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RESEARCH ARTICLE

The mechanisms of the widespread production of phosphorylated HSP25 after fatiguing muscle stimulation

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SUMMARY

We previously showed that a widespread heat shock protein (HSP) response to fatigue of a single hindlimb muscle was responsible for a global adaptive response to an acute localized stress. We also demonstrated that the HSP response resulted from the activation of nerve afferents from the stimulated muscle. However, we did not examine the role played by the different muscle afferents or the efferent arm of HSP response. In the present study we measured the changes in phosphorylated HSP25 (pHSP25) levels in resting hindlimb muscles and the diaphragm, kidney and brain in response to a fatiguing stimulation of one tibialis anterior muscle that was repeated in five series of experiments: (1) intact muscle innervation, (2) during the selective procaine block of conduction in group IV muscle afferents, (3) after muscle nerve transection to suppress all the sensory messages, and under pharmacological blockade of the (4) alpha-adrenergic or (5) glutamatergic neurotransmission. The data showed that: (1) the pHSP25 response in hindlimb muscles resulted from the stimulation of both group III and IV muscle afferents while the pHSP25 response in the diaphragm, kidney and brain resulted from the sole activation of the group IV fibres, and (2) the blockade of alpha-adrenergic, but not glutamatergic, neurotransmission suppressed the pHSP25 response in all explored tissues except the brain. The present study highlights the role played by the group III and IV muscle afferents in the fatigue-induced pHSP25 response and shows that the sympathetic nerve supply to the muscles and kidney represents the efferent arm of the pHSP25 activation. However, the pHSP25 changes in the brain cannot be explained by the pathways investigated here.

Key words: muscle fatigue, phosphorylated HSP25, adrenergic neurotransmission, glutamatergic neurotransmission, muscle afferents.

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INTRODUCTION

The heat shock proteins (HSP) function to protect cells against oxidative stress and provide protection against future insults (McArdle and Jackson, 2002). Several small HSPs, such as αB-crystallins and Hsp25, have been shown to enhance survival of cells subjected to different forms of stress (Benjamin and McMillan, 1998; Martin et al., 1997).

After a fatigue bout induced in one skeletal muscle by electrical stimulation in rats, we previously showed (Jammes et al., 2012) a widespread activation of the phosphorylated and non-phosphorylated HSP25 and HSP70 in the resting skeletal muscles and the diaphragm, kidney and brain. We showed an early increase in phosphorylated HSP25 (pHSP25) measured by enzyme-linked immunosorbent assay (ELISA), confirmed by western blot analysis, and also that muscle afferents activated by the fatiguing muscle stimulation played a key role in the widespread HSP response. Indeed, the denervation of the stimulated muscle prevented the response. Our observations suggested that the peripheral nervous system could trigger a global protective response against a stress limited to one skeletal muscle. However, in this previous work we could not identify the muscle afferents involved in the phosphorylated and non-phosphorylated HSP response nor the efferent arm of the widespread HSP response.

The skeletal and also the respiratory muscles contain ergoreceptors that are afferents sensitive to muscle contraction, differentiated into mechanoreceptors, sensitive to mechanical changes, and metaboreceptors, sensitive to metabolic changes. The muscle mechanoreceptors are mostly represented by the muscle spindles and Golgi tendon organs, and to a lesser extent by the group III thin myelinated fibres. The muscle metaboreceptors are free nerve endings connected to unmyelinated (group IV) fibres but some group III muscle afferents are also metabosensitive (Kandel et al., 2012). 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, 2002; Ling et al., 2003). The role played by the activation of the group III and IV muscle afferents in the widespread HSP production could be suspected. Indeed, the stressors that elicit the HSP activation (fatiguing muscle contraction, hypoxia, ischemia, and the reactive oxygen species) also stimulate these muscle afferents (Darques and Jammes, 1997; Decherchi et al., 1998; Delliaux et al., 2009; Rotto and Kaufman, 1988). Whitham and Fortes (Whitham and Fortes, 2008) have already hypothesized that the activation of muscle afferents might participate in a general HSP production but the role of these muscle afferents in triggering the HSP activation has never been investigated.

The efferent arm of the fatigue-induced widespread HSP response is unknown. It is well known that 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 responsible for an increase in arterial blood pressure (Kaufman, 2012). The exercise-induced 'pressor' reflex is not limited to the limb muscles but it also concerns the renal circulation (Koba et al., 2006). Animal observations suggest that the activation of α1 adrenergic receptors mediates the stress-induced elevations of the circulating phosphorylated HSP70 (Johnson et al., 2005). Thus, we hypothesized that the sympathetic stimulation might also affect the HSP25 levels. Moreover, the activation of the group IV muscle afferents also modulates the motor drive to the skeletal (Ling et al., 2003) and respiratory muscles (Decherchi et al., 2007; Jammes et al., 1986) through their projections on spinal motor neurones and brain stem neurones. Glutamate is the most abundant and potent excitatory neurotransmitter in the mammalian central nervous system (Collingridge and Lester, 1989) and it is also an established transmitter of excitatory motor pathways in the spinal cord (Gougis et al., 2002), including the phrenic motoneurones (Issa et al., 2010; Mantilla et al., 2012). Because glutamate is suspected to modulate the HSP expression in several brain structures (Ayala and Tapia, 2003) and in culture of spinal cord slices (Guzmán-Lenis et al., 2008), it could be a possible candidate for the widespread HSP response to tibialis anterior (TA) stimulation.

The present study in rats examined the involvement of activation of the group III and IV muscle afferents in electrically induced TA fatigue to trigger the widespread pHSP25 response in different tissues [resting TA, extensor digitorum longus (EDL), diaphragm, kidney and brain]. We also hypothesized that noradrenaline and/or glutamate could modulate the pHSP25 production to the muscle stimulation. Thus, electrically induced fatigue of one TA muscle was performed with: (1) an intact muscle innervation, (2) during the selective procaine block of conduction in the sole group IV nerve fibres, (3) after muscle nerve transection to suppress all the sensory messages, and after pharmacological blockade of the (4) adrenergic or (5) glutamatergic neurotransmission. The present study was limited to the measurements of the changes in pHSP25. Indeed, we have already shown that the pHSP25 level increased very early after the fatiguing muscle stimulation had ended, preceding the increase in HSP70 and non-phosphorylated HSP25 (Jammes et al., 2012).

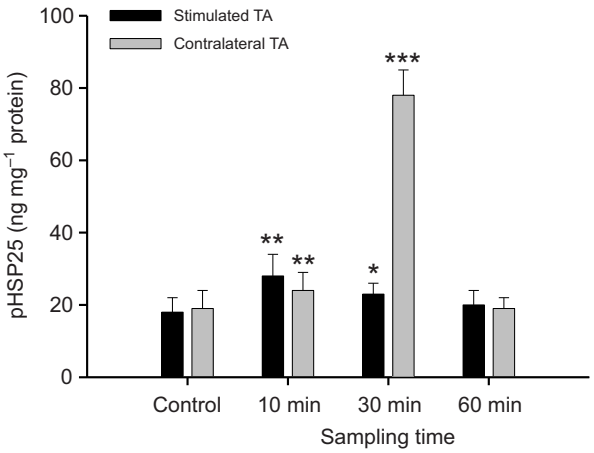

Fig. 1. The kinetics of the pHSP25 response in the stimulated tibialis anterior (TA) muscle and the contralateral TA. pHSP25 was measured before muscle stimulation (control) and then 10, 30 and 60 min after the stimulation bout had finished. The 10 min muscle stimulation consisted of 1 ms pulse trains delivered at a stimulation frequency of 10 Hz. The train duration was 500 ms and a 1000 ms rest period elapsed between two successive trains. Asterisks denote significant variations from data obtained in the control condition, where no muscle was stimulated (*P<0.05; **P<0.01; ***P<0.001).

MATERIALS AND METHODS

Ethical approval

The animal experiments were performed in 58 adult (8–10 weeks old, 310–345 g) Sprague-Dawley rats (Iffa-Credo, Les Oncins, France). The protocol was approved by the Jean Roche Research Institute ethics committee under licence number C 13-055-8. All the experiments, including surgery, monitoring and euthanasia, were conducted by physiologists authorized to perform animal surgery.

Animal care and general preparation

The rats were anaesthetized by an intra-peritoneal injection of sodium pentobarbital (Nembutal, 40 mg kg^{-1}, Sanofi-Aventis, France). The common carotid artery was cannulated to continuously measure the arterial blood pressure with an electromanometer (Gould Statham P23 Db, Hato Rey, Puerto Rico), which was continuously recorded (TA 4000 Gould recorder, Gould SA, Ballainvilliers, France). Heart rate (HR) and percutaneous oxygen saturation were continuously measured using the Mouse Ox apparatus (STARR Life Sciences Corporation, Oakmont, PA, USA). 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, The 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 anaesthesia was judged from the changes in blood pressure and HR 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 the end of the experiments and after muscle sampling, the rats were killed by an intra-arterial injection of a hyperosmolar potassium chloride solution.

Experimental setup

In a preliminary series of 18 rats, we determined the kinetics of pHSP25 variations in both the stimulated and contralateral TA muscles sampled 10 min (N=6), 30 min (N=6) or 60 min (N=6) after the stimulation bout had ended. As shown in Fig. 1, the pHSP25 activation in the contralateral pair muscle culminated at 30 min, and at 60 min the pHSP25 level did not significantly differ from that in control rats. We therefore measured the pHSP25 variations in tissues sampled 30 min after the stimulation bout had stopped.

To assess the role played by the group III and IV muscle afferents in the HSP25 response, the HSP25 response to single leg stimulation was studied in 24 rats in three circumstances: (1) stimulation of an innervated TA muscle (N=8); (2) stimulation of an innervated TA muscle with procaine block of conduction in unmyelinated (group IV) nerve fibres (N=8); and (3) stimulation of a denervated TA muscle which also suppresses the group III component (N=8).

To explore the efferent arms of the widespread HSP25 response to TA stimulation, TA stimulation was performed in eight rats after intravenous injections of α1 receptor antagonist (tamsulosin hydrochloride, 0.03 mg kg^{-1}; T1330, Sigma-Aldrich, Saint Quentin Fallaviers, France) and α2 receptor antagonist (SKF 86466, 8 ng kg^{-1};

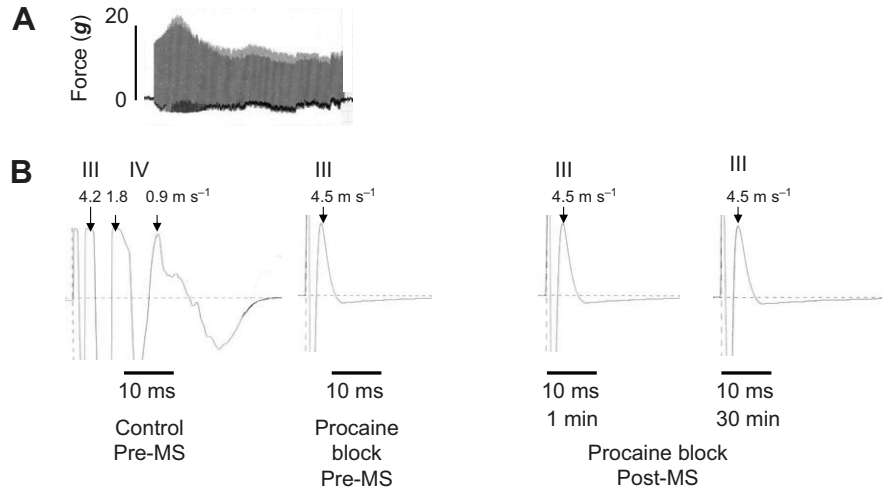

Fig. 2. Blockade of conduction in the group IV (unmyelinated) fibres in the peroneal nerve by local application of 5% procaine solution. Recordings of the compound potential evoked by distal nerve stimulation shows that the suppression of the group IV wave (B) began before the fatiguing muscle stimulation (MS) (A) and continued until 30 min post-MS. The conduction in group III (thin myelinated) fibres was left intact.

S1463, Sigma-Aldrich) and in eight other rats after intravenous injection of a competitive NMDA receptor antagonist (D-2-amino-5-phosphonopentanoate or D-AP-5, 3 μg kg^{-1}; 0106 Tocris, R&D Systems Europe, Lille, France) and a non-competitive NMDA receptor antagonist (MKA-801 hydrogen maleate, 2 mg kg^{-1}; 0924 Tocris, R&D Systems Europe). In each series, the successive injections of α1/α2 antagonists or competitive/non-competitive NMDA antagonists were performed at random before performing the fatiguing TA stimulation. In each situation, the tissues were sampled 30 min after the stimulation bout had ended.

Procaine block of conduction in the peroneal nerve

On the side of muscle stimulation, a dissection at the mid-thigh level was carried out to expose the common peroneal nerve, which innervates the TA muscle. The nerve was left intact and dissected into 4–5 cm lengths, so that it was free from surrounding tissues, and it was isolated with a rubber film to apply the procaine solution (5%). The nerve was stimulated caudal to this level *via* a pair of platinum electrodes connected to the isolation unit of a neurostimulator (Grass S8800, Quincy, MA, USA), which delivered single 1.0 ms rectangular pulses at 1.0 Hz frequency. The compound action potentials evoked by the nerve stimulation were recorded on the nerve trunk, proximal to the level of procaine application, using a monopolar tungsten electrode. The nerve activity was referred to a nearby ground electrode, amplified (50,000 to 100,000×) and filtered (30 Hz to 10 kHz) by a differential neuroamplifier. The nerve signal was displayed on a storage oscilloscope (DSO 400, Gould, Ballinvilliers, France) to average the nerve action potentials evoked by the distal nerve stimulation. The conduction velocity of each wave was measured considering the interelectrode distance, which varied from 2 to 2.5 cm among the rats. Under the local procaine application between the stimulating and recording electrodes, we ensured that the group IV wave (conduction velocity ≤1.8 m s^{-1}) totally disappeared before the muscle stimulation bout began, and also that the blockade of conduction in the unmyelinated fibres persisted during the 10 min stimulation bout and also the 30 min post-stimulation period. Fig. 2 gives an example of recordings of the compound action potentials and their changes throughout the same session. This clearly shows that during the procaine block, the action potentials recorded in the unmyelinated (group IV) nerve fibres were totally suppressed whereas the conduction in the thin myelinated (group III) fibres (conduction velocity between 3 and 6 m s^{-1}) was left intact.

Electrically induced muscle fatigue

As in our previous animal studies (Darques and Jammes, 1997; Decherchi et al., 1998; Decherchi et al., 2007; Delliaux et al., 2009; Jammes et al., 2012), muscle stimulation consisted of rhythmic contractions of the TA muscle. 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 each situation, 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 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 maximal fall of peak force (0.53±0.04 N) was 65±8%. We ensured that the electrical muscle stimulation never elicited the contraction of contralateral muscles.

Tissue sampling, biochemical analyses and immunoassays

Immediately after the sample collection, sampled tissues were cut into two or three aliquots (each aliquot corresponding to a specific assay), which were frozen in liquid nitrogen and then stored at –80°C until biochemical analyses. The mean size of each aliquot was 0.19±0.02 g for TA, 0.15±0.01 g for EDL, 0.40±0.04 g for diaphragm, 0.76±0.04 g for kidney and 1.23±0.18 g for brain.

The tissue extracts were homogenized in 5% trichloroacetic acid according to a 1:4 weight:volume ratio with an Ultra-Turrax T25 basic disperser (Ika-Werke, Staufen, Germany) at 7680 ***g***. The resultant mixtures were then centrifuged (10,000 ***g***) at 4°C for 15 min.

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^{++} to Cu^{+} in the presence of bicinchoninic acid and proteins in alkaline medium. The resulting chromophore exhibits a characteristic purple colour at 562 nm. Bovine serum albumin allows the measurement of total protein content (mg protein g^{-1} of wet tissue). As in our previous study (Jammes et al., 2012), pHSP25 levels were determined with a high-sensitivity ELISA kit dedicated to HSP

Table 1. Conduction velocity (m s^{-1}) of group III and IV fibres measured in the peroneal nerve in control conditions, under procaine block (PB) and after fatiguing muscle stimulation (MS) with maintenance of PB (Post-MS 2 min, 10 min and 30 min)

	Group III	Group IV
Control	4.05±0.48 (8/8)	0.88±0.13 (8/8)
PB	3.22±0.48 (8/8)	0.96 (1/8)
Post-MS 2 min	3.74±0.57 (8/8)	1.10 (1/8)
Post-MS 10 min	3.73±0.66 (8/8)	1.21 (1/8)
Post-MS 30 min	3.64±0.62 (8/8)	1.23 (1/8)

Values are means ± s.e.m. The number of rats in which the group III and IV evoked potentials could be recorded is indicated in parentheses.

measurement in rat tissues using polyconal IgG against HSP25 phosphorylated at Ser82. The capture antibody was GWB-E2BCF (5 μg ml^{-1} in coating buffer; Genway, San Diego, CA, USA) and the detection antibody was [pSer82] HSP25, mAb (5B9) (0.5 μl ml^{-1} in assay buffer; Enzo Life Science, Villeurbanne, France). We used lyophilized recombinant HSP25 standard (Enzo Life Science) and the calibration curve was performed in assay buffer. All measurements were made in duplicate by spectrophotometry on a Statfax 3200 microplate reader (Awareness Technology, Palm City, FL, USA) using a point-by-point method, which allows a better estimation of HSP levels. The HSP25 level was expressed in ng mg^{-1} protein content and the limits of detection of the assay were 0.38 ng ml^{-1}.

Data analyses

Values are expressed as means + 1 s.e.m. After the data were tested for homogeneity of variance, a two-way ANOVA was used to evaluate at the same time (30 min), in each situation, the changes in pHSP25 levels elicited in the stimulated muscle and the other tissues compared with the corresponding control levels. The first factor was the muscle status (stimulated TA *versus* tissue) and the second factor was the presence or absence of procaine block, unilateral nerve section, or blockade of noradrenergic or glutamatergic neurotransmission. A *post hoc* Student–Newman–Keuls test indicated the direction and the magnitude of differences between the different conditions. The analyses used in the five series of experiments were provided separately. Data processing was realized on absolute HSP level values with SigmaStat software (Jandel, Chicago, IL, USA). Differences were deemed significant at $P<0.05$.

RESULTS

Assessment of procaine block of conduction in unmyelinated peroneal nerve fibres

Table 1 shows the mean values of conduction velocities in group III and group IV fibres recorded in the eight rats in which the procaine block of nerve conduction was performed. This shows that the blockade of conduction in the group IV unmyelinated fibres was successful in seven out of eight rats.

Role of the different muscle afferents in the widespread pHSP25 response

In rats where the stimulated TA muscle was innervated, the pHSP25 level significantly increased in the contralateral (resting) TA (+359%) and EDL (+46%) muscles, as well as in the diaphragm (+62%), kidney (+16%) and brain (+38%) (Fig. 3). During the procaine block of conduction in the sole group IV nerve fibres, pHSP25 activation was reduced but persisted in the contralateral TA (+164%) and EDL (+23%), while it totally disappeared in the diaphragm, kidney and brain. After denervation of the stimulated TA, the pHSP25 response to TA stimulation disappeared in all the tested organs. The pHSP25 response in the contralateral resting TA was proportional to the muscle force developed by the stimulated TA at the beginning of the fatigue test (Fig. 4). This relationship persisted under the procaine block, though its slope was markedly and significantly reduced (0.19 *versus* 0.68; $P<0.01$), and it disappeared when the stimulated TA muscle was denervated.

Role of noradrenergic neurotransmission

The efficacy of the blockade of noradrenergic transmission was assessed by measurement of a significant blood pressure drop, which

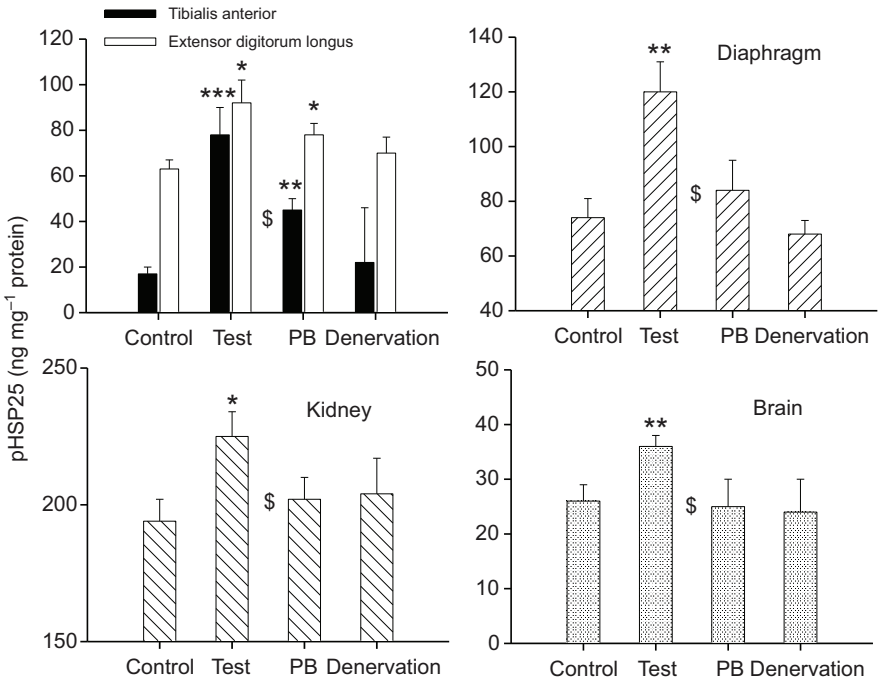

Fig. 3. HSP25 content in the different tissues measured using ELISA in the absence of muscle stimulation (control), after stimulation of the innervated tibialis anterior muscle (Test), after muscle stimulation performed under procaine block of nerve conduction in unmyelinated nerve fibres (PB), and after stimulation of a denervated tibialis anterior (TA) muscle (Denervated). pHSP25 levels were measured in the resting contralateral muscles (TA and extensor digitorum longus) and in the diaphragm, kidney and brain. Values are means ± s.e.m. Asterisks indicate that values significantly differ from controls (*$P<0.05$; **$P<0.01$; ***$P<0.001$). The $ symbol was used to depict significant differences between pHSP25 responses to TA stimulation with an intact innervation nerve and after procaine block of conduction in unmyelinated nerve fibres ($^{\$}P<0.05$). $N=8$ in each condition and each sampled tissue.

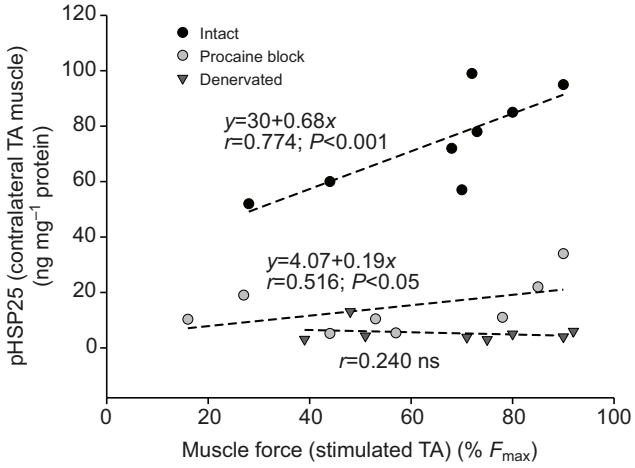

Fig. 4. pHSP25 content in the resting contralateral tibialis anterior (TA) muscle plotted against the muscle force elicited by electrical stimulation of the TA muscle, expressed as a percentage of the maximal force (F_{max}) that was developed. Significant least square regression lines were obtained in preparations where the peroneal nerve conduction was left intact or under procaine block of conduction in the unmyelinated nerve fibres, but no relationship was obtained after denervation of the stimulated TA muscle.

was accompanied by a bradycardia (Fig. 5). Fig. 6 shows that blockade of α1 and α2 adrenergic receptors abolished the pHSP25 response to TA stimulation in the contralateral TA and EDL, diaphragm and kidney, but not in the brain.

Role of glutamatergic neurotransmission

The injection of competitive and non-competitive NMDA receptor antagonists did not elicit changes in HR or blood pressure (Fig. 4). Fig. 5 shows that the widespread pHSP25 response to TA stimulation was significant in all sampled tissues. Indeed, under blockade of glutamatergic transmission, ΔpHSP25, expressed in percentage of control, continued to be significantly elevated, i.e. +268±24% and +140±22% in the TA and EDL, respectively, +60±9% in the diaphragm, +74±7% in the kidney and +213±18% in the brain.

DISCUSSION

The present study confirms our observations that fatigue of a single hindlimb muscle elicits a widespread pHSP25 response triggered by the activation of muscle afferents from the stimulated TA muscle (Jammes et al., 2012). The new findings are: (1) the groups III and IV muscle afferents from the stimulated TA muscle play key roles in the widespread pHSP25 response but the stimulation of group IV afferents is only able to induce pHSP25 activation in organs in the trunk (diaphragm, kidney) and brain; (2) the demonstration of the key role played by the adrenoreceptor simulation in the widespread pHSP25 response to TA stimulation in all samples tissues, except in the brain; (3) the absence of a glutamatergic pathway responsible for the HSP25 response.

The role played by muscle afferents in the pHSP25 response to muscle fatigue

Our data confirm our hypothesis of the key role played by the group III and IV muscle afferents in the widespread pHSP25 response to muscle stimulation which occurs early within the first 10 min following the fatiguing TA stimulation (Jammes et al., 2012). The HSP response probably results from the broad projections of the muscle afferents on the spinal and supraspinal structures, including

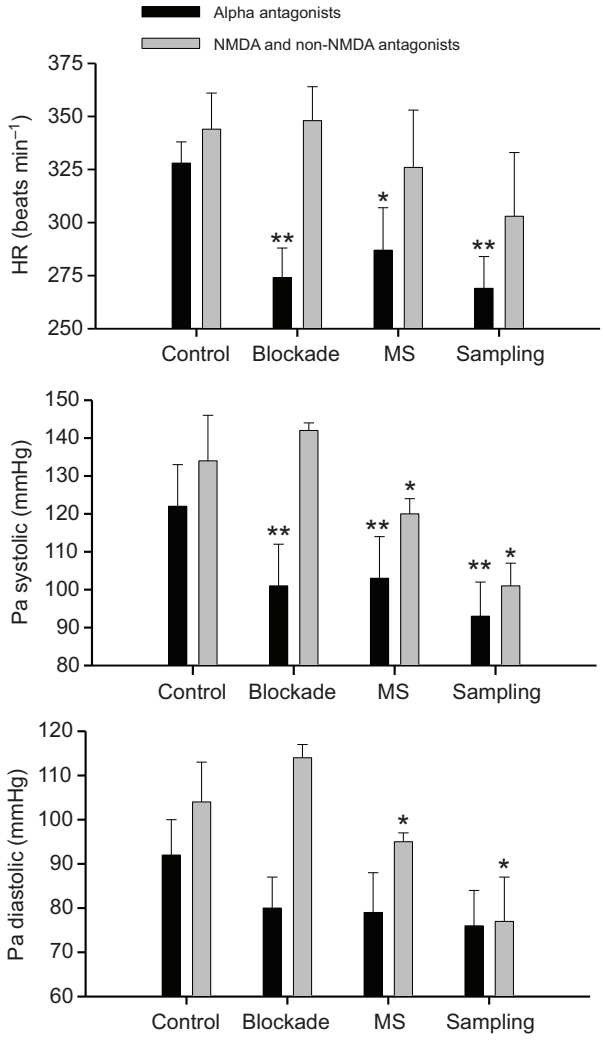

Fig. 5. Changes in heart rate (HR) and arterial blood pressure in response to injection of agents blocking the alpha-adrenergic neurotransmission or of competitive and non-competitive NMDA receptor antagonists. Significant variations were only measured under blockade of the adrenergic neurotransmission and the changes persisted until tissues were sampled. Asterisks denote significant variations from data obtained in the control condition, i.e. before drug injection (*P<0.05; **P<0.01). MS, muscle stimulation.

the brain. Indeed, data in the literature confirm the existence of spinal (Degtyarenko and Kaufman, 2002; Ling et al., 2003) and supraspinal projections (Decherchi et al., 2007) of the group III and IV limb muscle afferents. Muscle stimulation leading to fatigue probably activates the two categories of afferents. Indeed, the thinly myelinated group III muscle afferents are also responsive to mechanical stimuli (Kaufman et al., 1983). The unmyelinated group IV afferents are highly responsive to muscle acidosis, potassium efflux, oxidative stress and cytokines released during and after muscle fatigue (Decherchi et al., 1998; Delliaux et al., 2009; Rotto and Kaufman, 1988). Surprisingly, the present study reveals that only the activation of the group IV TA muscle afferents was responsible for the HSP25 response in the diaphragm, kidney and brain, while the activation of the group III muscle afferents participated in the HSP25 response in the contralateral hindlimb muscles. This suggests that only the group IV muscle afferents project on higher spinal segments and the brain.

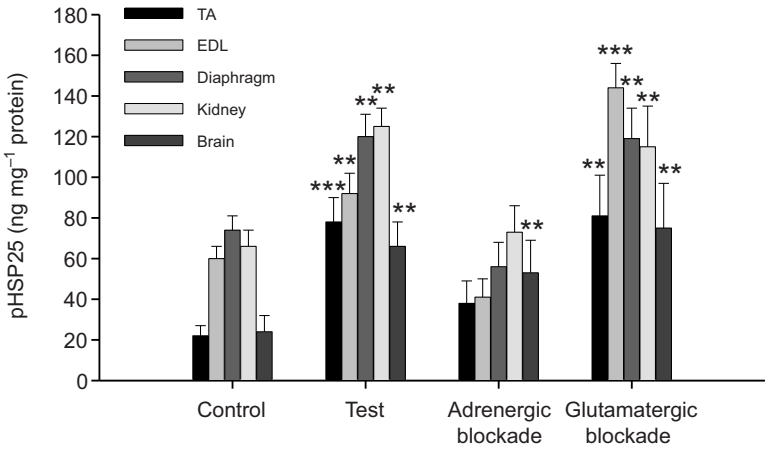

Fig. 6. pHSP25 content in the different tissues [tibialis anterior (TA), extensor digitorum longus (EDL), diaphragm, kidney and brain] sampled in control conditions, 30 min after the TA stimulation had ended without injection of adrenergic or glutamatergic antagonists (Test), or after blockade of the adrenergic or glutamatergic neurotransmission. The pHSP25 response did not occur (any more) in skeletal and respiratory muscles or in the kidney in rats pre-treated with α1 and α2 receptor antagonists, but it persisted in the brain. Blockade of the glutamatergic neurotransmission had no effect. Asterisks denote significant variations from data obtained in the control condition, i.e. in rats whose TA muscle was not stimulated (**P<0.01; ***P<0.001).

Pharmacological approaches to study the efferent arms of pHSP25 activation by muscle afferents

The other question addressed in the present study concerns the efferent arm of the widespread fatigue-induced pHSP25 response. The noradrenergic and glutamatergic pathways were explored because they are suspected to modulate HSP production. Indeed, observations in animals suggest that activation of α1 adrenergic receptors mediates the stress-induced elevations of circulating phosphorylated HSP70 (Johnson et al., 2005). Glutamate was also suspected to modulate the HSP70 expression in several brain structures (Ayala and Tapia, 2003) and the spinal cord (Guzmán-Lenis et al., 2008). The α1 adrenergic receptors have been identified in skeletal (Ives et al., 2012) and respiratory muscles (Aaker and Laughlin, 2002), kidney (Liu et al., 2011) and brain (Karczewski et al., 2012), and glutamate is the most abundant excitatory neurotransmitter in the central nervous system (Collingridge and Lester, 1989) and spinal cord (Gougis et al., 2002), including the phrenic motoneurones (Issa et al., 2010; Mantilla et al., 2012). The present study allows us to eliminate the possibility that glutamatergic neurotransmission is responsible for the widespread pHSP25 response to muscle fatigue. However, the noradrenergic neurotransmission seems to play a key role. Thus, under a complete pharmacological blockade of alpha-adrenergic neurotransmission, we observed a disappearance of the pHSP25 response to TA stimulation in the skeletal and respiratory muscles and the kidney. However, despite the fact that noradrenaline is also a central neurotransmitter (Arnsten and Pliszka, 2011; Berridge et al., 2012; Fang et al., 2011; Tomycz and Friedlander, 2011), we did not measure any significant reduction of pHSP25 response in the brain. This might signify that the noradrenergic-induced pHSP25 response to fatigue solely concerns the sympathetic innervations of vessels. Our data of a persistent HSP25 response in the brain under administration of α1 and α2 antagonists clearly indicate that the blood adrenaline does not participate in this response.

In conclusion, the present study confirms our previous observation on the intervention of muscle afferents in the widespread pHSP25 response to muscle fatigue and highlights the role played by group III and IV muscle afferents. The sympathetic nerve supply to muscles and the kidney represents the efferent arm of fatigue-induced pHSP25 response, but pHSP25 changes measured in the brain cannot be explained.

LIST OF ABBREVIATIONS

EDL	extensor digitorum longus
ELISA	enzyme-linked immunosorbent assay
HR	heart rate
HSP	heat shock protein
NMDA	*N*-methyl-D-aspartic acid
pHSP25	phosphorylated HSP25
TA	tibialis anterior

AUTHOR CONTRIBUTIONS

All the authors contributed to the conception, design, execution and interpretation of the findings published, and drafting and revising the article.

COMPETING INTERESTS

No competing interests declared.

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