Acute exercise boosts cell proliferation and the heat shock response in lymphocytes: correlation with cytokine production and extracellular-to-intracellular HSP70 ratio

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Received: 19 December 2016 / Revised: 24 January 2017 / Accepted: 31 January 2017 / Published online: 1 March 2017
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Abstract Exercise stimulates immune responses, but the appropriate “doses” for such achievements are unsettled. Conversely, in metabolic tissues, exercise improves the heat shock (HS) response, a universal cytoprotective response to proteostasis challenges that are centred on the expression of the 70-kDa family of intracellular heat shock proteins (iHSP70), which are anti-inflammatory. Concurrently, exercise triggers the export of HSP70 towards the extracellular milieu (eHSP70), where they work as pro-inflammatory cytokines. As the HS response is severely compromised in chronic degenerative diseases of inflammatory nature, we wondered whether acute exercise bouts of different intensities could alter the HS response of lymphocytes from secondary lymphoid organs and whether this would be related to immunoinflammatory responses. Adult male Wistar rats swam

for 20 min at low, moderate, high or strenuous intensities as per an overload in tail base. Controls remained at rest under the same conditions. Afterwards, mesenteric lymph node lymphocytes were assessed for the potency of the HS response (42 °C for 2 h), NF-κB binding activity, mitogen-stimulated proliferation and cytokine production. Exercise stimulated cell proliferation in an “inverted-U” fashion peaking at moderate load, which was paralleled by suppression of NF-κB activation and nuclear location, and followed by enhanced HS response in relation to non-exercised animals. Comparative levels of eHSP70 to iHSP70 (H-index) matched IL-2/IL-10 ratios. We conclude that exercise, in a workload-dependent way, stimulates immunoinflammatory performance of lymphocytes of tissues far from the circulation and this is associated with H-index of stress response,

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Electronic supplementary material The online version of this article (doi:10.1007/s12192-017-0771-3) contains supplementary material, which is available to authorized users.

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which is useful to assess training status and immunosurveillance balance.

Keywords Heat shock response · HSP70 · eHSP70/iHSP70 ratio · HSP70 H-index · Exercise · Lymphocyte · Immune function · Inflammation

Abbreviations

β-MCD	Methyl-β-cyclodextrin
APC	Antigen-presenting cell
BSA	Bovine serum albumin
Con A	Concanavalin A
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
FBS	Foetal bovine serum
HIER	Heat-induced epitope retrieval
HS	Heat shock
HSF1	Heat shock transcription factor-1
HSP70	70 kDa family of heat shock proteins
eHSP70	Extracellular HSP70
iHSP70	Intracellular HSP70
HRP	Horseradish peroxidase
IEM	Immunoelectron microscopy
IF	Immunofluorescence
IHC	Immunohistochemistry
IKKs	Inhibitor of nuclear factor κB (IκB) kinases
IL	Interleukin
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear transcription factors from the kappa light chain enhancer of activated B cells (κB) family
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
TCA	Trichloroacetic acid
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α

Introduction

Stress-inducible expression of the 70 kDa family of heat shock proteins (HSP70), alongside its accompanying heat shock (HS) response, is the most highly conserved genetic system ever known, evolving throughout the history of cellular life on Earth (Boorstein et al. 1994; Krenek et al. 2013) and existing in every organism in which it has been sought, from prokaryotes to eukaryotes, from plants to animals (Lindquist and Craig 1988; Locke and Noble 1995). The evolutive force of the HS response may be inferred from the striking homology between human (HSPA) and bacterial (e.g. DnaK) protein orthologs and prolificacy of the 13 genes so far identified in humans that are responsible for HSP70 family coding (Hageman et al. 2011; Kampinga et al. 2009). Despite the fact

that HSP70 chaperones and their partner co-chaperones had been named after the serendipitous discovery in heat-shocked *Drosophila busckii* cells (Ritossa 1962), HSP70 expression and function are associated with a variety of homeostatically threatening situations, not only heat. The HS response, i.e. the biochemical pathway centred mainly on heat shock transcription factor-1 (HSF1)-dependent activation of HSP70 expression (Lindquist 1986; Anckar and Sistonen 2011), works to protect thermally damaged proteins from aggregation as well as to unfold aggregated proteins and to refold damaged proteins or target them for efficient degradation (Verghese et al. 2012). This maintains intracellular protein homeostasis (proteostasis), thus avoiding the formation of toxic polypeptide aggregates that may trigger apoptosis or inflammation (Newsholme and Homem de Bittencourt 2014; Leite et al. 2016).

The HS response evolved to adapt organisms appropriately against several stressful insults, whether from heat, cold, oxidation, free radicals, toxins, hypoxia or metabolic stresses, including exercise (Hooper et al. 2014). Indeed, exercise, which is a strong homeostasis-threatening challenge, is one of the most powerful inducers of intracellular HSP70 (iHSP70) expression and HS response, comparable only to heat stress (Newsholme et al. 2009; Heck et al. 2011; Krause et al. 2015b; Leite et al. 2016). Moreover, HS response is also recruited from other branches of metabolism very far from proteostasis, at least a priori. This is the case of inflammation, energy preservation and immune responses (Newsholme and Homem de Bittencourt 2014). Importantly, by hampering the activation of nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB) family (NF-κB), at multiple regulatory levels, the HS response is anti-inflammatory in its very essence (Leite et al. 2016).

Impaired HS response, on the other hand, is a common feature of several chronic degenerative conditions associated with unresolved inflammation, such as cardiovascular diseases (CVD), obesity, type 1 (T1DM) and type 2 diabetes mellitus (T2DM), ageing and neurodegenerative diseases (Di Naso et al. 2015; Hooper et al. 2014; Krause et al. 2014a, 2014b; Krause et al. 2011; Krause and Homem de Bittencourt 2008; Leite et al. 2016). Although suppression of HSF1 expression associated with decreased amounts of iHSP70 is a hallmark of chronic inflammatory diseases (Chung et al. 2008; Rodrigues-Krause et al. 2012; Di Naso et al. 2015; Newsholme and Homem de Bittencourt 2014; Krause et al. 2015a, 2015b), iHSP70 levels, per se, are not the main issue; the impairment in tissue capacity to trigger a robust HS response in reaction to homeostatic challenges is the real problem. In fact, derangement of HS response associated with inadequate or decreased HSP expression and action is found in a number of diseases, including acute (sepsis, acute respiratory distress syndrome, extensive body burns, pancreatitis) and chronic degenerative illnesses of inflammatory

nature (obesity, T1DM and T2DM), chronic obstructive lung disease, colitis, psoriasis, neurodegenerative diseases and ageing itself (Török et al. 2014).

Under the same stressful conditions able to trigger intracellular HS response, however, cells may deliver HSP70 towards the extracellular milieu by non-canonical mechanisms (exosomes) (Chirico et al. 1988; Hightower and Guidon 1989; Lancaster and Febbraio 2005). Once secreted, extracellularly located HSP70 (eHSP70) works exactly in opposite manner as regards its intracellular function. eHSP70 signals to physiological systems for the presence of homeostatic challenges (Heck et al. 2011) after binding to Toll-like receptors (TLR-2, TLR-4 and TLR-7) in a variety of cells, leading to the activation of pro-inflammatory pathways (via MyD88 and TIRAP that signal downstream to NF- κ B through IRAK4, TRAF6 and IKK), and inducing JNK activation via MEKK4/7 (De Maio 2011). eHSP70 stimulates the immune system (Whitham and Fortes 2008) at several key points, for example, by increasing neutrophil microbicide capacity (Ortega et al. 2006) and chemotaxis (Ortega et al. 2009), by enhancing the recruitment of natural killer (NK) cells (Horn et al. 2007) and boosting cytokine release from different immune cells (Asea et al. 2000; Johnson and Fleshner 2006), eventually triggering pro-inflammatory responses that elevate immunosurveillance (Campisi and Fleshner 2003). High-affinity binding of eHSP70 to other surface receptors, including LRP/CD91, CD40, scavenger receptors and c-type lectins, has also been described (Calderwood et al. 2007).

Upon the previous circumstances, eHSP70 export has been documented in lymphocytes, macrophages, epithelial cells, dendritic cells, neuronal cells and hepatocytes (Hunter-Lavin et al. 2004; Ireland et al. 2007; De Maio 2011; De Maio and Vazquez 2013) in response to exercise and adrenergic stimulation (Walsh et al. 2001; Lacoste et al. 2001; Febbraio et al. 2002; Johnson et al. 2005; Johnson and Fleshner 2006; Giraldo et al. 2010; Henstridge et al. 2016). Not unexpectedly, serum eHSP72 concentrations are positively correlated with inflammation markers in humans [e.g. C-reactive protein, monocyte count, and TNF- α (Mayer and Bukau 2005; Njemini et al. 2004)], being regarded as a prognostic marker of the progression of immune-related diseases (Terry et al. 2004, 2006), clinical status and death (Gelain et al. 2011; Genth-Zotz et al. 2004; Jenei et al. 2013a, 2013b). Consequently, the ratio between eHSP70 and iHSP70 ($R = [\text{eHSP70}]/[\text{iHSP70}]$) correlates with organismal immunoinflammatory balance, whereas the rate of variation in R ratios between different conditions (a.k.a. H-index of HSP70 status) is regarded as an index of changes in overall immunoinflammatory balance (Krause et al. 2015a, 2015b; Leite et al. 2016; Schöler et al. 2016; Krause and Rodrigues-Krause 2011). Therefore, in the present study, we investigated, in the rat model, whether acute exercise bouts of different intensities could influence the HS response of lymphocytes, which

are a main source of both eHSP70 and iHSP70 under stressful situations (Hunter-Lavin et al. 2004; Ireland et al. 2007). It is of note that, although circulating leukocytes should be mostly affected by the changes imposed by exercise, as these cells are directly “stressed” by turbulent blood flow and hormonal alterations during exercise bouts, we chose mesenteric lymph nodes to be studied because lymphocytes obtained from different secondary lymphoid organs present basal and mitogen-stimulated proliferative responses to exercise that greatly differ between diverse sources (Vitorino et al. 2010), and this has never been investigated in relation to the HS response.

Methods

Animals and ethics

Sedentary male Wistar rats ($n = 60$ in total, 90 days old; weighing approx 250 g) obtained from The Federal University of Rio Grande do Sul Institute of Basic Health Sciences Animal Care Facility (CREAL), maintained under controlled temperature ($23 \pm 1^\circ\text{C}$) and humidity (60%) in a 12/12-h light/dark cycle (lights on at 0700 a.m.) and housed in plastic cages ($49 \times 34 \times 16$ cm) were used. Throughout the experiments, the animals had free access to water and were fed with standard pelleted laboratory chow (NUVILAB CR-1, Nuvital Nutrientes S.A., Curitiba, Brazil) ad libitum. After 1 week of acclimation (five rats per cage), the animals were randomly assigned to the experimental groups described in the following. The investigation followed all ethical rules established by Arouca's Act (Federal Law 11794/2008) and the Guide for Care and Use of Experimental Animals published by the National Institutes of Health (NIH publication no. 85-23, revised in 1996). All the procedures were approved by the Committee of Animal Welfare of the Federal University of Rio Grande do Sul (CEUA-UFRGS, protocol #2008110).

Study design and exercise protocol

All animals were accustomed to the water environment prior to exercise to avoid any stress response related to the new environment and situation. The adaptation period consisted of keeping the animals for 8 min in individual swimming pool chambers ($20 \times 20 \times 56$ cm) filled with water at $31 \pm 1^\circ\text{C}$ (50-cm depth), for three consecutive days, without any overload (avoiding any type of exercise training). Afterwards, overnight-fed animals (five per group) were randomly assigned for each round of swimming experiments according to exercise burden imposed by a lead overload (2, 4, 6 or 8% of body weight) attached to the base of the tail and were, then, submitted to an acute swimming exercise bout of 20 min under the same circumstances as during the adaptation, one rat

per chamber, as described (Rossato et al. 2014) with the adaptations introduced elsewhere (Schöler et al. 2016). Control animals remained at rest in shallow water (5-cm depth; 31 ± 1 °C, 20 min) during each experiment. Then, the following five groups were formed: rest, 2, 4, 6 and 8. Swimming experiments were always carried out between 8:00 and 11:00 a.m. Room temperature during the experiments was kept at 24 °C. All the procedures were in accordance with those prescribed in The American Physiological Society's Resource Book for the Design of Animal Exercise Protocols (Kregel et al. 2006), and an experienced researcher was present at all the times to avoid drowning. Swimming time (20 min) was chosen because this is the time limit within which an untrained animal really swims before learning how to perform bobbing, which is a survival strategy used to conserve energy without doing exercise. The animals were killed by decapitation immediately after the end of exercise sessions, and mesenteric lymph nodes were surgically excised (see details in the following section, please).

Swimming exercise is assumed to be a judicious choice to study the physiological impacts of physical exercise in rats, particularly by virtue of this influence on sympathetic nervous system (Baptista et al. 2008), whose activation is tightly involved in stress response and immune surveillance (Schöler et al. 2016; Rossato et al. 2014; Mastorakos et al. 2005; Febbraio and Pedersen 2005; Hackney 2006). Although treadmill running could be another choice, cardiovascular findings indicate that swimming and running protocols are not comparable (Nunes et al. 2015), while swimming exercise is a more uniform type of exercise for rats because it does not necessarily involve "stop-and-go" activity such as that found with treadmill running (Kregel et al. 2006).

Euthanasia

In order to prevent unavoidable stress, animal killing (by decapitation) always occurred in a separate laboratory environment where there is exhaustion (to remove fear pheromones in the air). Between the death of an animal and another, the guillotine and the rest of the material were completely sanitised with water, detergent and alcohol. The method induces immediate loss of reflexes producing minimal physiological changes in tissues. Considering the need to obtain peripheral blood for additional cellular and biochemical analyses, animal killing under anaesthesia, while desirable, is incompatible with the experimental goals because all anaesthetics commonly used in studies with experimental animals lead to an intense hyperglycaemia in rodents (Brown et al. 2005; Saha et al. 2005). Moreover, these anaesthetics interfere in the function of cells involved in the production of HSP70, such as leukocytes (Chang et al. 2005). Considering also that plasma concentrations of the major molecular object of the study (HSP70) are greatly affected (complete reduction) by

high levels of plasma glucose (Febbraio et al. 2004), animal killing was performed without anaesthesia.

Assessment of exercise effort

Exercise loads (as a percentage of body weight) were chosen to examine a broad range of intensity effects of swimming exercise over the physiological parameters assessed. Accordingly, we tested, for the same 20-min sessions, low-intensity (2%), moderate-intensity (4%), high-intensity (6%) and strenuous (8%) exercise bouts. It was assumed that, at 4% overload, oxygen consumption under the present conditions was ca. 65–70% of maximal oxygen consumption ($\dot{V}O_{2\max}$), which is estimated to be $85\text{--}100\text{ mL min}^{-1}\text{ kg}^{-1}$ (Kregel et al. 2006), so that the anaerobic threshold is reached at 6% overload in which maximal lactate steady state is around 5.5 mM (Gobatto et al. 2001; Voltarelli et al. 2002). Hence, we assessed exercise effort and intensity by measuring blood lactate (Accutrend® Lactate, Roche, caudal vein, approx 25 µL) just after the exercise session. These data were matched against glycogen contents in the gastrocnemius and soleus muscles, which are highly recruited during this type of exercise (Kregel et al. 2006). For glycogen measurements, approximately 200 mg of each gastrocnemius and soleus muscles was surgically excised and prepared soon after the end of the exercise session as described by Van Handel (1965). In brief, fresh muscle samples were dissected free of blood and connective tissue, homogenised in 30% (w/v) KOH solution in the presence of saturated Na_2SO_4 solution and led to dissolve in a boiling water bath (10 min). Afterwards, glycogen was precipitated, re-dissolved and recrystallised using 70% (v/v) ice-cold ethanol solution and warm water for three times. Sample glycogen and a standard curve (Sigma) were then hydrolysed to glycosyl units (Howarth et al. 2010) for 120 min in 2 N HCl at 100 °C and subsequently neutralised with 2 N Na_2CO_3 to be enzymatically (glucose oxidase-peroxidase coupling) assessed for glucose contents spectrophotometrically (Laborlab, São Paulo, Brazil). In total, the effectiveness of exercise was inferred from the increasing concentrations of blood lactate (supplemental Fig. S1a), paralleled by a proportional reduction in muscular hindlimb glycogen contents (supplemental Fig. S1b). It is of note that blood lactate concentrations varied linearly (Pearson's $r = 0.9511$, $p = 0.0129$) from 3.6 ± 0.2 mM (rest controls after 20 min in shallow water) to 9.6 ± 0.5 mM (8% overload after the 20-min swimming sessions). We believe that the relatively high lactate values of the present controls, as compared to the usual lower figures observed in our laboratory rats in their own cages (1.96 ± 0.94 mM), are due to the exploratory movement of the rats that remain always restless in the shallow water during the experiments.

Swimming performance in the rat is known to be dramatically influenced by water temperature and, if the water is much

warmer (e.g. $T > 42^\circ\text{C}$) or much cooler (i.e. $T < 20^\circ\text{C}$) than the rat's core temperature, then exercise performance is greatly reduced (Kregel et al. 2006), generating early fatigue (between 12 and 14 min) in such cases (Baker and Horvath 1964). Furthermore, the water temperature range chosen herein ($31 \pm 1^\circ\text{C}$) has been suggested to be the optimal water temperature to evaluate stress controllability effects in swimming rats (Mabry et al. 1995; Drugan et al. 2005). Because of this, we assessed core temperature of the animals with Minipa Digital Thermometer MT450 equipped with a rectal probe. As depicted in supplemental Fig. S2, before exercise, averaged animal core temperature was $36.6 \pm 0.2^\circ\text{C}$ and decreased to $32.7 \pm 0.2^\circ\text{C}$ after 20 min in all test animals, as compared to rest controls ($36.6 \pm 0.6^\circ\text{C}$; $p = 0.0077$ by ANOVA). However, it was observed no temperature difference within test groups ($p > 0.999$, repeated-measure ANOVA), so that no observable differences in experimental results could be attributed to temperature effects, but rather to exercise effort.

Lymphocyte preparation, culture and HS treatment

For the isolation of mesenteric lymph node lymphocytes, the rats were killed by decapitation and the cells were always obtained **immediately** after death from the mesenteric lymph nodes as previously described (Homem de Bittencourt et al. 1993) with the specific adaptations introduced later (Homem de Bittencourt et al. 1994). After killing, lymph nodes were surgically excised, freed of blood and connective tissue and maintained in sterile phosphate-buffered saline (PBS), pH 7.4, containing antibiotics (100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, Sigma) on ice to be processed in an autoclavable lymphocyte isolation grinder developed by us (Vieira et al. 1990), which consists of two concentric stainless steel cylinders ended with a steel mesh each. Afterwards, lymphocyte suspension was sterile-filtered (Whatman 105 lens cleaning tissue, GE Healthcare, USA) and centrifuged ($370\times g$ for 10 min) at room temperature (22°C) to be resuspended in PBS, pH 7.4, containing antibiotics (as described earlier) and then filtered and centrifuged again under the previous identical conditions. Finally, lymphocytes were suspended in RPMI 1640 culture medium (Sigma), pH 7.4, containing 11 mM glucose, 2 mM L-glutamine, 25 mM HEPES and 24 mM NaHCO_3 , supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Sigma) and antibiotics (as previously mentioned) to be cultivated (see in the following, please). These repeated washings and filtrations reduce the lymphocyte yield but ensure the elimination of platelets and phagocytes (Johnson et al. 1974), thus avoiding contaminating cell interference. A highly pure lymphocyte preparation (more than 99.5%) virtually platelet-free and having only traces ($<0.5\%$) of macrophages and polymorphonuclear leukocytes (PMNL), as measured by Romanowsky and crystal violet/acetic acid techniques, was thus obtained. There was no erythrocyte contamination. Total lymphocyte yield under these conditions was about 1--

2×10^8 cells/rat, and cell viability just after plating was $>98\%$, as measured by the Trypan blue dye exclusion technique.

Soon after preparation, lymphocytes were plated (1×10^7 cells/well in 2 mL) in the previous culture medium in Parafilm M®-wrapped six-well dishes. For heat shock (HS) treatments, the plates were submersed in a temperature-controlled water bath (Precision Scientific, USA) at $42 \pm 0.01^\circ\text{C}$ for 2 h (immersion depth = 2.5 cm; $t_{1/2} = 1.5$ min). Parallel control preparations were maintained in another water bath at 37°C for the same time period. After water bath incubations, cells from both control and HS groups were placed in a Forma™ model 3100 water jacket cell incubator (Thermo Scientific, USA) in 2 mL of fresh medium (as previously mentioned) at 37°C in a 5% (v/v) CO_2 humidified atmosphere in sterile-filtered air for the recovery period of 6 h in culture. Elapsed this time, heat-shocked cell viability was $>86\%$ in all groups.

As referred in the main text, the aim of studying the association of exercise with HS treatment was to investigate the physiological influences of an acute exercise bout on the physiological HS response of lymphocytes, as the HS response is anti-inflammatory. Accordingly, the present model of HS was chosen because, under fever-like (physiological) temperature stress, lymphocytes respond augmenting HSP70 expression significantly only if above 42°C , differently from monocytes and PMNL that show conspicuous rise in HSP70 at 39°C (Oehler et al. 2001). This may be related to the fact that lymphocytes and lymphocyte-like cells (e.g. lymphomas) virtually do not show any appreciable activation of HSF1 and DNA binding activity except above 41°C , in which range HSF1 is associated with the inhibition of NF- κB activity that is maximal between 43 and 45°C (Belardo et al. 2010). The same rationale was employed when fixing the duration of HS (2 h), as it is known that the release of eHSP70 from peripheral blood mononuclear cells (PBMC) towards culture media is high during the first 2 h of incubation (Hunter-Lavin et al. 2004), this same being observed in T lymphocyte-like cells (Jurkat) when heat-stressed for identical period of time (Ireland et al. 2007). Moreover, under the present stressful situation, iHSP70 expression peaks around 2–4 h returning to basal levels by 6 h post-HS (Lovell et al. 2007), so that during the 6 h recovery period, there is a robust accumulation of iHSP70 and export of eHSP70 to the media.

For every round of experiments, samples of lymphocyte preparations and cultures were taken at three time points: immediately after preparation (T_{zero}), just after the 2 h water bath (at 37 or 42°C) period ($T_{\text{after bath}}$) and after the recovery period of 6 h post-bath ($T_{6\text{ h}}$).

Lymphocyte function measurements

Lymphocytes need to proliferate following adequate stimuli in order to guarantee a strong immune response against non-self invaders and/or abnormal cells of the body. Therefore, lymphocyte proliferation studies furnish a good indication of overall

immune function, provided that complementary studies examining other aspects of immune function are carried out in parallel (Demas et al. 2011). Herein, we investigated the effects of exercise (and HS) over lymphocyte proliferative capability comparing the results with those from cytokine production and eHSP70 export when in culture.

In order to assess lymphocyte proliferative capacity after each of the time points chosen (as previously mentioned), cells were suspended in RPMI 1640 culture medium (as previously mentioned) containing 10% (v/v) FBS and cultured for 24 h at 37 °C in 24-well plates (1×10^6 cell/well in 450 μ L final volume) in a humidified atmosphere of 5% CO₂ in air. Mitogenic stimulus was provided with the T lymphocyte-specific lectin concanavalin A (Con A) from *Canavalia ensiformis*, type VI, highly pure lyophilised powder (Sigma, L7647), dissolved in culture medium (5 μ g/mL), as previously described (Homem de Bittencourt and Curi 1998). Control unstimulated cells received only the same volume of medium. After 24 h of culture, cells were pulse-labelled with [2-¹⁴C]-thymidine (PerkinElmer, NEC1560, 0.5 μ Ci/well, 1 μ Ci/mL final concentration) and left in culture for additional 18 h. Cell viability was subsequently checked again (>85%), and the reactions were then stopped on ice. Afterwards, the cell suspensions were transferred to microfuge tubes and washed three times with ice-cold PBS pH 7.4 (15,000 $\times$ g for 10 s at 4 °C). The cells were then resuspended in 200 μ L of cold PBS to be lysed with the addition of 200 μ L of trichloroacetic acid (TCA, 10% w/v final concentration in the tubes) on ice for 1 h.

After lysis, the samples were vortexed and centrifuged again (15,000 $\times$ g for 5 min at 4 °C), and the radioactivity incorporated into acid-insoluble material (thymidine taken up from the medium and incorporated into DNA) as well as acid-soluble one (thymidine taken up but not incorporated into DNA) was assessed by the rapid filtration technique described previously (Rozera et al. 1996) by using a manifold vacuum filtration system (model 1225 Millipore, USA) and a 0.22 μ m mixed cellulose ester membrane (Millipore, cat no. GSTF02500, 25 mm). Briefly, the samples were filtered under vacuum and TCA-soluble material saved for the calculation of total thymidine uptake. Then, the filters containing TCA-insoluble material were washed three times with 5% (w/v) ice-cold TCA (5 mL/wash) and left to dry under vacuum. Dried filters were dissolved in Ultima Gold F liquid scintillation cocktail (PerkinElmer, USA), and radioactivity was determined in Tri-Carb 2810TR Liquid Scintillation Analyser (PerkinElmer) and the accompanying software QuantaSmart. Disintegration-per-minute (dpm) values were assessed and converted into incorporations of [2-¹⁴C]-thymidine into DNA as femtomole/10⁶ cells. All the measurements were performed in duplicates for each experiment ($n = 5$ /group). Uptake (soluble + insoluble materials) and incorporations (TCA-insoluble) were measured, and incorporations were always proportional and similar to total uptake, so that the

results were presented as incorporations only. Additionally, proliferative response was also calculated as the stimulation index (SI) calculated by dividing mean incorporation values of mitogen-stimulated lymphocytes by the mean incorporations of unstimulated cells, as described by De Fries and Mitsunashi (1995).

In order to have further insight into lymphocyte function, culture media from lymphocyte cultivations were collected at $T_{\text{after bath}}$ and $T_{6 \text{ h}}$, centrifuged (370 $\times$ g for 10 min at 4 °C) and stored at -86 °C for cytokine and eHSP70 measurements. HSP70 exported to lymphocyte culture media (eHSP70) was measured by using high-sensitivity HSPA1A-specific HSP70 ELISA Kit (Enzo Life Sciences, EKS-715) in undiluted cell supernatant samples. It is expected that both the inducible HSPA1A and HSPA6 (HSP70B') forms as well as the cognate HSPA8 form of HSP70 should be delivered into the extracellular space of different cell types after appropriate stressful conditions. However, only HSPA1A ELISA kits have been sufficiently tested worldwide and proved to be of enough sensitivity (pg/mL range) to detect minute HSP70 quantities in culture media and sera. Additionally, previous results from this laboratory (Schöler et al. 2016) have indicated that the principal eHSP70 forms (HSPA1A and HSPA8) are secreted in similar amounts. Therefore, it was assumed that HSPA1A is representative of total eHSP70 secretion. Since there is a close correlation between extracellular HSP70 and the plasma levels of inflammation-related cytokines, we checked eHSP70 levels out in test groups comparing them with those of the interleukins IL-2 (a T_{h1} inflammatory cytokine), IL-10 (a T_{h2} anti-inflammatory) and IL-6 (a conditionally dual cytokine: mostly anti-inflammatory but also pro-inflammatory, depending on the type of membrane receptors in cells and accompanying co-stimuli (Scheller et al. 2011; Ye et al. 2014). Lymphocyte supernatant levels of IL-2, IL-10 and IL-6 were evaluated by using MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Panel-Immunology Multiplex Assay (Millipore, cat no. RCYTO-80K). Either of the procedures was performed according to the manufacturer's instructions.

H-index of immunoinflammatory status

Extracellular-to-intracellular HSP70 ratio index (H-index) measured in different tissues and cell types in relation to plasma or culture medium has commenced to be ascribed to as a novel and overall index of immunoinflammatory status of an individual (Krause et al. 2015a, 2015b; Heck et al. 2011). For example, by using identical swimming protocol as described herein, we have recently shown that H-index perfectly matches macrophage activation state and phagocytic capacity in a workload-dependent way (Schöler et al. 2016), so that we wondered whether exercise is able to produce similar responses in lymphocytes with regard to immunosurveillance assessed by cytokine production. The rationale for H-index is that the

higher are the eHSP70 amounts, the more inflammatory signals are coming into play, because eHSP70 is pro-inflammatory in its very nature. Conversely, for each particular situation, if cells are able to respond to stressful stimuli by enhancing iHSP70, then they tend, at the same time, to enter into a state of anti-inflammation. Therefore, if one takes $R_c = [eHSP70]_c/[iHSP70]_c$ as the HSP70 ratio in a control situation, whatever the techniques used to assess each eHSP70 and iHSP70, then H-index can be calculated as the quotient of any $R_j = [eHSPA]_j/[iHSPA]_j$ by R_c , which will be therefore considered as the unity ($R_c = 1$), normalising all the remaining results in this situation “j.” Hence, $H\text{-index} = R_j/R_c$ may allow for the comparisons between any stressful situation j and the situation assumed as the control one. H-index can be applied to estimate immunoinflammatory balance in many different situations, such as immune responses, diabetes and immunological impacts of exercise (Krause et al. 2015b).

SDS-PAGE and immunoblotting analysis of iHSP70 protein

Lymphocyte preparations (approx 10^7 cells) were taken either after exercise bouts (T_{zero}) or after water bath (at 37 or 42 °C) at the following time points in culture: just after the bath ($T_{after\ water\ bath}$) and 2, 4 and 6 h after bath. Then, for the preparation of whole cell lysates to be analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), lymphocyte suspensions were transferred to 1.5 mL microfuge tubes, washed in ice-cold PBS (370×g for 10 min at 4 °C) and disrupted on an ice bath in a model UIS250V ultrasonic processor (Hielscher Ultrasonics GmbH, Germany) equipped with an LS24d3 sonotrode operating at 24 kHz and 75% of maximal potency (= 9 W final power in the tubes), for 30 s in pulses of 0.5 s each. Lysis buffer consisted of 0.1% (w/v) SDS containing protease and phosphatase inhibitor cocktail (Sigma, freshly prepared) comprising of (final concentrations) leupeptin (4.2 µM), aprotinin (0.31 µM), *N*-tosyl-L-lysine chloromethyl ketone (TLCK, hydrochloride; 20 µM), phenyl-methyl-sulfonyl fluoride (PMSF, 100 µM), sodium orthovanadate (Na_3VO_4 ; 1 mM), sodium molybdate (Na_2MoO_4 ; 1 mM) and β-glycerophosphate (1 mM). Cell lysates were stored at −86 °C until use when they were defrosted and processed as described by Kolberg et al. (2006) with modifications (Rodrigues-Krause et al. 2012). Briefly, 10 µL samples from whole cell lysates were saved for protein determination (Bradford 1976) and equivalent amounts of protein from whole-cell lysates (~40 µg) were mixed with 2× Laemmli's loading buffer [50 mM Tris, 10% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 2 mg/mL bromophenol blue, final concentrations] in a ratio of 1:1, boiled for 5 min and electrophoresed in a 10% polyacrylamide minigel for 4 h 15 mA/gel.

After that, proteins were transferred onto nitrocellulose membranes (GE Healthcare) according to the electrotransfer (Bio-Rad) manufacturer's instructions (2 h, 100 V) and transferred bands were visualised with 0.3% (w/v) Red Ponceau S (Sigma) in 3% (w/v) TCA solution to be photodocumented (ImageQuant 350, GE Healthcare). For immunoblotting procedures, SNAP i.d. (Merck Millipore) quick immunoblot vacuum system was used (Di Naso et al. 2015). The membranes were washed with water and then blocked in 0.5% (w/v) non-fat dry milk (Nestlé) in wash buffer [50 mM Tris, 5 mM EDTA, 150 mM NaCl (TEN)-Tween 20 (0.1% w/v) solution, pH 7.4] for 30 s. The membranes were then washed three times (30 s each) with wash buffer and incubated for 10 min with mouse anti-human HSP70 monoclonal antibody (Sigma H5147, clone BRM-22 ascites fluid), which recognises both the 73 kDa constitutive HSC70 (*hspa8* gene) and the 72 kDa inducible HSP70 (*hspa1a* gene) forms, at 1:1000 dilution. Alternatively, the 72 kDa inducible HSP70 expression was investigated by using the antibody (Abcam, Cambridge, UK, cat. no. ab47455) clone C92F3A-5 (IgG₁ produced in mouse), which only and specifically recognises the *hspa1a* gene product (1:1000). After three washes with washing buffer (30 s each), membranes were probed with horseradish peroxidase (HRP)-labelled secondary antibody (anti-mouse IgG whole molecule, Sigma A9044, 1:10,000) for 10 min. Membranes were then washed three times (30 s each) with washing buffer, and protein detection was performed by the enhanced chemiluminescence method (ECL Prime, GE Healthcare). As a gel loading control, peroxidase-labelled mouse anti-human β-actin monoclonal antibody (Sigma A3854) was used at 1:25,000 dilution. Protein bands were photodocumented for 600 s (60 frames, one photo each 10 s) and quantified in ImageQuant™ 350 chemiluminescence system (GE Healthcare) and the accompanying online stacking imaging software ImageQuant TL 7.0. The data are given as the means ± SD of immunocontents normalised in terms of β-actin.

NF-κB binding assay

In order to investigate the combined effects of exercise and HS over NF-κB binding to DNA, nuclear extracts from lymphocytes (from the same samples assessed for immunoblotting) were processed to be analysed by the Combo p50/p65 NF-κB Transcription Factor Assay kit (Cayman Chemical, cat no. 10011223), which detects the binding of the transcription factor to a specific double-strand DNA sequence containing the NF-κB response element immobilised onto the wells of a 96-well plate. For the present experiments, we focused on p65 (RelA) because the kit detects rat, mouse and human RelA binding. Hence, following the manufacturer's instructions, after lymphocyte preparation or water baths and 6 h cultures (T_{zero} , $T_{after\ bath}$ and $T_6\ h$, respectively), 1 mL of each cell suspension (ca. 1×10^7 cells) was harvested from the dishes

and centrifuged in pre-chilled tubes ($300\times g$ for 5 min at 4°C) and the pellets were suspended in 5 mL cold PBS containing protease and phosphatase inhibitors (as previously mentioned). Then, the cells were spun again under the same conditions and the new pellets were resuspended in 500 μL of ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.5 containing 4 mM NaF, 10 μM sodium EDTA and 1 μM Na_2MoO_4 , Cayman, cat. no. 10009277). The pellets were further gently mixed by pipetting and then transferred to a fresh pre-chilled 1.5 mL microfuge tube. The cells were incubated on ice for 15 min allowing to swell. Afterwards, 50 μL of 10% (v/v) Nonidet P-40 (1% final concentration) was added to the preparation and gently mixed by pipetting again. The samples were then centrifuged (pulse spin) at 4°C for 30 s, and the entire supernatant (cytosolic) fraction was saved and stored at -86°C until use. The pellets containing the nuclei were disrupted in freshly prepared Complete Nuclear Extraction Buffer consisting of (final concentrations in the $1\times$ solution) 10 mM HEPES, pH 7.9, containing 100 μM sodium EDTA, 1.5 mM MgCl_2 , 420 mM NaCl, 10% glycerol, 1 mM DTT and inhibitors of protease (5 μM bestatin, 4.2 μM leupeptin, 1 μM pepstatin A, 0.8 μM aprotinin, 20 μM TLCK and 100 μM PMSF) and phosphatase (1 mM β -glycerophosphate, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4) activities. After the previous treatment, nuclei were vortexed for 15 s at the highest setting and left to gently rock for 15 min in a shaking platform at 4°C . This procedure was repeated once with a 30 s vortexing and additional 15 min of shaking at 4°C . Finally, the samples were centrifuged ($15,000\times g$ for 10 min at 4°C), and the supernatant fractions (containing disrupted nuclei) were transferred to a fresh chilled tube and flash-frozen (liquid nitrogen) to be stored in working aliquots at -86°C until use. A small aliquot of each sample was saved for protein measurements [Bradford 1976]; using bovine serum albumin (BSA) prepared in Complete Nuclear Extraction Buffer as the standard] before freezing. Equal amounts of nuclear protein extracts were assayed in the ELISA plates that were washed in Model 1575 ImmunoWash microplate washer (Bio-Rad) and quantitated in a model Benchmark thermostatted ELISA reader (Bio-Rad).

Immunohistochemistry, immunofluorescence and immunoelectron microscopy for HSP70 and NF- κB location

For the evaluations by microscopy, samples of lymphocyte preparations were taken immediately after preparation (T_{zero}), just after the 2 h water bath (at 37 or 42°C) period ($T_{\text{after bath}}$) and after the recovery period of 6 h post-bath ($T_{6\text{ h}}$). At each time point, cells were harvested from the dishes, transferred to 1.5 mL microfuge tubes, washed three times in ice-cold PBS, pH 7.4 ($370\times g$ for 10 min at 4°C), and fixed

in 4% (w/v) PBS-buffered *p*-formaldehyde. The preparations were maintained at room temperature until use.

In order to assess membrane-located HSP70, the cells were processed for immunofluorescence (IF) and immunoelectron microscopy (IEM) without detergent treatment, unless otherwise stated. In order to expose membrane-bound HSP70 in lymphocytes, heat-induced epitope retrieval (HIER, at 95°C , for 5 min in a boiling water bath) was performed by washing (pulse spin, max. $2000\times g$, for 10 s at room temperature) fixed cells with PBS (pH 7.4) and treating them with 500 μL of 10 mM sodium citrate buffer, pH 6.0, without detergent. For IF studies, the cells were washed twice with PBS (pulse spin for 10 s at room temperature each) and unspecific binding sites were blocked by resuspending the cells in 50 μL of IHC blocking solution [1% (w/v) BSA in PBS, pH 7.4, containing 300 mM glycine, freshly prepared on a daily basis] for a 30-min incubation at room temperature. The cells were then centrifuged (pulse spin, max. $2000\times g$, for 10 s at room temperature), and the supernatant was carefully discarded for the cell pellet to be resuspended and incubated for 1 h (room temperature, gently rocking) in anti-HSP72 monoclonal antibody solution (mouse anti-*hspa1a*-specific FITC-labelled IgG₁, Enzo Life Sciences, ADI-SPA-810-F, 1:100 in IHC blocking solution). The cells were subsequently washed with 500 μL of PBS ($370\times g$ for 10 min at room temperature) and resuspended in 20 μL of PBS to be directly examined by epifluorescence microscopy (exc. 450–480 nm; emiss. 520–800 nm) in a motorised semi-confocal phase-contrast (PC)/differential interference contrast (DIC) Olympus model IX81 inverted microscope (dichroic mirror cubes) equipped with a xenon lamp (X-cite EXFO-Lumen Dynamics) and ColorView III Peltier-cooled CCD 5 Mpixel (Soft Imaging) camera. The data were processed via Olympus Cell[^]F software while cell counts and fluorescence quantifications were conducted by using ImageJ software and accompanying plugins (<http://imagej.nih.gov/>).

Alternatively, cells were prepared for IEM just after antigen retrieval (HIER, previously mentioned) to give more insight into membrane-associated HSP70 immunodetection. Accordingly, lymphocytes were resuspended in 50 μL of IHC blocking solution containing (or not) 2.5 mM methyl- β -cyclodextrin (β -MCD, Sigma) and incubated for 30 min at 37°C . β -MCD is a lipid raft-disrupting detergent (Broquet et al. 2003) that removes membrane cholesterol without compromising cellular viability (Vega et al. 2008). Thereafter, the preparations were pulse-spun (max. $2000\times g$, 10 s at room temperature) and the cell pellets incubated (gently rocking at room temperature) for 1 h with 50 μL of anti-HSP70 monoclonal solution (mouse anti-HSP70 IgG₁ clone 3A3, Santa Cruz Biotechnologies, sc-32239, 1:100 in detergent-free blocking solution), which recognises both *hspa1a* and *hspa8* forms of HSP70. Negative controls were processed identically except that mouse anti-HSP70 IgG was replaced with 3.5 μg of pre-immune mouse IgG serum

(Sigma). After three pulse-spin washings, the cells were incubated for 1 h (gently rocking at room temperature) with HRP-labelled secondary antibody (rabbit anti-mouse IgG, Sigma A9044, 1:100 in blocking solution) without any previous endogenous peroxidase-blocking treatment (e.g. H_2O_2). Afterwards, the samples were pulse-spun, washed three times with PBS and incubated at room temperature for 15 min with 50 μL of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) reagent [0.05% (w/v) DAB in 10 mM Tris-HCl, pH 7.6, containing 154 mM NaCl and 0.001% (w/v) H_2O_2]. The samples were then washed three times with PBS, resuspended in 20 μL of PBS and mounted onto glass slides with coverslips to be analysed by negative PC and DIC microscopy, previously to be further processed for IEM. Another lot of DAB-treated cells was subsequently post-fixed for 90 min in 2% (w/v) glutaraldehyde in PBS (pH 7.4) containing 1% (w/v) OsO_4 , being dehydrated in ascending alcoholic series and pre-embedded in a propylene oxide (Fisher Scientific)/Araldite (Durcupan ACM, Fluka) mixture to be finally embedded in Araldite as described by Dos Santos et al. (2005). The preparations were then mounted onto glass slides and left to polymerise for 2 days at 56 °C. Semi-thin sections (1 μm) were cut with the ultramicrotome MT 6000-XL (RMC, Tucson, USA) using glass knives and stained with 1% (w/v) toluidine blue solution in 2% (w/v) sodium borate for the evaluation of section quality under PC light microscopy. Ultrathin sections (70 nm) were further obtained with the ultramicrotome Ultracut UCT 2.0 employing a diamond knife (Diatome). After that, the sections were treated with 2% (w/v) uranyl acetate (Merck), for counterstaining of lipid and proteins, followed by 1% lead citrate (proteins and glycogens) also purchased from Merck, thus allowing clear identification of exosomes and other membrane-associated structures. Ultrathin sections were mounted onto copper grids (200 mesh, Sigma) and examined under a 40–120 kV transmission electron microscope (JEM 1200 EXII model JEOL, Japan) equipped with a CCD camera and companion Gatan software for image analyses. Since the insoluble brown polymer that is produced by the reaction of DAB with H_2O_2 in the presence of IgG-labelled peroxidase is highly osmiophilic (Hanker 1979), HSP70-labelled ultrastructures can be easily visualised under electron microscopy.

For the visualisation of HSP70 intracellular location and tests for the eventual colocation with p65-NF- κB , *p*-formaldehyde fixed cells were processed for antigen retrieval (HIER) as described earlier and, after washing, lymphocyte pellets were resuspended in 50 μL of IHC blocking solution (as previously mentioned) containing 0.25% v/v Triton X-100 (Sigma) and incubated at room temperature in microfuge tubes for 30 min in order to permeabilise the cells. Afterwards, the cells were centrifuged (pulse spin, max. 2000 $\times$ g, for 10 s at room temperature) and incubated for 1 h at room temperature in 50 μL of a mixture containing mouse

anti-HSP70 (H5147 Sigma, which recognises HSP72 and HSP73; 1:100) and rabbit anti-p50 NF- κB (from Cayman Combo Transcription Factor Assay Kit #10011223; 1:100) in IHC blocking solution with gentle rocking. After being washed three times with 100 μL of IHC blocking solution, the cells were incubated for 1 h at room temperature with gentle rocking in 100 μL of a mixture containing FITC-labelled donkey anti-mouse IgG (Abcam ab97029; 1:100) and biotin-labelled goat anti-rabbit (Sigma, B8895 1:100) antibodies, respectively, in IHC blocking solution. After that, the cells were washed three times (pulse spin, max. 2000 $\times$ g, for 10 s at room temperature) with 100 μL volumes of blocking solution and incubated with Cy3-labelled streptavidin (GE Health Care, PA43001; 1:1000) for 1 h in order to detect NF- κB bound to biotin-labelled antibodies. The cells were then washed three times in IHC blocking solution as described earlier and resuspended in 50 μL of the fluorescence stabilising reagent Fluorshield™ with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Sigma, F6057) during 5 min for counterstaining nuclei. The preparations were mounted onto gelatine-coated slides with coverslips, and the images were acquired in a Olympus model IX81 epifluorescence microscope (detailed earlier). Negative controls with either no primary or secondary IgGs were processed in parallel identically, except that monoclonal antibodies were replaced with 3.5 μg of either pre-immune mouse or rabbit IgG serum (Sigma). Negative controls for endogenous biotin were also performed by using only Cy3-labelled streptavidin and no secondary antibodies.

Quantitative morphometric analysis

Data reported are the results from five separate experiments. Thin sections were cut from three or more randomly selected samples for each experimental condition in each experiment, and at least, five different thin sections were examined under ME as previously described (Smith et al. 1991). IHC and membrane-bound HSP70-DAB labelling analyses were carried out in at least five randomly assigned optical fields for each experimental condition in each experiment. Although there was some cell-to-cell variation in cell diameter and depth of cytoplasm, when pooled data of ten cells were examined, no significant differences were detected between experimental conditions or different experiments. The data were processed via the Olympus Cell[^]F software while cell counts, areas and mean integrated density of fluorescence (IntDen) quantifications were conducted by using ImageJ software (<http://imagej.nih.gov/>) and accompanying plugins, after background subtraction. The results were expressed as means $\pm$ SD of the quantifications from at least three photomicrographs from each experimental group.

Statistical analysis

Preceding any statistical analysis, all outcome variables were assessed for normality through the Kolmogorov-Smirnov test. Data are presented as the mean $\pm$ SD except if otherwise stated. Concerning molecular biology analyses and IF microscopy, the minimum calculated sample size to detect differences in magnitude equalling 1.41 standard deviation units, keeping $\alpha = 0.05$ and test power of 80%, was $n = 5$ animals in each group, as inferred from previous studies (Kolberg et al. 2006). Comparisons between test groups were performed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. A two-way (time-by-trial) repeated-measure ANOVA was performed to test significance between and within trials when necessary. For time-line experiments, repeated-measure one-way ANOVA was used. Individual p values for each experiment were provided alongside data presentation. Correlations were tested by using the parametric Pearson's product-moment correlation and the non-parametric Spearman's rank correlation followed by bi-tailed Student's t test. Pearson's r and Spearman's ρ ($\pm\sigma$) coefficients were also calculated and given appropriately. For the correlation between H-index and interleukin ratios, we first fit the data using a robust form of non-linear regression, based on the assumption that scatter follows a Lorentzian distribution (Press et al. 1988) and asymmetric five-parameter logistic (5PL), as described (Giraldo et al. 2002). Fit statistics for 5PL was performed as described by Gottschalk and Dunn (2005). After curve fitting, correlations were predicted (extrapolated) by using the online MyCurveFit software (<https://mycurvefit.com/>). Data were also tested for the presence of outliers as described (Motulsky and Brown 2006). We used adjusted R^2 that quantifies how well a model fits the data. The adjusted R^2 accounts for the number of parameters fitted by the regression and so can be compared between models with different numbers of parameters (Miles 2014). The risk α for type I errors was settled for $p < 0.05$. GraphPad Prism 6.0 for Windows was used.

Results

Exercise boosts lymphocyte HS response and stimulates eHSP70 delivery from plasmalemma

Just after exercise, lymphocytes were submitted to HS (42 °C for 2 h) or maintained at 37 °C for the same period to be cultured for an additional 6 h recovery period. As shown in Fig. 1, exercise enhanced eHSP70 secretion into culture medium by up to 3.3-fold (8% group), which was much enhanced by HS treatment (up 6.9-fold in relation to 8% control group). At the same time, exercise boosted lymphocyte HS response (assessed by the increment in iHSP70 expression in

Fig. 1 Combined effects of exercise and heat shock (HS, 42 °C, 2 h) treatment on lymphocyte iHSP70 accumulation and eHSP70 release into the culture medium during the 6 h recovery time in culture. **a** HSP70 released (eHSP70) into culture medium. **b** Intracellular HSP70 content (iHSP70) from lymphocytes obtained from mesenteric lymph nodes of rats after 20 min of different exercise workloads (as the % of body weight) or maintained at rest. White bars, controls; black bars, HS-treated cells. For the comparisons (ANOVA), * $p < 0.05$ vs all lower workload groups; # $p < 0.05$ vs respective control 37 °C groups; § $p < 0.05$ vs all groups. Data are presented as means $\pm$ SD of five experiments with five animals per group. A representative gel is given

response to HS treatment), in a workload-dependent fashion, by roughly 60% in relation to 37 °C groups (Fig. 1b). Interestingly, however, iHSP70 expression was maximal at 6% load in both 37 °C controls and HS cells, decreasing afterwards. This occurred in antagonism to which was observed in relation to exported eHSP70 in 8% group (Fig. 1a). Because eHSP70 can be liberated from exosomes under stressful conditions, we labelled the cells with FITC-anti-HSP70 antibodies without detergent permeabilisation in order to check whether the source of eHSP70 could be lymphocyte plasmalemma. In fact, exercise strikingly decreased membrane HSP70 labelling (Fig. 2a) in a workload-dependent

correlation ($r = -0.8911$; $p = 0.0424$; Fig. 2b). Cultivating the cells for 2 h at 42 °C decreased HSP70 membrane labelling even further, but there was no more correlation with exercise load, up to 6 h in culture (Fig. 2c; please see also Supplemental Table S1 for raw fluorescence data). Since HSP70 release into culture medium may be due to cell death, we investigated lymphocyte viability and nuclear morphology under the same previous situations. As shown in Fig. 2d and e, none of the conditions or treatments promoted nuclear fragmentation and shrinkage, or chromatin condensation, and this was in parallel with unchanged cell viability (Trypan blue, as described in the “Methods” section). Interestingly, however, nuclear size decreased with culture time ($r = -0.9085$; $p = 0.0328$) independent of cell treatment or exercise effort (Fig. 2f).

In order to provide further insight into membrane location, lymphocytes were treated (or not) with the lipid-raft-disrupting detergent, methyl- β -cyclodextrin (β -MCD), and processed for HSP70 membrane immunohistochemistry (Fig. S3 and Table S1) and IEM (Fig. 3). The results mimicked those from IF studies (as previously mentioned) in that exercise conspicuously decreased the detection of HSP70 in lymphocyte membrane microdomains (Fig. 3a–c), whose presence was also abolished by β -MCD (Fig. 3d–f). Culturing cells for longer times did not modify this pattern any longer (Fig. 3g–j). Surprisingly, however, it was observed that HSP70 immunolabelling accumulates in discrete portions of nuclear envelope (arrowheads in Fig. 3), which were resistant to both detergent (β -MCD) and temperature treatment, but completely abolished by exercise (cf. Fig. 3a, c) and time in culture (Fig. 3h–j).

The degree of HS response is inversely correlated with NF- κ B activation and directly associated with exercise load

eHSP70 export to lymphocyte culture media (T_{6h}) was found to be enhanced in cells obtained from strenuously exercised animals (Fig. 1a), while iHSP70 indeed decreased in the same group (Fig. 1b), irrespective of HS treatment. Hence, we speculated that this could be related to the observational timing. Then, we analysed the time course of iHSP70 contents in both control and HS cells. As depicted in Fig. 4a, exercise markedly enhanced the HS response of the cells, up to moderate loads (4% groups), in a time-dependent way, without affecting basal iHSP70 levels. However, at high intensity (6% groups), exercise increased both basal (by up to 68%) and HS-induced (up to 2.75-fold) iHSP70 expression; i.e. exercise enhances the HS response of lymphocytes even without heat treatment. Conversely, at strenuous exercise loads, there was no change in either basal or HS-elicited iHSP70. As the HS response (and HSP70 itself) blocks NF- κ B activation, we chased up NF- κ B binding activity to DNA HS element during the same

time. The results (Fig. 4b) showed that, as compared to rest animals, lymphocytes from exercised rats retained NF- κ B binding activity up to moderate loads, while HS-treated cells decreased binding activity proportionately to their respective HS responses (Fig. 4a). Nevertheless, in the high-intensity group, NF- κ B binding increased by up 68% in control cells (just after water bath), whereas a deeper fall was observed in HS-treated ones. Strenuous exercise did not alter either basal or HS-dependent binding. Therefore, we sought out whether this finding could be matched by IF analyses of detergent-permeabilised cells. In fact, as shown in Fig. 5, exercise induced a workload-dependent reorganisation of iHSP70 location within lymphocytes in nuclear stress granules and cytoplasm that was followed by NF- κ B co-location (please see raw fluorescence data in Table S1). Most importantly, at higher loads, iHSP70 was strongly associated with NF- κ B in the cytosol, which might impede migration of the nuclear factor into the nucleus. This is even more evident in HS-treated cells. Similarly to that found for nuclear measurements (Fig. 2f), averaged lymphocyte areas decreased with culture time ($r = -0.9720$; $p = 0.0056$) independent of cell treatment or exercise effort (Fig. 5f).

Exercise modulates lymphocyte proliferation and modifies immunoinflammatory status in a workload-dependent way

In order to understand the repercussion of the previous results for lymphocyte function, we investigated the effects of exercise loads on basal and mitogen-stimulated lymphocyte proliferation and cytokine production in culture. Accordingly, exercise did not affect basal proliferative responses (Fig. S4) but dramatically and workload-dependently modulated cell proliferation, in order that stimulation index (SI) peaked at moderate exercise loads (Fig. 6a). Additionally, HS treatment significantly reduced SI as per T_{6h} group data. Since NF- κ B activation commands pro- and anti-inflammatory cytokine production by lymphocytes, we argued whether there would exist any connection between exercise, HS and interleukin (IL-2, IL-6 and IL-10) release by the cells. When analysed individually, none of the ILs had the production appreciably affected (Fig. S5). However, when IL-2/IL-10 ratios were calculated, it became clear that both exercise and HS treatment shifted cytokine status to a more inflammatory one (Fig. 6b). In fact, at strenuous workloads, exercise increased IL-2/IL-10 ratio by 140% under control (37 °C) condition while enhancing it by up to 15.5-fold in HS-treated cells. This result cogently matched H-indices of extra to iHSP70 concentrations (please see explanation in “Methods” section), as depicted in Fig. 6c. Indeed, we found a strong sigmoidal correlation ($R^2 = 0.9868$; $p = 0.000019$) between H-index and IL-2/IL-10 ratio (Fig. 6d): the higher is the H-index, the more that the immune status tends to be pro-inflammatory.

Fig. 2 Combined effects of exercise and heat shock (HS, 42 °C, 2 h) treatment on HSP70 membrane location in lymphocytes. Lymphocytes were fixed and prepared for membrane-directed immunofluorescence (with no detergent pre-treatment) just after exercise (T_{zero}), just after the 2 h water bath (at 37 or 42 °C) period ($T_{after\ bath}$) and after the recovery period of 6 h post-bath (T_{6h}). **a** Fluorescence due to HSP70 (*hsp70*) membrane location (given as the $\log_{10}$ of integrated density of fluorescence per cell) progressively decreases with exercise load (**b**) in

T_{zero} lymphocytes, but not in $T_{after\ bath}$ or T_{6h} groups or treatments (**c**). DAPI labelling of lymphocyte nuclei indicates no alteration in nuclear morphology or apoptosis-related nuclear fragmentation and swelling (**d**, **e**). Conversely, a negative correlation between nuclear area and time in culture is observed after water bath treatments, irrespective of exercise load or culture condition (**f**). Representative photomicrographs and fields are given. Original magnification $\times 960$. Scale bar = 10 μm

Discussion

A robust tissular HS response is sine qua non for the maintenance of a balanced inflammatory status, i.e. the capability of efficiently triggering an inflammatory response that evolves towards its own resolution, thus avoiding chronic inflammatory conditions (Newsholme and Homem de Bittencourt 2014). Although low iHSP70 contents in metabolic tissues (e.g. adipose tissue, skeletal muscle, liver) are closely related to poor resolution of inflammation and the persistence of

chronic inflammatory diseases (Leite et al. 2016), the failure in arming a robust HS response under stressful situations is a much more serious concern (Török et al. 2014). Therefore, the observation that a single 20-min exercise session was capable of enhancing lymphocyte HS response in lymph nodes is sound. It is also noteworthy that lymphocytes responded to exercise, delivering membrane-associated HSP70 into culture medium, most alike that which has been observed in peripheral blood mononuclear cells (PBMC) submitted to heat stress (Hunter-Lavin et al. 2004; Clayton et al. 2005; Ireland et al.

Fig. 3 Immunoelectron microscopy of HSP70 location on lymphocyte membranes after different intensities of exercise and heat treatments. The *arrows* indicate strong electron-dense structures related to anti-HSP70 (*hspa1a* + *hspa8*) DAB-peroxidase labelling in lymphocyte membranes from rats maintained at rest with **no** detergent treatment (**a** $\times 25,000$ and **b** $\times 120,000$). *Arrowheads* in **a**, **d**, **e**, **f**, **h** indicate nuclear envelope labelling. (**c** $\times 30,000$). Lymphocytes from the 8% group just after exercise showing **no** clear evidence (*arrows*) of HSP70 labelling (also with **no** detergent treatment) (**d** $\times 12,000$). Rats were maintained at rest and lymphocytes were soon taken and treated with the lipid rafts/caveolae disrupting detergent methyl- β -cyclodextrin (β -MCD) showing no longer plasma

membrane immunostaining, as evidenced by the *arrows* in higher magnifications (**e** $\times 25,000$ and **f** $\times 120,000$). Conversely, perinuclear HSP70 labelling (*arrowheads*) was not affected (**e**, **f**). Lymphocytes were also examined 2 h after water bath at 37 °C (**g** $\times 25,000$) or 42 °C (**h** $\times 10,000$) and 6 h after water bath at 37 °C (**i** $\times 15,000$) or 42 °C (**h** $\times 40,000$). *Arrows* indicate the persistence of plasmalemmal HSP70 labelling just after water bath at 37 °C (**g**) that was much weaker after a 42 °C water bath, in which perinuclear (*arrowheads*) labelling is still present (**h**). Plasmalemmal HSP70 labelling (*arrows*) is virtually absent in lymphocytes incubated for 6 h, whether at 37 or 42 °C (**i**, **j**). Representative photomicrographs and fields are given

Fig. 4 Time course of intracellular HSP70 (iHSP70, *hspa1a* + *hspa8*) immunocontents and p65/NF- κ B DNA binding activity in lymphocytes obtained from rats submitted to different exercise workloads. For iHSP70 expressions normalised by β -actin immunocontents (**a**), lymphocytes were taken immediately after exercise (T_{zero}), just after the water bath period of 2 h ($T_{after\ water\ bath}$) or following 2, 4 and 6 h in culture after the water bath period at 37 °C (*dashed lines with open circles*) or HS at 42 °C (*continuous lines with closed circles*). A representative gel from each group is furnished. For NF- κ B binding activity (**b**), lymphocytes

(from the same samples assessed for immunoblotting) were taken immediately after exercise (T_{zero}), just after the water bath period of 2 h ($T_{after\ water\ bath}$) or after 6 h in culture following to the water bath period ($T_{6\ h}$) at 37 °C (*dashed lines with open circles*) or HS at 42 °C (*continuous lines with closed circles*). Data are presented as means $\pm$ SD of three separate experiments. Comparisons (repeated-measure one-way ANOVA): * p < 0.05 between 37 and 42 °C groups at the same time points; # p < 0.05 for the comparison with T_{zero} at the same temperature group

Fig. 5 Combined effects of exercise and heat shock (HS, 42 °C, 2 h) treatment on intracellular HSP70 (*hspa1a* + *hspa8*) and p65/NF- κ B location in lymphocytes. Lymphocytes were fixed and detergent-permeabilised for immunofluorescence analyses just after exercise (T_{zero}), just after the 2 h water bath (at 37 or 42 °C) period ($T_{after\ bath}$) and after the recovery period of 6 h post-bath (T_{6h}). T_{zero} . **a** HSP70. **b** NF- κ B and **c** merged images. **d** HSP70, $T_{after\ bath}$ and T_{6h} under both

temperature conditions. **e** NF- κ B, $T_{after\ bath}$ and T_{6h} under both temperature conditions. **f** Negative correlation between averaged fluorescent areas and time in culture that is observed after water bath treatments, irrespective of exercise load or culture condition. Representative photomicrographs and fields are given. Original magnification $\times 960$. Scale bar = 10 μ m

2007). In fact, ectocytotic secretion of Triton X-100-resistant β -MCD-soluble HSP70-containing exosomes (Broquet et al. 2003; Lancaster and Febbraio 2005; Mambula et al. 2007) has been described in a variety of cell types (De Maio and Vazquez 2013), not only in PBMC. These findings may also suggest that exercise and heat treatment share common pathways that boost HSP70 expression and secretion. Because eHSP70 induces the activation of macrophages, monocytes, dendritic cells and NK cells, while modulating endotoxin signalling and increasing microbicidal capacity and chemotaxis of neutrophils (De Maio and Vazquez 2013), exercise effects over HSP70 physiology described herein in a secondary

lymphoid organ may have important implications for balancing immune responses. Of note, it does not matter whether these lymph node-harboured lymphocytes came from circulation during (or after exercise) or were already residing in the lymph nodes previous to exercise: a single bout of 20-min exercise was capable of dramatically changing lymphocyte function that was associated with HSP70 status. Additionally, although such effects could be seen 6 h after exercise sessions (e.g. HSP70 expressions), the cells in study were obtained immediately after exercise, so that it is clear that exercise produces strong and rapid effects on immune cells that are linked to the HS response. Interestingly, at time zero

Fig. 6 Combined effects of exercise and heat shock (HS, 42 °C, 2 h) treatment on lymphocyte stimulation index of proliferation, IL-2 to IL-10 ratios and eHSP70 to iHSP70 index (H-index). Lymphocytes from exercised rats (or rest controls) were obtained just after exercise (T_{zero}), just after the 2 h water bath (at 37 or 42 °C) period ($T_{\text{after bath}}$) and after the recovery period of 6 h post-bath ($T_{6 \text{ h}}$). **a** Cells were cultivated for the assessment of [^3H]-thymidine incorporation into DNA, and stimulation index (SI) of proliferation was calculated as the ratio between counts from Con A-stimulated cells and Con A-unstimulated ones as described in the “Methods” section. Data were calculated from the original values depicted in Supplemental Fig. S4. **b** Supernatant fractions from lymphocytes cultured during the 6-h recovery period post-bath ($T_{6 \text{ h}}$) at 37 °C (white bars) or 42 °C (black bars) were taken for the measurement of the ratio between the pro-inflammatory IL-2 and the anti-inflammatory IL-10, whose original values are given in Supplemental Fig. S5, while the relative ratios between the pro-inflammatory HSP70 (eHSP70) and the anti-inflammatory HSP70 (iHSP70) based on rest control values (at 37 °C) were expressed as H-index (**c**), whose original values were obtained from Fig. 1. **d** Sigmoidal correlation between IL-2/IL-10 ratio and H-index is given. In this case, dashed line indicates extrapolated data that continue in Supplemental Fig. S6. All data are presented as means $\pm$ SD (propagated appropriately after quotient calculations) of five experiments with five animals per group. Statistics: for SI (**a**), p values indicate differences between 4% group and the remaining ones in each test time, while $*p < 0.05$ vs all lower workload groups within each test time by repeated-measure ANOVA (Bonferroni post-test); $\#p < 0.05$, in relation to T_{zero} and $T_{6 \text{ h}}$ at 37 °C at the same exercise loads by two-way ANOVA (Bonferroni). In **b**, **c**, $\#p < 0.05$ vs control 37 °C groups; $\$p < 0.05$ vs all groups (one-way ANOVA, Student-Newman-Keuls post hoc test). SPL fit statistics was employed in **d**

(i.e. just after exercise), immunoelectron perinuclear HSP70 labelling is observed only in lymphocytes from resting animals (Fig. 3a, d, e, f), but not from strenuously exercised ones (Fig. 3c). Such perinuclear pattern of labelling is also observed in the rest group just after the 2 h water bath, but only in the 42 °C treated cells, not in the controls. Curiously, after 6 h in culture, we could not observe this perinuclear labelling in any of the test groups or controls. Although we have at present no experimental evidence, we speculate that this change of location pattern could be related to workload-dependent reorganisation of iHSP70 location within lymphocytes in nuclear stress granules (Sandqvist and Sistonen 2004), as observed in fluorescent images of Fig. 5. Whether temperature rise and/or α -adrenergic stimulation observed during exercise bouts takes part in exercise-induced alterations described earlier as well as in the activation of the HS response in immune cells, as observed in related works (Matz et al. 1996; Johnson et al. 2005; Whitham et al. 2006; Ogura et al. 2008; Gibson et al. 2014), is now under investigation in our laboratory.

It is unarguable that physical exercise introduces health benefits that include cardiovascular, neuroendocrine, gut microbiome and mental and immune gains. Whatever the volume and intensity employed, exercise clearly provides health benefits to humans that subsist even with short sessions beneath recommended intensity and duration (Eijssvogels and Thompson 2015). At present, however, there is no definitive

congruity on the “dose” of exercise that may positively impact immune function, especially concerning immunoinflammatory balance. Notwithstanding, in response to acute exercise, a rapid interchange of immune cells between peripheral lymphoid tissues and the circulation occurs and this response depends on many factors, including the intensity, duration and mode of exercise (Nieman 1997; Walsh et al. 2011a, 2011b). Herein, we showed that exercise enhances lymphocyte HS response (but not necessarily iHSP70 contents) up to moderate intensity, this being paralleled by decreased NF- κ B binding activity (Fig. 4) associated with NF- κ B cytosolic location (Fig. 5) and corresponding to both a maximal proliferative response (Fig. 6a) and minimal ratios for IL-2/IL-10 and H-indices (Fig. 6b, c). In other words, moderate exercise induces the best proliferative response with the minimal pro-inflammatory status. At high-intensity workload (6% group), however, exercise induced a rise in both basal and heat-stimulated HS responses paralleled by a conspicuous depression of NF- κ B activation and decreased proliferative response. Interestingly, at strenuous doses of exercise, it was observed a dramatic shift of lymphocyte pattern into one remarkable pro-inflammatory (high IL-2/IL-10 and H-index ratios) to the detriment of cell proliferation (Fig. 6). Therefore, at least in relation to immunoinflammatory status, the predicted (Walsh et al. 2011a) “inverted-U” relationship between exercise volume and immune responses (or the “U-shaped” association between physical activity and resistance to disease) is confirmed by the present results.

A crucial corollary of the previous observations is that H-index, obtained from tissue or circulating cells against extracellular eHSP70, stratifies different clinical conditions as per their immunoinflammatory states. We have prepared an extensive review of works in which H-indices could be calculated to summarise this point (please see Table S2). Accordingly, despite the tissue or cell type used to measure iHSP70 and regardless eHSP70 being assessed in serum, plasma or culture media, H-index was encountered to be enhanced in total PBMC as well as in lymphocytes, monocytes and NK cells in relation to plasma of both humans and rodents submitted to acute exercise sessions (Horn et al. 2007; Ruell et al. 2014; Lancaster and Febbraio 2005; Schöler et al. 2016), similar to that observed in the present work. The same was inferred from muscle iHSP70 samples in relation to plasma of acutely exercised humans (Walsh et al. 2001). Conversely, exercise training appears to always impose a state of diminished H-index (PBMC $\times$ plasma) in relation to control subjects (Magalhães et al. 2010; Yamada et al. 2007), which is exacerbated by training in the heat (Bruchim et al. 2014). Exercise training also reduces H-index in metabolic tissues of rats submitted to inescapable stress, which is correlated with inflammatory cytokine patterns (Speaker et al. 2014). As observed in the present study, heat treatment of isolated cells, on the other hand, tends to remarkably enhance H-index in different cell types,

especially in PBMC of human and rodent origin (Hunter-Lavin et al. 2004; Lancaster and Febbraio 2005). H-index was found to be positively associated with systemic markers of injury and plasma LDH in humans (Yang et al. 2007) and obese mice exposed to environmental pollutants (Goettems-Fiorin et al. 2016), in which H-index reflects increased pancreas dysfunction in obesity-induced diabetes. H-index is also correlated with the severity of human peripheral arterial disease (Madden et al. 2010) and homeostatic model assessment-insulin resistance (HOMA-IR) in obese diabetic patients (Rodrigues-Krause et al. 2012). Finally, H-index is correlated with pro-inflammatory cytokine profile in hypoglycaemic rats (Ludwig et al. 2014).

Taken as a whole, the present findings and the previous results depicted in Supplemental Table S2 lead us to conclude that exercise training is always associated with $H < 1.00$, whereas if training is performed in the heat, H values are even lower. Acute moderate exercise presents $1.00 < H < 2.00$, while acute high intensity to strenuous exercise promotes $H > 5.00$. On the other hand, basal H-index values obtained from patients and animal models of chronic degenerative diseases of inflammatory nature were found to be always elevated and higher than 5.00. High basal H-indices are associated with high HOMA-IR (Rodrigues-Krause et al. 2012), visceral obesity and insulin resistance (Goettems-Fiorin et al. 2016), and elevated ultra-sensitive C-reactive protein (CRP) levels (Madden et al. 2010). Moreover, in the latter conditions, high H-indices directly correlate with high control-normalised IL-2/IL-10 values, which in turn are positively associated with pro-inflammatory states (Table S3). Strikingly, by plotting H-indices obtained from Table S2 versus IL-2/IL-10 ratios (Table S3) under different conditions, we confirmed the sigmoidal correlation between H-index and IL-2/IL-10 ratios (Fig. S6a) that approaches maxima at H-index of 542.9 and IL-2/IL-10 ratio of 4734. In addition, we realised that the utmost point depicted in Fig. 6d ($x = 20.13$; $y = 31.66$), which appears to be an outlier, is not so. On the contrary, it is situated near the inflexion point ($x = 19.188$; $y = 28.345$) of the whole curve (Fig. S6b). The physiological significance of such fitting and these maxima in vivo are currently under investigation in our laboratory.

Finally, the potential of the whole organism to efficiently trigger a robust HS response can be evaluated by the ability of PBMC from the donor to respond to heat treatments with $H > 2.00$ (Table S2). This is remarkable, having an enormous clinical repercussion, as H-indices can be assessed with ease by examining plasma eHSP70 and iHSP70 expression in heat-treated PBMC (against control cells maintained at 37 °C) after an appropriate time (Krause et al. 2015b). In addition, as judged from the present data in secondary lymphoid organ, it is plausible that H-index obtained by assessing plasma and PBMC HSP70 values fully matches individual tissue H-index, as suggested by the comparisons made in Table S2. This possibility is currently under evaluation in our laboratory.

Clinical perspectives

In the present study, we confirmed the expectation that acute exercise, in a workload-dependent manner, modulates cytokine production and cell proliferation of lymphocytes from secondary lymphoid organ in an inverted-U fashion, in which moderate exercise presents maximal stimulation, thus constituting the best exercise regimen to be clinically applied if immune balance is to be targeted. Moreover, exercise strikingly boosts the HS response in lymphocytes, whereas the kinetics of iHSP70 expression is directly correlated with suppression of NF- κ B binding activity and nuclear localisation, in a workload-dependent way. The comparative ratios of eHSP70 to iHSP70 (H-index) accurately match IL-2/IL-10 ratios, so that H-index may be used as a robust marker of immunoinflammatory status. In total, exercise-induced enhancement of lymphocyte HS response is translated into a dramatic increase in H-index after HS treatment.

These findings are sound because H-index, obtained from the ratios of iHSP70 in tissue or circulating cells against eHSP70 (plasma or culture media), may easily stratify training status (low H-indices relative to untrained profile), overtraining (higher H-indices in relation to well-trained ones) and different clinical conditions according to their immunoinflammatory states. Indeed, H-index emerges as a potentially new biomarker for the overall effects of exercise on immune system and as a useful and precocious marker of immunoinflammatory imbalances. Therefore, in addition to monitoring exercise effects on immunosurveillance, H-index may predict clinical status of patients bearing chronic degenerative diseases of inflammatory nature in which H-index may be severely enhanced in basal assessments and much reduced (poor HS response) under heat-stressful conditions.

Acknowledgements TGH and CMS were supported by a fellowship from CAPES-Brasília. SPS, PRN and GSS were supported by fellowships from CNPq. The authors are grateful to Silvia Barbosa and Christiane Lopes for technical support in sample preparation for electronic microscopy. We also appreciate the free access to the facilities of The Federal University of Rio Grande do Sul Electronic Microscopy Centre (CME/UFRGS).

Author contribution TGH and PIHBJ designed the study. TGH, SPS, PRN and AB completed all the experiments described in this manuscript. CMS and GSS performed experiments on gene expressions. MCFH, TGH and PIHBJ designed immunoelectron microscopy analyses and MCFH blindly analysed all the samples. All authors were involved in analysing the results. TGH, CMS and PIHB supervised statistical analyses. TGH, SPS, MK and PIHBJ co-wrote the paper. PIHBJ, MK, RBB and RC provided experimental advice and helped with manuscript revision. PIHB prepared the figures, performed microscopy quantifications and revised the final version of the manuscript. All the authors had final approval of the submitted and published versions.

Compliance with ethical standards All the procedures performed in studies involving the animals followed the ethical rules established by Arouca's Act (Federal Law 11794/2008) and the Guide for Care and Use

of Experimental Animals published by the National Institutes of Health (NIH publication no. 85-23, revised in 1996). The procedures were approved by the Federal University of Rio Grande do Sul Ethics Committee on Animal Experimentation (CEUA #2008110), according to the guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

Funding PIHBJ and RC were responsible for grant support with respect to The Brazilian National Council for Scientific and Technological Development [CNPq, grant #563870/2010-9 to RC and grants #5510987/2007-8, 402626/2012-5, 402364/2012-0 to PIHBJ], while TGH was responsible for grant support from CNPq [#382692/2011-0] and [FAPERGS no. 002106-2551/13-5] which funded the present work.

Conflict of interest The authors declare that they have no conflicts of interest.

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