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HSP70 expression: does it a novel fatigue signalling factor from immune system to the brain?

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Integrative physiology studies have shown that immune system and central nervous system interplay very closely towards behavioural modulation. Since the 70-kDa heat shock proteins (HSP70s), whose heavy expression during exercise is well documented in the skeletal muscle and other tissues, is also extremely well conserved in nature during all evolutionary periods of species, it is conceivable that HSP70s might participate of physiologic responses such as fatigue induced by some types of physical exercise. In this way, increased circulating levels of extracellular HSP70 (eHSP70) could be envisaged as an immunomodulatory mechanism induced by exercise, besides other chemical messengers (e.g. cytokines) released during an exercise effort, that are able to binding a number of receptors in neural cells. Studies from this laboratory led us to believe that increased levels of eHSP70 in the plasma during exercise and the huge release of eHSP70 from lymphocytes during high-load exercise bouts may participate in the fatigue sensation, also acting as a danger signal from the immune system. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS — HSP70; immune system; exercise; fatigue

INTRODUCTION

Historically, fatigue is an intricate point of human biology.[1,2] This phenomenon has gained different explanations ranging from the catastrophic theory (also termed peripheral fatigue, i.e. peripherally based, metabolite-induced failure of skeletal muscle contractile function), to central governor theory, in which fatigue would occur only after a summation of different sensory inputs towards the central nervous system (CNS) which 'would decide' how long and how much physical activity should be done without threatening homeostasis.[2–5]

More recently, an integrative view has been established in which it is apparent that an imperative communication between the CNS and remaining systems must attempt to maintain homeostasis during an exercise demand. In this way, fatigue may be considered a 'conscious' manifestation of subconscious CNS process.[6]

Integrative physiology studies have shown that immune system and CNS have a close relation to behavioural modulation.[7] This integration occurs by inflammatory and anti-inflammatory mediators acting as signals that can modify the stress behaviour to prevent damage.[8] This commentary focuses on the participation of one of the most conserved proteins expressed during the fatigue process: the stress regulated family of 70-kDa heat shock proteins (HSP70s).

Since fatigue is related to a pre-existent stress or to a physiological behaviour that prevents stress during a homeostasis threatening situation, understanding the stress response of HSP70 within immune cells may unravel new avenues for the comprehension of fatigue, its effects and its underlying mechanisms. Indeed, recent studies from our laboratory led us to propose that increased levels of extracellular HSP70 (eHSP70) secreted towards the plasma during physical exercise and the huge release of eHSP70 from lymphocytes during high-load exercise bouts may both participate in the fatigue sensation by the CNS.

HEAT SHOCK PROTEINS

HSPs are highly conserved proteins in both eukaryotic and prokaryotic organisms. The first report about them was documented in salivary gland cells of *Drosophila buskii* after a serendipitous heat shock by Ritossa.[9–11] Their molecular entities, however, were only characterized later in 1974.[9–11]

HSPs are categorized in families according to their molecular sizes and include HSP110, HSP100, HSP90,

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HSP70, HSP60 HSP30 and HSP10 subclasses. The most studied (due to its evident high expression in mammalian cells under stress conditions) and conserved is the 70-kDa family (HSP70), which comprises a number of related proteins whose molecular weights range from 66 to 78 kDa. HSP70 isoforms are encoded by a multigene family consisting of, at least, 13 distinct genes in humans so far studied.[12] For the rationalization of the current nomenclature, human HSP70 genes (rat and mouse, also) have given the locus symbol HSPAx, where A defines members of HSP70 family and X designates the individual loci. In this sense, HSPA8 is the human gene that encodes a 73-kDa constitutive form of HSP70 (HSP73 or HSC70, the cognate form), while HSPA1A gene, located at the major histocompatibility complex (MHC) III region, encodes an inducible form (HSP72 or simply HSP70). In humans, but not in the rat or the mouse, there is an even higher inducible form (HSP70B') encoded by HSPA6 gene. Other representative members, besides mitochondrial (HSP75) and endoplasmic reticulum (HSP78) members of HSP70 family, are found in the intracellular space.[13]

HSP70s are known to function as intracellular molecular chaperones that facilitate protein transport, prevent protein aggregation during folding, and protect newly synthesized polypeptide chains against misfolding and protein denaturation. Molecular chaperone property of such proteins allow them to assist the non-covalent assembly/disassembly of other macromolecular structures without being permanent components of such structures when they are performing their normal biological functions.[12] Additionally, molecular chaperones assist the unfolded protein to achieve its single correct three-dimensional configuration (by whatever mechanism it has evolved to generate this folded state), without becoming a constituent of the final folded protein.[12]

While the constitutive form is expressed in a wide variety of cell types at basal levels (being only moderately inducible), the so-called inducible HSP70 forms (which are barely detectable under non-stressful conditions) could be promptly synthesized under a condition of 'homeostatic stress', this being any 'homeostasis threatening' condition, such as heat, glucose deprivation, lack of growth factors and so forth. Habitually, research groups indistinctly use HSP70 as a unified term for both constitutive and inducible form.[13–15] However, HSP70 is the preferable form to be used when one refers to the inducible HSP72 protein encoded by HSPA1A gene.

All HSP70 proteins share the same overall structure. They are composed of an actin-like N-terminal nucleotide binding/ATPase domain of 45 kDa, a substrate-binding domain (SBD) of approximately 15 kDa and a C-terminal domain of approximately 10 kDa that is involved in co-chaperone binding.[16,17] It is of note that N- and C-terminal domains have expressive relevance to antigen presentation, an important way by which HSP70s participate in immune responses.[18]

Many different events can induce HSP expression, among them are environmental, pathological and physiological factors, such as heavy metal exposure, UV radiation, amino acids analogous, bacterial or viral infection, inflammation, cyclo-oxygenase inhibitors (including acetylsalicylic acid), oxidative stress, cytostatic drugs, growth factors, cell differentiation and tissue development, as reviewed early.[19]

The functions of HSP70s (both inducible and constitutive forms) are regulated by ATP hydrolysis. The chaperon activity (cycles of binding and release of native proteins during refolding process) depends on the ATP-binding state. While binding to ATP, HSP70s couple with low affinity to its substrates, but in the ADP-bound state, HSP70s bind with higher affinity to them and the ATPase activity of HSP70s is inherently weak. Cooperatively, HSP40 (a 40 kDa family of HSPs) catalyzes this reaction working as a nucleotide exchange factor, because it facilitates the ADP release. Other HSP70-interacting proteins have also been demonstrated.[20]

INTRACELLULAR FUNCTION OF HSP70

Initially, HSP70s have been described essentially in studies that addressed molecular chaperone action of such proteins, or HSP70s were shown to limit protein aggregation, facilitating protein refolding and maintaining structural function of proteins.[11] Intracellular HSP70s have further been demonstrated to be anti-inflammatory (for review, see Ref.[21]), providing cytoprotection through anti-apoptotic mechanisms, inhibiting gene expression and regulating cell-cycle progression.[22]

Besides the now classical molecular chaperone action, the most remarkable intracellular effect of HSP70 is the inhibition of nuclear factor κB (NF-κB) activation, which has profound implications for immunity, inflammation, cell survival and apoptosis. Indeed, HSP70 blocks NF-κB activation at different levels. For instance, HSP70 inhibits the phosphorylation of inhibitor of κB (IκBs), while heat-induced HSP70 protein molecules are able to directly bind to IκB kinase gamma (IKKγ) thus inhibiting tumour necrosis factor-α (TNFα)-induced apoptosis.[23] In fact, the supposition that HSP70 might act intracellularly as a suppressor of NF-κB pathways has been raised after a number of discoveries in which HSP70 was intentionally induced, such as the inhibition of TNFα-induced activation of phospholipase A_2 in WEHI-S murine fibrosarcoma cells,[24] the suppression of astroglial inducible nitric oxide (NO) synthase (iNOS, encoded by *NOS-2* gene) expression paralleled by decreased NF-κB activation[25] and the protection of rat hepatocytes from TNFα-induced apoptosis by treating cells with the nitric oxide (NO)-donor SNAP, which reacts with intracellular glutathione molecules generating *S*-nitrosoglutathione (SNOG) that induces HSP70, and, consequently, HSP70 expression.[26]

HSP70 confers protection against sepsis-related circulatory fatality via the inhibition of iNOS (*NOS*-2) gene expression in the rostral ventrolateral medulla through the prevention of NF-κB activation, inhibition of IκB kinase activation and consequent inhibition of IκB degradation.[27] This is corroborated by the finding that HSP72 assembles with liver NF-κB/IκB complex in the cytosol thus impeding

further transcription of NF-κB-depending *TNF-α* and *NOS-2* genes that worsen sepsis in rats.[28] This may also be unequivocally demonstrated by treating cells or tissues with HSP70 antisense oligonucleotides that completely reverses the beneficial NF-κB-inhibiting effect of heat shock and inducible HSP70 expression (see, for instance, Refs.[26,27]). Hence, HSP70 is anti-inflammatory *per se*, when intracellularly located, which also explains why cyclopentenone prostaglandins (cp-PGs) are powerful anti-inflammatory autacoids.[29–31]

Another striking effect of HSP70 is the inhibition of apoptosis. Caspases form an apoptotic cascade by an intrinsic pathway characterized by the release of mitochondrial pro-apoptotic factors into the cytosol, while stimulation of cell surface receptors triggers the extrinsic pathway by external signalling factors that may induce the apoptotic process. The inhibitory potential of HSP70 over apoptosis occurs via many intracellular downstream pathways (e.g. JNK, NF-κB and Akt), which are both directly and indirectly blocked by HSP70 either, besides the inhibition of Bcl-2 release from mitochondria. Together, these mechanisms are responsible for HSP70 anti-apoptotic function in cells under stress conditions.[31]

In conclusion, intracellularly activated HSP70 are cytoprotective and anti-inflammatory by avoiding protein denaturation and excessive NF-κB activation which may be damaging to the cells.

EXTRACELLULAR FUNCTION OF HSP70

After HSP70s had been found in the circulation, researchers have commenced to study the correlation between HSP70 blood levels and the prognosis in patients suffering from several diseases, usually related to oxidative stress. While healthy people usually have low plasma levels of HSP70, the association of increased blood concentrations of such proteins with illness and disease progression has been hypothesized; contrarily, longevity and health have been attributed to low levels of plasma HSP70.[32] In this way, oxidative stress, inflammation, cardiovascular disorders and pulmonary fibrosis have been directly correlated with HSP70 concentration in the bloodstream.[33] On the other hand, glutamine supplementation, which rises circulating HSP70 levels in critically ill patients, is associated with lower hospital treatment period.[34] Therefore, these studies may suggest that elevation of HSP70 levels could be an important immunoinflammatory response against physiological disorders or disease.

Since HSP70s exist in the extracellular space, molecular interactions with cell surface receptors may occur and signalling pathways could be triggered in many cell types, whereas there are a variety of receptors to HSP70 binding, amplifying the possible targets to these extracellular molecules.[35,36] However, the function of circulating HSP70 is incompletely understood yet. HSP70s are released towards the extracellular space by special mechanisms that include pumping across cell membranes through the highly conserved ABC cassette transport proteins. Recent studies have demonstrated that exosomes provide the major pathway for the vesicular secretory release of HSP70s and that heat stress strikingly enhances the amount of HSP70 secreted *per* vesicle, but does not influence the efficiency of stress-induced rate of HSP70 release and the number of exosomes neither.[37–39] A similar profile was observed in our hands (T.G. Heck and P.I. Homem de Bittencourt, manuscript in preparation), in which lymph node lymphocytes from exercised rats submitted to a further (other than the exercise bouts) challenge (heat shock) presented an HSP70 accumulation into the culture medium that is dependent on previous exercise load. Apparently, systemic eHSP70 could arise from many tissues and different cell types and this may involve distinct mechanisms of release (including necrosis) and a large variety of inducing factors.[40] Finally, HSP72 is clearly the major component of the secreted eHSP70 found in the circulation, although recent evidence suggests that other forms may also be released into the blood, as recently pointed out by De Maio.[41] eHSP70 has been shown to bind to type 2 and 4 toll-like receptors (TLR2 and TLR4) on the surface of antigen-presenting cells (APCs) similarly to lipopolysaccharides (LPS), inducing the production of the pro-inflammatory cytokines IL-1β and TNF-α, as well as NO (a product with prominent anti-microbial activity), in an NF-κB-dependent fashion.[42–45] Interestingly, however, the component of *Salmonella typhimurium* responsible for the aggregation of the bacterium to the colonic mucus has been found to be a 66-kDa protein which is correlated with the severity of the disease, while monoclonal antibodies anti-HSP65 of *Mycobacterium leprae*, as well as a polyclonal antibody against the 66-kDa protein of *S. typhimurium*, may cause dose-dependent inhibition of the aggregation of *S. typhimurium* by crude mucus preparations.[46] Because of this and other similar findings, HSP70 is considered a virulence factor of different pathogens.[47] On the contrary, it has been noticed that different *Helicobacter pylori* strains do induce downregulation of HSC70 (*HSP8*), HSP70 (*HSP1A*) and HSF-1, the main HSP70-inducible transcriptional activator in both *in vivo* and *in vitro* models.[48] Indeed, Axsen *et al*.[48] have also argued that HSPs may dampen the host's ability to trigger an inflammatory response, reinforcing the idea that, for *H. pylori*, and probably many other bacterial pathogens, inflammation is neither good nor bad, but it is rather a highly regulated and intrinsic part of chronic infection.

HSP70 INDUCED BY EXERCISE

Exercise and its inherent physiological alterations induce HSP70 expression in many tissues and cell types, not only in the muscle cells. The breakdown of cell homeostasis produced by modifications in temperature, pH, ion concentrations, oxygen partial pressure, glycogen/glucose availability, and ATP depletion are among the factors that activate HSP70 synthesis during exercise.[49]

Rise in core and muscle temperature during exercise seems an obvious way to induce HSP70. However, while skeletal muscle sustains HSP70 expression in the absence of

heat stimulus, the heart is not able to do the same, thus suggesting that the mechanisms of HSP70 protein synthesis are specifically driven in each tissue,[50–53] and that augmented temperature is insufficient to elicit HSP70 synthesis during exercise. Moreover, the susceptibility of tissues to be stressed by the environmental changes elicited by exercise varies enormously and other protective pathways may be activated in the heart, as we have shown for MRP/GS-X pump ATPases whose expression seems to prevent HSP70 expression in the heart after exercise bouts.[54]

In spite of free radicals may be produced under normal conditions, a burst in reactive oxygen species does occur during exercise.[55] Besides enzymatic and non-enzymatic antioxidant apparatus, studies in both animal models and humans implicate HSP70s as a complementary protection against oxidative damage,[56–58] particularly because HSP70s may recover oxidatively denatured proteins.

After an acute exercise session, skeletal muscle,[59] cardiac muscle[60] and other tissues, such as the liver,[61,62] have shown a state of oxidative stress, concomitantly to high concentrations of intracellular HSP70.[63] Even though oxidative stress is a strong factor to induce HSP70s in response to exercise, free radical production is not the only pathway involved in this process, since sexual hormones and adrenergic stimuli may modulate HSP70 response[64–67] and circulating monocytes from acutely exercised rats do not show appreciable changes in erythrocyte GSSG to GSH ratio and plasma TBARS, even in a state of high-profile synthesis of hydrogen peroxide.[68]

More recently, however, it has been demonstrated the presence of HSP70s in the circulation in response to exercise.[69] Since exercise is able to induce high concentrations of HSP70s in both muscle and plasma, the most obvious hypothesis was, primarily, that skeletal muscle should be the releaser of HSP70 during exercise. However, further studies have revealed that this is not the case, at all. Postural muscles express high levels of HSP70s under basal conditions, which has led to the belief in a preventive role for these proteins against muscle damage through the stabilization of ionic channels,[70] as well as myotube development.[71] HSP70s were also believed to be an important way to preserve low twitch (oxidative) muscle phenotype after frequent activation, as in physical training.[72,73] Preservation of intracellular muscular function during different exercises, venous-arterial HSP70 differences in different territories,[74] and the lack of evidence supporting the proposition that the muscle could be the major source of circulatory eHSP70 precluded the 'muscle hypothesis' and suggested that other tissues/cells should be responsible for the increase of eHSP70 in the circulation. Once HSP70 protein release from muscle to extracellular fluid could eventually happen by lysis process, and considering that the lysis of muscle fibre occurs only under severe cellular stress condition, the presence of eHSP70 during moderate exercise was found to be unfeasible. Though it had been shown that both the intensity and duration of exercise have effects in plasma[75] and muscle[76] HSP70 concentration, this rise in circulating levels of HSP70 precedes, however, any gene or protein expression of HSP70 in skeletal muscle,[77] which is another strong argument against the 'muscle hypothesis'.

As stated above, other tissues synthesize HSP70s during physiological challenges to the homeostasis, as in an acute physical exercise bout. In this way, after treadmill exercise protocol, the rat liver has been found to enhance the expression of HSP70s.[61] Moreover, and finally, human study featuring leg and hepatosplanchnic venous-arterial HSP70 difference in response to exercise has unequivocally demonstrated that the contracting muscle *does not* contribute to HSP70 circulating levels, while hepatosplanchnic viscera release eHSP70 from undetectable levels at rest to 5.2 pg min^{-1} after 120 min of exercise.[74] Additional studies have shown that oral glucose administration may exclusively reduce HSP70 release from the liver without any effect on muscle glycogen content or intracellular expression of HSP70.[78] Taken together, these results suggest that other cells may release eHSP70 during exercise, as verified during an experiment that analyzed cerebral venous-arterial HSP70 difference.[79] Although the liver seems to participate in this process, the nature of eHSP70-releasing cell during exercise remains to be established.

HSP70 AND IMMUNE SYSTEM

Although immune system cells (mainly lymphocytes and macrophages) are able to synthesize and release HSP70, there is yet no evidence about the participation of these cells in maintaining HSP70 circulatory levels during exercise. As discussed above for other cell types, besides chaperone-like functions, HSP70s present a dual effect on leukocytes depending on its cellular location, being anti-inflammatory when intracellular and pro-inflammatory when acting extracellularly. Indeed, immune cells are extremely susceptible to HSP70 inducers (for review, see Ref.[80]), so that HSP70 may be considered a target molecule for treating immune-related diseases. Moreover, strong HSP70 inducers, such as electrophilic cp-PGs dramatically inhibit viral replication (including HIV-1) in a way that completely depends on HSP70 synthesis for the entire suppression of viral life cycle.[81] Anti-viral properties of intracellular HSP70 are in association with the modification of viral protein synthesis and inhibition of viral fusion and maturation. As HSP70 synthesis precedes viral protein synthesis, HSP70 expression is not a response induced by denatured viral protein accumulation, as one might expect once HSP70s have chaperone activity. In this way, suppression of HSP70 synthesis abolishes the anti-viral features of cp-PGs.[82] Anti-viral cp-PG activity is, indeed, mediated by HSP70s through the inhibition of NF-κB translocation, which is essential for HIV-1 replication.[83,84] The equilibrium of NF-κB translocation between virus and host cell can also be a target of intracellular immune effects of HSP70.[82,85]

The secretion of exosomes with high amounts of HSP70s by peripheral blood mononuclear cells occurs in response to heat shock whereas lymphocytes of T and B types, macrophages and platelets are able to secrete exosomes as

well.[40,84] The increase in eHSP70 during the exposure to stresses has also been demonstrated to be the result of the activation of the sympathetic nervous system via alpha-adrenergic receptors leading to eHSP70 export and increased eHSP70 serum concentration.[38] Thus, even though the necrotic cell death might result in the appearance of HSP70 within the extracellular milieu, an increasing number of studies suggest that this is not the major rule but, on the contrary, physiological effectors (e.g. fever, hypoglycemia and sympathetic stimulation) are the true excitatory signals for the eHSP70 exocytotic pathway. Specifically, it has been suggested that lymphocytes are the major releasers among mononuclear cells, being responsible for nearly 100% of total eHSP70 release from immune system cells. It is of note that, although cell death may bring about the delivery of the cytosolic protein content to the plasma, the liberation of eHSP70 from lymphocytes towards the extracellular space is not associated with cell damage process. In fact, in experiments in which eHSP70 release by peripheral mononuclear cells was evaluated, the cell death counts registered have been shown to be of *ca.* 0.1% only.[11] Corroborating these data, a study in Jurkat cells has shown that lymphocytes synthesize and release HSP70 in a larger scale than monocytes/macrophages without any vestige of cell death.[80]

There is a growing body of evidence indicating that proteins of the complexes of toll-like receptor (TLR, belonging to the superfamily of the interleukin-1/toll-like) TLR2 and/or TLR4 act as cellular surface receptors to eHSP70, which 'informs' an inflammatory signal to cells of the innate immune response (macrophage/dendritic cells/neutrophils). Under stimulation of TLRs, eHSP70 signalizes cells of the innate immune response by increasing the expression of the protein of first response of myeloid differentiation inflammatory 88/kinase, which is associated to IL-1 receptor/over signal transduction of NF-κB. Asea and co-workers, have shown that eHSP70 induces NF-κB activation and the production of inflammatory cytokines in a process that requires CD14, in addition to TLR2 and TLR4. This has led to the concept that CD14 could work as a co-receptor to eHSP70.[44,86] Interestingly the binding of TLR2 and/or TLR4 selective receptor agonists (Pam3Cys that binds TLR2, or taxol that binds TLR4) to CD14 results in synergic increase of NF-κB activation,[43] thus suggesting that the release of eHSP70 into the blood after exposure to stressor agents (e.g. exercise, and even psychological stressing situations) could result in disseminated inflammation.[87]

eHSP70 stimulates the proliferation of $TCD4^+$ lymphocytes and changes their phenotype to a more cytotoxic one, since the mediators found in inflammation IL-6 and IL-8 are produced in response to eHSP70. On the other hand, $TCD8^+$ lymphocyte properties are not affected significantly. However, eHSP70 binding to $TCD3^+$ lymphocytes, via TLR2 receptors, can result in adaptive and innate immune response activation, specifically assisting $CD8^+$ lymphocyte cytotoxic activity and promoting the proliferation of natural killer (NK) cells.[11] Circulatory eHSP70 can produce cellular effects by binding to cell surface receptors in APCs, via the interaction with TLR2, TLR4, CD40, CD91, CD14, CCR5 receptors and scavenger receptors LOX-1 and SREC-1. Nevertheless, few information does exist about HSP70 receptors in lymphocytes, in active or inactive states.[35,39,88]

HSP70s bind to several macrophage surface receptors and regulate specific and non-specific key functions of antigens, including cytokine release, phagocytosis, tumour rejection and upregulation of co-stimulatory molecules.[36,45,89–94] HPS70s are also intimately implicated in innate immune response, as many, if not all, class I MHC proteins present HSP ligands for immune inspection.[95]

Since HSPs are a sort of proteins extremely well conserved in nature during the evolution of all species, one can, teleologically, suppose that HSPs shall participate in many primitive physiologic responses of the organisms. The cytoprotection given by the production of intracellular HSP72 in response to the thermal stress is a good example.[96] The link of eHSP70 with receptors that are highly preserved during the evolution, such as TLRs, offers another clue in that these proteins, beyond its role as molecular chaperones, may participate as a danger signal from the immune system to the whole body. It has also been hypothesized that the ability of leukocytes to express HSP72s could be an indicative of a good prognosis in different conditions. For instance, it has been shown that maintaining physical activity during aging can preserve the ability of leukocytes to induce HSP72 in response to physiological stress, this being associated with good health parameters.[81]

EXTRACELLULAR HSP70 AND IMMUNE FUNCTION DURING PHYSICAL EXERCISE

The role of eHSP70s in immune responses[18,97,98] has been studied in several homeostasis threatening conditions apart from exercise.[69,74,78,80] Many, if not all of them suggest that circulating eHSP70 could be an immunomodulatory mechanism induced by exercise, being of primordial relevance for the comprehension of the origin of such a protein, as well as the immune cell responses during higher eHSP70 plasmatic levels during exercise.

At rest, thereabout 50% of immune cells are found in the bloodstream, while another half is adhered to the vascular wall. At the start of an exercise effort, the majority of immune cells unfix from the endothelium by sympathetic activation. Concomitantly, noradrenaline induces an increase in eHSP70 levels by the interaction with α-adrenergic receptor.[75] Neutrophil, lymphocyte and NK cell numbers can increase, two-, four- and ninefold, respectively, during exercise when compared with rest values, and these modifications are dependent on exercise intensity. In trained subjects, where cortisol levels during moderate exercise are lower than in untrained ones, the mild stress (due to exercise) to the immune system does not result in great leukocytosis. On the other hand, during the recovery phase from exercise, a decrease in NK cell number occurs in the bloodstream. Similar effects are shown by lymphocyte during first 48 h after exercise. In mononuclear cells, HSP70

expression was significantly increased immediately after the ultra-marathon, remaining at high levels 2 h post-exercise, returning to rest (low) levels 24 h after the trial.[11] This reduction could be attributed to hormonal response, which can produce 'homing' effects, in other words, the permanence of high amount of immune cells inside the organs and lymph nodes, which worsens the defense of the organism against infection. Concomitantly, in the early phase after high-intensity exercise, eHSP70 is elevated in peripheral blood. Afterwards, eHSP70 blood concentration returns to the lower basal levels as soon as 2 h after the end of the physical effort, remaining practically undetectable for 24 h.[11,99] This high susceptible period is named 'open window', when airway infections are facilitated, which is the second reason for training interruption in elite athletes.

Moderate-intensity exercise when regularly practiced, on the other hand, may improve immune defense by an increase in macrophage activity[100] and lymphocyte stimulation. In animals infected with herpes virus, moderate exercise decreases mortality and morbidity rates, while strenuous exercise has the opposite effect.[68] Although $CD4^+$ lymphocyte (and also other immune cells) number increases during both intense and moderate exercise, the downfall of immune defenses during the 'open window' period (which can last for 3–72 h after exercise) occurs only following high-intensity physical activity.[101] Interestingly, exercise is able to increase lymphocyte proliferation and cytokine production, while increasing vaccine efficacy in experimental models.[102] The spread effect of exercise is found in other immune system cells, as observed during the stimulatory effect on macrophage phagocytosis.[103] The HSP70 stimulating effect over monocytes/macrophages leads to the expression of the NOS-2, thus promoting NO production,[104] which is microbicidal. In addition to TNF-α production, NO production has been shown to be the principal tumouricidal mechanism of activated macrophages both *in vitro* and *in vivo*.[68] Phagocytosis is also increased in macrophages treated with HSP70 *in vitro*.[105] On the other hand, results from our laboratory have shown an increase of phagocytosis by macrophages from rats submitted to the 'stress' of moderate physical exercise.[89] These considerations and the fact that moderate loads of exercise do increase intracellular HSP70s in lymphocytes, whereas higher loads increase eHSP70 release from these cells, as stated above, suggest that eHSP70 may also function as an actual immune signal to target organs and this may be a physiological universal during exercise efforts.

MOLECULAR BASES OF FATIGUE WITHIN THE CNS

Physical exercise is recommended for the treatment of many chronic metabolic diseases or simply to 'release stress', with many types of exercises. Despite over 100 years of scientific inquiry into the mechanisms of muscle fatigue, many issues remain unclear, particularly because of the initial definitions: 'fatigue is the failure to maintain the required force or power for the task'. Typically, this is a focus of researchers who perform isolated experiments, in muscle motor units, for example. However, there are considerable biochemical and physiological levels of cross-regulation and integration between central and peripheral organs. Since fatigue was perceived also as a physiological process that occurs within CNS, many factors were included in the above *a priori* simplistic definition to allow the understanding of fatigue during exercise, such as motivation, central command failure and motor unit behaviour.[68,106]

Gradual brain cortical activity is present during fatiguing contractions, when the increase of central drive to the fatiguing muscles results in the spread of cortical activation to promote some help to neighbour muscles in which depletion of acetylcholine within motor end plate is found. Furthermore, the CNS has the ability of roaming the activity of the motor neurons by changes in the recruitment order and discharge rate during submaximal contractions. Hypoxemia, hypotension and hyperthermia, are all rare situations that may also contribute to the sensation of fatigue, besides a possible failure in the function of sarcolemmal transverse tubules at the neuromuscular junction.[106,107]

The interpretation of CNS participation during fatiguing process has many experimental biases: the type of muscle contraction required (concentric and eccentric contractions have different neuronal modulation),[107] the intensity of task (maximal or submaximal exercise) and duration of activity are some of them which are present in many fatigue studies. Thence, the multifactorial phenomenon named as fatigue, not necessarily involves all individual process of muscle failure, metabolic deviations from the homeostatic points or CNS processes[106,108] in order to the exercise be terminated by the fatigue sensation. Isolated control of fatigue, as previously argued, makes little sense from an evolutionary perspective. It is not correlated with the 'flight-or-fight' principle that governs vertebrate physiology. An integrative process is much more plausible to explain body control over a vast majority of physical demands.

Central factors may have important roles to promote exercise termination. In this sense, 'motivation' is an essential factor to improve performance during an exercise test. If the subject is not 'motivated', this will cause a premature termination of muscle contraction and the companion sensation of 'muscle fatigue'. Some conditions can modify the motivation, such as pain and discomfort, but it could also be modified by the exercise situation (recreational or competitive) and could be modulated by variation in the levels of neuromodulator, such as epinephrine and serotonin (5-HT). Evidence suggests a role of neurotransmitters, hormones and amino acids in regulating fatigue during an exercise session.[107,109–112] An increase in brain concentrations of 5-HT can be observed during physical exercise and repetition of this stimulus (training) leads to an adaptation or desensitization of central 5-HT1A serotonin receptors (5-HT1A), which influence physical performance and the well-being in general, thus promoting higher exercise tolerance.[109,110,113] These observations suggest that exercise may promote the modification of gene transcription as well.[112] Accordingly, 5-HT in the

brain is also affected by exercise and modifies the expression of pre-synaptic receptors and 5-HT auto-receptors. While moderate physical exercise does not affect free L-tryptophan metabolism (which is presented itself at high concentrations in an exercise situation), some adaptations on the blood brain barrier occurs.[111,114,115] Angiotensin II appears to assist in the delay for the establishment of central fatigue by promoting heat dissipation, decreasing heart rate and lowering brain temperature during physical stress. Several works point out that the activation of AT1 angiotensin receptor is involved in thermoregulation and postponement of central fatigue by promoting a significant brain protection against thermal damage,[112,116] thus allowing the individual to proceed with the physical demand. However, conflicting studies have shown no significant differences on sports performance at low 5-HT levels.[117] Inhibition of norepinephrine uptake (i.e. a more pronounced noradrenergic effect) was shown to significantly increase the time elapsed before the establishment of a drop in exercise performances under normal-temperature environments and heat as well, although there is also evidence suggesting that catecholamines may not affect the income of the fatigue status.[118]

Physical exercise has many effects on the CNS, much more than mood influence. Peripheral signals generated during and after an exercise session, such as IL-6 and IL-10, decrease endoplasmic reticulum stress markers at hypothalamic level, an effect related to the decrease in NF-κB activation,[119] similarly to that observed by intracellular HSP70 expression. Additionally, an association between increased NF-κB gene expression and activation in human leukocytes from cancer patients and a persistent fatigue state has been reported.[120] Therefore, one cannot exclude the possibility that eHSP70 could be signalling a pro-inflammatory status from the immune system to CNS in order to impose a 'fatigue behaviour'.

As in the case of other cell types, CNS cells present stress inducible heat shock response. In response to fever or fever-like augmentations in body temperature, glial cells show induction of HSP70s while higher constitutive levels of these proteins impose a state of acquired thermotolerance, which is characterized by lower heat-induced expression of HSP70 following a further episode of hyperthermia or ischaemic events.[121] Additionally, some type of neuronal cells, such as motor and sensory neurons, usually show a particularly high threshold for HSP70 induction, which was found to be associated with a failure in the activation of HSF-1.[20] It has also been noticed that HSPs may be transferred between cell types in the nervous system. Accordingly, HSP70s synthesized in glial cells may be rapidly transported into adjacent axons as a mechanism of fast delivery of neuroprotectoral agent. The post-synaptic neuron that 'receives' HSP70 exhibits an enhanced tolerance to stress while the *in vivo* administration of HSP70 results in inhibition of motor and sensory nerve degeneration.[122] A thermotolerant state is also observed after prior expression of HSP70 in neurons that results in protection of synaptic neurotransmission against damage insults.[20] Secretion of HSP70s by CNS cells appears to be mediated by exosomes formed at lipid rafts, which is reinforced by the finding that the brain is enriched in lipid rafts.[20]

Although a conspicuous body of evidence indicates that the brain behaves as a major HSP70 releaser to the blood stream, there is yet no study approaching the opposite, that is, that CNS cells may take up HSP70 from the circulation and that this may function as a stress/danger signal from of peripheral cells. This prediction is currently under consideration in our laboratory.

IMMUNE CELL-TO-CNS SIGNALLING NETWORK FOR FATIGUE

The processes of building certain behaviours and control of them can be analyzed under the optics of neuroimmunomodulation. The expression of 'sickness behaviour' can be induced by immune modifications and immune capacities that are associated with distinct behaviour in mammals. Studies on animal models show that 'submissive behaviour' is associated with reduction of oxidative burst and cytotoxicity of NK cells, which are signals of impaired immune function and may represent an increased susceptibility to disease development.[7,123] In this sense, it is clear the participation of mediators including TNF-α, interleukin-1β (IL-1 β), and IL-6, through the inhibition of noradrenalin-induced melatonin production, the activation of hypothalamus–pituitary–adrenal axis and the impairment of the transcription of enzymes required for neurotransmission.[7,8] In parallel, research in the physical exercise field has been progressively showing the participation of cytokines during exercise demands. For instance, the release of skeletal muscle-derived IL-6 into the blood is the most remarkable alteration in cytokine pattern observed during exercise so that IL-6 is now considered as an exercise factor, a 'myokine',[8,124] not just an inflammatory mediator. Additionally, as previously hypothesized,[125,126] exercise-evoked IL-6 may also act on the CNS to induce the fatigue sensation. Although IL-6 is always produced during the course of an inflammatory response, it has a truly anti-inflammatory action and, when produced during exercise, IL-6 may indeed exert a protective role. In other words, the skeletal muscle must be considered as an auxiliary endocrine organ that interacts with the immune system and CNS, so that IL-6 is a robust exercise marker.

Myokine signals are correlated with sensation of fatigue, and alongside other cytokines, such as IL-1 and TNF-α, myokines have been demonstrated to be inducers of sleep or illness response and pyrogenic behaviour.[125,127] Also, brain macrophages have been shown to contribute to the increase in brain IL-1β and the 'fatigue behaviour' that is associated with the recovery from exercise-induced muscle damage,[127,128] a clear demonstration of cross-talk between immune and 'fatigue sensation systems'. Similarly to the cytokines released by immune cells during exercise, serum HSP70 concentration does rise after exercise sessions, mainly because of the contribution of lymphocytes.[128] As a corollary, lymphocyte-derived HSP70s may interplay with CNS to induce the state of 'fatigue behaviour'. Con-

sequently, HSP70 (intra or extracellularly located) is also a strong and unbiased exercise marker, especially if compared to circulating levels of IL-6.

Enhanced thermotolerance of larval neuromuscular transmission conferred by heat shock-induced HSP70 overexpression has been observed.[130] However, the involvement of HSP70 in heat shock-mediated protection remains unclear. On the other hand, HSP70 has been proved to protect CNS against cerebral ischaemic injury and to mitigate neurodegenerative disorders.[129] Indeed, overall neuronal function is affected prior to hyperthermic cell death while a previous heat shock treatment is able to sustain synaptic performance via pre- and post-synaptic modifications that occur in parallel with HSP70 induction.[20] Additionally, incubation of mouse brainstem slices with inducible HSP70 has been found to relieve the effects of thermal stress neural transmission, which indicates that targeting HSP70 to motor neurons is sufficient to induce thermoprotection. However, directing HSP70 to the skeletal muscle results in no difference of performance.[129,130] Summarizing, motor neurons must be considered the critical neuronal targets for eliciting HSP70-mediated thermoprotection during locomotion (or during the capital flight-or-fight behaviour) whereas peripheral sensory neurons, dopaminergic and serotoninergic neurons alone are not sufficient to impose an HSP70-mediated thermoprotective behaviour.[129,130]

CONCLUDING REMARKS

As summarized in Figure 1, physical exercise promotes stimulation of autonomic nervous system leading the increased catecholamine plasma concentrations which modify redox status in target organs with higher metabolic demands during exercise, such as the liver and skeletal muscle. Contracting muscle and circulating lymphocytes 'see' exercise-evoked rise in vascular shear stress and circulating 'danger signals', such as IL-1, IL-6 and angiotensin II. The oxidative modification of intracellular milieu of such territories induces intracellular HSP70 expression that prevents cell damage and represents an important defense against injury induced by intense metabolism and associated protein denaturation. eHSP70 release by the liver may be a metabolic response to plasma glucose alterations as well as to adrenergic stimulation triggered by physical activity, while lymphocytes may release eHSP70 towards the extracellular space following induction by muscle signals, such as IL-6 and glutamine.

Within lymphocytes, intracellular HSP70 (increased by chronic adaptions to exercise training) plays an anti-inflammatory role, while eHSP70 binding to toll-like receptors (TLR-2/4) represents a pro-inflammatory stimulus that may result in a fatigue signal to the CNS during the higher load bouts of acute or chronic exercise. Indeed, chronic adaption of HSP70 synthesizing machinery works as a thermotolerant state for the muscle, immune cells and CNS. This may explain why HIV/AIDS patients subjected to exercise training protocols show an evident 'health behaviour'[130–132] associated to a rise in immune system function:[108,133] exercise induces HSP70 expression and the consequent HSP70-dependent blockade of viral NF-κB activation reduces viral proliferation, while exercise-induced liberation of eHSP70 into the circulation evokes a state of inflammation that stimulate immune system function as a whole.

Besides the above considerations, eHSP70 released from immune cells during exercise, mainly from lymphocytes, fulfil all the features to be an immune signal from the periphery to the CNS, inasmuch as eHSP70 has proven to exert a marked influence on motor neurons and deeper structures of CNS (e.g. hypothalamus) leading to the 'fatigue sensation/behaviour'. In other words, after eHSP70 concentrations had reached a maximum, further exercise load would be dangerous and the immune system signals that to CNS in order to impose fatigue sensation and shutdown of exercise. Moreover, and still most important, the ratio between lymphocyte HSP70 content and lymphocyte-derived eHSP70 blood levels during exercise sessions and along exercise training protocols may represent a novel and unbiased diagnostic tool to impose the limit of the exercise load to immunosuppressed patients in order to maintain their immune function without fatigue.

ABBREVIATIONS

5-HT	5hydroxytryptamine serotonin
5-HT1A	5HT receptor 1A
APC	antigen-presenting cell
CCR5	C-C motif chemokine receptor type 5
CNS	central nervous system
cp-PG	cyclopentenone prostaglandin
GSH	glutathione
GSSG	glutathione disulfide
HIV-1	human immunodeficiency virus-1
HSP70	the 70-kDa family of heat shock proteins
HSC70	heat shock cognate proteins the constitutive forms of HSP70 gene family
eHSP70	extracellular HSP70
HSF-1	heat shock transcription factor-1
IKKs	the inhibitor of nuclear factor (B (I(B) kinases
IL	interleukin
JNK	c-Jun N-terminal kinase
LOX-1	lectin-type oxidized LDL receptor-1 also known as oxidized low-density lipoprotein receptor 1 (OLR-1)
LPS	lipopolysaccharides
MHC	major histocompatibility gene complex
MRP/GS-X	the glutathione S-conjugate export ATPases of the multidrug resistance-associated gene family
NF-(B	nuclear factor (B
NK	natural killer lymphocytes
NO	nitric oxide free radical ($NO^{\cdot}$)
NOS	NO synthase
SNOG	*S*-nitrosoglutathione

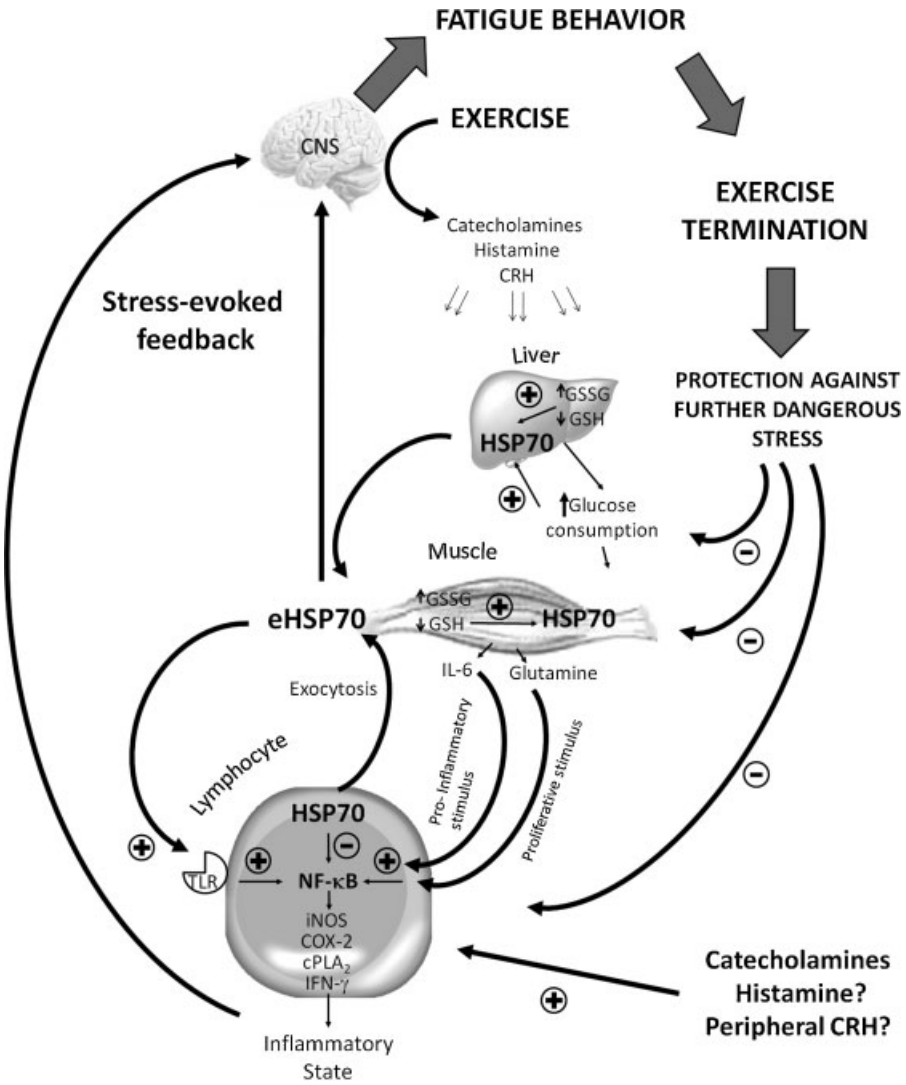

Figure 1. Possible role of HSP70 as a fatigue signal during exercise. Exercise promotes stimulation of autonomic nervous system leading to increased catecholamine plasma concentrations which modify redox status in target organs with higher metabolic demands during exercise, such as the liver and skeletal muscle. Also, peripheral corticotropin-releasing hormone (CRH) and histamine produced at sympathetic post-ganglionic terminals may be involved in lymphocyte stimulation during exercise bouts. The consequent oxidative modification induces intracellular HSP70 expression that prevents tissue damage. HSP70 release by the liver may be a metabolic response to plasma glucose alterations as well as to adrenergic stimulation, while lymphocytes may release HSP70 towards the extracellular space (eHSP70) following induction by muscle signals, such as interleukin-6 (IL-6) and glutamine. Within lymphocytes, intracellular HSP70 (increased by chronic adaptions to exercise training) plays an anti-inflammatory role, while eHSP70 binding to toll-like receptors (TLR-2/4) represents a pro-inflammatory stimulus that may result in a fatigue signal to the CNS during the higher load bouts of acute or chronic exercise. Then, the balance between intracellular HSP70 in lymphocytes and its ability of exporting eHSP70 towards the circulation determines the optimum of exercise-dependent stimulation of immunoinflammatory function before fatigue has been attained.

SREC-1	type F scavenger receptor expressed by endothelial cells
TBARS	thiobarbituric acid-reactive substances
TLR	toll-like receptor
TNF-a	tumour necrosis factor-a

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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