[Frontiers in Bioscience 13, 1328-1339, January 1, 2008]

Heat shock protein 72 : release and biological significance during exercise

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1. ABSTRACT

The cumulative stressors of exercise manifest themselves at a cellular level by threatening the protein homeostasis of the cell. In these conditions, Heat Shock Proteins (HSP) are synthesised to chaperone mis-folded and denatured proteins. As such, the intracellular HSP response is thought to aid cell survival in the face of otherwise lethal cellular stress. Recently, the inducible isoform of the 70Kda heat shock protein family, Hsp72 has been detected in the extracellular environment. Furthermore, the release of this protein into the circulation has been shown to occur in response to a range of exercise bouts. The present review summarises the current research on the exercise Hsp72 response, the possible mediators and mechanisms of extracellular (e)Hsp72 release, and the possible biological significance of this systemic response. In particular, the possible role of eHsp72 in the modulation of immunity during exercise is discussed.

2. HEAT SHOCK PROTEINS – INTRODUCTION

Exercise induces a wide range of stressors at the cellular level which ultimately challenge homeostasis. These stressors (e.g. heat, oxidative stress, glucose deprivation, hypoxia, lowered pH, increased Ca^{2+} concentration (1,2)) stimulate the synthesis of heat shock proteins (HSPs). HSPs have strong cytoprotective effects, acting as molecular chaperones to maintain cellular homeostasis. While there are many HSP's that are typically classified into families based on their molecular weight, the focus of the current review is on the inducible isoform of the 70kDa heat shock protein family, Hsp72. The induction of Hsp72 depends on the heat shock element (HSE) in the promoter region of the HSP gene binding heat shock transcription factor -1 (3). Hsp72 mRNA is then transcribed, followed by Hsp72 protein synthesis. Many signals have been identified that result in the expression of intracellular Hsp72; for example, adrenocorticotropin hormone (ACTH)

(4), corticosterone (5), catecholamines (6,7), glycogen deprivation (8), oxidative stress (9) and heat/hyperthermia (10). Hsp72 is capable of binding ATP and polypetides, with the N-terminal responsible for binding ATP, whilst the C-terminal binds polypeptides (11). The aim of this review is to describe the exercise extracellular (e)Hsp72 response, review the literature examining the mechanisms and signals of eHsp72 release and discuss the potential role this protein may have in modulating immune function.

3. EXTRACELLULAR (e)Hsp72 AND EXERCISE

Previous evidence has shown that stressors characteristic of exercising muscle such as acidosis, increased temperature, free radical formation, ischemia and glucose deprivation all induce the *intracellular* expression of Hsp72 (12). Furthermore, exercise itself induces the expression of Hsp72 in a number of central and peripheral tissues. This led Walsh *et al* (13) to investigate the possible effects of exercise on the extracellular expression of Hsp72. Since Hsp72 had been demonstrated *in vitro* to have potential immuno-stimulatory capabilities (and therefore systemic roles) (14-16) and that Hsp72 had been detected in the circulation of humans (17), the authors hypothesized that an acute bout of exercise would increase Hsp72 protein expression in contracting skeletal muscle which would be released into the extracellular environment increasing the concentration of serum Hsp72. Following 60 minutes of treadmill running at 70% VO_{2max}, serum eHsp72 was indeed elevated, but the increase preceded the increase in mRNA and protein Hsp72 synthesis within the muscle tissue, prompting the authors to assert that muscle cells are not responsible for the extracellular release of eHsp72. Regardless of the origin of eHsp72, this study was the first to suggest that exercise stress was capable of stimulating Hsp72 release. Subsequent to this original finding, further studies have corroborated these data in a range of exercise modes and intensities (Table 1). Furthermore, on investigation of the eHsp72 responses to marathon running (~260 mins at ~65% VO_{2max}), various duration and intensity treadmill running (120 mins at 60% VO_{2max} and 60 mins at 75% VO_{2max}) and high-intensity interval running (10 x 1000m, ~35 mins at ~88% VO_{2max}), Fehrenbach *et al* (18) were able to assert that the exercise eHsp72 response is both intensity and duration dependent. Therefore, these studies reveal that the cumulative stressors of exercise result in an increase in eHsp72 in humans and have initiated much interest in heat shock protein physiology to determine not only the mechanism, releasing signal and origin of extracellular Hsp72 release, but perhaps more importantly, the biological significance of the presence of Hsp72 in the extracellular environment.

4. RELEASING MECHANISMS OF eHsp72

For Hsp72 to be present in the peripheral circulation, it must be released from a cell/organ. There are two potential mechanisms for the cellular release of Hsp72. Firstly, Hsp72 is passively released from an intra-cellular pool following cellular lysis or death. Alternatively, or perhaps additionally, Hsp72 is actively released due to a receptor-mediated exocytotic pathway (19,20). One further plausible explanation related to this is that Hsp72 is chaperoning other molecules that have been excreted from the cell (21).

4.1. eHsp72 release and cell death

It was first suggested by Galluci *et al.* (22) that heat shock proteins are released only under pathological circumstances resulting in necrotic death and not programmed or apoptotic death. This was substantiated in two *in vitro* studies whereby induced cellular necrosis (through repeated freeze/thaw exposures, hypotonic lysis or virally induced lysis) released HSPs into the culture supernatant, whereas simulated apoptosis via exposure to ultraviolet radiation did not (23,24). Tissue/cellular necrosis releases Hsp72 into the extracellular milieu via lysis of the cellular membrane with the protein contents of the cell 'spilling' into the surrounding space. Exercise that results in damage to the sarcolemma reflects necrosis and there is some *in vivo* supporting evidence linking elevated eHsp72 with markers of muscle damage. Running a marathon (~4 hours) resulted in a greater eHsp72 response than shorter continuous runs at 60 and 75% VO_{2max} or interval training, and was accompanied by a greater concentration of post-exercise plasma creatine kinase concentration (CK) (18). Following an ironman triathlon, Suzuki *et al.*(25) showed a 22 fold increase in eHsp72 concentration that was accompanied by changes in classical markers of muscle damage such as decreases in muscle strength and squat jump height, and increases in delayed onset of muscle soreness (DOMS) and CK, although no correlations were reported. Interestingly, total bilirubin and alkaline phosphatase were also elevated post race demonstrating partial evidence for haemolysis. Damage to erythrocytes (which contain Hsp72, (26)) through repeated footstrikes may partially account for the elevated eHsp72 concentrations, particularly as exercise duration appears to be a main function of increased eHsp72 (18). Extensive haemolysis consequent of poor venepuncture has also been shown to elevate eHsp72 concentration (27). Whilst damage to muscle tissue from eccentric exercise may result in elevated eHsp72 concentrations through passive release from cells, the majority of evidence to date suggests that any effect of this is only marginal. No known studies have reported a direct correlation between eHsp72 levels and markers of muscle damage, whilst downhill running failed to induce a greater eHsp72 response than running on a flat gradient (28). Furthermore, repeated eccentric elbow flexor contractions failed to elevate eHsp72 concentrations above baseline levels (29). Perhaps importantly, exercise modalities that involve no eccentric component and at relatively low intensities such as semi recumbent cycling and underwater running result in an elevation in circulating Hsp72 (30,31). Hsp72 is released into the peripheral circulation within 10-30 minutes of stressor onset which is too quick for the classical protein induction/necrosis pathway and since increases in the blood can be considerable (table 1), it is unlikely that such a large number of cells would simultaneously die a necrotic death (19). It therefore seems unlikely that tissue/cellular necrosis accounts for a large proportion of the increase in eHsp72 seen with exercise.

Table 1. Summary of studies investigating the effects of exercise on the extracellular Hsp72 response in humans

Mode of exercise	Intensity	Duration, distance or time	Serum plasma	Effect on eHsp72. % change from pre-ex baseline to immediately post ex	Notes	Ref
Semi-recumbent cycling	~62% VO_{2max}	120 mins	S	0 – 0.88 ng/ml[1]	*n*=7.	30
Two-legged knee extensor exercise	40% of leg peak power output	4 – 5 hrs	S	None	*n*=7. Unable to detect eHsp72 in any samples.	8
Semi-recumbent cycling	~65% VO_{2max}	120 mins	S	0 – 0.9 ng/ml[1]	*n*=6.	58
Competitive marathon Interval training Flat treadmill run “ “ “	~65% VO_{2max} ~88% VO_{2max} 75% VO_{2max} 60% VO_{2max} 80% VO_{2max} 60% VO_{2max}	42.2km 260 mins 10x1000m 35 mins 60 mins 120 mins 24 mins 24 mins	P P P P S S	↑ ~725% ↑ ~150% ↑ ~170% ↑ ~140% ↑ ~1100% ↑ ~250%	*n*=17 *n*=10 *n*=7 *n*=7 *n*=10 *n*=10.	18
Two-legged knee extensor exercise	50% of leg peak power output	180 mins	S	↑ ~ 300%	*n*=7.	61
Cycling	60% VO_{2max}	180 mins	S	↑ ~150%	*n*=6. Carbohydrate ingested throughout trial[2].	72
Cycling	43% VO_{2max}	120 mins	S	↑ ~32%	*n*=7. Hot and humid conditions (38°C, 60%RH).	75
Flat treadmill run “ Downhill (-10%) treadmill run	60% VO_{2max} 80% VO_{2max} 60% VO_{2max}	60 mins 60 mins 45 mins	P P P	↑ 47% ↑ 88% ↑ 147%	*n*=9.	28
Competitive running	Variable	14km ~58 mins ~64 mins	P	Control ↑ ~850% EHI[3] ↑ ~2900%	*n*=7. *n*=22.	56
Competitive ironman triathlon: Swimming Cycling Running	Variable	3.8km ~57mins 180km ~311mins 42.2km ~231mins	P	↑ ~2100%	*n*=9. Muscle damage evident.	25
Treadmill running	70% VO_{2max}	60 mins	S	↑ 685%	*n*=6.	13
Underwater running Core temp clamped Un clamped	59% VO_{2max} 59% VO_{2max}	120 mins 120 mins	P	↑ 129% ↑ 212%	*n*=11.	31
Cycling	74% VO_{2max}	90 mins	P	↑ 146% ↑ 180%[5]	*n*=10. [5]with caffeine supplement (6mg/kg)	66
Cycling	VO_{2max} test followed by ~ 40min time trial	~20 mins + 40 mins	S P P	0.2 ng/ml[4] 2.7 ng/ml (heparin)[4] 6.4 ng/ml (EDTA)[4]	*n*=9. Time trial in hot conditions (34.6°C)	27

Note: Where exact eHsp72 values have not been reported in the published journal article, approximate changes from baseline have been calculated. Serum derived eHsp72 concentrations tend to be lower than that derived from plasma. Hence, with very low baseline eHsp72 levels, % change tends to be very high compared with plasma. [1] eHsp72 was virtually undetectable at rest thus unable to calculate exact % change. [2] Carbohydrate has been shown to blunt the eHsp72 response to exercise (58). [3] EHI: exertional heat illness, those competitors who collapsed during the race with core temperatures exceeding 39°C (mean T_{re} ~41°C). [4.] Only post exercise values reported.

It has been suggested that cellular necrosis is highly unregulated and only likely during trauma cases (32), and indeed, trauma has been shown to elicit very large concentrations of eHsp72 (33,34). In addition, pathological conditions such as atherosclerosis (35), peripheral and renal vascular disease (36), septic shock (37) and prostate cancer (38) all elevate circulating Hsp72. Whilst a passive release through necrosis may be an important mechanism in pathological circumstances and trauma, the fact that psychological stressors such as predatory fear and electric shock evoke a stress induced eHsp72 release (39-41) add further evidence to the suggestion that a pathway other than necrosis is also evident.

4.2. Active release of eHsp72

The classical protein transport pathway involves targeting of newly synthesized proteins to the endoplasmic reticulum, followed by transfer to the golgi apparatus where the protein is ‘packaged’ into secretory vesicles which then fuse with the plasma membrane allowing the contents to be transported into the extracellular environment (42). An active release mechanism for HSPs in the absence of lysis was first demonstrated by Hightower and Guidon (43) where stimulated cultured rat embryo cells released a number of heat shock proteins. Interestingly, this release was not blocked by either monensin[1] or colchicines[2] (inhibitors of the classical protein transport pathway), leading the authors to suggest the involvement of a non-classical secretory pathway. More recently, glia-like cells have been shown to actively release Hsp70 upon heat shock *in vitro* (44), prompting recent investigations into the mechanism of this selective exocytotic release.

It is documented that a number of proteins (e.g. IL-1β, macrophage inhibitory factor and fibroclast growth factor-2) are secreted by non-classical secretory pathways by one

of a number of different processes, e.g. within secretory lysosomes, secretory exosomes and/or through plasma membrane resident transporters (42). Broquet *et al.* (45) demonstrated the presence of Hsp72 within plasma membrane detergent-resistant microdomains (also known as lipid rafts) of epithelial cells which increased significantly when exposed to heat shock. Furthermore, treatment with Brefeldin A (which inhibits the classical transport pathway) had no effect on this response, whereas treating the cells with the cholesterol[3] depleting agent methyl-β cyclodextrin (MBC), blunted the elevation in Hsp72 under heat shock conditions. This demonstrated a potential involvement of lipid rafts in Hsp72 release from cells. The same finding has been shown in peripheral blood mononuclear cells (PBMC's) in culture, with ~70% of release of Hsp72 from B-lymphocytes and ~30% from T-lymphocytes via a non-classical pathway possibly involving lysosomal lipid rafts (46). It should be noted however that controversy surrounds the use of cholesterol-depleting agents to identify lipid raft functions, as these agents may interfere with the integrity of structures/processes within the cell, such that the loss of a particular cellular process after cholesterol depletion cannot be contributed solely to lipid raft dysfunction (47). The uncertainty about the role of lipid rafts in Hsp72 release was heightened by the fact that in PBMC's, treatment with MBC did not blunt the increase in Hsp72 concentration in the culture medium (48).

Recently, much interest has revolved around the role of exosomes in the selective release of Hsp72. Much of this interest is based on findings that exosomes contain many immuno-stimulatory molecules such as major histocompatability complex (MHC) I and II, co-stimulating molecules and adhesion molecules (49), suggesting that exosomes could provide an exocytotic pathway for other potential immuno-stimulatory molecules such as Hsp72. Exosomes are small membrane-bound vesicles (60 to 100nm) that are secreted by a number of eukaryocytes as a consequence of fusion of multivesicular bodies with the plasma membrane (50). Several hematopoetic cells have been shown to secrete exosomes, e.g. dendritic cells, macrophages, T and B-lymphocytes, and platelets (50), and they are excreted under the basal state as a result of changes in intracellular calcium levels (51). Hsp72 has been detected within exosomes in a variety of cell types, although it appears that cellular specificity exists with regards to exosomal Hsp72 release/expression. For example, Clayton *et al.* (52) demonstrated that exosomes derived from B-lymphocytes were positive for Hsp70 under normal conditions which increased after 3-hours of heat shock, and that this increase was as a result of an increase in the number of exosomes secreted This is in contrast to findings of Lancaster and Febbraio (48), who showed that the temperature dependent increase of Hsp72 in PBMC's when exposed to heat shock was due to an increase in Hsp72 concentration within each exosome and not due to an increase in the number of exosomes secreted. Furthermore, Hsp72 was detected in the lumen and on the cell surface of tumor derived exosomes (53), but only within the lumen of B-cells (52).

Whilst the supporting evidence to date suggests that the majority of Hsp72 is actively released via an exocytotic pathway into the circulation from cells under times of stress, and it is likely that exosomes/lysosomes are involved, further research is needed to clarify the factors involved in Hsp72 release.

5. STIMULATION AND RELEASING SIGNALS OF eHsp72

Exercise results in a multitude of physiological changes which individually and collectively impose stress on the body's regulatory system; for example, increases in core/local muscle temperature, reduction in blood glucose, progressive dehydration, oxidative stress etc. A number of these stressors associated with exercise are known to alter the concentration of intracellular Hsp72, thus may provide answers as to what triggers the specific release of eHsp72.

5.1. Heat

The very term "heat shock protein" clearly implies heat as a primary stimulator of the heat shock protein response. Indeed, using an isolated working heart model at different temperatures, Staib *et al* (54) implied that elevated temperature was a requirement for myocardial Hsp72 expression and data suggests the regulator of Hsp72 synthesis, HSF-1, is directly activated by temperature (55). However, it is presently unclear as to the effect of heat stress on Hsp72 release into the extracellular environment. Using a thermal clamp protocol in our laboratory (whereby core temperature is 'clamped' by exercising in cold water), we were able to investigate the independent and combined effects of exercise and rises in core temperature on eHsp72 (31). Unsurprisingly, combined exercise and increases in core temperature generated the greatest eHsp72 response, whilst exercise alone and passive heating alone both induced significant eHsp72 responses. Thus, increases in eHsp72 during exercise could not solely be contributed to increases in core temperature. Furthermore Ruell *et al.* (56) demonstrated that only 42% of the variance in eHsp72 was explained by increases in core temperature in runners who had suffered from exertional heat illness during a 14km race (mean rectal temp ~41^{o}C). This suggests that other factors are involved in the eHsp72 response.

5.2. Glucose and hepatic stress

Prolonged exercise results in a decrease in muscle glycogen and blood glucose if glucose is not replaced by exogenous sources (57). In order to maintain blood glucose levels, increased glycogenolysis in the liver and skeletal muscle occurs, and since the liver has been shown to release a number of acute phase proteins it was suggested that HSPs may originate from this organ. Indeed, arterial-venous difference cannulation techniques have highlighted a significant release of Hsp72 from the hepatosplanchic viscera during semi-recumbent cycling (30). Furthermore, it has been shown that this release is attenuated (but not completely eliminated) by glucose ingestion (58). The authors concluded that ingestion of glucose reduced hepatic stress as hepatic glucose production during exercise is reduced to basal levels when glucose is ingested (59). Further evidence that blood

glucose may regulate eHsp72 production was presented by Oglesbee *et al* (60). Upon admission to hospital, diabetic ketoacidosis (DKA)[4] patients presented with high blood glucose and high eHsp72 levels (compared to matched controls). Upon successful treatment, blood glucose and eHsp72 had lowered to normal values. Importantly, there was a strong correlation between change in blood glucose and change in eHsp72 (r = 0.93), thus, 86% of the variance in eHsp72 concentration was explained by changes in blood glucose.

5.3. Oxidative stress

Exercise results in increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that challenge cellular integrity. ROS have been shown to induce the expression of HSPs within lymphocytes (9). Furthermore, 28 days of supplementation with combined vitamin C and the vitamin E isoform γ-tocopherol blunted the circulating Hsp72 response to exercise (61). This affect was most likely mediated by a reduction in ROS and RNS generated during exercise.

Taken together, these findings demonstrate that heat, glucose availability and oxidative stress all play a role in the generation of an eHsp72 response to exercise. However, these studies do not imply cause and effect, and any association may be mediated through a different signal, particularly if an active exocytotic pathway is the likely release mechanism from the cell.

5.4. eHsp72 and hormonal releasing signals

It has been suggested that eHsp72 is part of the normal stress response (62). Thus it seems justifiable that eHsp72 release may be mediated by one (or more) of the signals associated with the hypothalamic-pituitary-adrenal (HPA) or sympathoadrenal medullary (SAM) axis (Figure 1), particularly as a number of stress-induced signals originating from the anterior pituitary and adrenal glands are known to increase the *intra*-cellular concentration of Hsp72, e.g. ACTH (4), corticosterone (5) and the catecholamines adrenaline (6) and noradrenaline (7). Indeed, using adrenalectomy, hypophosectomy and adrenergic receptor blockade in animal models of eHsp72 induction, Fleshner *et al* have been able to assert a probable role for catecholamines but not glucocorticoids or ACTH in the stimulated release of eHsp72 (39,41,62). In particular, pharmacological blocking of α_1-adrenergic receptors by prazozin completely attenuated the stress induced eHsp72 response, suggesting a strong role for noradrenaline in the stimulation of eHsp72 release Furthermore, whilst α_1-adrenergic receptor stimulation has been shown to increase intra-cellular Hsp72 expression in a variety of tissue/cell types such as brown adipose tissue (6), myocardium (63) and immune cells (64), α_1-adrenergic receptor stimulation also results in an intra-cellular calcium flux (65). Recall that changes in intra-cellular calcium can affect the release of exosomes (51). Thus one current hypothesis is that under times of stress, increases in noradrenaline acting upon α_1-adrenergic receptors results in a calcium flux within the cell and a subsequent release of Hsp72 within exosomes (62) (Figure 1).

Whilst it appears that the release of eHsp72 in rodents is mediated by noradrenaline stimulation of α_1-adrenergic receptors, to date there has been little investigation into the releasing signals of eHsp72 in humans. Unpublished findings by Fleshner *et al* (In (40)) reported that humans exposed to uncontrollable hand shocks had elevated levels of circulating Hsp72 compared to control, and that this co-incided with increases in circulating adrenaline, noradrenaline, ACTH and cortisol. Importantly, only noradrenaline correlated with circulating Hsp72 (r = 0.467). More recently, Whitham *et al*. (66) investigated the effect of supplemented caffeine upon the eHsp72 response to prolonged exercise. Caffeine is a known stimulator of sympathetic activity, and results in an elevated adrenaline and to a lesser extent noradrenaline response to exercise. This attribute of caffeine provides a useful *in vivo* model to investigate the role of catecholamines in eHsp72 release. Using a randomized counterbalance design, 10 trained males performed two trials cycling at a constant work rate (74% VO_{2max} for 90mins), separated by one week – one with caffeine supplementation (CAFF - 6ml/kg body mass) and the other with placebo. Exercise in the CAFF trial not surprisingly resulted in greater serum caffeine, which was also accompanied by a significantly greater eHsp72 response compared to placebo. Circulating adrenaline was significantly greater in CAFF, whilst the greater concentration of noradrenaline observed in this trial, did not reach statistical significance. This data supports the contention suggested from animal data, that catecholamines are involved in the eHsp72 release. However, it also suggests a more prominent role for adrenaline under the conditions encountered in this study and may reflect different patterns of sympathetic activation both across species and between different stressors. Current work being undertaken in our laboratory utilizing adrenergic blockade aims to further clarify a role for catecholamines in the eHsp72 response to stress in humans.

5.5 Stimulation of catecholamines and eHsp72

Given the possible role of catecholamines in the stimulation of eHsp72 release, it is tangible that any stimulation of sympathetic activity such as the metaboreflex (67) and chemoreflex (68) has the potential to stimulate eHsp72 (Figure 1). Interestingly, initiation of a mental stress during exercise induces a greater cardiovascular response (69) and since psychological stress has been shown to stimulate eHsp72 in animals (39), heightened mental stress during exercise may be additive in inducing a greater eHsp72 response. Furthermore, curarization and anaesthesia studies, which essentially block or reduce afferent feedback from peripheral tissues, suggest a contribution of central command on sympathetic activity (70,71). Therefore, this provides a potential mechanism through which central drive may stimulate eHsp72, independent of afferent feedback.

6. ORIGINS OF eHsp72

The possible contribution that hepatosplanchnic tissue derived Hsp72 has on the total circulating concentration during exercise has already been discussed.

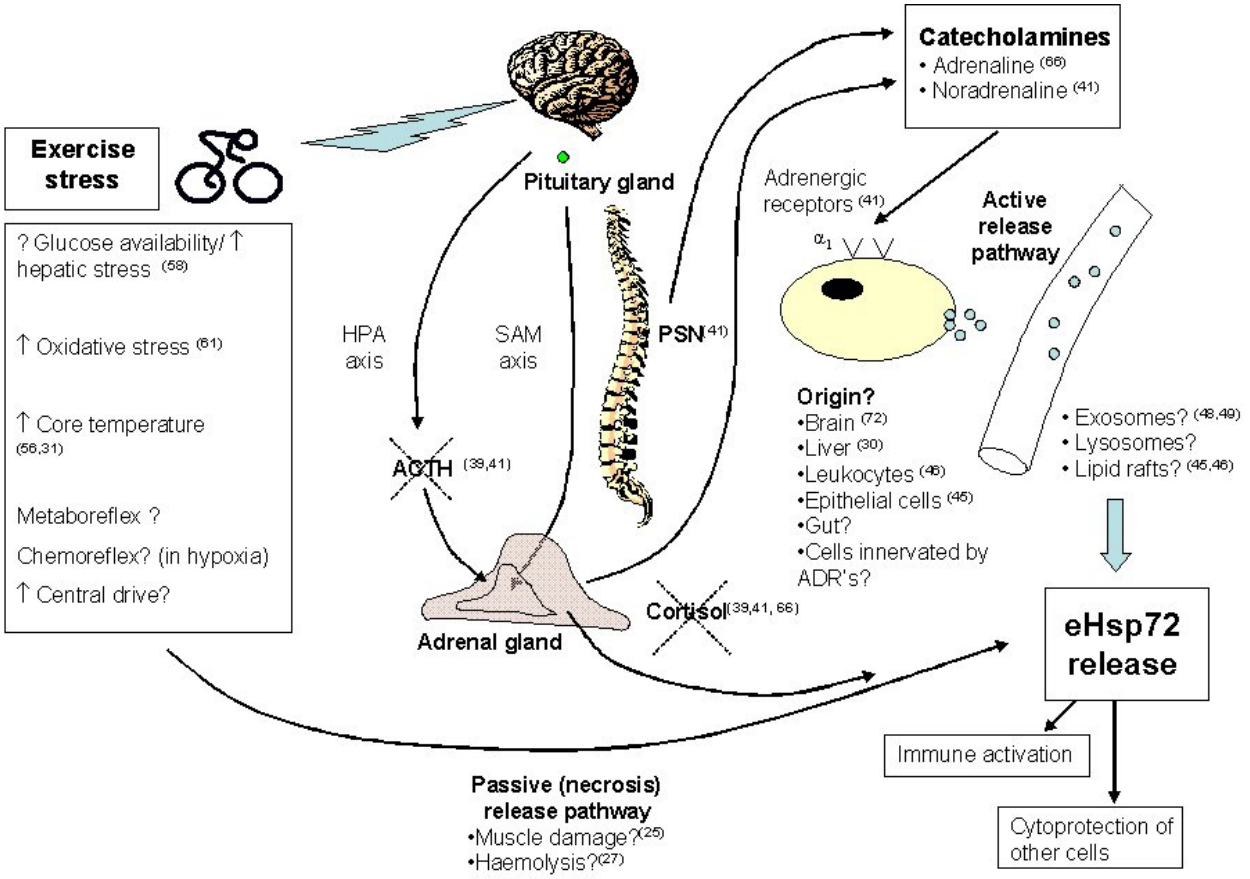

Figure 1. Model for the exercise induced release of eHsp72. Depending on the mode, intensity and/or duration of exercise, eHsp72 may be released by one of two methods: through cellular necrosis (muscle damage or haemolysis), or through an active exocytosis mechanism. Stressful stimuli associated with exercise such as a reduction in blood glucose or increases in oxidative stress and core temperature activates a stress response leading to the release of hormones from the pituitary gland via the hypothalamic-pituitary-adrenal (HPA) axis or sympatho-adrenalmedullary (SAM) axis. Evidence from rodent models suggests that neither adrenocorticotropic hormone (ACTH) nor cortisol is responsible for eHsp72 release. Catecholamine stimulation of adrenergic receptors from either the adrenal medulla or from direct innervation with postganglionic sympathetic neurons (PSN) has been associated with the accumulation of Hsp72 in the circulation, probably via an α_1-adrenergic dependent pathway. It is currently unclear as to the origin of eHsp72 but the mechanism for release likely involves exosomes, lysosomes or lipid rafts. Upon release into the extracellular environment, eHsp72 may act as an endogenous danger signal, serving to enhance immune responses, or aid other distant cells that are incapable of either synthesizing or inducing *intracellular* Hsp72 themselves.

Furthermore, there are convincing data that immune and epithelial cells are also capable of releasing Hsp72 into the extracellular environment (45,46,48). However, it is also worth considering other potential tissue sources of eHsp72 during exercise. Since the skeletal muscle is a highly active tissue during exercise, and an exercise induced *intracellular* Hsp72 response has been widely demonstrated, one might imagine skeletal muscle cells to be an origin of eHsp72. However, as already mentioned, the appearance of eHsp72 preceded the increase in skeletal muscle Hsp72 mRNA and protein concentration (13) in exercising humans. Furthermore, eHsp72 was undetectable after 4-5 hours of knee extensor exercise at 40% maximal leg power output in a muscle glycogen depleted state (8). This led the authors to assert that Hsp72 is only released when muscle is damaged and that intact muscle membranes are impermeable to Hsp72. However, eHsp72 in the latter study was undetectable in serum using an assay with a detection limit of 0.8ng/ml. On comparison of post exercise eHsp72 concentrations from plasma and serum (27), EDTA treated plasma was, on average 6.5 ng/ml higher than serum concentrations using the same commercially available assay as previous studies (13,30,58). With this in mind, it is suggested that further work measuring plasma Hsp72 concentrations is necessary to determine the contribution of skeletal muscle Hsp72 to the circulating concentration.

Additional work using cannulation techniques revealed a possible release of Hsp72 from the brain following 180 minutes of cycling at 60% VO_{2max} (72), though this response demonstrated significant subject

specificity. Bearing in mind the possible involvement of sympathetic activation on Hsp72 release (41,66) and the apparent ubiquity of Hsp72 expression, it is conceivable that Hsp72 can be released from any cells served by the sympathetic nervous system as already alluded to (Figure 1)

7. SIGNIFICNACE OF THE EXERCISE eHsp72 RESPONSE: POTENTIAL ROLES IN IMMUNITY

A greater understanding of the signalling mechanisms behind intracellular and extracellular Hsp72 expression becomes more significant when considering the potential roles heat shock proteins may have in the function of the immune system. Since the original observation by Srivastava *et al* (73) that immunization of mice with tumor derived HSPs induced anti-tumor immune responses, immunologists have been interested in the role of HSPs in key immune steps such as antigen presentation, dendritic maturation and activation of innate immunity.

7.1. Hsp72 and cytoprotection

Initiation of an intracellular heat shock protein response results in stress tolerance and protection against otherwise lethal stressors.. In particular, there is some evidence to suggest a role for Hsp72 in the protection of immune cells in the cellularly stressful condition of inflammation. Oehler *et al* (74) demonstrated higher Hsp72 responses to heat shock in phagocytic leucocytes than other immune cell types and attributed this heightened response to phagocytes' main role in producing reactive oxygen species. Additionally, resting and post exercise eHsp72 concentrations decrease during the initial stages of heat acclimation (75). Thus it may be that there is an increased uptake of eHsp72 by cells in order to improve stress tolerance to subsequent heat exposures. During times of stress therefore, Hsp72 may protect cells not only from external stressors, but also from the cytotoxicity of the immune system itself. This is particularly interesting if eHsp72 has the potential to be taken up by distant cells that may be undergoing stress and/or incapable of inducing Hsp72 themselves. For example, motor neurons contain basal Hsp72 but are incapable of synthesizing it when exposed to heat shock. However, exogenous application of Hsp72 to the cell culture promoted motor neuron survival (76). Glial cells also release Hsp72, and due to their close proximity may provide a means by which neurons can enhance their stress tolerance (44). This attribute of Hsp72 may be of particular relevance to the treatment of neurodegenerative diseases associated with destabilization of protein structure (77).

7.2. Heat shock proteins and antigen presentation

Bearing in mind the intracellular protein chaperone roles of Hsp72, it is perhaps appropriate that this protein would be involved in binding antigenic proteins. Hsp72 binds immunogenic peptides generated within cells and assists in the presentation of these peptides by major histocompatibility complex (MHC) class 1 molecules (78). These Hsp-peptide complexes may well play a role in the pathology of autoimmune disorders (79) and as such, manipulation of the Hsp response may offer treatment avenues. Furthermore, tumor derived exogenous Hsp72 has been shown to chaperone immunogenic peptides and selectively bind to antigen presenting cells (APC) (80). Following receptor mediated endocytosis, the HSP-chaperoned peptides are re-presented on MHC class 1 molecules and a tumor specific cytotoxic T cell response ensues (80). Therefore, HSPs in this regard are promising candidates for continued research into cancer immunotherapy (for review see (81)). In addition to the chaperoning of "self" antigens, HSPs are also able to bind to microbial peptides in order to facilitate their detection by the immune system. For example, HSPs are able to bind to unmethylated CpG motifs of bacterial DNA (82) and assist in the amplification of the immune response to LPS (83).

7.3. Extracellular Hsp72 and innate immunity

Aside from the first discovery of HSPs involvement in tumor derived antigen presentation, in the absence of these chaperoned immunogenic peptides, Hsp72 is thought to also stimulate innate immunity (84). Whilst a number of cell types have been shown to release Hsp72, eHsp72 must bind to a receptor thereby setting in motion a cascade of events resulting in the initiation of an immune response. A number of leucocytes have been shown to bind eHsp72, for example, natural killer (NK) cells (85), monocytes (14), macrophages (86) and dendritic cells (87). Also, a number of pattern recognition receptors (PRR's) have been shown to bind eHsp72 e.g. toll like receptors (TLR) 2, 4 and 7, CD14, CD40, CD36, CD91, LOX-1 and SR-A (88). Asea *et al.* (14) demonstrated for the first time a cytokine like action of eHsp72. Hsp72 bound with high affinity to the plasma membrane of human monocytes resulting in an intracellular calcium flux, the activation of nuclear factor kappa B (NF-κB) and upregulation of inflammatory cytokines TNF-α, IL-1β and IL-6. The term "chaperokine" has been coined, reflecting the unique chaperone and cytokine function of this protein (84). More recently, binding of Hsp72 to the surface of monocytes increased matrix metalloproteinase-9 (MMP-9) expression also through the NF-κB pathway and enhanced cell motility (89). This is pertinent as MMP-9 plays a crucial role in extravasation of monocytes during inflammation. A number of studies have now demonstrated *in vitro* that various sources of eHsp72 can stimulate immune effectors from APC's, such as cytokines TNF-α, IL-1β, IL-6 and IL-12 (14,87,90), a number of chemokines such as RANTES[4], macrophage inflammatory protein 1α and 1β (91), and nitric oxide (90,92). In addition Hsp72 is known to activate the complement cascade independently of antibody (15). Interestingly, the induction of NF-κB and proinflammatory cytokines by eHsp72 appears dependent upon the expression of CD14 (14). An upregulation of innate immunity by eHsp72 may therefore only occur in the presence of bacterial activation of CD14. Furthermore, since the interaction between CD40 on APC and CD40L on T cells is an important costimulatory pathway (93), that Hsp72 binds to, and stimulates CD40+ cells places Hsp72 as a potential key player in the interphase between innate and adaptive immunity.

7.4. Endotoxin contamination controversy

The bacterial stimulant LPS and Hsp72 appear to have similar cytokine affects on immune cells (14). Furthermore, these affects both appear to be mediated by the same pattern recognition receptors (94,95). As a consequence, there is much debate within this field as to whether the cytokine inducing actions of eHsp72 are due to 'naked' Hsp72, or to bound endotoxin contamination of the recombinant preparations used. A series of experiments from a selection of laboratories have shown that low LPS containing recombinant Hsp72 preparations failed to stimulate inflammatory cytokine release from immune cells *in vitro* (62,96-98). Furthermore, the activation of T-cells by recombinant Hsp72 has been ascribed to flagellin contamination (99). However, studies have been conducted that have purified and isolated Hsp72, showing that the immune activation of eHsp72 is via a different pathway to LPS (14,92). For example, the calcium flux that was induced within 10 seconds of eHsp72 binding to the surface of monocytes does not occur with LPS stimulation and is an important signalling step that separates these two pathways (14). It has since been suggested that due to the complex forming ability of Hsp72, 'naked' Hsp72 *in vivo* is actually unlikely (21,62). Indeed, exercise can cause increased gastrointestinal permeability (100,101) and since physical and psychological stressors have been shown to stimulate bacterial translocation from the gut, Johnson and Fleshner (62) have suggested that under times of stress, eHsp72 may be chaperoning LPS released from endogenous gut bacterial flora, thereby stimulating both innate and acquired immunity (102). Whilst there is still a debate over endotoxin contamination in recombinant preparations, evidence exists that eHsp72 can stimulate aspects of the immune system. Furthermore, this debate does not invalidate much of the *in vivo* evidence linking stress induced increases in eHsp72 with improved immune function.

7.5. eHsp72 and immunity *in vivo*

Much of the *in vivo* work has been conducted in rodent models. For example, elevations in eHsp72 concentration in response to tail-shock were associated with reduced inflammation and quicker time to recovery following a subcutaneous *e-coli* injection, whilst physically active rats recovered quicker than sedentary rats (90,103). In addition, *in vivo* delivery of Hsp72 into mice accelerated wound closure by 60% compared to control-treated mice (104) which was likely due to enhanced macrophage phagocytosis of wound debris, a finding that has been confirmed by a recent study (105).

Conclusive evidence in humans is currently lacking though studies have demonstrated relationships between elevated eHsp72 and improved prognosis/outcome. Pittet *et al* (34) found that high levels of eHsp72 derived from patients admitted to hospital with trauma were associated with improved survival, whereas all of the patients who died of their injuries had low (<15ng/ml) serum Hsp72 concentrations. Furthermore, it is known that concentrations of eHsp72 decline with advancing age (106), which may be indicative of an age-related reduced ability to respond to stress that may partially account for the increased morbidity and mortality seen with ageing.

7.6. Hsp72 and blood clotting

One potential area of future research derives from some useful methodological data collected in our laboratory. Concentrations of eHsp72 published in the literature tend to vary dependent on the matrix in which it is measured. Since Hsp72 is thought to chaperone intracellular aggregated proteins, we hypothesised that postexercise plasma-derived Hsp72 concentrations would be higher than serum derived Hsp72, because Hsp72 would bind to aggregated clotting proteins in serum (27). As expected, blood treated with EDTA or heparin contained significantly higher concentrations of Hsp72 than serum. While this has implications for the methodological assessment of eHsp72, that Hsp72 binds to clotting or wounded tissue *in vivo* and performs immuno-stimulatory roles is an interesting concept that warrants further investigation.

8. PERSPECTIVE

The current review article has summarised the current literature on the eHsp72 response to exercise. While the biological significance of exercise induced eHsp72 expression is yet to be determined, an increased level of eHsp72 in response to exercise may prepare the immune system for the stressors associated with exercise and potential pathogenic challenge (62). The *in vitro* immune data reviewed here add support to the hypothesis that heat shock proteins act as "danger" signals to alert the immune system during times of stress (107). Furthermore, it is tangible that elevated eHsp72 is able to aid other cells that may be incapable of inducing Hsp72 themselves, in order to maintain homeostasis under times of stress. These attributes of the HSP response place these proteins at an interesting focus in areas of immunotherapy where immunoenhancement or immunosuppression is desired.

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Footnotes: [1] Monensin – a sodium ionophore that disrupts the structure of the golgi apparatus, thus preventing vesicle transport, [2] Colchicine – an alkaloid that blocks microtubule assembly. [3] Cholesterol is a crucial component of lipid rafts, thus depleting cholesterol results in a disruption in raft integrity, [4] DKA is characterised by abnormally high levels of blood glucose. [1] RANTES – Regulated upon activation normal T cell expressed and secreted

Key Words: Stress, Hyperthermia, Immunity, Inflammation, Cytoprotection, Exercise, Heat Shock Protein, Review

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