Mechanisms of stress-induced cellular HSP72 release: implications for exercise-induced increases in extracellular HSP72

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Abstract

The heat shock proteins are a family of highly conserved proteins with critical roles in maintaining cellular homeostasis and in protecting the cell from stressful conditions. While the critical intracellular roles of heat shock proteins are undisputed, evidence suggests that the cell possess the necessary machinery to actively secrete specific heat shock proteins in response to cellular stress. In this review, we firstly discuss the evidence that physical exercise induces the release of heat shock protein 72 from specific tissues in humans. Importantly, it appears as though this release is the result of an active secretory process, as opposed to non-specific processes such as cell lysis. Next we discuss recent ***in vitro*** *evidence that has identified a mechanistic basis for the observation that cellular stress induces the release of a specific subset of heat shock proteins. Importantly, while the classical protein secretory pathway does not seem to be involved in the stress-induced release of HSP72, we discuss the evidence that lipid-rafts and exosomes are important mediators of the stress-induced release of HSP72.*

Introduction

The heat shock proteins (HSP) are a family of highly evolutionary conserved proteins found in all eukaryotes and prokaryotes. Members of HSP family are primarily classified according to the their molecular size, e.g. HSP110, HSP90, HSP70 and HSP40, and contain both constitutive and stress-inducible members. HSP are quintessential intracellular proteins whose primary function is to interact with naïve and denatured proteins to prevent the aggregation of aberrantly folded proteins, facilitate the folding of naïve proteins, facilitate the refolding of denatured proteins, and to aid intracellular protein trafficking (11). One of the most fascinating aspects of HSP biology is that induction of the heat shock response confers cytoprotection and thermotolerance to subsequent, and otherwise lethal, cellular stressors. These aspects of HSP biology underscore the critical role of HSP within the intracellular environment; however, a growing body of literature is providing evidence that specific HSP can be actively released from cells.

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While this idea may seem counterintuitive, evidence from additional studies has demonstrated that extracellular HSP can exert important biological functions (1, 2, 25, 26). Several years ago, we demonstrated that the circulating concentration of a specific member of the HSP family, HSP72 – the stress-inducible member of the HSP70 family, was significantly increased following a bout of moderate-intensity exercise. Importantly, this increase was observed in the absence of any overt tissue damage and suggested that exercise stress may stimulate the active release of certain HSP from intracellular locales, to the extracellular environment. This review will firstly discuss the data pertaining to the effect of exercise on extracellular HSP72, and secondly, discuss recent data that has elucidated some of the mechanisms by which cellular stress induces the release of HSP72 from mammalian cells.

Exercise and extracellular HSP72

The first evidence that exercise induced the release of HSP72 and subsequent accumulation in the systemic circulation was provided by Walsh and colleagues (27). It was demonstrated that 60 min of running exercise at 70% of maximal oxygen uptake resulted in a marked increase in the circulating level of HSP72. While it had been demonstrated previously that circulating levels of HSP72 are increased in several disease states (22), this study was the first to demonstrate that a physiological intervention resulted in an increase in extracellular HSP72 levels. Importantly, this increase occurred in the absence of any changes in plasma creatine kinase levels, suggesting that the increase in the circulating HSP72 level was not the result of exercise-induced tissue damage and subsequent leaking of intracellular HSP72 into the extracellular environment. The term heat shock protein is somewhat of a misnomer as many cellular stressors induce the expression of HSP. Indeed, skeletal muscle contraction is associated with a number of cellular stresses that may induce the heat shock response, e.g. increases in muscle temperature, changes in muscle pH, oxidative stress, mechanical stress and substrate depletion. The skeletal muscle would therefore appear to be an attractive candidate tissue source for the increase in the systemic HSP72 concentration. However, increases in HSP72 mRNA and protein content within the contracting skeletal muscle occur considerably later than increases in the systemic circulation (27), suggesting that the contracting skeletal muscle is not the tissue source of the exercise-induced increase in the circulating HSP72 concentration. This correlative interpretation of the data was subsequently tested in a later study (7) in which the arterial-venous balance of HSP72 over the contracting leg was determined. Despite an increase in the arterial plasma HSP72 concentration during exercise, no leg release of HSP72 was observed, thus confirming that the contracting skeletal muscle does not contribute to the exercise-induced increase in the circulating HSP72 concentration.

It has been shown that exercise induces the expression of numerous HSP, including HSP72, in the liver of exercised rodents (17, 23). To examine whether liver HSP72 release contributed to the exercise-induced increase in the systemic HSP72 concentration, Febbraio and colleagues (7) tested the hypothesis that hepatosplanchnic tissues release HSP72 during exercise. Therefore, the arterial-venous balance of HSP72 was determined via catheterisation of the brachial artery and hepatic vein. The results of this study demonstrate that the hepatosplanchnic tissues release HSP72 during exercise (semi-recumbent cycling at 62% maximal oxy-

gen uptake) and that this release contributes, in part, to the exercise-induced increase in the systemic HSP72 concentration. Importantly, a full blood analysis revealed no signs of liver damage or dysfunction as a result of the experimental protocol. Therefore, it was concluded that exercise induces the release of HSP72 from liver via a specific exocytotic pathway, as opposed to non-specific processes such as cell lysis.

Collectively, these studies demonstrate that physical exercise results in an increase in the systemic HSP72 concentration and that this increase is not the result of tissue damage. Furthermore, recent data has shown that this increase is dependent on both the duration and intensity of exercise performed (8). Importantly, while the contracting skeletal muscle is subject to numerous stressors, it does not appear to release HSP72 during exercise. However, the hepatosplanchnic tissues, most likely the liver, do indeed release HSP72 during prolonged exercise and contribute to the increase in the systemic HSP72 concentration. These results raise several intriguing questions; firstly, what is the exercise-associated stimulus promoting the release of HSP72, secondly, by what exocytotic mechanism is HSP72 released from the cell, and thirdly, what is the biological function of the exercise-induced increase in the systemic HSP72 concentration?

While the focus of this review is the mechanism/s by which cells actively release HSP72 in response to cellular stress, recent data suggest a possible stimulus that may promote the release of HSP72 during exercise and we shall briefly discuss these results. Recently, Fleshner and colleagues examined the potential role of stress hormones in mediating stress-induced elevations in the systemic HSP72 concentration (15). Their results demonstrate that neither adrenalectomy nor hypophysectomy had any effect on stress-induced elevations in the extracellular HSP72 levels, suggesting that corticosterone, ACTH and adrenaline do not play a role in stress-induced elevations in extracellular HSP72. Importantly, while propranolol (a specific $\beta 1/\beta 2$-adrenoreceptor antagonist) did not affect the stress-induced increase in extracellular HSP72, both labetalol and prazosin (a non-specific adrenoreceptor antagonist and a specific $\alpha 1$-adrenoreceptor antagonist, respectively) blocked the stress-induced increase in extracellular HSP72. Although Fleshner and colleagues used tail shock as the model stressor, exercise, like tail shock, results in an intensity and duration dependent increase in numerous stress hormones (16). Therefore, given the critical role of $\alpha 1$-adrenoreceptors in mediating tail shock-induced increases in the systemic HSP72 concentration, it would seem reasonable to suggest that during exercise sympathoadrenergically innervated tissues may be stimulated to release HSP72.

Cellular stress induces the release of specific HSP from mammalian cells: mechanisms of action

While the observation that exercise results in a significant elevation in the circulating HSP72 concentration is relatively recent, the discovery that mammalian cells have the capacity to actively release specific HSP was originally made over 15 years ago. In 1989, Hightower and Guidon (14) provided the first evidence that mammalian cells possessed the capacity to release selective HSP in both the basal and stress-induced state. In this study, culture medium from rat embryo cells, incubated at either 37 or 45˚C for 10 minutes followed by a 2h recovery period, was collected

and subjected to two-dimensional polyacrylamide gel electrophoresis. It was shown that cultured cells released a selective panel of proteins in the basal state, and in response to heat shock HSP72 was readily detectable in the cell culture medium. To address the cellular mechanism by which these proteins were released cells were treated with pharmacological inhibitors of the common secretory pathway. Intriguingly, neither monensin (a Na^{2+} ionophore that disrupts the structure of the Golgi apparatus and inhibits vesicular transport) nor colchicine (an inhibitor of microtubule assembly) had any effect on basal, or stress-induced, HSP release. Importantly, further experiments provided strong evidence that the observed HSP release is indeed an actively regulated process as opposed to a non-specific release mechanism such as cell lysis (14). While these data convincingly demonstrate that mammalian cells are indeed capable of releasing stress proteins, the cellular mechanism/s facilitating this transport remained, until recently, unknown.

Cells are able to secrete proteins via either classical or non-classical secretory pathways. Protein transport through the classical pathway occurs via the targeting of newly synthesised proteins to the endoplasmic reticulum and subsequent transfer to the Golgi apparatus where the protein may undergo modification before being packaged into secretory vesicles. These vesicles then fuse with the plasma membrane thus allowing the protein to exit the cell and interact with the extracellular environment. Evidence from several independent laboratories supports the notion that stress-induced HSP72 release from cells is not dependent upon the classical pathway, as is evidenced by the inability of inhibitors of the classical pathway to block stress-induced HSP72 release (3, 14, 18).

Several proteins are secreted through non-classical secretory pathways, e.g. interleukin-(IL)-1β, macrophage inhibitory factor, fibroblast growth factor-2 and members of galactin family (21). Recently, it was demonstrated that plasma membrane-associated microdomains, also termed lipid-rafts, expressed HSP72, and that heat shock resulted in a marked elevation in lipid-raft HSP72 content (3); this effect was insensitive to treatment with Brefeldin A (an inhibitor of the classical transport pathway). To examine the role of lipid rafts in mediating stress-induced cellular HSP72 release, cells were treated with the cholesterol depleting agent methyl-β-cyclodextrin (cholesterol is an integral component of lipid rafts and its removal from the cell severely compromises raft integrity). Crucially, cellular stress-induced (heat shock for 1h at 43°C) HSP72 release, compared to control (37°C for 1h), was markedly inhibited by methyl-β-cyclodextrin treatment, providing evidence that lipid rafts play an important role in mediating the stress-induced release of HSP.

Evidence that exosomes play a role in stress-induced HSP72 release

In our experiments, we aimed to determine whether heat shock at physiological/pathophysiological temperatures, *i.e.* during febrile states, stimulated the release of HSPs, and whether stress-induced HSP72 release displayed cell specificity. Initially, we confirmed that stress-induced HSP72 release occurred independently of the classical secretory pathway (based on the inability of Brefeldin A treatment to inhibit stress-induced HSP72 release), and that non-specific processes such as cell lysis could not account for stress-induced release of HSP72 (18). However, and in contrast to the results of Broquet and co-workers, we were unable to confirm

a role for lipid rafts in stress-induced HSP72 release. Using methyl-β-cyclodextrin (the cholesterol depleting agent) to disrupt lipid raft function, our results demonstrate that lipid rafts do not play a role in stress-induced HSP72 release from human peripheral blood mononuclear cells (PBMCs) (18).

Almost 10 years ago (20) it was hypothesised that small vesicles, termed 'exosomes' (9), secreted following the fusion of multivesicular bodies (MVBs) with the plasma membrane may provide a secretory pathway allowing cells to actively release specific HSPs. In support of this notion, Johnstone and colleagues, using an antibody against the constitutive (HSC70) and inducible (HSP72) forms of HSP70, provided evidence that exosomes contain members of the 70kDa family of HSP (19). Furthermore, recent work has identified HSP90, HSP90 α and HSC70 in exosomes derived from tumour cells (13). These studies led us to investigate the possibility that, in response to stressful cellular conditions, cells may release HSP72 via an exosomal pathway. Cells secrete exosomes in the basal state, and their rate of release is affected by changes in intracellular calcium levels (24). Therefore, we investigated whether heat shock *per se* increased exosome secretory rate, thus facilitating an increase in cellular HSP72 release. However, heat shock (1h at either 40 or 43˚C followed by 4h recovery at 37˚C) had no effect on exosomal secretory rate compared to control conditions (5h at 37˚C) (18). Next, we examined whether heat shock increased exosomal HSP72 content. Importantly, heat shock resulted in a marked increase in the level of HSP72 within isolated exosomes (18). These data demonstrate that exosomes do indeed provide a secretory vesicle facilitating the release of HSP72 in response to cell stress. Further support for the idea that exosomes facilitate HSP72 release in response to cellular stress was recently provided by Clayton and colleagues (5) who provide evidence that in response to heat shock, exosomes derived from a variety of B-cell lines have a markedly elevated HSP72 content.

Collectively, the current data demonstrate that exosomes provide a secretory vesicle facilitating the exocytosis of HSP72 in response to stressful cellular conditions. This effect is primarily mediated via a heat shock-induced increase in exosomal HSP72 content, although it has been demonstrated that heat shock stimulates an increase in exosomal secretory rate (5) – albeit to only a small extent – and this may also contribute to increases in cellular HSP72 release. Furthermore, the work of Broquet and colleagues clearly demonstrates that lipid rafts play an important role in HSP72 export from heat shocked epithelial cells (3). Exosomes are secreted from several hematopoietic cells, *e.g.* T- and B-lymphocytes, dendritic cells, macrophages, and platelets (6), and it is possible that stress-induced HSP72 release via exosomes may be specific to these cell types. However, given the cellular ubiquity of HSP72 release (3, 12, 18), future studies will be required to confirm the involvement of specific exocytotic pathways in HSP70 release from different cell types.

Conclusion

These *in vitro* data (3, 18) raise the interesting question of how specific cells/tissues release HSP72 in response to stress/exercise. The existent data strongly suggest that cell death cannot account for exercise-induced increases in the systemic HSP72 concentration. This point is further emphasised in studies that have used a psychological, as opposed to physical, stressor (10). The evidence clearly demonstrates that

exercise/stress results in an increase in the extracellular level of HSP72. Recent work suggests that activation of α_1-adrenreceptors is critical in mediating stress-induced elevations in extracellular HSP72 (15), and *in vitro* work provides a mechanistic basis for the observation that stressful cellular conditions promote the release of HSP72, i.e. via exosomes (18) and lipid rafts (3). Whether the activation of α_1-adrenreceptors is critical to exercise-induced elevations in the systemic HSP72 concentration, and whether lipid rafts and/or exosomes contribute to the exercise/stress-induced increase in extracellular HSP72, remains to be determined. Finally, and perhaps most importantly, what is the biological function of exercise/stress-induced elevations in extracellular HSP72. It has been hypothesised that actively released HSP may initiate the activation of specific components of the immune system (4), and, in this regard, the reviews by Asea and Multhoff in this issue of Exercise Immunology reviews (EIR 11, 2005) are of importance.

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