role in baseline HSP regulation. The very early increase in HSP25 level can not result from an increase in protein synthesis, which needs at least one hour, but must be secondary to the HSP activation by phosphorylation. Indeed, our immunologic methods evaluate the phosphorylated (activated) form of HSP. Our results indicate that this activation occurs in the 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 needs several hours to be significant. Also, we did not examine the potential sources

for the HSP activation that are the nerve terminals, blood vessels, and the muscle fibres themselves. As in previous rat (Inaguma et al., 1993; 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 completed by measuring the changes in non phosphorylated HSP content using Western blotting. HSP phosphorylation rapidly occurs in response to a variety of stresses, including the hydrogen peroxide and other oxidants, and the phosphorylation status determines the capacity of HSP 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 indicates that the phosphorylated HSP25 transiently increased at the 10^{th} min in all the sampled tissues whereas the phosphorylated HSP70 content increased at the 60^{th} min. On the other hand, WB shows a delayed elevation of non phosphorylated HSP25 and HSP70 in all the sampled tissues one hour 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 measurements of phosphorylated and non phosphorylated HSPs in muscles. Indeed, most of previous studies used WB 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 contents 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 the 60^{th} min (Jammes et al., 2009). The early increase in phosphorylated HSP content in tissues might result from

their damage. This might occur in the stimulated TA muscle and could also concern the contralateral muscles if the electrical stimulation would spread over the contralateral limb. Because we demonstrated that no increase in muscle force and no oxidative stress were measurable in the contralateral TA during fatiguing stimulation of the pair muscle, any damage of the resting TA (and also probably EDL) can be discarded. Moreover, we also measured a significant increase in phosphorylated HSPs levels in the diaphragm, kidney, and brain after TA stimulation and there was no reason for any damage of these organs in stable anesthetized animal preparation. An increase in HSPs content could happen in hypoxic tissues during prolonged anesthesia. This could partly explain the increased HSP content in all sampled tissues at the 60^{th} min. However, hypoxia-induced HSP changes should be also measured in our rats where the stimulated TA muscle was denervated and no significant HSPs variations were then measured. Thus, at the present time we can not propose any satisfactory explanation for the obtained differences between both ELISA and WB 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 HSP response in contralateral denervated TA muscle demonstrated the existence of a motor arm of this reflex loop. In the absence of rat series demonstrating the key role played by the muscle innervation in the HSP response to exercise, it would be tempting to speculate that the circulating catecholamines might explain the increase in HSP70 level in the different tissues. Indeed, it was already shown that the activation of alpha1-adrenoceptors resulted 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 solely an elevated HSP25 content in 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 groups 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 anesthetized 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 here reported that the unilateral transection of the peroneal and sciatic nerves, which must transiently activates all nerve fibres, elicited a modest but significant increase in the mean arterial blood pressure (+ 12 + 5%). Thus, the aforementioned observations converge to show up that muscle nerve stimulation activates the sympathetic system in anesthetized rats.

The present study also demonstrates that the muscle innervation plays a key role in the baseline HSPs levels in resting muscles. Indeed, an 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 underlined that the effects of muscle denervation were localized to the hind limbs and absent in the other sampled tissues. The 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 the norepinephrine. Only long term effects of muscle denervation on HSPs levels were reported in the literature. Huey et al. (2005) reported a significant decrease in phosphorylated HSP24 in denervated resting rat *plantaris* muscles sampled 1 to 28 days after nerve transection. Kato and co-workers (2002) also found that transection of the sciatic nerve trunk abolished the HSP27 response in disused muscles. The aforementioned studies, including ours, clearly showed the existence of nerve-activity dependent processes for an optimal HSP expression.

Our study shows that nerve pathways arising from an electrically stimulated muscle trigger HSP variations in contralateral resting skeletal muscles (TA, EDL), in respiratory muscle, and also in the kidney and brain. A very recent study in rat pups (Daniels et al., 2012), using proteomic analysis, reports that exercise training in 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 precise the role played by the different muscle afferents and to verify the hypothesis of a participation of sympathetic activation in the HSP response to fatiguing muscle contraction. Anyway, the presence of spread out activation of chaperone proteins after leg exercise might have a high clinical significance in sport and rehabilitation medicine. It must be underlined that the exercise-induced phosphorylation of HSP, reported in the present study, might constitute a potential mechanism of controlling cytoskeletal assembly and remodeling (Ganote and Armstrong, 1993).

References

- Ayala, G.W. and Tapia, R. (2003). Expression of heat shock protein 70 induced by 4-aminopyridine through glutamate-mediated excitotoxic stress in rat hippocampus in vivo. *Neuropharmacology* **45**, 649-660.

- Bai, L., Koopmann, J., Fiola, C., Fournier, P., Schirrmacher, V. (2002). Dendritic cells pulsed with viral oncolysates potently stimulate autologous T cells from cancer patients. *Int. J. Oncol*. **21**, 685-694

- Brerro-Saby, C., Delliaux, S., Steinberg, J.G., Boussuges, A., Gole, Y., Jammes, Y. (2010). Combination of two oxidant stressors suppresses the oxidative stress and enhances the heat shock protein 27 response in healthy humans. *Metabolism* **59**, 879-886.

- By, Y., Durand-Gorde, J.M., Condo, J., Lejeune, P.J., Fenouillet, E., Guieu, R., Ruf, J. (2010) Monoclonal antibody-assisted stimulation of adenosine A2A receptors induces simultaneous downregulation of CXCR4 and CCR5 on CD4+ T-cells. *Hum. Immunol.* **71**, 1073-1076

- Daniels, W.M.U., Marais, L., Stein, D.J., Russell, V. (2012). Exercise normalizes altered expression of proteins in the ventral hippocampus of rats subjected to maternal separation. *Exp. Physiol.* **97**, 239-247.

- Darques, J.L., Bendahan, D., Roussel, M., Giannesini, B., Tagliarini, F., Le Fur, Y., Cozzone, P.J., Jammes, Y. (2003). Combined in situ analysis of metabolic and myoelectrical changes associated with electrically induced fatigue. *J. Appl. Physiol.* **95**, 1476-1484.

- Darques, J.L. and Jammes, Y. (1997). Fatigue-induced changes in group IV muscle afferent activity: differences between high- and low-frequency electrically induced fatigues. *Brain Res.* **750**, 47-54.

- Decherchi, P., Darques, J.L., Jammes, Y. (1998). Modifications of afferent activities from tibialis anterior muscle in rat by tendon vibrations, increase of interstitial potassium or lactate concentration and electrically-induced fatigue. *J. Periph. Nerv. Syst.* **3**, 267-276.

- Decherchi, P., Dousset, E., Jammes, Y. (2007). Respiratory and cardiovascular responses evoked by tibialis anterior muscle afferent fibers in rats. *Exp. Brain. Res.* **183**, 299-312.

- Degtyarenko, A.M. and Kaufman, M.P. (2002). Spinoreticular neurons that receive group III input are inhibited by MLR stimulation. *J. Appl. Physiol.* **93**, 92-98.

- Delliaux, S., Brerro-Saby, C., Steinberg, J.G., Jammes, Y. (2009). Reactive oxygen species activate the group IV muscle afferents in resting and exercising muscle in rats. *Pflugers Arch* **459**, 143-150.

- Ganote, C. and Armstrong, S. (1993). Ischaemia and the myocyte cytoskeleton: review and speculation. *Cardiovasc. Res.* **27**, 1387-1403.

- Garland, S.J. and Kaufman, M.P. (1995). Role of muscle afferents in the inhibition of motoneurons during fatigue. *Adv. Exp. Med. Biol.* **384**, 271-278.

- Graf, G.L.A., Hudson, A.J., Strickland, K.P. (1965). Changes in the relative amounts of ascorbic acid and glycogen in denervated rat gastrocnemius muscle. *Biochem. Cell. Biol.* **43**, 705-710.

- Hashimoto, K., Tomitaka, S., Bi, Y., Narita, Y., Iyo, M. (1997). Rolipram, a selective phosphodiesterase type-IV inhibitor, prevents induction of heat shock protein HSP-70 and HSP-79 mRNA in rat retrosplenial cortex by the NMDA receptor antagonist dizocilpine. *Eur. J. Neurosci.* **9**, 1891-1901.

- Huang, W., Fileta, J.B., Filippopoulos, T., Ray, A., Dobberfuhl, A., Grosskreutz, C.L. (2007). Hsp27 phosphorylation in experimental glaucoma. *Invest. Ophthalmol. Vis. Sci.* **48**, 4129-4135.

- Huey, K.A., Hyatt, J.P.K., Zhong, H., Roy, R.R. (2005). Effects of innervation state on Hsp24 content and phosphorylation in inactive rat plantaris muscle. *Acta Physiol. Scand.* **185**, 219-227.

- Huey, K.A., Burdette, S., Zhong, H., Roy, R.R. (2010). Early response of heat shock proteins to functional overload of the soleus and plantaris in rats and mice. *Exp. Physiol.* **95**, 1145-1155.

- Jammes, Y., Steinberg, J.G., Delliaux, S., Brégeon, F. (2009). Chronic fatigue syndrome combines increased exercise-induced oxidative stress and reduced cytokine and Hsp responses. *J. Intern. Med.* **266**, 196-206.

- Johnson, J.D., Campisi, J., Sharkey, C.M., Kennedy, S.L., Nickerson, M., Fleshner, M. (2005). Adrenergic receptors mediate stress-induced elevations in extracellular Hsp72. *J. Appl. Physiol.* **99**, 1789-1795.

- Kato, K., Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y. (2002). Innervation-dependent phosphorylation and accumulation of αB-crystallin and HSP27 as soluble complexes in disused muscle. *The FASEB Journal* **16**, 1432-1434.

- Kaufman, M.P. and Hayes, S.G. (2002). The exercise pressor reflex. *Clin. Auton. Res.* **12**, 429-439.

- Koba, S., Yoshida, T., Hayashi, N. (2006). Renal sympathetic and circulatory responses to activation of the exercise pressor reflex in rats. *Exp. Physiol.* **91**, 111-119.

- Lancaster, G.I., Moller, K., Nielsen, B., Secher, N.H., Febbraio, M.A., Nibo, L. (2004). Exercise induces the release of heat shock protein 72 from the human brain in vivo. *Cell Stress Chaperones* **9**, 276-280.

- Ling, L.J., Honda, T., Shimada, Y., Ozaki, N., Shiraishi, Y., Sugiura, Y. (2003). Central projection of unmyelinated (C) primary afferent fibers from gastrocnemius muscle in the guinea pig. *J. Comp. Neurol.* **461**, 140-150.

- Locke, M., Noble, E.G., Atkinson, B.G. (1990). Exercising mammals synthesize stress proteins. *Am. J. Physiol. Cell. Physiol.* **258**, C723-C779.

- Maickel, R.P. (1960). A rapid procedure for the determination of adrenal ascorbic acid: application of the Sullivan and Clarke method to tissues. *Anal. Biochem.* **1**, 498–501.

- Matt, P., Fu, Z., Carrel, T., Huso, D.L., Dirnhofer, S., Lefkovits, I., Zerkowski, H.R., Van Eyk, J.E. (2007). Proteomic alterations in heat shock protein 27 and identification of phosphoproteins in ascending aortic aneurysm associated with bicuspid and tricuspid aortic valve. *J. Mol. Cell. Cardiol.* **43**, 792-801.

- McArdle, A., Pattwell, D., Vasilaki, A., Griffiths, R.D., Jackson, M.J. (2001). Contractile activity-induced oxidative stress: cellular origin and adaptive response. *Am. J. Physiol. Cell. Physiol.* **280**, C621-C627.

- McArdle, A. and Jackson, M. (2002). Stress proteins and exercise-induced muscle damage. In *Exercise and Stress Response. The Role of Stress Proteins* (ed. M. Locke and E.G. Noble), pp 137-150, Boca Raton, FL: CRC.

- Morton, J.P., Don P.M., Cable, N.T., Bongers, T., Griffiths, R.D., Campbell, I.T., Evans, L., Kayani, A., McArdle, A., Drust, B. (2006). Time course and differential response of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J. Appl. Physiol.* **101**, 176-182.

- Paulsen, G., Vissing, K., Kalhovde, J.M., Ugelstad, I., Bayer, M.L., Kadi, F., Schjerling, P., Hallén, J., Raastad, T. (2007). Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R844-853.

559 - Rotto, D.M. and Kaufman, M.P. (1988). Effect of metabolic products of muscular
560 contraction on discharge of group III and IV afferents. *J. Appl. Physiol.* **64**, 2306-2313.
561 - Rowell, L.B. and O'Leary, D.S. (1990). Reflex control of the circulation during exercise:
562 chemoreflexes and metaboreflexes. *J. Appl. Physiol.* **69**, 407-418.
563 - Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., Garrido, C. (2007). Intracellular
564 and extracellular functions of heat shock proteins: repercussions in cancer therapy. *J.*
565 *Leukoc. Biol.* **81**,15-27.
566 - Skidmore, R., Gutierrez, J.A., Guerrio, V., Kregal, K.C. (1995). HSP70 induction during
567 exercise and heat stress in rats: role of internal temperature. *Am. J. Physiol. Regul. Integr.*
568 *Comp. Physiol.* **268**, R92-R97.
569 - Steinberg, J.G., Faucher, M., Guillot, C., Kipson, N., Badier, M., Jammes, Y. (2004).
570 Depressed fatigue-induced oxidative stress in chronic hypoxemic humans and rats. *Respir.*
571 *Physiol. Neurobiol.* **141**, 179-189.
572 - Tupling, A.R., Bombardier, E., Stewart, R.D., Vigna, C., Aqui, A.E. (2007). Muscle fiber
573 type-specific response to HSP70 expression in human quadriceps following acute isometric
574 exercise. *J. Appl. Physiol.* **103**, 2105-2111.
575 - Uchiyama, M. and Mihara, M. (1978). Determination of malonaldehyde precursor in
576 tissues by thiobarbituric acid test. *Anal. Biochem.* **86**, 271-278.
577 - Walsh, R.C., Koukoulas, I., Garnham, A., Moseley, P.L., Hargreaves, M., Febbraio, M.A.
578 (2001). Exercise increases serum HSP-72 in humans. *Cell Stress Chaperones* **6**, 386-393.
579 - Whitham, M. and Fortes, M.B. (2008). Heat shock protein 72: release and biological
580 significance during exercise. *Frontiers in Bioscience* **13**, 1328-1339.
581 - Wilson, J.X., Dixon, S.J., Yu, J., Nees, S., Tyml, K. (1996). Ascorbate uptake by
582 microvascular endothelial cells of rat sketelal muscle. *Microcirculation* **3**, 211-221.
583

Legends of illustrations:

Figure 1: Tracings of muscle force recorded in both tibialis anterior (TA) muscles during the 10-min muscle stimulation of one TA and the blood pressure measured in a carotid artery. Panels A and B respectively show the responses to the stimulation of an innervated or denervated TA muscle. Mean values of systolic and diastolic blood pressures measured before the stimulation bout, at the instant of maximal variations, and 1 min after the stimulation had ended are indicated.

Figure 2: Intramuscular concentrations of TBARS and RAA measured in resting TA muscles (C10 and C60) and after the stimulation bout in the stimulated (St10 and St60) and contralateral TA muscles (Contra 10 and Contra 60) in series in which the nerve supply to the stimulated muscle was left intact or suppressed. Asterisks denote significant differences with controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 3: Absolute values of HSP25 and HSP70 protein levels 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 reported control HSP levels in resting TA muscles (C10, C60)) and HSP content measured after the stimulation bout in the stimulated (St10 and St60) and contralateral TA muscles (Contra 10 and Contra 60). Asterisks denote significant differences between stimulation-induced HSP changes in stimulated or contralateral muscles and the corresponding control values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 4: HSP25 content given by immunoassays in innervated contralateral resting TA muscles, sampled 10 min after electrical stimulation of the pair stimulated 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.

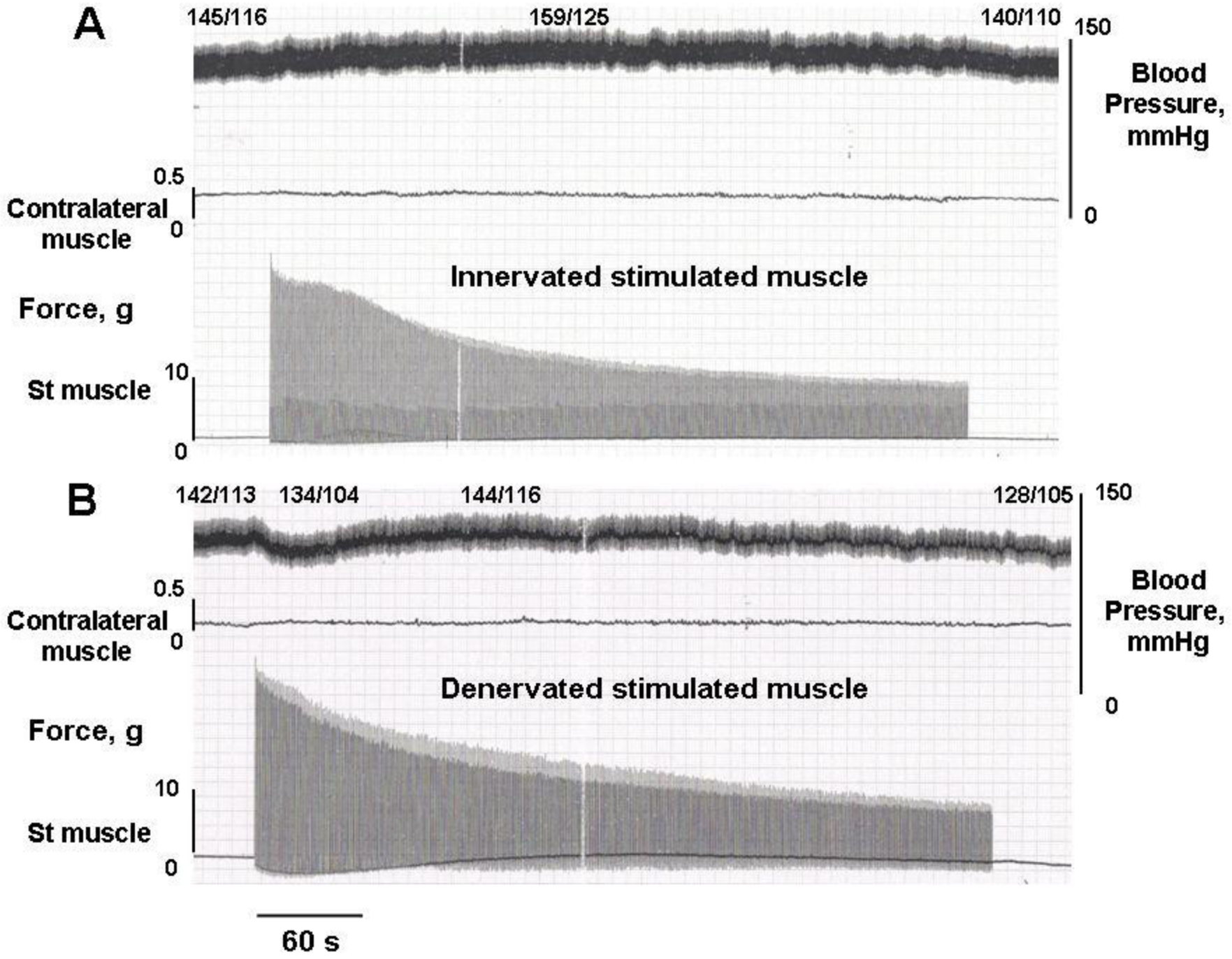
Figure 5: ELISA measurement of HSP25 and HSP70 contents in different tissues (*extensor digitorum longus* or EDL, diaphragm, kidney, brain) sampled 10 or 60 min after electrical stimulation of one innervated or denervated tibialis anterior (TA) muscle. Asterisks denote significant differences between stimulation-induced HSP changes (Test condition) and the corresponding control values (C10 or C60) (** $p < 0.01$; *** $p < 0.001$).

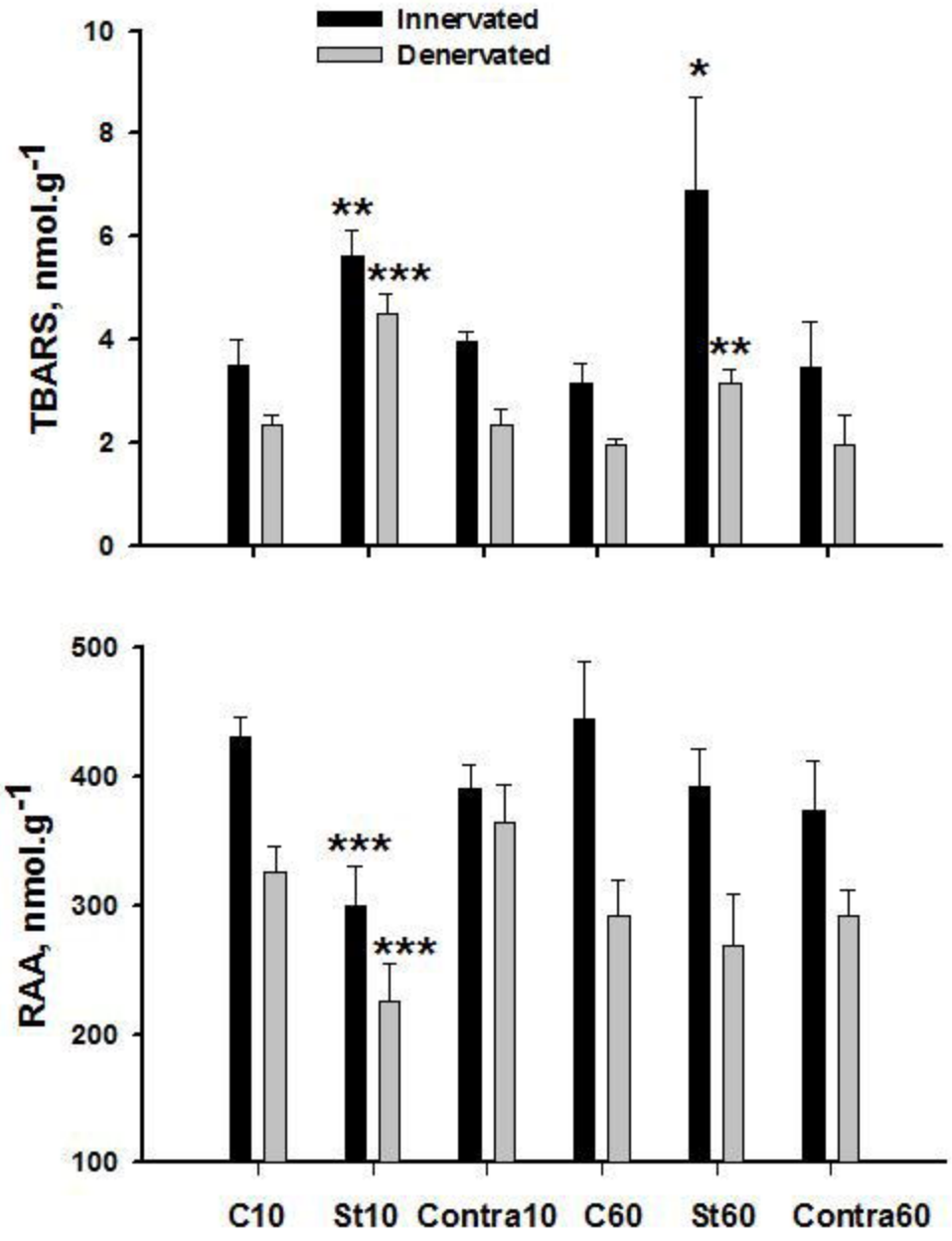
Figure 6: Example of measurements of HSP25 and HSP70 content using Western blotting in one stimulated innervated (left panels) or denervated (right panels) *tibialis anterior* (TA) muscles (St) and the resting contralateral pair muscles (Contra). Sampling was performed 10 min or 60 min after the stimulation bout had ended for HSP25 and HSP70, respectively, i.e., at the instants of major changes in these protein levels. The relative HSP variations after TA stimulation are expressed in percentage of baseline values measured in resting TA.

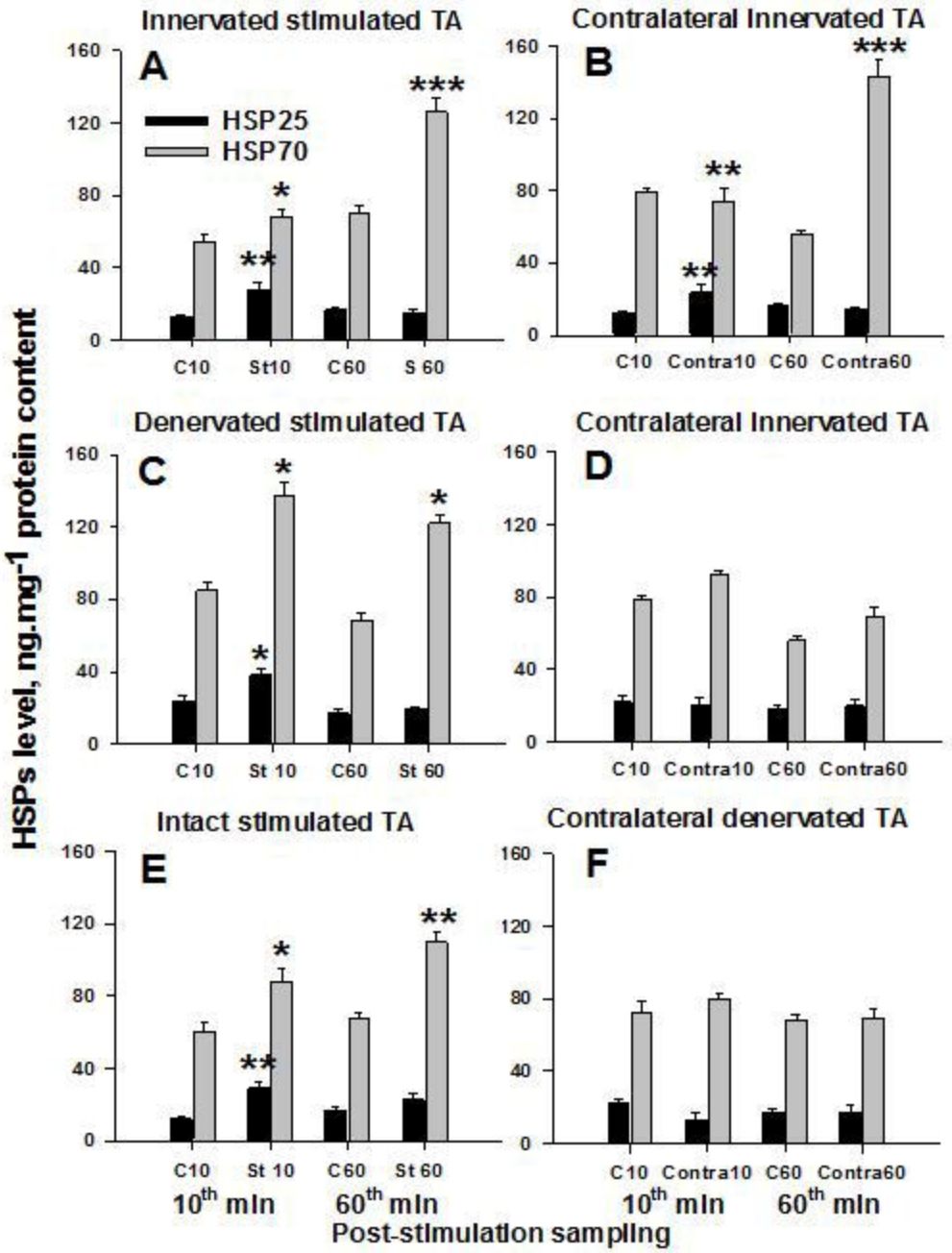
Figure 7: Measurements of HSP25 and HSP70 using Western blotting in contralateral TA and EDL muscles, diaphragm, kidney, and brain 10 min (Test 10) and 60 min (Test 60) after unilateral stimulation of an innervated (left panels) or denervated (right panels) TA muscle. Asterisks denote significant differences between stimulation-induced HSP changes (Test condition) and the corresponding control values measured at the 10^{th} min (C10) or 60^{th} min (C60) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

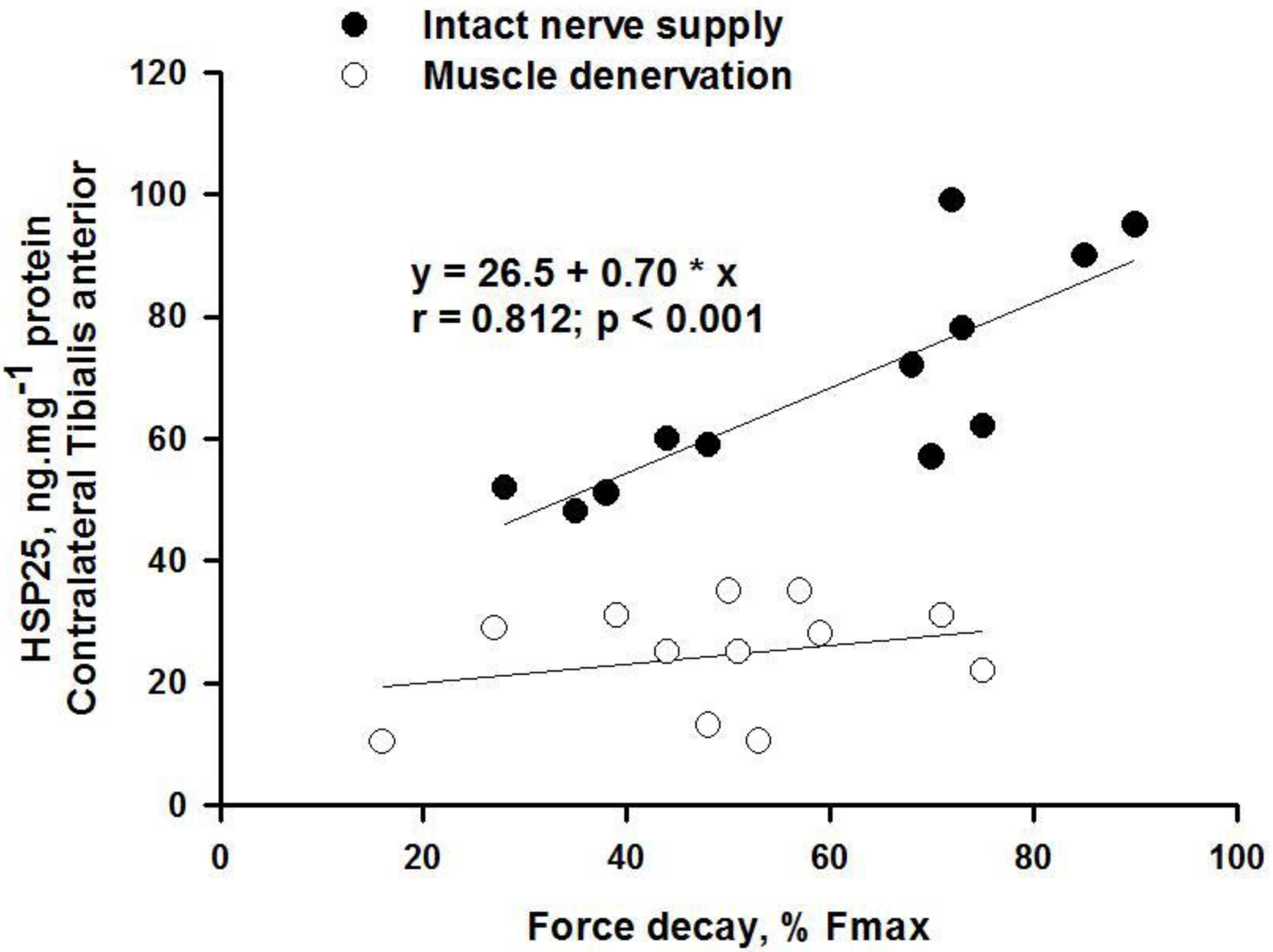
Table 1: Control values of HSP25 and HSP70 contents measured by immunoassays (ELISA) in the different tissues sampled at the 10^{th} or 60^{th} min when one limb was innervated or denervated. Symbol # indicates significant effect of denervation compared to the intact preparation (# $p < 0.05$; ## $p < 0.01$). Symbol $ indicate significant difference in HSP values measured at the 60^{th} min compared to the 10^{th} min ($ $p < 0.05$; $$ $p < 0.01$). Values are the mean ± SEM.

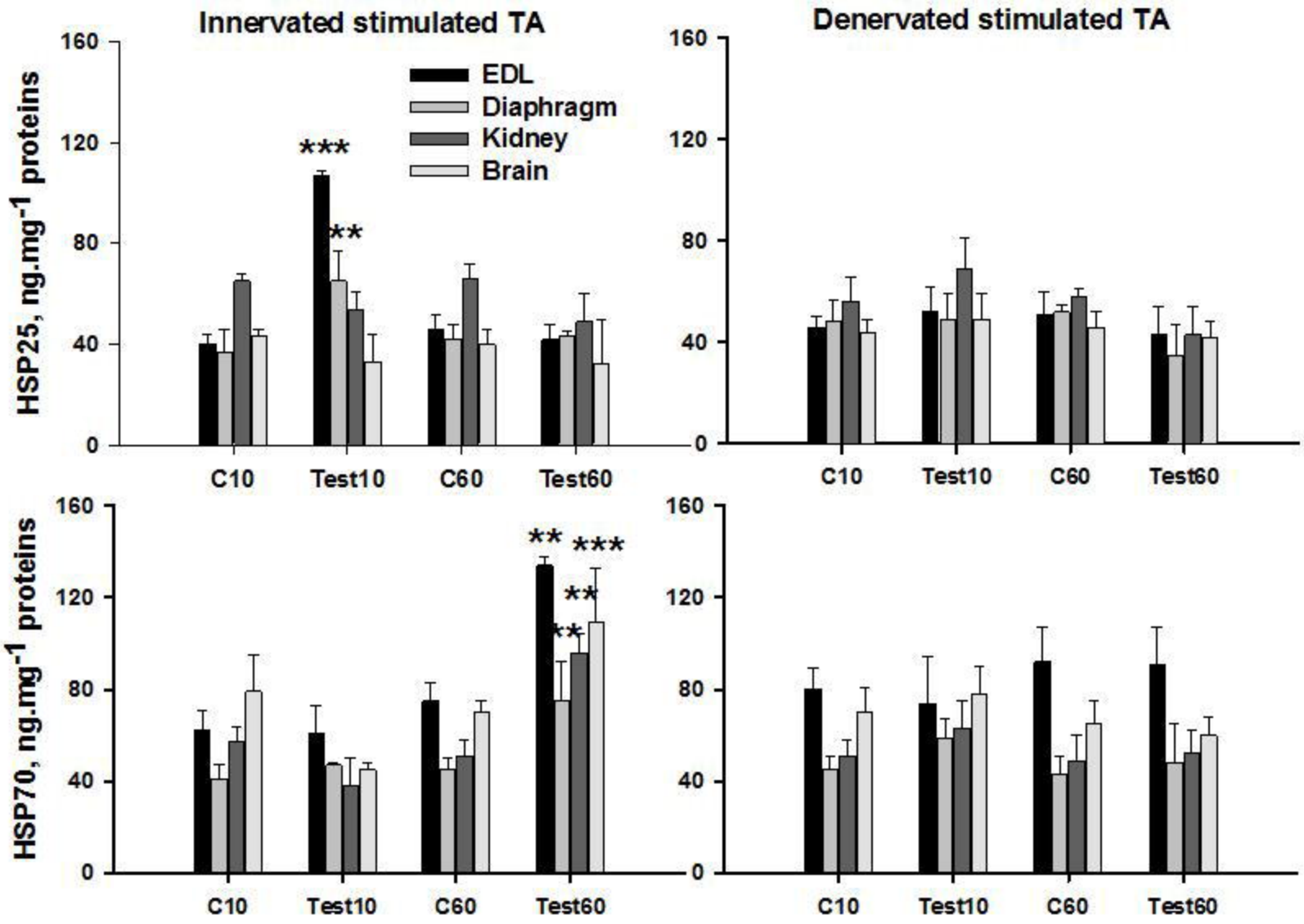
	Rat number	10^{th} min		60^{th} min	
		HSP25	HSP70	HSP25	HSP70
		ng.mg^{-1} proteins content			
Intact nerves	16	13 ± 1	54 ± 4	16 ± 2 $	70 ± 5 $
Tibialis anterior					
Severed nerves	16	24 ± 3 #	85 ± 4 ##	17 ± 2 $	68 ± 4 $
Intact nerves	8	40 ± 4	62 ± 9	46 ± 3 $	80 ± 11 $
Extensor digitorum longus					
Severed nerves	8	49 ± 4 #	75 ± 9 #	51 ± 3 $	92 ± 10 $
Intact nerves	8	37 ± 9	41 ± 6	48 ± 7	45 ± 6
Diaphragm					
Severed nerves	8	42 ± 6	45 ± 6	52 ± 10	43 ± 8
Intact nerves	8	65 ± 3	57 ± 7	56 ± 9	51 ± 7
Kidney					
Severed nerves	8	68 ± 5	51 ± 7	58 ± 3	49 ± 11
Intact nerves	8	43 ± 3	79 ± 16	44 ± 6	70 ± 5
Brain					
Severed nerves	8	40 ± 5	70 ± 15	46 ± 6	65 ± 10

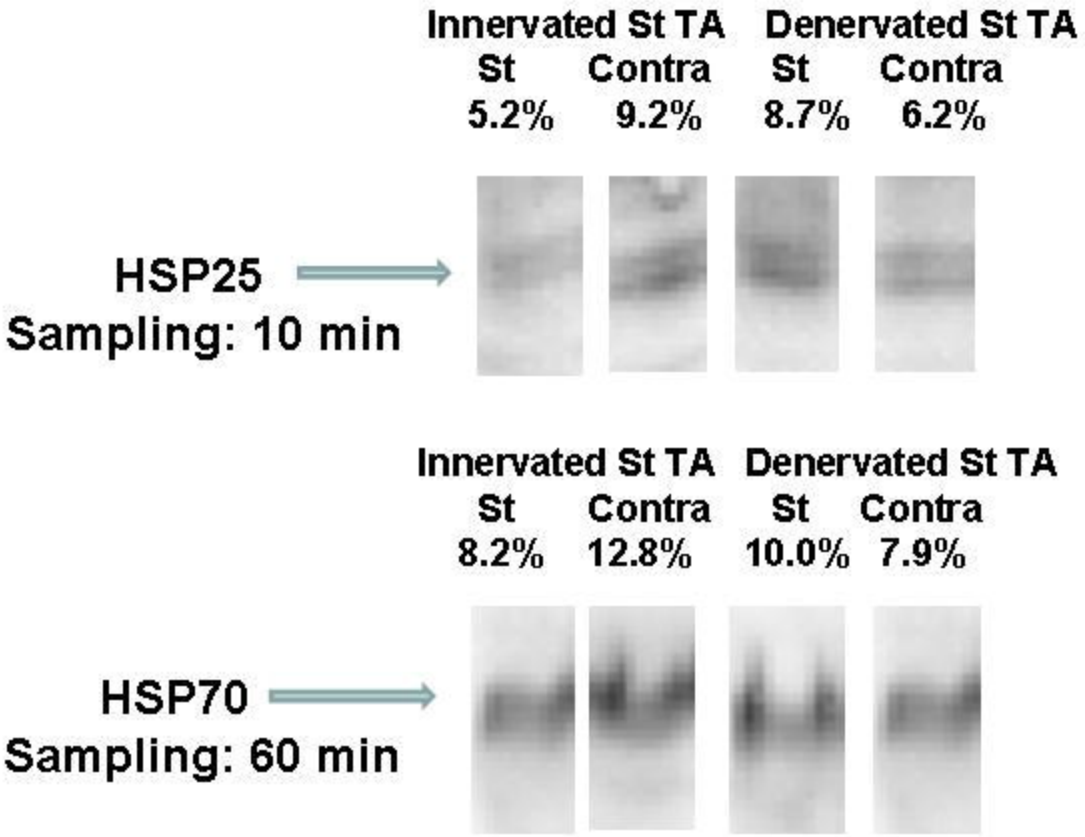

A
145/116
159/125
140/110
150
Blood Pressure, mmHg
0
0.5
Contralateral muscle
0
Innervated stimulated muscle
Force, g
St muscle
10
0
B
142/113
134/104
144/116
128/105
150
Blood Pressure, mmHg
0
0.5
Contralateral muscle
0
Denervated stimulated muscle
Force, g
10
St muscle
0
60 s

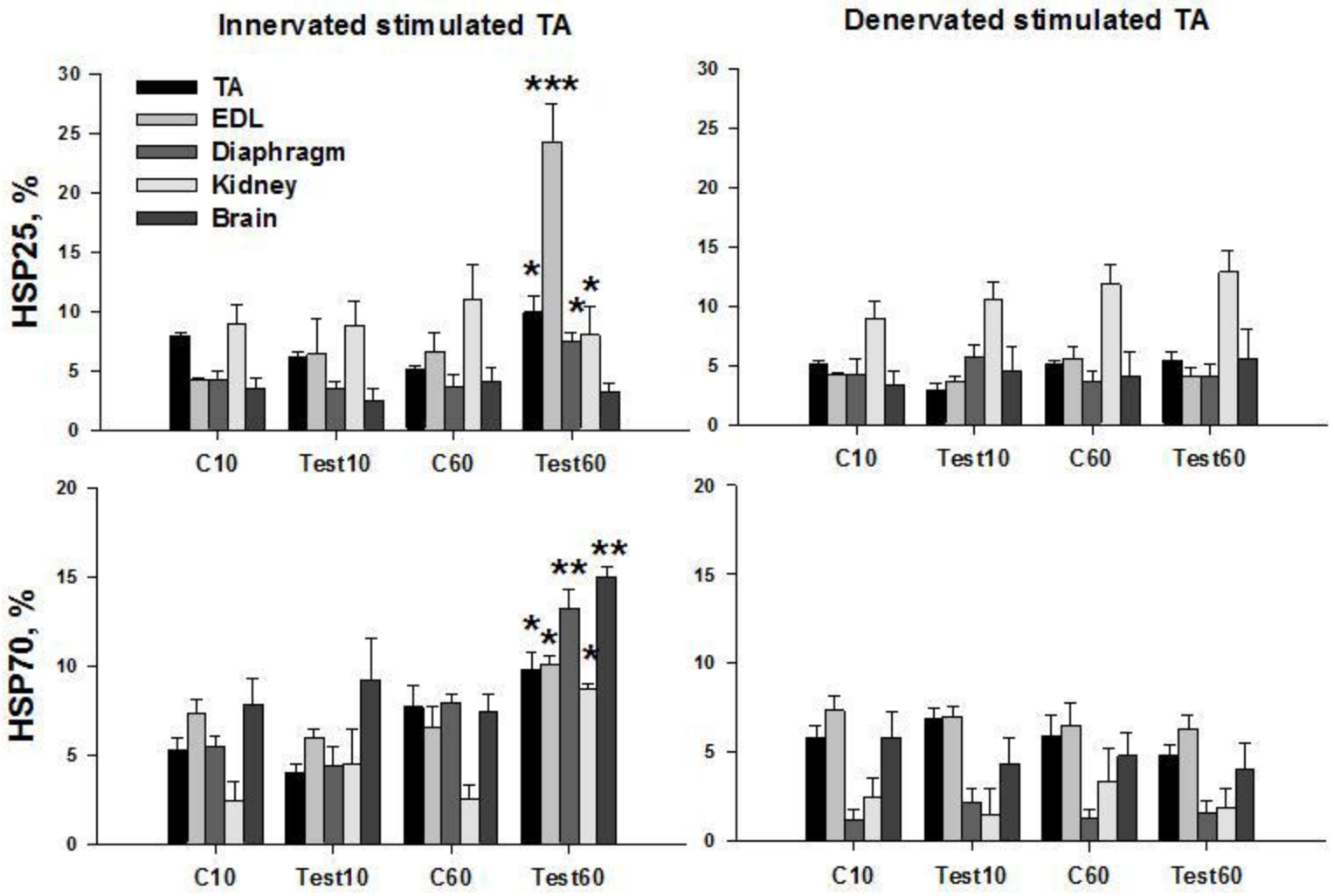

Innervated
Denervated
TBARS, nmol.g-1
10
8
6
4
2
0
**
***
*
**
RAA, nmol.g-1
500
400
300
200
100
***
***
C10
St10
Contra10
C60
St60
Contra60


Innervated stimulated TA
Contralateral Innervated TA
Denervated stimulated TA
Contralateral Innervated TA
Intact stimulated TA
Contralateral denervated TA
A
B
C
D
E
F
HSP25
HSP70
HSPs level, ng.mg-1 protein content
C10
St10
C60
S 60
Contra10
Contra60
St 10
St 60
10th min
60th min
Post-stimulation sampling


Intact nerve supply
Muscle denervation
y = 26.5 + 0.70 * x
r = 0.812; p < 0.001
HSP25, ng.mg-1 protein
Contralateral Tibialis anterior
Force decay, % Fmax
0
20
40
60
80
100
120


Innervated stimulated TA
Denervated stimulated TA
EDL
Diaphragm
Kidney
Brain
HSP25, ng.mg-1 proteins
HSP70, ng.mg-1 proteins
0
40
80
120
160
C10
Test10
C60
Test60
***
**
**
**
***
**


Innervated St TA
St
5.2%
Contra
9.2%
Denervated St TA
St
8.7%
Contra
6.2%
HSP25
Sampling: 10 min
Innervated St TA
St
8.2%
Contra
12.8%
Denervated St TA
St
10.0%
Contra
7.9%
HSP70
Sampling: 60 min


Innervated stimulated TA
Denervated stimulated TA
TA
EDL
Diaphragm
Kidney
Brain
HSP25, %
HSP70, %
C10
Test10
C60
Test60
*
**
***